ADDING LOCALLY SOURCED EFFECTIVE MICROORGANISMS (LEM): EFFECTS ON COMPOST CHARACTERISTICS, SOIL NUTRIENT DYNAMICS AND NUTRIENT DENSITY IN WHEAT GRAIN, EDAMAME BEAN AND PUMPKIN PULP AND SEED

by

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(Under the Direction of Dorcas H Franklin)

ABSTRACT

Animal manure or organic waste are used frequently by growers to maintain productivity of agricultural soils. Composting is increasingly being used in agriculture due to its' contribution to the disposal of waste materials. Nitrogen (N) and phosphorus (P) content in these wastes are in organic forms and must undergo mineralization or hydrolysis reactions to become available to crops as a nutrient source upon application to the soil. Microbial communities are crucial in waste decomposition, regulating nutrient cycling in soil and crop productivity. Use of bio-inoculum agriculture is becoming of more interest to organic and conventional producers. We adapted a locally sourced bio-inoculate for the temperate region, local effective microorganisms (LEM), a fermented mixture of actively decomposing leaf litter and a nutrient rich solution (carbohydrates). We identified the microbial communities in LEM and field studies were carried out to evaluate the efficiency of LEM in reducing N losses as ammonia volatilization during composting, it's role in enhancing soil N mineralization and P solubilization in soil where edamame (*Glycine max*), wheat (*Triticum aestivum*) and pumpkin (*Cucurbita maxima* Duchesne) were being grown. We also investigated the effect of LEM in improving macronutrient [e.g.,

calcium (Ca), magnesium (Mg) and potassium (K)] and micronutrient [e.g., zinc (Zn)] density in edamame bean, wheat grain, and pumpkin (pulp and seed). Our results indicate that LEM contains a wide range of microbial functional groups that includes photosynthetic bacteria, nitrogen cycling microorganisms, phosphorus-solubilizing bacteria (PSBs), and actinomycetes. Significantly greater plant available N was found in the second (2016) and third (2017) years of the study in soils that received LEM, grown with edamame, compared to control (non-chlorinated, well water). Greater plant available P was found in soil during wheat and pumpkin growing seasons compared to the control. Nutrient density of wheat grain, edamame bean and pumpkin pulp and seed showed promising results in response to applications of LEM, especially Ca and Zn.

INDEX WORDS: Compost, local effective microorganisms, ammonia volatilization, nitrogen mineralization, phosphorous, nutrient density

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INTRODUCTION

Life in all forms on earth is not only dependent on agricultural and food security but also on the soil characteristics (Gouda et al. 2018). The global human population is projected to reach 8.9 billion by 2050 and most of the increase will be observed in the developing countries of Asia and Africa (Wood, 2001). This population growth has imposed a higher resource demand on agriculture, generating a greater global use of natural resources and a significant decline in ecosystem services (Gianinazzi et al., 2010). High-intensity agriculture has been mainly focusing on higher productivity, instead of integrating natural resource management into food security. Furthermore, monocultures and increased use of synthetic inputs, such as chemical fertilizers and pesticides, have reduced soil fertility (Foley et al., 2005). One of the challenges facing agriculturalists today is how to increase food production by 70-100% to feed the growing population in the face of dwindling resources (Pretty et al. 2010). Therefore, now it is vital to develop a sustainable agricultural progression model that is socially just and that will encourage more durable and more resilient forms of agriculture, will adopt technologies that are sustainable, affordable, easily replicable, have low carbon foot print, and finally will favor local and regional biodiversity (Pimentel et al. 1995; Singh et al. 2011 and Chavarria et al. 2018).

Soil fertility can be defined as the capacity of soil to provide physical, chemical and biological needs for the growth of plants for productivity, reproduction and quality (Abbott and Murphy, 2007). Nitrogen and phosphorus are the most essential nutrients in crop growth and yield and constitute most applied fertilizers. Despite increasing crop production, commercial nitrogen (N)-fertilizers show low recovery in soil–plant systems, with recovery rates seldom

exceeding 50% of applied N, while the remainder is lost (Kaleem and Shah 2003). Phosphorus (P), the second most essential macronutrient, P, is mined from limited rock phosphate reserves and used as commercial fertilizer. Future demand for phosphorus is expected to rise with increasing world population and the associated increase in food production. The United States Geological Society projects an increase in world consumption of P-fertilizers from 42.2 Megatons in 2014 to 45.9 Mt by 2018 (USGS 2015), with an estimated world reserve of 300 billion tons. Recent estimates predict a world-reserve lifespan of 50-100 years (Cordell et al. 2009). With this increase in P-demand, agriculturalists require a way to increase P-availability from sustainable sources, such as animal manures or other forms of organic wastes enriched in N and P.

Macro/micronutrient availability in soil for plant uptake is of critical importance. Human population require more than twenty-two minerals in their everyday diets. These include macronutrients such as calcium (Ca), magnesium (Mg), potassium (K) and several other micronutrients such as iron (Fe), zinc (Zn), manganese (Mn) or selenium (Se). If these minerals are not in bioavailable form in soil or if not ingested in adequate amount through food then severe health risks can occur especially in resource-poor women, infant and child. Because of increased population pressure globally, especially in developing countries, many global food systems are not providing enough macro/micronutrients into their diets and as a result, nutrient deficiencies, especially micronutrient deficiencies afflict more than three billion people (Welch 2002).

Globally, there is an urgency to intensify agricultural food production in a sustainable way. However, the major challenge is to use these agro-bio-systems efficiently that takes the entire agroecosystem's bio-chemical diversity into account and utilizes their potential to alleviate

negative effects of low soil nutrient content, biotic and abiotic stress (Tilman et al. 2011 and Timmusk et al. 2017). The concept of sustainable agriculture is broad-based rather than adopting a specific methodology. Ideally sustainable agriculture would produce good crop yields with minimal impact on ecological factors such as soil fertility (Tilman, 1999). Sustainable agriculture involves successful management of agricultural resources to satisfy human needs while maintaining environmental quality and conserving natural resources for the future. Today, the drive for productivity is increasingly combined with the desire and even the demand for sustainability. Global food security issues will have to rely on the fusion of innovation, development and delivery of technologies both on farm and in the laboratory.

Innovative and efficient technologies in agriculture have been adopted in recent years such as smart irrigation, smart-fertilization, integrated fertilization, disease biocontrol, imaging and sensor-based technologies for monitoring crop yield, and agricultural microbial technologies. Use and development of microbial consortium technologies (essentially a polymicrobial population of beneficial microorganisms) in agriculture could enhance plant-microbe and microbiome rhizophere interactions and may be a viable and sustainable approach in sustainable agricultural management systems. Their use can offer redundancy in function, improve crop productivity and both regenerate and preserve soil health. These suits of microbial communities can help us better comprehend the complex plant-microbe-soil continuum (Singh et al. 2016). These exogenous microbial communities can also play important roles in treating animal waste or other organic amendment or crop residues and thereby accelerate the release plant available nutrients from them. Thus, upon their addition, nutrients will be more readily available for plant assimilation. Thus, the combined use of these microbial consortium in conjunction with organic resources is a promising field in sustainable agroecosystem management.

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CHAPTER 1

LITERATURE REVIEW

ECOSYSTEM SERVICES FROM SOIL

The sustainable use and management of global soils is one of the greatest challenges for the future (Morel et al. 2015). Soils are a major components of terrestrial ecosystems and fulfill a range of functions of vital importance (European Commission 2010). Functions of natural soils include (1) supporting plant growth (nutrients and water supply, support of roots), (2) maintaining biodiversity pool (habitats, species, and genes), (3) filtration, storage and transformation of substances (carbon, water, nutrients) and (4) physical and cultural support for human activities (habitat, transport, landscape, waste disposal, transport of energy, and water). Interactions between biotic and abiotic components in nature results in soil functioning. Those functions generate products and services which are useful to human well-being (de Groot et al. 2002, 2010). Ecosystem services (Costanza et al. 1997) provided by soils are the result of (a) inherent properties and (b) manageable properties. While physical support, food, wood, fiber, and raw materials are termed as provisioning services of soils, regulating services are maintaining water quality, biocontrol of pest and diseases, recycling of waste, carbon storage, and regulation of GHG emissions (Morel et al. 2015 and Millennium Ecosystem Assessment 2005). Supporting processes includes nutrient and water cycling, and soil biological activity. Cultural services are related to spirituality, knowledge, and aesthetic.

SOIL MICROBIAL COMMUNITIES

Soil microbial populations are instrumental to fundamental processes that drive stability and productivity of agroecosystems. Sustainability of agricultural systems is critically dependent upon one of the key players in sustainable agriculture which is soil microbial communities. The nutrient cycling and the movement of energy fluxes in the terrestrial ecosystems are closely related to the turnover of organic matter. In farming systems where, organic sources of nutrients are used, soil respiration and the release and availability of nitrogen (N) and phosphorus (P) are dependent on the action of soil microbial communities. Soil microbial biomass pools are important component of the decomposer subsystem that regulates nutrient cycling, energy flow and plant and ecosystem productivity (Pankhurst et al., 1996). Even though, small in mass, the microbial biomass is among the most labile pools of organic matter and thus serves as an important reservoirs of plant nutrients, such as N and P (Jenkinson and Ladd 1981; Marumoto et al. 1982). An understanding of microbial processes is important for the management of farming systems, particularly those that rely on organic inputs of nutrients. Microbiological processes occurring in the soil constitute the basis on which agroecological farming is sustained (Faria and Franco 2002). Soil microbial communities have a fundamental role in soil nutrient cycling, soil organic matter decomposition (Burton et al. 2010), however, their abundance, role and function are often poorly accounted for in plant facilitation and appear to be the missing link in understanding plant growth, nutrition and their interactions with the plant's immediate environment (Lemanceau et al. 2014, Philippot et al. 2013). Soil microbial communities can act as an ecosystem indicator because they can rapidly respond to changes in land use (Singh, 2015). Soil microorganisms in the plant rhizosphere may have positive, negative or no visible effect on plant growth. Although most rhizosphere microbes appear to be benign, deleterious

microorganisms include pathogens and microbes producing toxins that inhibit root growth or those that remove essential substances from the soil (Pereg and McMillan 2015). By contrast the main mechanisms for plant growth promotion include biocontrol, biofertilization and phyto-stimulation (Martinez-Viveros et al., 2010, Bhattacharyya and Jha, 2012). In addition, the indigenous soil microbial population of soil can also be influenced by root exudates (Haichar et al. 2008), management practices like, tillage, season, crop residue retention and incorporation, residue burning, plant species selection, as well as soil type (Berg and Smalla 2009, Reeve et al. 2010).

WASTE MANAGEMENT IN AGRICULTURE

Manures and by-products of cattle, dairy, poultry, seafood and other agricultural industries have been used by growers to maintain productivity of agricultural soils for millennia (Lazarovits, 2001). Carbon is required for the biological components in soils as an energy source, but a phenomena termed “chemical drip” for the past 50 years with little organic energy input to the system has been observed (Bailey and Lazarovits 2003). This has led to detrimental changes in soil health and water quality, leading to an increase in plant diseases and other pest problems, all within a relatively short period of time (Pimental and Zepp 1991, Hoitink and Boehm 1999).

Organic fertilizers and manures primarily contain organic forms of N and P, (urea and inositol phosphates, respectively, are the most common) which must undergo mineralization (N) or hydrolysis (P) reactions to become available to crops as a nutrient source. The general ratio of N: P (2:1 to 4:1) found in manure-based fertilizers often leads to excessive P-application, as fertilizers are applied to meet the nitrogen requirement (Havlin et al. 2016)]. Organic nitrogen and phosphorus not released is often lost to runoff or accumulates in soil as unavailable,

insoluble precipitates. The use of organic waste as an amendment to agricultural soils can be beneficial not only to crop production and soil quality but also a viable and cost-effective method of utilizing waste (He et al. 2000). Land application of organic wastes, such as manure, is quite common in sustainable agricultural practices (Ajwa and Tabatabai 1994) and provides us with an efficient way to utilize the waste as nutrient sources in agricultural production systems which otherwise would be discarded. (Westerman and Bicudo 2005). Recent studies have highlighted that nutrient cycling of N and P depends on the activities of soil microbial biomass and on those of their microbial grazers, such as protozoa and nematodes (Irshad et al. 2013). Thus, soil microbial biomass pools are important component of the decomposer subsystem that regulates nutrient cycling, energy flow and plant and ecosystem productivity (Pankhurst et al. 1996).

BROILER LITTER AS FERTILIZER

Due to lower water content and high N percentage compared to other organic manures, broiler litter is an ideal alternative fertilizer (Stephenson et al. 1990). Broiler production (*Gallus gallus domesticus*) makes up 70% of poultry production (USDA 2014). Annually producing a staggering 1.3 billion birds (15.2 billion kg of broilers) Georgia ranked number one in 2014 in broiler production (USDA 2014). Broiler production brings economic growth to this region but the generation of huge amount of litter as a waste product leads to serious issues for growers, communities in the farm vicinity, and the environment. Thus, determining optimal methods of utilizing and managing this large amount of broiler litter produced is of utmost importance. Major problem in managing broiler litter includes ammonia volatilized as ammonia (NH_3). N content in broiler litter varies highly and NH_3 volatilization of ammonia can range from 4 to 60% of the total N applied in laboratory and field experiments (Brinson et al. 1994; Cabrera and Chiang 1994; Marshall 1998). High N losses through NH_3 volatilization often leads to decreased

crop yields and cast doubts on the efficacy of broiler litter as an organic amendment (Moore, 1998; Paramasivam et al. 2009). Nitrogen in litter is present in both organic and inorganic forms (Cabrera and Chiang 1994; Paramasivam et al. 2009). The majority of plant-available N in broiler litter is in organic form that needs to be mineralized. That is why the mineralization organic-N litter is important for plant-available N and microbial communities and enzymes present in both litter and soil are critical to NH_3 and NH4+ production and subsequent NH_3 volatilization.

COMPOSTING OF ANIMAL MANURES

Compost is a form of organic amendment and it is increasingly being used in agriculture due to their contribution to the disposal of waste materials and consequently to the environment preservation. Composting is not a new technology. The use of composts as plant nutrient-sources or as soil conditioners on farmlands, public and private gardens, parks, highway embankments, landscaping and for environmental reclamation (Erickson et al. 2009) represents an age long practice (Tognetti et al. 2011; Pan et al. 2012). Compost is regarded as one of the important low-cost inputs used for meeting nutrient requirements for plant growth (Zameer et al. 2010). Mature compost eliminates or reduces the risk of pathogens, parasites and weed seeds associated with direct land application of waste. Composting ensures a final product that is sanitized and stabilized, and which can be used to improve and maintain soil health, quality and fertility (Larney and Hao, 2007). Composting can be an efficient way to better handling, transporting and managing the sheer volume of waste generated during livestock management in agriculture. Composting is a reliable way to dispose of organic waste and can be a viable source of organic matter and other nutrients such as nitrogen (N) if incorporated into soil (de Guardia et. al. 2010). It is a microbially-driven bio-oxidative process dominated by an aerobic environment and an

enriched source of diverse microbial communities breaking down organic matters into a more stabilized and hygienic final product (He et al. 2018). The main forms of N for crop uptake are ammonium and nitrate, which are produced through microbially mediated processes (Schlesinger 1997; Jones et al. 2005). The process by which ammonium is released from organic N forms is called N mineralization or ammonification. The uptake of ammonium and nitrate by the soil microbial community to build their biomass is termed immobilization. The availability of these inorganic N forms to crops is, therefore, dependent on the balance between N mineralization and immobilization (Myrold and Bottomley 2008). Nitrogen is not the only important, growth limiting nutrient that soil microbes mediate. It is well known that some microbes in soil have the potential to greatly enhance the rate of organic P (Po) or inorganic (Pi) cycling (i.e. by solubilizing insoluble organic and mineral-bound P) (Jones, 2011).

Even though the incorporation of composted waste with high organic matter content could improve soil quality and fertility and reduce waste materials (García et al. 1991 and Marinari et al. 2000), it could also produce toxicity problems that inhibit seed germination and plant development if inadequately matured composts are used (García et al. 1991, Marambe and Ando 1992). Due to degradation of nitrogenous substances, detrimental amount of ammonia (NH_3) is emitted during composting (Guo et al. 2016). Ammonia (NH_3) is a major source of atmospheric nitrogen and farming contributes to 50% of the global ammonia emissions (Schlesinger and Hartley 1992). Volatilization of ammonia during composting not only reduces the value of the compost as a nutrient source but also causes environmental pollution (Li et al. 2013). NH_3 volatilization during composting is due to the active decomposition of organic matter (Chan, Selvam and Wong, 2016). Alkaline conditions (pH > 8.0) in association with

thermophilic temperatures result in a loss of N through NH_3 volatilization (Jiang et al., 2016 and Awasthi et al., 2017).

In addition, organic phosphorus not released is often lost to runoff or accumulates in soil as unavailable, insoluble precipitates and could create eutrophication. When animal waste is surface applied, the efficiency of ammonium (NH4+) as a source of nitrogen to crops can be variable, due to volatilization of ammonia (NH_3) (Jarvis and Pain 1990). Ammonia volatilization occurs due to the conversion of ammonium-N (NH_4^+- N) in the manure or solution to dissolved ammonia gas. The reaction is as followed-

$NH_4^+ - N \leftrightarrow NH_3 (g) + H +$

(This reaction produces more ammonia as pH increases or temperatures rises, or the ammonium-N concentration increases)

While composting occurs naturally, efficient composting requires the control of several factors to avoid nuisance problems such as odors and dust, and for obtaining a quality agricultural product. The controlled conditions are basic for a composting procedure, distinguishing it from aerobic fermentation. In using compost as a biological amendment for soil health reclamation, certain aspects should be kept in mind and among those compost maturity and compost stability are of most value. Compost maturity and stability are two terms commonly used to define the decomposition degree of organic matter during the composting process, however, these two terms are not synonymous. Compost maturity refers to the degree of decomposition of phytotoxic organic substances produced during the active composting stage (Wu et al., 2000). On the other hand, compost stability refers to the level of activity of the microbial biomass and can be assessed by measuring O2 uptake rate, CO2 production or by the heat released because of microbial activity (Iannotti et al., 1994; Wu et al., 2000). However,

while discussing compost, both stability and maturity should be considered, since in an unstable compost, the phytotoxic elements are generated by the microbial populations (Zucconi et al., 1985). Maturity is, in part, affected by the relative stability of the composting material but also describes the impact of other compost chemical properties on plant development. An immature compost can lead to several hazards such as limited seed germination and root developments and high levels of free ammonia. The physical properties of composts like odor, color and temperature of the composting pile can be a good indicator but not sufficient enough to ascertain the compost maturity and analyzing different chemical properties of the compost will give us proper assessment of the compost which will include measurement of the C/N ratio in the solid phase (Bernal et al., 1998; Iglesias-Jimenez and Perez-Garcia, 1992), nitrification (NH4-N concentration and NH_4-N/NO_3-N ratio; Bernal et al. 1998; Finstein 1985; Zucconi 1987), cation exchange capacity (CEC) (Harada and Inoko, 1980) and the degree of OM humification (Jimenez and García 1992). Compost stability it can be assessed with respirometry (Iannotti et al., 1994; Scaglia et al., 2000). It reflects the degree to which the organic matter has been stabilized during composting (Chen, 2003). Moreover, by assessing relationship between the enzymatic activity and quantity and quality of organic matter information on compost stability (Garcia et al., 1993) can be extracted.

COMPOSTING PHASES ARE DRIVEN BY MICROBIAL DIVERSITY IN FEEDSTOCK

During composting, different microorganisms play different functional roles in the transformation and stabilization of organic substances. Composting process involves four phases and during these phases different groups of microbial population plays different roles. The phases are (1) Mesophilic phase; it is the first phase that starts with an initial breakdown of the organic matter. Dominant microbial families involved are Pseudomonaceae, Erythrobacteraceae,

Comamonadaceae, Enterobacteriaceae, Streptomycetaceae, and Caulobacteraceae, among others, which grow between 15^0C and 35^0C. Soluble and readily assimilable compounds such as simple sugars, amino acids, and lipids are broken down in this phase (Bernal et al., 2009; Insam and de Bertoldi, 2007). (2) Thermophilic phase; it triggers from the continued metabolic activates in mesophilic phase. The temperature can rise to 55^0C to 65^0C. Notable microbial population are actinobacteria (mostly belonging to Thermoactinomycetacea, Thermomonosporaceae, and Pseudonocardiaceae families). These families degrade recalcitrant substances such as, cellulose, hemicellulose, lignin and proteins. This phase is very important because higher temperature of this phase sanitizes the compost of pathogens (Bernal et al., 2009; Insam and de Bertoldi, 2007). (3) Cooling phase; in this phase temperature recedes and the remaining simple sugars and carbohydrates are broken down by mesophilic microbes (Insam and de Bertoldi, 2007). (4) Curing or maturation phase; finally, the precursors of the humic substances are formed in this phase (Bernal et al. 2009; De Bertoldi et al. 1983; Sharma et al. 1997; Shilev et al. 2007; Zapata 2009; Zeng et al. 2010).

USE OF MICROBIAL INOCULUM IN AGRICULTURE

Soil microbial community abundance influence nutrient availability to plants (Alloway 2009; Rengel et al. 1999). Plant growth-promoting rhizobacteria (PGPR) or plant growth-promoting microbes (PGPM) (Kloepper and Schroth1978) are involved in plant growth and development through the release of substances (Kloepper and Schroth 1981) which ensure cycling of essential plant nutrients (Behera etal. 2014). These microbes are in part responsible for improved soil health, increased crop yield (Suslow and Schroth 1982), and improved plant nutrient density. As an alternative means of synthetically produced agrochemicals, PGPR is increasingly gaining popularity among producers (Shaik and Sharaf 2017). Their function in the

rhizosphere include: solubilizing, mineralizing and immobilizing nutrients such as phosphorus (Bhattacharyya and Jha 2012). Some examples are phosphorus solubilizing bacterial communities (PSBs) such as, Pseudomonas, Bacillus, Rhodococcus, Arthrobacter, Xanthomonas, Enterobacter, Klebsiella (Sharma et al. 2013; Chen et al. 2006; Wani et al. 2005 and De Freitas et al. 1997), direct fixation of nitrogen (Zahran 2001), producing phytohormones (Pérez-Flores et al. 2017), biofortification of macronutrients such as Ca, K and Mg (Rana et al. 2012), and Zn content in cereal crops such as wheat, cereal rye (Alloway 2009). For instance, Zn is one of the deficient micronutrient in human diet, especially in developing countries. It is also an imperative microelement required for optimum plant growth. Pseudomonas, Bacillus and Rhizobium spp are known to solubilize Zn by producing organic acids, producing siderophores and protons, oxido-reductive sytems on cell membrane and ligand chelation. PGPM have also been reported as being responsible for greater productivity in a variety of different crops, vegetables and fruits, such as, wheat, soybean, rice, tomatoes, lettuce, and apple (Deshmukh et al. 2016; Etesami and Alikhani 2016; Bhattacharyya and Jha 2012).

MICROBIAL INOCULATION OF COMPOST

The inoculation of compost with microorganisms is a sustainable process that contributes to the increase in N and P levels and allows the suitable utilization of the microbial diversity involved during the composting. Composting process has been subjected to thorough research worldwide, however many scientific and technological issues are still to be addressed to improve the efficiency of the process and quality of the final compost. To improve compost quality and composting efficiency, for example, enhanced N and P mineralization, suppressing NH_3 volatilization etc. application of exogenous microorganisms is often sought after and are becoming increasingly popular. Inoculated compost may become act as a low-cost bio-fertilizer,

Thus, it is important to pay considerable attention to the formation and application of exogenous microorganisms because of their progressive effects on compost quality and maturity. For instance, to conserve and enhance the nutritional content of compost, inoculating compost by bacteria that fixes atmospheric N to enhance the N content in the compost (Pepe et al. 2013) or bacteria that oxidizes NH_3-N into nitrites and nitrates (Ahmad et al. 2008) which in turn offsets NH_3 volatilization. Among N fixers that are supplemented during composting, Stenotrophomonas, Xanthomonas, Pseudomonas, Klebsiella, Alcaligenes, Achromobacter, and Caulobacter (Pepe et al. 2013) are common and can fix atmospheric N by an enzymatic complex called nitrogenase composed of di-nitrogenases and di-nitrogenase-reductase, which reduce the atmospheric N to NH_3. After the fixation of atmospheric N in the form of NH_3, different microbial groups like the ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) convert the NH_3 into nitrites and nitrates through action of the ammonia monooxygenase and hydroxylamine oxidoreductase enzymes (Sánchez et al. 2017). For instance, microbial families, like Nitrosomonadaceae, Nitrospiraceae and Nitrososphaeraceae (Yang et al. 2019) have the potential to suppress NH_3 volatilization via enhanced ammonia oxidation.

Many soil microorganisms can transform insoluble forms of P from mineral-P complex and organic P into an accessible soluble form, thus contributing to plant nutrition through exhibiting other beneficial traits such as production of organic acids, siderophore indole acetic acid (IAA) and production of hydrogen cyanide (HCN). Microorganisms involved in the composting process are also related to the solubilization of phosphates. Number of these microbes vary depending on the soil type and bacteria constitute 1–50% and fungi 0.5–0.1% of the corresponding total population (Khan et al. 2007). These microorganisms solubilize phosphates by similar mechanisms in both soil and compost and some of these microorganisms

involved in the transformation of organic matter during composting are also related to the P mineralization (Khan et al., 2007; Wickramatilake et al., 2011; Zayed and Abdel-Motaal, 2005). They generally release P species in amounts higher than these microorganisms can utilize (Fernández et al. 2005; Restrepo-Franco et al. 2015). It has been reported that when compost supplemented and inoculated with microorganisms leads to higher solubilized P when applied to the soil. Bacteria solubilize this P mainly by lowering the pH of the medium through the synthesis of organic acids, such as 2-ketogluconic acid, gluconic acid, maleic acid etc. that act on the insoluble P (Sanchez et al. 2017). Major P solubilizing bacterial families involve Pseudomonaceae, Acetobacteraceae, Bacillaceae etc. Fungi in compost solubilize via production of phytase that transform organic P into inorganic P. Major fungi in compost P solubilization from organic P are Penicillium spp. by acid and alkaline phosphatase (Gómez-Guiñán, 2004).

Potassium (K) can also be solubilized in compost with the help of potassium solubilizing microorganism such as Bacillus mucilaginosus, Enterobacter hormaechei, Bacillus edaphicus and Aspergillus fumigatus (Sanchez et al. 2017). The fundamental mechanisms of K solubilization is the excretion of organic acids such as citric acid or oxalic acid that lowers pH of the medium and finally releasing K in the soluble form (Guevara 2010; Basak and Biswas 2009; Sugumaran and Janarthanam 2007 and Liu et al. 2006).

EFFECTIVE MICROORGANISMS

The concept of effective microorganisms (EM) was first developed in 1971 by Professor Teruo Higa, University of the Ryukyus, Okinawa, Japan (Higa and Wididana, 1991). Its' spread as a microbial inoculum started in 1989. It is reported that this commercially available EM^{TM} contains culture of naturally occurring beneficial and synergistic microorganisms such as photosynthetic bacteria, ammonia oxidizers, actinomycetes, lactic acid bacteria, yeasts and fungi

(Higa 2000; Mupondi et al. 2006). EM microorganisms are mostly heterotrophic, which means they require organic sources of carbon and nitrogen. Studies has shown that EM has been most effective when applied in combination with organic amendments to provide carbon, nitrogen and energy (Yamada and Xu 2001). According to the EM Research Organization, EM^{TM} is reported to enhance composting efficiency by reducing composting period and by decreasing compost turning time. Some other services provided by EM^{TM} is reduced odor and pest control from animal waste and compost. Reducing odor from animal waste would mean the reduction through ammonia loss and thereby decreasing nutrient loss in the form on N (Higa and Parr 1994). There have been several studies done in different parts of the world looking at the effects of EM on productivity of agricultural crops. The reported results of EM^{TM} efficacy are contrasting. Enhanced soil fertility and crop yield, reduced pest infestation, accelerated organic matter decomposition and influx of beneficial microorganisms in soil have been reported (Asian-Pacific Natural Agriculture 1996; http://www.royalgcol.ac.uk/research/conferences/tabo.htm; http://www.emtrading.com; http://www. emtechnologynetwork.org; http://www.emrousa.com). Similarly, Daly and Stewart (1999) reported that application of EM to onion, pea and sweet corn increased yields by 29%, 31% and 23%, respectively. A few studies found that there is no minimal beneficial effects from incorporating EM^{TM} into production systems (Schenck and Müller 2009; Vliet et al. 2006; Formowitz et al. 2007; Daiss et al. 2008). In Switzerland, Mayer et al. (2010) examined the effectiveness of EM^{TM} only and with manure and fermented organic matter on crop yields. They did not find any significant effect of EM^{TM} on yields of potatoes, barley, wheat, or alfalfa nor in the soil microbial biomass, microbial community structure in soil. Other research focusing on only N loss by van Vliet et al. (2006) in the Netherlands examined the efficacy of EM^{TM} added to “cow manure slurry”. They found no effect of EM^{TM} on bacterial

diversity in the manure slurry, chemical composition of the manure, and grass production. Vliet el al. (2006) used DNA bands to examine the survivability of EM^{TM} in the slurry and found that dominate DNA-bands in the EM^{TM} only treatment was not present, suggesting that beneficial microorganisms did not survive in the slurry.

LOCAL EFFECTIVE MICROORGANISMS (LEM)

Throughout much of the Caribbean and Central America producers utilize a locally sourced bio-inoculate called microorganisms of the mountains (MM) which is a modified version of EM^{TM}. While the EM^{TM} is cultured in laboratory and sold commercially, Local Effective Microorganisms or LEM is sourced locally from actively decomposing upland forest litter, followed by an anaerobic fermentation with a mixture of un-pasteurized, antibiotic-free goat milk; semolina; molasses; charcoal; chlorine-free water; and dry yeast. LEM contains a wide range of functional microbial groups typical of EM^{TM}, except LEM is collected from the surrounding environment and can be cultured on farm; therefore, easily adapts to the environment and can be used to fortify the nutrient status of animal waste while improving microbial diversity (Mahmud et al. 2019 in review; Ney et. al. 2018). This on-farm, locally-sourced bio-inoculum could be a potential tool to increase or re-establish soil biodiversity and ecosystem functioning in perturbed agroecosystem. Since, LEM contains a suite of microorganisms, it has the potentiality to offer a wide range of ecosystem functioning in the agroecosystem and functional redundancy within the microbial communities. LEM can also be used in fortifying nutrients, especially nitrogen in organic waste such as broiler litter or compost. The presence of ammonia oxidizers in LEM utilizes the ammonia volatilized from broiler litter and oxidize it to ammoniacal-N (Mahmud et al. 2019 in review). In addition, to nitrogen utilizing

bacterial families, several phosphorus solubilizing and zinc solubilizing bacterial families have been identified in LEM.

RATIONAL AND JUSTIFICATION FOR THE RESEARCH

Local effective microorganisms are collected locally, produced on-farm by using readily available raw materials, harbors a wide range of microbial population thus have functional redundancy. These microbial consortium can be applied directly to soil, can be used to inoculate organic waste for fortifying nutrients, can be applied on foliage for possible pest control etc. If LEM is used to inoculate composted manure, litter, or effluent, this will keep inorganic fertilizer costs lower for producers and provides a sustainable route for waste processing. So, harvesting local microorganisms and fermenting the population in an economically viable growth medium and applying the resulting inoculum is one possible route to increase nutrient availability and reduce the overall loss of nutrients from manure. If more nutrients are available for plants for the immediate growing season, a crop may produce a higher yield or a more nutritional yield, resulting in less land and resources used overall.

LEM might play a major role in developing countries such as India, Pakistan, Nepal and Bangladesh where macro and micronutrient deficiency is severe among resource-poor women, infant and child. LEM contains several strains of P, Zn and Ca solubilizing families that can solubilize and enhance the uptake of those nutrients. As opposed to commercially available microbial additives, LEM contains a wide array of microbial communities which gives the agroecosystem functional redundancy. This functional redundancy in microbial population is very important in ecosystems that are under biotic or abiotic stress. LEM has the potentiality to re-establish microbial populations in degraded or barren ecosystem. LEM could also be a potential source of organic matter which greatly improves overall soil quality and fertility.

Finally, it should be kept in mind that LEM is not a substitute for other management practices, but is an additive for optimizing all other amendments and practices used for crop production, meaning amending agricultural soils with LEM-treated composted manures or litters or effluents makes the most efficient use of resources by effectively reducing the amount of farm waste, increase the release of unavailable N and P nutrients found in organic waste.

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CHAPTER 2

ROLE OF LOCALLY SOURCED BIO-INOCULA IN IMPROVING COMPOSTING BROILER LITTER CHARACTERISTICS

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ABSTRACT

Adding bio-inoculates to animal waste composts is of increasing interest to organic and conventional poultry producers. We adapted a locally sourced bio-inoculate for the temperate region, local effective microorganisms (LEM), a fermented mixture of actively decomposing leaf litter and a nutrient rich solution (carbohydrates) which may minimize N losses and improve compost characteristics. The objectives of this study were to evaluate the effects of adding LEM, false LEM (F-LEM) and water (Control) to composting broiler litter (*Gallus gallus domesticus*) by evaluating pH, C/N, total phosphorus, plant available phosphorus (P), germination bioassays and ammonia volatilization. The microbial diversity of LEM inoculum and LEM-inoculated compost was also characterized. Plant available P was significantly lower in LEM-treated composts than in F-LEM and Control in Year-2 ($p < 0.05$). Ammonia loss was reduced from LEM compost piles 22% in Year-1 and 18% in Year-2 compared to Control. Microbial community richness was greater in LEM inoculum which was predominantly *Bacillales, Lactobacillales, Pseudomonales, and Rhizobiales* along with *Nitrosomonadales, Nitrospirales, Actinomycetales,* and *Acidobacterales* compared F-LEM inoculum which was predominantly *Lactobacillales*. These results suggest that inoculating composting broiler litter with LEM can improve microbial community richness and decrease nitrogen losses through ammonia volatilization.

Keywords**:** Ammonia volatilization, compost, germination index, local effective microorganisms, microbial community

INTRODUCTION

Composting is a reliable way to dispose of organic waste and can be a viable source of organic matter and other nutrients such as nitrogen (N) if incorporated into soil (de Guardia et. al., 2010). It is a microbially-driven bio-oxidative process dominated by an aerobic environment and an enriched source of diverse microbial communities breaking down organic matters into a more stabilized and hygienic final product (He et al., 2018). However, due to degradation of nitrogenous substances, detrimental amount of ammonia (NH_3) is emitted during composting (Guo, Lu and Li, 2016). Volatilization of NH_3 not only reduces the value of the compost as a nutrient source but also causes environmental pollution (Li et al. 2013).

To improve compost quality and composting efficiency, suppressing NH_3 loss has been explored using both chemical and biological means. Chemical approaches involve the use of alum or zeolite, (Awasthi et al., 2016) and biological attempts explores the use of NH_3 assimilating (Wakase et al., 2008) or lignocellulose degrading (Jurado et al., 2015) microbial communities. Ammonia (NH_3) volatilization during composting is due to the active decomposition of organic matter (Chan, Selvam and Wong, 2016). Alkaline conditions (pH > 8.0) in association with thermophilic temperatures result in a loss of N through NH_3 volatilization (Jiang et al., 2016 and Awasthi et al., 2017). Microbial families, like *Nitrosomonadaceae, Nitrospiraceae* and *Nitrososphaeraceae* (Yang, Liu and Wang, 2019) have the potential to suppress NH_3 volatilization via enhanced ammonia oxidation**.** Furthermore, during composting, ammonia loss is correlated with the temperature of the composting piles (Awasthi et al., 2017). A considerable attention has been given to the formation and application of exogenous microorganisms because of their progressive effects on compost quality and maturity. During composting, different microorganisms play different functional roles in the transformation and

stabilization of organic substances, however, most of the research has been concentrated on the effects of simple exogenous microorganisms, such as lignocellulytic bacteria or fungi (Zeng et al., 2007, Sarkar et al., 2010 and Wang et al., 2011) or actinomycetes (Wei et. al., 2019), or NH_3 assimilating bacteria, rather than on complex microbial communities on the composting process. Many farmers are using commercial additives, for instance, Effective Micro-organisms® (EM^{TM}), Euro Mest-mix® (Mx), and Agrimest® (Am) (Van et. al., 2007) that are available in the market, however, microbial communities present in those ready to sell additives are seldom disclosed by the manufacturers (Wakase et. al., 2008).

Throughout much of the Caribbean and Central America producers utilize a locally sourced bio-inoculate called microorganisms of the mountains (MM) which is a modified version of EM^{TM} by Higa and Wididana (1991) in Japan. The EM^{TM} is cultured in laboratory and sold commercially. It contains specific strains of beneficial and synergistic microorganisms such as ammonia oxidizers, actinomycetes, lactic acid bacteria, yeasts and phototrophic bacteria (Higa, 2000; Mupondi, Mnkeni and Brutsch, 2006). In Central America MM is applied to suppress pests and odors of manure and compost. We adapted the MM formulas for temperate climates which resulted in two bio-inoculates that can be grown on-farm or near-by and utilized on-farm by individual producers. We call these inoculates Local Effective Microorganisms (LEM) and False-LEM (F-LEM). The LEM bio-inoculum is gathered from actively decomposing upland forest litter, followed by an anaerobic fermentation with a mixture of un-pasteurized, antibiotic-free goat milk; semolina; molasses; charcoal; chlorine-free water; and dry yeast. LEM contains a wide range of functional microbial groups typical of EM^{TM}, except LEM is collected from the surrounding environment and can be cultured on farm; therefore, easily adapts to the environment and can be used to fortify the nutrient status of animal waste while improving

microbial diversity (Ney et. al., 2018). The goal of our research was to: a) investigate the effect of inocula: LEM and F-LEM and Control (water only) on broiler litter compost pH, carbon/nitrogen ratio (C/N ratio), total Kjeldahl phosphorus, plant available phosphorus (Mehlich-1), germination index and ammonia loss through volatilization, b) assess the microbial community of LEM and F-LEM inocula, and c) assess microbial community composition of LEM and F-LEM inoculated compost.

MATERIAL AND METHODS

Raw Materials

Organic broiler litter (anti-biotic free) from a local organic broiler (*Gallus gallus domesticus*) house was used to make compost in this study. Each year we obtained fresh litter from the same antibiotic-free farm. Compost from broiler litter was made in two consecutive years, from Dec 2014 to May 2015 (Year-1) and from Nov 2015 to May 2016 (Year-2).

Local Effective Microorganisms

The LEM was made by collecting the O horizon (loose and actively decomposing leaf litter) from upland, healthy, well established mixed pine and hardwood forests. This is the microbial inoculant. The LEM inoculum was then combined with 23 kg of organic semolina, 250 gm of baker's yeast, 10 kg of crushed natural hardwood charcoal, and 4 L each of molasses, antibiotic-free raw goat milk, and chlorine-free water to make a solid mixture. This solid mixture was then placed into a container and sealed for 6 weeks to allow for anaerobic fermentation. After first solid phase fermentation, a liquid phase fermentation was carried out for at least 2 weeks. Liquid phase fermentation: 3 kg of solid phase material is placed into a porous sack and suspended in a sealed 120 L tank with 60 L of water and 4 L of molasses.

False-Local Effective Microorganisms

Because the growing media (molasses and semolina, yeast and raw milk) contains nutrients and microbes, the False-LEM (F-LEM) was also prepared with the growing media at the same ratio as the LEM but without the forest microbial inoculum.

Experimental Design

The broiler litter was thoroughly mixed and weighed into nine piles in Year-1 and 12 piles in Year-2. In Year-1, each of the nine composting piles had 180 kg and in Year-2, each of the twelve composting piles had 90 kg of broiler liter. Each pile was housed in a mini hoop house (1.7 x 2.7 x 2.4 m^3) and prevented from freezing with 350-w lightbulbs. This study had three treatments: 1) Control (Chlorine-free Water), 2) F-LEM, and 3) LEM. Treatments were assigned in a randomized complete block design. Each of the treatments was applied to three broiler litter piles in Year-1 and four piles in Year-2.

Composting process

The treatments, liquid inoculants (F-LEM and LEM) and Control, were sprinkled on composting broiler litter to maintain a water content of approximately 0.44 g/g on a dry mass basis. The total volume of liquid LEM or F-LEM applied to compositing broiler liter in Year-1 was 151 L per 91 kg over 5 months (164 d) and in Year-2 was 136 L per 91 kg over 6 months (184 d). Year-1 required more liquid to maintain composting water content, as Year-2 was cooler and had a higher relative humidity. Treatments were applied approximately every 28 day immediately following compost turning for facilitating aeration and maintaining desirable moisture regime. Field temperature probes were inserted into each composting pile. In Year-1, second application of treatments was delayed to day 41 because of inclement weather and equipment malfunction.

Compost Sampling

During composting, compost samples were taken at three phases. Baseline sampling at day 0 (fresh broiler litter before the start of the composting), Mid- stage sampling (at 82nd day of composting in Year-1 and 90th day of composting in Year-2) and Final-stage sampling (164th and 183rd day of composting, in Year-1 and Year-2, respectively). In Year-1, five cored (30- cm depth) samples were taken from fresh broiler litter before the litter was placed into huts and the treatments were assigned. During composting, five cored (30 cm depth) samples were taken from each pile at Mid-stage and finally at Final-stage. In Year-1, treatment samples were composited for analysis. In Year-2, fresh litter was placed into huts, one sample from each of the 12 litter piles was taken at Baseline, treatments were assigned and applied and, individual piles were sampled separately at Mid-stage and Final-stage.

Compost Analysis

Baseline samples were taken from fresh broiler litter both years. In Year-1, Mid-Stage and Final-Stage samples were composited by treatments and in Year-2, compost samples were collected from each pile by treatments. Compost samples were then air-dried (65^0C), ground and sieved (<2 mm) for determining total carbon (C) and total N and elements such as, calcium (Ca), potassium (K), magnesium (Mg), sulfur (S) and zinc (Zn) at Baseline, Mid-Stage and Final-Stage (except for the Year-2 Baseline elemental analysis). In both years, pH, carbon dioxide (CO_2) respiration, NH_3 volatilization, phosphorus (P) fractions, such as, total Kjeldahl phosphorus (TKP) and plant available phosphorus (Mehlich-1P), and % germination index (% GI) was analyzed for Baseline, Mid-Stage and Final-stage. Method descriptions for all the parameters can be found below.

Ammonia Volatilization and Carbon dioxide Respiration

NH_3 volatilization and CO_2 respiration were measured from each composting broiler litter pile throughout each year of composting. Measurements were made on days 0, 1, 3, 7, 14 and 28 after each treatment application (except in Year-1, when second application of treatments was delayed to day 41 due to inclement weather and equipment malfunction). In each broiler litter pile, two gas trap chambers (20-cm ID PVC pipe open at the bottom containing two metal rings inside with airtight removable lid), were utilized on each sampling date to capture both CO_2 and NH_3 emissions from composting piles. Within each of the chambers, the two metal rings held glass jars, one with alkali (100 mL 2 M NaOH) to trap CO_2 and one with acid (100 mL 1 M H_3BO_3) to trap NH_3. On each measuring day, the chambers were inserted 5 cm into the piles, alkali and acid traps were placed into the metal rings and the lids were closed for 24 hours. After, 24 hours the jars were collected and brought back to the lab for chemical analysis. CO_2 was measured by placing a 10-mL aliquot from the glass jars into centrifuge tubes and adding 8 ml $BaCl_2$ followed by centrifugation (1500 rpm for 3 minutes) and subsequent back titrating the aliquot with 1M HCl (standardized) (Yiqi and Zhou, 2010). NH_3 was measured by the change in pH of the H_3BO_3 in the glass jars followed by back titration with a weak HCl (0.0025M HCl) to initial pH of 1 M H_3BO_3 (4.8 to 5.0) (Bremner and Mulvaney, 1982). A thermophilic index was calculated to better understand if microbial inoculates impacted the time in thermophilic stage, which is the product of number of days ≥40 °C and duration of thermophilic conditions (hours) divided by 1000 as described by Beffa et al. (1996) and Varma, Muthusamy and Rajendran (2018). An empirical mathematical expression; $pK_a = 0.0901821 + 2729.92/T_k$ (where T_k is temperature in degrees Kelvin, T_k = °C + 273.2) related to equilibrium constant and temperature

(holding temperature constant at 40^0C) was also used to explain ammoniacal-N loss as NH_3 volatilization.

Total Kjeldahl Phosphorus and Mehlich-1 Phosphorus

TKP compost samples were determined by Kjeldahl method (Taylor, 2000). Plant available phosphorus fraction was extracted by Mehlich-1 method according to Mehlich (1953), followed by a modified single solution blue dye method (Murphy and Riley. 1962) using Tecan Infinite® 200 PRO Series, 2014 (Tecan Trading AG, Switzerland) for the determination of plant available phosphorus fraction.

Germination Bioassays

The germination index (GI) was analyzed by following a modified procedure described in (Zucconi et al, 1981). Zucconi et al. (1981) and Tang et. al. (2006) have shown that a GI above 50% is indicative of a mature compost. First, 15 g of compost (dry weight basis) was mixed with 150 ml of with deionized water and shaken for 48 hours. The compost-water mixture was centrifuged at 3000 rpm for 15 minutes and supernatant was extracted through 0.45-µm cellulose nitrate filters under vacuum filtration. The extracts were used to germinate 30 seeds of cucumber (*Cucumis sativus*) and 30 seeds of radish (*Raphanus raphanistrum*). The seeds were placed in a Petri dish with 10 ml of extract for each compost in triplicate for cucumber and radish seeds. Controls were prepared in triplicates using only deionized water. The cucumber seeds were incubated at 22^0C for 7 days and the radish seeds were incubated at 25^0C for 72 hours. At the end of the experiment germination percentage and the root elongation were measured to calculate the % Germination Index as follows:

% seed germination = (mean germination in compost extract)/ (mean germination in Control (in our case water)) × 100 (equation 1)

% root growth = (mean root length in compost extract)/ (mean root length in Control) × 100 (equation 2)

% Germination Index= (% seed germination in compost extract × % root growth in compost extract)/ (% seed germination in Control × % root growth in Control) (equation 3)

Microbial Community Analysis

Microbial DNA was extracted from compost samples (composited by treatment for each year) using QIAGEN DNeasy PowerSoil Kit (DNeasy PowerSoil Kit Handbook, May 2017, Qiagen, Valencia, California, United States) and extracted DNA samples were further amplified by Ion 16S Metagenomics™ kit to amplify the V4 and V5 hyper variable regions of bacterial 16S rRNA. The sequencing was done in Coastal Palins Soil, Water, and Plant Research Center, United State Department of Agriculture Agricultural Research Service, Florence South Carolina. Specific bacterial universal primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) were used where the adapter sequences barcode sequences had been combined. The sample libraries were prepared by Fusion Method as described in Ion Amplicon Library Preparation (Ion Torrent Systems Incorporated, Guilford, Connecticut, United States of America). Each 50 µl reaction mixture was performed as follows: 1 µl of primer stock mix (containing 5415F and 806R primers), 4 µl of genomic DNA and 45 µl of Platinum® PCR SuperMix High Fidelity were added to 50 µl. The amplification condition was as follows: high-temperature pre-denaturation or activate enzyme phase was set at 94 ^{0}C for 3 min, then denaturation for 30 s at 94 ^{0}C, followed by an annealing step at 58 ^{0}C for 30s, and extension step at 68 ^{0}C for 60 sec (denature-anneal-extend cycling – 40 cycles) and finally holding stage at 4 ^{0}C until they are taken out for amplicon purification. Further purification of amplicon libraries was conducted as followed: 50 µl amplified genomic DNA and 90 µl of Agencourt® AMPure® XP

Reagent. This mixture was repeatedly (5 times) on Agencourt® SPRIPlate 96R Magnet Plate for 2 minutes. For clonal amplification of the library, emulsion PCR was performed using the Ion PGM™ Hi-Q™ template kit and the OneTouch™ 2 instrument. The template positive enriched products were loaded on the 316™-chip using the Ion PGM™ Hi-Q™ sequencing kit and sequenced in the Ion PGM™ sequencer (Ion Torrent Systems Incorporated, Guilford, Connecticut, United States of America). Obtained sequence reads were analyzed using Ion Reporter™ software with the default parameter setting. For the reference database, both MicroSEQTM(R) 16S Reference Library (v2013.1) and Greengenes (v13.5) were selected (Ney et. al., 2018).

Statistical Analysis

Analysis of variance, JMP PRO 13 software (SAS Institute, 2013), was used to determine differences between treatments by each compost stage for pH, C/N, germination index, total Kjeldahl P, plant available P, organic P, and elemental analysis ($p < 0.05$). Differences in cumulative NH_3 loss between treatments at final stage were also evaluated with analysis of variance as described above. Comparisons between multiple means was determined with Tukey's HSD ($p < 0.05$).

RESULTS

Compost pH and C:N ratio

The pH of broiler litter at Baseline was 7.7 in Year-1 and 8.8 in Year-2 (mean pH). The pH of Final-stage composts was always more alkaline than the Baseline in both years (Table 2.1). There were no differences in pH among treatments in any year. In Year-2 mean Baseline litter pH dropped to 8.2 and increased to a Final-stage compost pH was 9.0.

Nutrient Content and Germination Index of Compost

Compared to Baseline, in Year-1, all composts showed an increase in Ca, Mg, and Zn content and in Year-2, regardless of treatments, Ca, K, Mg, S and Zn increased from Mid-stage to Final-stage compost (Table 2.1). These increases were not statistically significant (Year-2) and no differences between treatments were found. In Year-1, none of the composts treated with different treatments showed germination index greater than 50% for both cucumber and radish seeds (Table 2.1). In Year-2, LEM was the only treatment that had a percent germination index above 50% ($p < 0.05$), and this difference was found only with cucumber seeds (Table 2.1).

Total Phosphorus, Plant Available Phosphorus and Organic Phosphorus

Significant differences in TKP content of composts varied depending on year and compost maturation stage (Table 2.2). Sampling of Mid-stage compost showed TKP in LEM treated compost was significantly greater than Control in Year-1 and significantly greater than F-LEM in Year-2 ($p < 0.05$). Year-1 Final-stage composts had no significant differences between the treatments but in Year-2, Control had significantly greater TKP than F-LEM, and LEM was not different from either Control or F-LEM ($p < 0.05$). Mehlich-1P extraction of Mid-stage compost indicated no significant differences between treatments. At Final-stage in Year-2, LEM Mehlich-1P was significantly lower than either Control or F-LEM (Table 2.2).

Microbial Characterization of Applied LEM and F-LEM

The bacterial 16S rRNA sequencing of liquid LEM from both years showed a diverse microbial community distribution that included the phyla: proteobacteria, actinobacteria, nitrospirae, cyanobacteria, acidobacter and firmicutes while in F-LEM bacterial phylum were predominately firmicutes. Ammonia oxidizing bacterial (AOB) communities such as, *Nitrosomonadaceae* and *Nitrospiraceae*; ammonia oxidizing archaeal (AOA) community such

as, *Nitrososphaeraceae* and PSBs, such as, *Pseudomonadaceae*, *Bacillalaceae*, *Acetobacteraceae, Lactobacillacceae* etc. were identified in LEM, whereas, *Lactobacillacceae* were identified in F-LEM (Table 2.3).

Microbial Characterization of Year-1 and Year-2 Baseline litter and compost

Proteobacteria was the dominant phylum in Year-1 Baseline litter, however, post treatment application, at Final-stage, only Control had higher proteobacteria compared to F-LEM and LEM both of which showed dramatic increase in firmicutes populations (Figure 2.1a). The presence of major bacterial and archaeal families associated with ammonia oxidation, like *Nitrosomonadaceae, Nitrospiraceae* (AOB*) a*nd *Nitrososphaeraceae* (AOA) were found in LEM compost. Year-2 Baseline litter had higher proteobacteria and at Final-stage, N-utilizing bacterial families showed similar trends in abundance as Year-1 in the composts, except for *Nitrosomonadaceae* (Table 2.4 and Table 2.5). We also found that: 1) firmicutes populations differed between Year-1 and Year-2 (Figure 2.1a and 2.1b), 2) Final-stage, firmicutes was found to be higher in all the treatments compared to Baseline in Year 1, 3) proteobacteria was the dominant phyla in Baseline broiler litter in both years, and 4) proteobacteria was dominant in Final-stage compost in Year 2.

Ammonia Volatilization

In both years of composting, N loss as NH_3 was lower from the LEM applied composting piles compared to the other treatments. Compared to Control, ammoniacal-N loss from LEM was 22% less in Year-1 and 18% less in Year-2 (Figure 2.2a and 2.2b). In both years, LEM treated piles started with lower NH_3 volatilization (Figure 2.2a and 2.2b, respectively) and continued to show this trend until the end of composting. Thermophilic index, Figure 2.3a**,** demonstrates that composts inoculated with LEM experienced the least thermophilic conditions compared to either

F-LEM or Control. Within the composting piles, in Year-1, the reduction of NH_3 volatilization in the LEM composts could be attributed to immobilization and oxidation of NH_3 by the microbial communities like, *Acetobacteraceae*, *Nitrosomonadaceae* and *Nitrospiraceae* found in the applied LEM inoculate and the Final-stage LEM compost (Table 2.3 and 2.4). As shown above, there was a greater abundance of proteobacteria in Year-2 LEM compost and significantly less NH_3 volatilization (Figure 2.2b) from the LEM composting piles.

DISCUSSION

In Year-2 of our study, the drop of pH by mid-stage is an indication of organic acid production and the pH increase by Final-stage is likely due to organic N mineralization. A study on co-composting litter with tobacco waste exhibited an initial decline in pH with a later pH increase (Adediran et al., 2004). C/N values below 20 indicate a mature compost (Ren et. al., 2018). While all C/N values in our study were well within the desirable agronomic C/N values, GI index indicated that Year-1 composts were not mature. When considering GI index, LEM was the only treatment that had a percent germination index above 50% suggesting a mature compost, although only in Year-2 and as measured with cucumber seeds. The improved cucumber % GI in Final-stage LEM compost might be a contribution of *Methylobacteriaceae* family (Table 2.1), which was absent in Year-2 Baseline litter but was found in higher percentage in Year-2, Final-stage LEM composts and has been reported to stimulate seed germination (Madhaiyan, Poonguzhali and Sa, 2007). By Mid-stage, in Year-1 and Year-2, there was a decline in C/N ratio in all the treatments compared to Baseline broiler litter (Table 2.1). Decreasing C/N ratio could be attributed to less NH_3 volatilization (Sanchez-Monedero et. al., 2001) and increased N content (Table 2.1). The increase in macronutrient content could also be attributed to compost pH by the end of the composting process (Chandna et. al., 2013).

Analysis of liquid LEM revealed diverse array of bacterial and archaeal communities that offered greater functional redundancy (Waksman, Cordon and Hulpoi, 1939) compared to F-LEM inoculum (Table 2.3). While the presence of phosphorus-solubilizing bacterial (PSBs) families: *Bradyrhizobiaceae, Pseudomonadaceae, Bacillalaceae, Acetobacteraceae, Lactobacillacceae* and *Xanthomonadaceae (*Gyaneshwar et al., 2002) were identified in greater abundance in LEM inoculant in both years (Table 2.3), this was not the case for F-LEM inoculants. In both years, *Lactobacillacceae* was identified as the dominant bacterial family in F-LEM inoculant. In the compost some PSBs applied in the inoculants did not survive or were not identified in Final-stage compost (Table 2.4 and 2.5). With greater PSBs in the LEM one could expect a greater amount of Mehlich-1P, however, if Mehlich-1P were taken up by the microbiome within the compost (greater abundance) then a greater amount of TKP would be expected, as we found in Year-1. In a review, Sanchez et al. (2017) indicated that compost supplemented with P had increased populations of both PSBs and fungi. In both years, LEM composts increased TKP at Mid-stage which might suggest increased bacterial and fungal biomass. In Year-1, Final-stage LEM compost was richer (greater diversity) in PSBs than F-LEM (Table 2.4). For instance, all the PSBs listed above were present in Final-stage LEM compost while in F-LEM compost only *Bacillalaceae, Lactobacillacceae* and *Xanthomonadaceae* were present. In Year-2, Final-stage compost LEM had significantly less Melich-1P and had the same PSBs as F-LEM but the percent PSBs abundance at phyla level was 75% for LEM and 82% for F-LEM which could explain the significantly greater plant available phosphorus in Year-2 Final-stage F-LEM (Table 2.5).

Lesser degree of ammonia volatilization was observed from the LEM compost. Microbial communities like *Nitrosomonadaceae* and *Nitrospiraceae* possess analogous *amo*A genes that

express ammonia monooxygenase subunits for NH_3 oxidation (Ren et. al., 2018; (Yang, Liu and Wang, 2019)), they can reduce NH_3 loss by producing ammoxidation products (NO_2^--N and NO_3^-N) (Tourna et. al., 2011). A combination of the lack of ammonia oxidizing communities and higher thermophilic index accounted for some of the greater loss of N from F-LEM compost (Figure 2.2a; Figure 2.3a and Table 2.4). Although, ammonia oxidizing microbial families survived by Final-stage of composting, Control compost demonstrated similar trends as F-LEM, thus the greatest loss of N from this treatment might have been the sole function of higher thermophilic index (Figure 2.2a; Figure 2.3a and Table 2.4). *Lactobacillaceae* was higher and *Bacillaceae* (*Bacillus spp.*) was lower in abundance in Final-stage LEM due to the lower thermophilic index (Andrews and Harris, 1986) compared to Control and F-LEM (Table 2.1 and Table 2.4). In addition to the above-mentioned ammonia oxidizing bacterial families, *Chromatiaceae* were present in greater percentage in Final-stage LEM compost, which are reputed to consume ammonia (Pfenning and Trüper, 1992), filling in the role of ammonia oxidation in absence of *Nitrospiraceae* and *Nitrosomonadaceae* (Table 2.5). The difference in firmicutes population between Year-1 and Year-2 could be explained by the thermophilic index (Figure 2.3a and 2.3b) during composting. In Year-1, higher firmicutes population could be attributed to the higher thermophilic index. *Bacillalaceae* was present at Final-stage composting (regardless of treatments) indicating an attainment of higher thermophilic index and a better adaptation of those *Bacillus spp* to higher temperatures as they can produce endospores at temperatures ≥40 °C (Tian et.al, 2013). However, Year-2 composts did not experience as much time in thermophilic conditions (indicating lower temperatures) which would encourage the growth and thriving of proteobacteria (Bakermans et. al., 2006).

Ammonia (NH_3) volatilization during composting is due to the active decomposition of organic matter (Chan, Selvam and Wong, 2016). Alkaline conditions (pH > 8.0) in association with thermophilic temperatures (Figure 2.3a and 2.3b) result in a greater loss of N through NH_3 volatilization (Jiang et al., 2016 and Awasthi et al., 2017). The pH of our compost piles ranged from 7.7 to 8.6 in Year-1 (164 days of composting) and 8.8 to 9.1 in Year-2 (183 days of composting), NH_3 volatilization is common at these pH levels especially with periodic aeration (Dias et al., 2010). As described above, many factors influence NH_3 volatilization. In our study, several factors contributed to lower NH_3 volatilization: 1) during the composting periods, Year-2 ambient temperatures were colder than Year-1 and while none of the composts froze during the composting period, colder temperatures likely inhibited attainment of thermophilic conditions; 2) some ammonifying bacteria (*Bacillus* and *Pseudomonas*) were identified to a lesser degree in Year-1 than Year-2 in the LEM composts; and 3) ammonia oxidation during composting is not only a function of the bacteria but also ammonia-oxidizing archaea (AOA) communities (Zeng et al., 2011), which are widely found in agronomic soils as well (Habteselassie et al., 2013). The detection of *Crenarchaeota* in Final-stage LEM compost suggests the presence of AOA, which might have oxidized volatilized NH_3 and might have reduced N loss. While it is unclear which of these above factors were dominant, the combination of slightly lower compost temperatures and pH (in Year-1, 8.1in LEM and 8.5 in F-LEM and Control at Final-stage and in Year-2, 9.0 in LEM and 9.1 in Control) in the LEM compost piles is in part responsible for the lower amounts of cumulative ammonia volatilization. For example, using the empirical mathematical expression $pK_a = 0.0901821 + 2729.92/T_k$ (where T_k is temperature in degrees Kelvin, $T_k = °C + 273.2$) related to equilibrium constant and temperature and holding temperature constant at 40^0C, Year-1 Final-stage LEM pH of 8.1 would result in 13.5% of the ammoniacal-N was in the form of

NH_3 and Final-stage F-LEM pH of 8.5 would result in 33.1% of the N ammoniacal-N being present as NH_3. In Year-2, pH differences were not as great the difference in percent ammoniacal-N present based on pH 9.0 and 9.1 was 60.9 % and 66.3%, respectively.

CONCLUSION

The loss of N as NH_3 was significantly lower from LEM inoculated composts in both years compared to either the Control or the F-LEM composts. The lesser degree of NH_3 volatilization from Final-stage LEM was a combined function of lower thermophilic index, lower pH, and the presence of ammonia oxidizing bacterial families. Only Year -2 Final-stage LEM compost attained desirable % GI with cucumber seeds. Therefore, the potential use of LEM as a locally-made-on-farm management tool might be a viable option to enrich and retain the compost N value.

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TABLES AND FIGURES

Table 2.1. Values of pH, C/N ratio, GI (%) for cucumber and radish seeds at different composting stages and elements of broiler litter treated with LEM, F-LEM, or water (Control) in two years.

Parameters	pH	C: N	GI Cucumber	GI Radish	Ca	K	Mg	S	Zn
			-------- % ----------		---------- mg kg^{-1} ----------				
					Year-1 Baseline				
Composite	**7.7**	**9.0**	**1**	**0**	**14700**	**32500**	**5700**	**9600**	**446**
					Year-1 Mid-Stage				
Control	**8.5**	**7.9**	**1**	**5**	**10199**	**16278**	**3463**	**5731**	**313**
F-LEM	**8.6**	**6.2**	**0**	**0**	**8873**	**13926**	**3281**	**3822**	**265**
LEM	**8.5**	**6.0**	**3**	**5**	**8302**	**11838**	**2816**	**3975**	**245**
					Year-1 Final-Stage				
Control	**8.5**	**8.3**	**5**	**0**	**20604**	**31099**	**6475**	**11182**	**548**
F-LEM	**8.5**	**6.5**	**0**	**4**	**21732**	**28295**	**7612**	**10145**	**596**
LEM	**8.1**	**7.8**	**1**	**0**	**19114**	**26393**	**6264**	**9543**	**509**
					Year-2 Baseline				
Control	**8.8a (0.02)**	**9.1 ab (0.08)**	**0.35 a (0.2)**	**19 a (7.4)**	**----------Composite Before Treatments ----------**				
F-LEM	**8.8a (0.05)**	**9.0 b (0.06)**	**0.40 a (0.1)**	**27 a (7.6)**	**26000**	**52000**	**9300**	**12900**	**739**
LEM	**8.7a (0.01)**	**9.5 a (0.2)**	**0.69 a (0.5)**	**20 a (7.6)**					
					Year-2 Mid-Stage				
Control	**8.2a (0.11)**	**7.7 a (0.22)**	**22 a (10.0)**	**3 a (2.4)**	**12893 a (1520)**	**22634 a (1731)**	**5016 a (454)**	**6106 a (552)**	**400 a (47.0)**
F-LEM	**8.2a (0.01)**	**7.9 a (0.14)**	**5 a (3.2)**	**4 a (1.2)**	**11487 a (466)**	**20601 a (970)**	**4386 a (202)**	**5549 a (254)**	**362 a (17.2)**
LEM	**8.2a (0.06)**	**8.2 a (0.29)**	**17 a (5.8)**	**4 a (2.7)**	**11803 a (370)**	**22505 a (982)**	**4703 a (323)**	**5993 a (307)**	**383 a (12.3)**
					Year-2 Final-Stage				
Control	**9.1a (0.08)**	**7.5 a (0.12)**	**24 b (8.9)**	**12 a (1.0)**	**15625 a (1486)**	**29875 a (3814)**	**6125 a (822)**	**6625 a (764)**	**508 a (51.0)**
F-LEM	**9.0a (0.10)**	**7.2 a (0.27)**	**33 b (5.6)**	**16 a (7.1)**	**17850 a (581)**	**34300 a (1342)**	**6850 a (104)**	**7200 a (196)**	**537 a (26.0)**
LEM	**9.0a (0.02)**	**7.3 a (0.29)**	**57 a (13.9)**	**21 a (7.1)**	**16950 a (731)**	**34900 a (1418)**	**6975 a (485)**	**7375 a (295)**	**536 a (36.0)**

Note: 1) Locally sourced microbial inoculant (LEMs; predominantly *Bacilli*, *Lactobacilli, Pseudomonales and Rhizobiales* and 2) False-LEM; predominantly *Lactobacilli.* Lower letters indicate significant differences between treatments ($p < 0.05$) and mean standard error is given in parenthesis. In Year-1 post treatments samples were composited by treatment for analysis. LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Table 2.2. Total Kjeldahl (TKP) and Melich-1P at different composting stages of broiler litter treated with LEM, F-LEM, or water (Control) in two years.

Parameter	Baseline	Mid-stage	Final-stage
Year-1			
TKP (mg P kg^{-1})	7929	Control – 7180 b (1853.3) F-LEM – 8018 ab (1067.6) LEM – 12545 a (1152.3)	Control – 6624 a (576) F-LEM – 7784 a (665) LEM – 7440 a (631.3)
Melich-1P (mg P kg^{-1})	6455	Control – 3037 a (55.3) F-LEM – 3089 a (88.0) LEM – 3013 a (69.0)	Control – 4710 a (96.4) F-LEM – 4348 a (435.3) LEM – 4776 a (235.1)
Year-2			
TKP (mg P kg^{-1})	7906	Control – 8669 ab (388) F-LEM – 8233 b (291) LEM – 9791 a (1208.2)	Control – 8207 a (408.4) F-LEM – 6955 b (289.2) LEM – 7200 ab (245.3)
Melich-1P (mg P kg^{-1})	6585	Control – 3396 a (72.4) F-LEM – 3333 a (102) LEM – 3438 a (80.3)	Control – 5408 a (101) F-LEM – 4818 b (247) LEM – 3412 c (62.3)

Note: 1) Locally sourced microbial inoculant (LEMs; predominantly *Bacilli, Lactobacilli, Pseudomonales and Rhizobiales* and 2) False-LEM; predominantly *Lactobacilli.* Lower letters indicate significant differences between treatments ($p < 0.05$) and mean standard error is given in parenthesis. LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Table 2.3. Prominent microbial families (%) present in liquid LEM and F-LEM in Year-1 and Year-2.

Phylum	Class	Order	Family	Year-1 LEM	Year-2 LEM	Year-1 F-LEM	Year-2 F-LEM
Proteobacteria	α-Proteobacteria	Rhizobiales	*Hyphomicrobiaceae* (Pht)	46 (8)	45 (6)	Abs	Abs
			Bradyrhizobiaceae (N-fix)	37 (2)	35 (5)	Abs	Abs
			*Methylobacteriacea*e (PGP)	5 (0.9)	1 (0.2)	Abs	Abs
			Rhizobiaceae (PSB) (N-fix)	3 (0.5)	6 (0.9)	Abs	Abs
			Xanthobacteraceae (N-fix)	1 (0.2)	4 (0.5)	Abs	Abs
		Rhodospirillales	*Rhodospirillaceae* (N-fix)	53 (2)	72 (2)	Abs	Abs
			*Acetobacteracea*e (N-fix) (PSB)	47 (2)	28 (0.9)	Abs	Abs
		Rhodobacterales	*Rhodobacteraceae* (AnPhtB)	100 (0.1)	100 (0.2)	Abs	Abs
		Caulobacterales	*Caulobacteraceae* (Pdtx)	100 (2)	100 (2)	Abs	Abs
	β-Proteobacteria	Nitrosomonadales	*Nitrosomonadaceae* (AOB)	100 (2)	100 (0.5)	Abs	Abs
		Burkholderiales	*Burkholderiaceae* (PSB)	11 (1)	60 (4)	Abs	Abs
			Comamonadaceae (DnB)	8 (0.8)	23 (1)	Abs	Abs
		Pseudomonadales	*Pseudomonadaceae* (PSB) (BC)	18 (6)	3 (2)	100	Abs
	γ - Proteobacteria	Xanthomonadales	*Xanthomonadaceae* (PP)	90 (7)	61 (2)	Abs	Abs
		Chromatiales	*Chromatiaceae* (PrSlfB) (AOB)	82 (3)	27 (1)	Abs	Abs
			Ectothiorhodospiraceae (PrSlfB)	18 (0.5)	68 (0.5)	Abs	Abs
	Delta- Proteobacteria	Desulfobacterales	*Desulfobacteraceae* (SlfRB)	71 (0.2)	93 (0.9)	Abs	Abs
			Desulfobulbaceae (SlfRB)	29 (0.09)	7 (0.06)	Abs	Abs
		Desulfuromonadales	*Geobacteraceae* (FeRB)	100 (0.1)	Abs	Abs	Abs
Actinobacteria	Actinobacteria	Actinomycetales	*Frankiaceae* (N-fix)	4 (3)	0.4 (0.2)	Abs	Abs
			Streptomycetaceae (BC)	16 (11)	11 (7)	Abs	Abs
Nitrospirae	Nitrospira	Nitrospirales	*Nitrospiraceae* (NOB)	100 (100)	Abs	Abs	Abs
Cyanobacteria	Oscillatoriphycideae	Oscillatoriales	*Phormidiaceae* (Pht)	100 (100)	100 (97)	Abs	Abs
Acidobacter	Acidobacteriia	Acidobacterales	*Acidobacteriaceae* (NitR)	72 (60)	67 (55)	Abs	Abs
			Koribacteraceae (UD)	Abs	Abs	Abs	Abs
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae* (PSB)	100 (98)	100 (42)	98 (98)	100 (100)
		Bacillales	*Bacillalaceae* (PSB) (BC)	43 (0.5)	32 (13)	Abs	Abs
	Erysipelotrichi	Erysipelotrichales	*Erysipelotrichaceae* (UD)	100 (0.7)	Abs	Abs	Abs
	Clostridia	Clostridiales	*Clostridiaceae* (PP)	25 (0.09)	27 (3)	Abs	Abs
			Heliobacteriaceae (UD)	11 (0.02)	Abs	Abs	Abs
Crenarchaeota	Thaumarchaeota	Nitrososphaerales	*Nitrososphaeraceae* (NOA)	100 (100)	100 (100)	Abs	Abs

Abbreviations for bacterial functions within LEM and F-LEM are: AnPhtB, anaerobic photosynthetic bacteria; AOB, Ammonia oxidizing bacteria; BC, bio-controller; DnB, denitrifying bacteria; FBC, fungal bio-controller; FeRB, iron reducing bacteria; N-fix, nitrogen fixing bacteria; NOA, nitrite oxidizing Archaea; NOB, nitrite oxidizing bacteria; NitR, nitrate reducers; ; Pdtx, Phosphate detoxifier; PGP, plant growth promoter; Pht, phototrophic Bacteria; PP, Plant Pathogen; PrSlfB, purple sulfur reducing bacteria; PSB, phosphorus solubilizing bacteria; SlfRB, sulfur reducing bacteria and UD, undetermined. *Abs = Absent. Percent abundance of order is given in parenthesis for respective families. LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Table 2.4. Prominent microbial families (%) belonging to different orders in Year-1 broiler litter and composted broiler litter.

Phylum	Class	Order	Family	Year-1 Baseline	Year-1 Control	Year-1 F-LEM	Year-1 LEM
Proteobacteria	α-Proteobacteria	Rhizobiales	*Hyphomicrobiaceae* (Pht)	64 (7)	49 (4)	Abs	45 (11)
			Bradyrhizobiaceae (N-fix)	19 (2)	16 (1)	Abs	35 (7)
		Rhodospirillales	*Rhodospirillaceae* (N-fix)	100 (5)	92 (3)	100 (3)	72 (4)
			Acetobacteraceae (N-fix) (PSB)	Abs	8 (0.3)	Abs	28 (1)
		Rhodobacterales	*Rhodobacteraceae* (AnPhtB)	100 (0.7)	8 (0.2)	Abs	100 (0.3)
		Caulobacterales	*Caulobacteraceae* (Pdtx)	100 (0.6)	100 (0.7)	100 (0.4)	100 (0.4)
	β-Proteobacteria	Nitrosomonadales	*Nitrosomonadaceae* (AOB)	100 (0.6)	100 (1)	Abs	100 (1)
		Burkholderiales	*Burkholderiaceae* (PSB)	12 (0.7)	29 (1)	Abs	60 (1)
			Comamonadaceae (DnB)	3 (0.2)	27 (1)	Abs	23 (2)
	γ - Proteobacteria	Pseudomonadales	*Pseudomonadaceae* (PSB)	5 (1)	5 (3)	Abs	3 (7)
		Xanthomonadales	*Xanthomonadaceae* (PP)	98 (17)	100 (2)	100 (13)	61 (11)
		Chromatiales	*Ectothiorhodospiraceae* (PrSlfB)	63 (0.4)	53 (0.08)	100 (1)	27 (2)
Actinobacteria	Actinobacteria	Actinomycetales	*Thermomonosporaceae* (UD)	16 (15)	0.4 (0.4)	Abs	13 (0.09)
			Streptomycetaceae (BC)	12 (11)	4 (4)	Abs	11 (2)
Cyanobacteria	Oscillatoriphycideae	Oscillatoriales	*Phormidiaceae* (Pht)	100 (100)	100 (99)	Abs	100 (98)
Acidobacter	Acidobacteriia	Acidobacterales	*Acidobacteriaceae* (NitR)	35 (35)	61 (47)	Abs	67 (33)
			Koribacteraceae (UD)	65 (65)	39 (30)	Abs	33 (31)
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae* (PSB)	85 (8)	11 (1)	18 (0.6)	100 (1)
		Bacillales	*Bacillaceae* (PSB) (BC)	60 (44)	88 (75)	99 (96)	32 (91)
	Clostridia	Clostridiales	*Clostridiaceae* (PP)	43 (7)	18 (0.9)	Abs	27 (0.3)
Crenarchaeota	Thaumarchaeota	Nitrososphaerales	*Nitrospiraceae* (NOA)	Abs	Abs	Abs	100 (100)

Abbreviations for bacterial functions within LEM and F-LEM are: AnPhtB, anaerobic photosynthetic bacteria; AOB, Ammonia oxidizing bacteria; BC, bio-controller; DnB, denitrifying bacteria; FBC, fungal bio-controller; FeRB, iron reducing bacteria; N-fix, nitrogen fixing bacteria; NOA, nitrite oxidizing Archaea; NOB, nitrite oxidizing bacteria; NitR, nitrate reducers; ; Pdtx, Phosphate detoxifier; PGP, plant growth promoter; Pht, phototrophic Bacteria; PP, Plant Pathogen; PrSlfB, purple sulfur reducing bacteria; PSB, phosphorus solubilizing bacteria; SlfRB, sulfur reducing bacteria and UD, undetermined. *Abs = Absent. Percent abundance of order is given in parenthesis for respective families. LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Table 2.5. Prominent microbial families (%) belonging to different orders in Year-2 broiler litter and composted broiler litter.

Phylum	Class	Order	Family	Year-2 Baseline	Year-2 Control	Year-2 F-LEM	Year-2 LEM
Proteobacteria	α-Proteobacteria	Rhizobiales	*Hyphomicrobiaceae* (Pht)	45 (4)	16 (33)	100 (0.2)	100 (0.6)
			Bradyrhizobiaceae (N-fix)	39 (4)	Abs	Abs	Abs
			Methylobacteriaceae (PGP)	Abs	79 (2)	Abs	100 (1)
		Rhodospirillales	*Rhodospirillaceae* (N-fix)	94 (3)	100 (7)	100 (1)	100 (1)
			Acetobacteraceae (N-fix) (PSB)	6 (0.2)	Abs	Abs	Abs
		Rhodobacterales	*Rhodobacteraceae* (AnPht)	100 (7)	100 (5)	100 (2)	Abs
	β-Proteobacteria	Nitrosomonadales	*Nitrosomonadaceae* (AOB)	100 (0.3)	Abs	Abs	Abs
		Burkholderiales	*Burkholderiaceae* (PSB)	23(1)	Abs	Abs	Abs
			Comamonadaceae (DnB)	15 (0.9)	Abs	Abs	Abs
			Alcaligenaceae (FBC)	44 (3)	100 (1)	100 (1)	100 (0.7)
		Pseudomonadales	*Pseudomonadaceae* (PSB)	20 (9)	75 (4)	100 (60)	100 (53)
		Xanthomonadales	*Xanthomonadaceae* (PP)	89 (6)	100 (8)	100 (1)	100 (2)
	γ - Proteobacteria	Chromatiales	*Ectothiorhodospiraceae* (UD)	43 (0.9)	72 (0.9)	35 (0.6)	28 (0.2)
			Chromatiaceae (PrSlfB) (AOB)	39 (0.8)	28 (2)	65 (0.2)	72 (0.4)
			Halomonadaceae (UD)	Abs	96 (33)	95 (12)	95 (27)
		Alteromonadales	*Alcanivoracaceae* (UD)	Abs	3 (1)	0.7 (0.5)	2 (0.2)
			Alteromonadaceae (UD)	Abs	61 (14)	64 (3)	64 (5)
			Idiomarinaceae (UD)	Abs	18 (4)	14 (0.6)	10 (1)
			Streptomycetaceae (BC)	6 (6)	Abs	Abs	Abs
			Bogoriellaceae (UD)	Abs	90 (34)	25 (46)	25 (25)
			Corynebacteriaceae (UD)	Abs	10 (4)	14 (8)	14 (14)
		Thermoleophilales	*Thermoleophilaceae* (UD)	100 (0.9)	Abs	Abs	Abs
Cyanobacteria	Oscillatoriphycideae	Oscillatoriales	*Phormidiaceae* (Pht)	100 (100)	100 (100)	Abs	Abs
Acidobacter	Acidobacteriia	Acidobacteriales	*Acidobacteriaceae* (NitR)	60 (52)	Abs	Abs	Abs
			Koribacteraceae (UD)	40 (35)	Abs	Abs	Abs
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae* (PSB)	14 (5)	Abs	Abs	Abs
			Carnobacteriaceae (UD)	Abs	100 (38)	96 (40)	98 (63)
		Bacillales	*Bacillaceae* (PSB) (BC)	81 (22)	47 (26)	60 (21)	60 (20)
	Clostridia	Clostridiales	*Clostridiaceae* (PP)	16 (6)	8 (0.5)	32 (7)	24 (1)
Crenarchaeota	Thaumarchaeota	Nitrososphaerales	*Nitrospiraceae* (NOA)	Abs	Abs	Abs	Abs

Abbreviations for bacterial functions within LEM and F-LEM are: AnPhtB, anaerobic photosynthetic bacteria; AOB, Ammonia oxidizing bacteria; BC, bio-controller; DnB, denitrifying bacteria; FBC, fungal bio-controller; FeRB, iron reducing bacteria; N-fix, nitrogen fixing bacteria; NOA, nitrite oxidizing Archaea; NOB, nitrite oxidizing bacteria; NitR, nitrate reducers; ; Pdtx, Phosphate detoxifier; PGP, plant growth promoter; Pht, phototrophic Bacteria; PP, Plant Pathogen; PrSlfB, purple sulfur reducing bacteria; PSB, phosphorus solubilizing bacteria; SlfRB, sulfur reducing bacteria and UD, undetermined. *Abs = Absent. Percent abundance of order is given in parenthesis for respective families. LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution

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Figure 2.2. The cumulative nitrogen loss (kg N ha^{-1}) as volatilized ammonia from composting piles in Year-1 (a) over a period of 164 days and Year-2 (b) over a period of 183 days. Lower letters indicate significant differences between treatments ($p < 0.05$). Note: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

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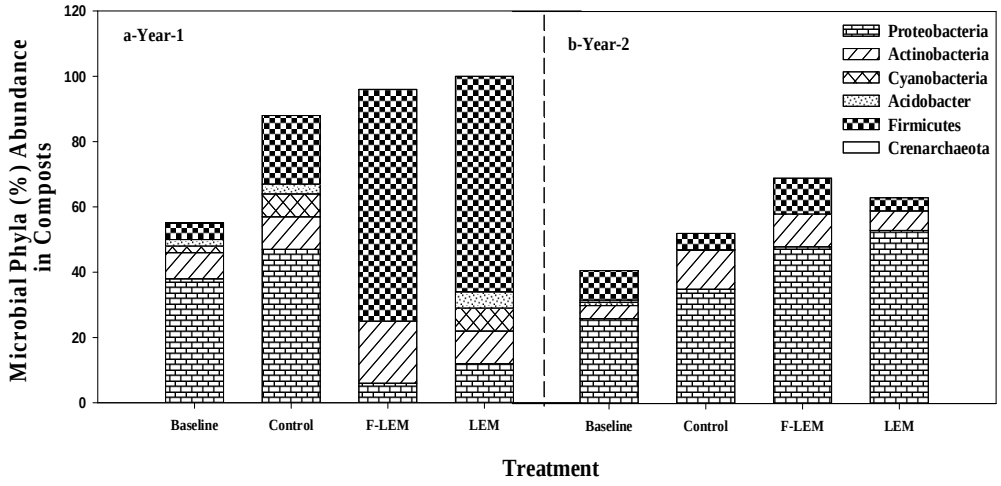

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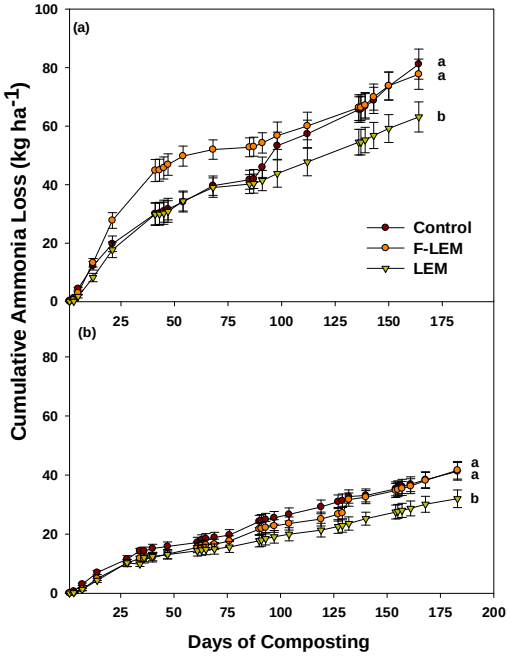

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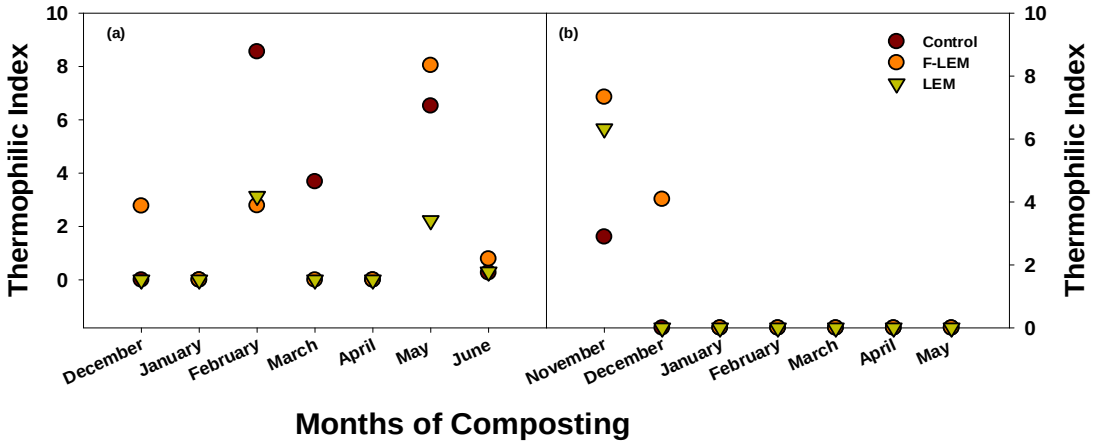

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CHAPTER 3

IMPROVING PLANT AVAILABLE NITROGEN IN SOIL AND NUTRIENT DENSITY OF EDAMAME BEAN IN THREE CONSECUTIVE SUMMERS BY UTILIZING A LOCALLY-SOURCED BIO-INOCULA

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ABSTRACT

An important key player in the development of sustainable food production system is the soil microbial communities. These soil microbes are responsible for macro- and micro-nutrient mineralization and solubilization in soil. Local effective microorganisms (LEM) is a locally sourced bio-inoculate, made from a fermented mixture of actively decomposing forest litter and a nutrient rich growing media containing carbohydrate, milk and water. It can be made on farm and have a suite of bacterial communities that includes phototrophic bacteria, phosphorous-solubilizers, ammonia-oxidizers, nitrogen fixers, actinomycetes etc. The objective of the study was to investigate the effect of a locally sourced bio-inoculum in releasing plant available nitrogen in three consecutive summers. Additionally, this study investigated protein, carbohydrates, fat, fiber, ash, macro and micro-nutrients density in edamame. Plant available nitrogen was significantly higher in LEM plots compared to Control (non-chlorinated water) in second and third year of the study. Sucrose content in LEM edamame was higher compared to both Control and False-LEM inoculated (F-LEM; contains only the growing media as LEM but no forest litter) edamame ($P < 0.05$). Significantly enhanced biofortification was observed in calcium, magnesium and zinc density in edamame bean in different years ($P < 0.05$).

Keywords: Compost, Microbial Communities, Potential Nitrogen Mineralization, Plant Available Nitrogen, Edamame, Micro Nutrient Density

INTRODUCTION

Animal manures or organic wastes can be a good source of soil nutrients. Soil nutrient accessibility for plant uptake, especially nitrogen (N), from these organic sources is affected by the diversity of microbial communities within the rhizosphere (Masunga et al. 2016). Rates of N mineralization varies for different organic sources, for instance, composted broiler litter releases N very slowly as opposed to fresh broiler litter, which releases N at a faster rate (Tyson and Cabrera 1993). Slow release of N from composts may not be enough for many crops and bio fortification may be beneficial.

Microbial communities known to have beneficial effects on plant growth and health, are collectively known as plant growth-promoting microorganisms (PGPM) (Kloepper and Schroth 1978). They include nitrogen-fixing bacteria, ammonia oxidizing bacteria and archaea, phosphorus-solubilizing bacteria and mycorrhizal fungi. Producers have begun to utilize biological amendments to improve plant availability of nutrients from animal and plant sources. One of the popular approaches include use of commercially available microbial inoculums. However, the success of these commercially available products depends on their performance in the field where the dynamic soil biogeochemical environment is different than a laboratory or greenhouse conditions. There is concern that these inoculum do not adapt or establish well in foreign agroecosystems with possibly differing moisture, temperature and soil environments, and may have difficulty coexisting with indigenous microbial communities (Verbruggen et al. 2013). These inoculums must also integrate themselves not only with the native microbiome, but with the food web (Morrien 2016).

Legumes are important components of everyday diet, especially, in developing parts of the world where health risks are associated with malnutrition and wasting is prevalent (Jager et

al. 2019). Legumes contain appreciable amount of protein (20-30%), fiber and carbohydrates (Sánchez et al. 2015) and their ability to fix atmospheric nitrogen helps reduce the utilization of nitrogen (N) fertilizer requirements (Giller et al. 2013). In addition, legumes are richer in key macro and micronutrients such as, calcium (Ca), magnesium (Mg), iron (Fe), potassium (K) and zinc (Zn) (Stadlmayr et al. 2012). Nutrient uptake by legumes and their concentration in legumes vary greatly (Sanderberg et al. 2002 and Hurrell et al. 2003). Among different legumes, a widely produced legume is soybean (Glycine max), which contains more protein per unit area than most other cereal and grain crops (Vollmann 2016). Soybean will play a crucial role in global food security by 2050 as one of the most important protein sources (Philis et al. 2018). Soybean as a protein source is highly valued because its production requires less land and because the same amount of protein from animal sources has a bigger carbon footprint (Tessari et al. 2016).

The process of making "effective microorganisms (EM^{TM}) originated in Japan by Higa and Wididana (1991) and a home-grown version has been adapted by many producers in Central America and Caribbean called organisms of the mountains (MM). This bio-inoculant is widely used primarily to control pest and odor from manures and compost. Further studies are required to fully comprehend how locally sourced bio-inoculants influence plant availability of nitrogen and other macro/micro nutrients from organic nutrient sources inoculated with these locally sourced microbial inoculum. In our study, we adapted the MM formulas for temperate climates which resulted in two bio-inocula that can be grown and utilized by individual producers. We have called these bio-inocula Local Effective Microorganisms (LEM) and False-LEM. LEM bio-inoculum is gathered from actively decomposing upland forest litter. In our study, we applied composted broiler litter inoculated with LEM, F-LEM or Water (Control) prior to planting butterbean edamame soybeans (Glycine max.). The goal of our research was to a) investigate

microbial diversity in LEM, F-LEM bio-inocula, b) identify bacterial and archaeal communities in soil after bio-inoculation with LEM, F-LEM, or Control c) assess inorganic nitrogen in the soil and d) evaluate macro and micro-(Ca, Mg, K, and Zn) nutrient density in edamame bean grown with composted broiler litter inoculated with LEM, F-LEM or Water (Control).

MATERIALS AND METHODS

Site Description

This study was conducted in the southeastern United States at the J. Phil Campbell Research and Education Center in Watkinsville, Georgia, (33°52′ N, 83°27′ W). The soil at the site is a fine kaolinitic, thermic Typic Kanhapludults in the Cecil sandy loam series with a 2–6% slope (Soil Survey Staff 2014.). The region has 123 cm average annual rainfall and an average minimum and maximum temperature of 10.4°C and 22.5°C, respectively.

Microbial Inocula Treatments and Experimental Design

Broiler litter was composted with LEM, F-LEM or Water only and was applied to plots as the fertilizer source to grow edamame. The treatments compared were 1) Local effective microorganisms (LEM); the LEM was made by collecting the O horizon (actively decomposing leaf litter) from upland, healthy, well established mixed pine and hardwood forests; 2) False-LEM (FLEM), made with the same growing media as LEM but without the O-horizon, and 3) Control (non-chlorinated, well water). The detailed process of making LEM and F-LEM is described in Appendix 3.1.

Treatments were arranged in a completely randomized design with four replications. Inoculum treatments were applied to twelve, 18 m^2 (3 m × 6 m) plots. The experiment was carried out for three consecutive summers; 2015, 2016 and 2017. In 2015 and 2016, composts inoculated with LEM, F-LEM or water (Control compost) were applied to according to the

soybean N requirement (62 kg N ha^{-1}) in 2015 and according to the P requirement (~ 38 kg N ha^{-1}) in 2016 (Table 3.1). Immediately after compost application 2 L m^{-2} of liquid inoculums was applied on top of the composts. Plots were then immediately tilled to incorporate amendments. In 2017, only microbial inoculant was added in liquid form (no compost). Liquid treatments were surface applied at a rate of 2 L m^{-2}. In all three years, incorporation of treatments was done with a BCS rototiller to a depth of 15 cm (BCS America, 14151 Fir Street, Oregon City, Oregon, United States of America), after which, the soil was smoothed and planted with edamame (Glycine max) at a depth of 2.5 cm and a row spacing of 38 cm spacing (target population of 175000 edamame plants per ha). Treatments application rates in 2015 and are shown in Table 3.1.

Soil Sampling

Baseline soil sampling was done prior to treatment application in 2015 by collecting 8 to 10 cores (2.5-cm diameter) to a depth of 15 cm (labeled as Baseline). In 2015, 2016, NS 2017, additional soil samples were collected one week and four weeks after treatment applications (labeled as YR 2015 wk1 and YR 2015 wk4, YR 2016 wk1 and YR 2016 wk4, and YR 2017 wk1 and YR 2017 wk4). All soil samples were kept refrigerated at 4ºC until they were air-dried, ground and passed through a 2-mm sieve for further chemical analysis. For microbial analysis, soil samples were separated and kept at -20ºC until the soil genomic DNA was extracted.

Microbial Community Analysis of LEM, F-LEM and soil samples

Microbial DNA was extracted from liquid LEM and F-LEM for 2015, 2016 and 2017. Genomic DNA was also extracted from inoculated compost applied in 2015 and 2016 and from soil samples at 4th week in 2015 and 2016. Resources did not allow us to do microbial DNA analysis of 2017 soil samples. For extracting DNA, QIAGEN DNeasy PowerSoil Kit (DNeasy

PowerSoil Kit Handbook, May 2017, Qiagen, Valencia, California, United States) was used and extracted DNA samples were further amplified by Ion 16S Metagenomics™ kit to amplify the V4 and V5 hyper variable regions of bacterial 16S rRNA. Specific bacterial universal primers 515F (5/-GTGYCAGCMGCCGCGGTAA-3/) and 806R (5/-GGACTACNVGGGTWTCTAAT-3/) were combined with barcoded adapter sequences. The sample libraries were prepared by Fusion Method as described in Ion Amplicon Library Preparation (Ion Torrent Systems Incorporated, Guilford, Connecticut, United States of America). Enriched products were loaded on the 316™-chip using the Ion PGM™ Hi-Q™ sequencing kit and sequenced in the Ion PGM™ sequencer (Ion Torrent Systems Incorporated, Guilford, Connecticut, United States of America). Sequence reads were analyzed by Ion Reporter™ software with the default parameter setting by MicroSEQTM(R) 16S Reference Library (v2013.1) and Greengenes (v13.5) as reference database. The sequencing was done in Coastal Palins Soil, Water, and Plant Research Center, United State Department of Agriculture Agricultural Research Service, Florence South Carolina. To measure the extent of functional groups within a treatment, we developed a Measure of Extent (ME). Functional groups considered were phosphorus-solubilizing bacteria (PSBs), ammonia-oxidizing bacteria (AOB), nitrogen-fixing bacteria (N-fix), phototrophic bacteria (Pht), nitrite-oxidizing bacteria (NOB), nitrite-oxidizing archaea (NOA) and Bio-controller (BC). We calculated ME as the summation of abundance (percentage family within its order) for all families considered to be within a specific functional group. For instance, in 2015 bio-inoculant, F-LEM sample, *Pseudomonadaceae*, a family within the PSB functional group comprised 100% of the order Pseudomonadales and *Lactobacillaceae* is another PSB comprised 98% of the order Lactobacillales the sum of which is 198 or a ME of 198 The larger the ME the greater the presence of this group (Supplemental Material 1).

Soil respiration (CO_2) and Ammonia (NH_3) Volatilization

CO_2 respiration and NH_3 volatilization were measured from each plot in 2015, 2016, and 2017. Measurements were made on days 0 (day before application), 1(immediately following application), 3, 7, 14 and 28 days after each treatment application. On each day, two static gas trap chambers (20-cm ID PVC pipe open at the bottom and containing two metal rings inside with airtight removable lid) were installed in each plot. Within each of the chambers, the two metal rings held one glass jar with alkali (100 mL 2 M NaOH) to trap CO_2 and one jar with acid (100 mL 1 M H_3BO_3) to trap NH_3. The chambers were inserted 5 cm into soil, alkali and acid traps were placed into the metal rings and the lids were closed for 24 hours, after which, the jars were brought back to the lab for chemical analysis. CO_2 was measured by placing a 10-mL aliquot from the glass jars into centrifuge tubes and adding 8 ml $BaCl_2$ followed by centrifugation (1500 rpm for 3 minutes) and subsequent back titration with 1M HCl (standardized) (Yiqi and Zhou 2010). NH_3 was measured by the change in pH of the H_3BO_3 in the glass jars followed by back titration with a weak HCl (0.0025M HCl) to an initial pH of 1 M H_3BO_3 (4.8 to 5.0) (Bremner and Mulvaney 1982).

Soil pH and Inorganic Nitrogen

Soil pH was determined in water (1:5) for all soil sampling phases. Baseline soil samples along with 1st week and 4th week soil samples after treatment applications were extracted for inorganic N by shaking 3 g with 20 mL of 2 mol L-1 KCl for 30 minutes (Maynard and Kalra 1993). All extractants were frozen until they could be analyzed spectrophotometrically on a Tecan Infinite® 200 PRO Series (Tecan Trading AG Switzerland). The ammonium (NH_4^+) content was determined by using salicylic acid method (Kempers and Zweers 1986) and the

nitrate (NO_3^-) content was determined by vanadium (III) chloride method (Doane et al. 2003). Inorganic nitrogen was calculated by adding NH_4^+ and NO_3^- fractions.

Productivity and Nutritional Value of Edamame

Edamame was hand harvested each season and fresh bean and pod weight was recorded. Above ground biomass for each plot was also harvested, weighed, and dried at 60°C for 48 h. The stabilized dry weights were recorded and used in dry matter (DM) correction of fresh weights. Edamame beans were freeze dried and then ground using mortar and pestle. In 2016 and 2017, edamame beans were analyzed for protein, sugar, ash, fat and fiber content by using near-infrared spectroscopy (NIR; model DA 7250, Perten Instruments). In addition, ground edamame beans were digested by the Kjeldahl digestion method (Taylor 2000), and the digests were analyzed for Ca, K, Mg and Zn using atomic absorption spectrometer, model AAnalyst200 (Perkin Elmer, 11695 Johns Creek Parkway, Suite 150, Johns Creek, Georgia, 30097-188.1, United States of America) at 423 nm, 766 nm, 285 nm and 214 nm, respectively.

Statistical Analysis

Analysis of variance with JMP PRO 13 software (SAS Institute, 2013) was used to determine differences between treatments by each soil sampling stage for pH, inorganic nitrogen and nitrate content (*$P < 0.05$). Analysis of variance was also used to determine Ca, Mg, K and Zn density in edamame beans (*$P < 0.05$). Comparisons between multiple means of different nitrogen fractions and the nutrient density of edamame beans were done with Tukey's HSD (*$P < 0.05$).

RESULTS AND DISCUSSIONS

Microbial Communities in Applied Liquid LEM and F-LEM Inoculants

Liquid LEM had greater functional redundancy compared to liquid F-LEM inoculum as indicated by an array of diverse bacterial and archaeal community distribution. Prominent microbial families (%) present in liquid LEM and F-LEM in 2015, 2016 and 2017 are given in Supplemental Material 1. In 2015, the prominent bacterial phylum in LEM was firmicutes (58% of the sample sequenced), in 2016, proteobacteria was dominant (63% of the sample sequenced), and in 2017, firmicutes was the dominant phylum (89% of the sample sequenced). Several ammonia oxidizing bacterial (AOB) families such as, *Nitrosomonadaceae* and *Nitrospiraceae*; ammonia oxidizing archaeal (AOA) families such as, *Nitrososphaeraceae*, nitrogen fixing bacterial families such as, *Bradyrhizobiaceae, Rhizobiaceae, Rhodospirillaceae* and *Acetobacteraceae* were evident in liquid LEM in all years (Supplemental Material 1). Phosphorus-solubilizing bacterial (PSBs) families: *Bradyrhizobiaceae*, *Rhizobiaceae*, *Acetobacteraceae*, *Burkholderiaceae, Pseudomonadaceae*, *Bacillalaceae* and *Lactobacillaceae* were identified in LEM inoculums in all years. In liquid F-LEM, all three years, firmicutes was the dominating phylum (> 95%) and *Lactobacillaceae* was the prominent family. Microbial community abundance of bio-inoculated compost in 2015 and 2016 are listed in Supplementary Material 2 and 3. Several major bacterial and archaeal families associated with ammonia oxidation, like *Nitrospiraceae* (AOB) and *Nitrososphaeraceae* (AOA) were identified in LEM inoculated compost in 2015 and 2016 (Supplemental Material 2 and 3).

Microbial Community Abundance in Soil at 4th Week of Sampling

In 2015 and 2016, several plant growth promoting microorganisms (PGPM) families were identified in soil. Microbial community abundance in soil at 4th week in 2015 and 2016 are listed in Supplementary Material 4. When considering all treatments, abundance of bacteria in soil, in 2015 and 2016, from greatest to lowest abundance was as follows: α-, β- and γ-

proteobacteria > Actinobacteria > Firmicutes > Bacteroidetes > Acidobacteria > Cyanobacteria > Nitrospirae > Crenarchaeota (Supplementary Material 4). In 2015 and 2016, LEM plots had greater abundance of nitrogen-fixing bacterial families, like, *Bradyrhizobiaceae, Rhodospirillaceae, Rhizobiaceae*, *Xanthobacteraceae* and *Acetobacteraceae* compared to both F-LEM and Control. In 2015, the Measure of Extent (ME) of functional group PSB in 4th week soil for LEM, F-LEM, and the Control was 274, 228, and 214, respectively (Table 3.2) and the families present in all were *Rhizobiaceae*, *Lactobacillaceae*, *Bacillalaceae*, *Acetobacteraceae*, and *Burkholderiaceae*. In 2016, PSB ME for LEM, F-LEM, and Control was 125, 127, and 107, respectively (Table 3.2).

Soil respiration (CO_2) and Ammonia (NH_3) Volatilization

In 2015, cumulative ammonia loss by Day 28 was significantly greater from Control treatment than from F-LEM and LEM (*$P < 0.05$) treatments (Figure 3.2a). Greater cumulative NH_3 loss in Year 2015 in Control plots may have been due to higher pH in Control plots (Figure .1). In 2016 and 2017, there was no difference between treatments in cumulative NH_3 loss (Figure 3.2b and 3.2c). Cumulative CO_2 emission from soil was not significantly different between treatments (data not shown) in any of the years.

Soil pH and Inorganic Nitrogen

Soil pH, in LEM plots, was significantly lower in 2015 at four weeks after treatment applications compared to F-LEM and Control (Figure 3.1). The abundance of PSBs in soil taken in 2015 was greater in LEM plots than in either F-LEM or Control plots. In 2016, four weeks after treatment applications F-LEM and LEM PSB abundances were more similar, and there were no significant differences between LEM and F-LEM pH values. In 2017, soil pH in LEM and F-LEM plots was significantly lower than in control plots 1 week after treatment

applications but not four weeks after treatment applications. This significantly lower soil pH in LEM plots compared to Control post treatment sampling phases may be the contribution of higher abundance of PSBs communities such as, *Bacillus* spp., *Lactobacillus* spp., *Pseudomonas* spp. These microbes are reported to lower soil pH by producing organic acids (Ndakidemi et al. 2011).

In 2016 at week 4 and in 2017 at both weeks 1 and 4, soil inorganic nitrogen was significantly greater in soils that received LEM treatment compared to Control samples (*$P < 0.05$) (Figure 3.3a). With the exception of the fourth week sample in 2016, where LEM had significantly greater nitrate than Control or F-LEM, no significant differences in nitrate content between treatments were observed (Figure 3.3b). In the first year of the study (2015), we found no difference in inorganic nitrogen between treatments while in subsequent years (2016 and 2017) LEM had greater N mineralization (greater inorganic in LEM) in soil. According to Fatunbi and Ncube (2009) utilization of effective exogenous microorganisms in soil is most effective when applied in conjunction with an organic source. In our study, we found that direct application of LEM (influx of microbial consortium) after plant emergence in 2017 led to rapid N mineralization. Thus, direct application of LEM after plant emergence may be proposed to growers for rapid N availability for plants.

Productivity and Nutritional Value of Edamame

There were no significant differences between treatments in edamame biomass and yield (Table 3.3). While the amount of increased inorganic N was significant, it was not large enough to influence yield. We speculate that these results may have management implications, so more research is needed to determine timing and rate of LEM and F-LEM applications. Protein, fiber and fat percentage in edamame beans were not significantly different between treatments (Table

3.4). However, there was a greater concentration of sucrose in 2016 and 2017 in LEM beans (*P < 0.05) compared to both Control and F-LEM beans. Additionally, there was a greater concentration of ash in 2016 LEM edamame compared to Control (*P < 0.05) (Table 3.4). This greater ash content in LEM bean led to further elemental analysis to determine nutrient density of bean for Ca, Mg, K and Zn. In 2015 and 2017, Ca, Mg and Zn density in LEM edamame beans were significantly greater (*P < 0.05) compared to Control (Table 3.5). In 2016, Ca and Zn content in edamame bean grown with LEM was significantly greater than Control (*P < 0.05) (Table 3.5). There was no significant difference in K density between treatments in any year. Greater macro and micro-nutrient density (Ca, Mg and Zn) in LEM plots compared to Control may have been a combined contribution of higher PSBs abundance in LEM bio-inocula (Table 3.2) and low soil pH (Rokhbakhsh-Zamin et al. 2011). Several PSBs families: Bradyrhizobiaceae, Pseudomonadaceae, Bacillalaceae, Acetobacteraceae, Lactobacillacceae and Xanthomonadaceae were identified in greater abundance in LEM inoculant (Table 2). These groups of microbiome have the capability of solubilizing complexed macro and micro minerals by excreting organic acids (Fankem et. al., 2006; Marra et. al., 2011) and lowering soil pH (Gonita-Mishra et al. 2017), thereby, releasing nutrients for plant use. Addition of F-LEM which had predominately *Lactobacillaceae* as the functional microbial group also demonstrated improved Ca and Mg density in edamame compared to the Control in 2015 and 2016 (Table 3.5). Some L*actobacillacceae* have the potential to increase nutrient uptake by accelerated organic matter decomposition (Lamont et al. 2017). As opposed to LEM, F-LEM lacks functional abundance and diversity and the resulting redundancy in function.

CONCLUSION

A broader range of microbial functional groups were identified in LEM bio-inoculum compared to F-LEM. Significantly greater plant available N was found in the second and third year of the study in soils that received LEM as treatment. While there were no significant differences observed in edamame yield and biomass, edamame grown with LEM showed increased sucrose content and Ca and Zn density. A greater abundance of PSBs in LEM inoculum may have been responsible for the increased Ca and Zn density in edamame beans. We conclude that this locally sourced bio-inoculum, LEM, offers functional redundancy as evident from a suite of microbial communities, enhances N mineralization in soil, and bio-fortifies the nutrient density of edamame.

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TABLES AND FIGURES

Table 3.1. Nitrogen, calcium, magnesium, potassium and zinc (kg ha^{-1}) from applied compost in Year 2015, 2016 in edamame growing plots.

Treatment	**Year 2015**					**Year 2016**				
	----------kg ha^{-1}----------									
	N	**Ca**	**Mg**	**K**	**Zn**	**N**	**Ca**	**Mg**	**K**	**Zn**
Control	62	41	13	62	1	40	30	12	56	1
F-LEM	62	43	15	56	1	37	34	13	66	1
LEM	62	38	12	52	1	38	30	14	63	1

Note: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Table 3.2. Measure of the Extent of functional groups (ME). It is the sum of percent (%) abundance of prominent functional families present in liquid LEM and F-LEM bio-inocula in years 2015, 2016 and 2017 and present in soil at 4th week of soil sampling in 2015 and 2016

Functional Groups	ME of Functional Groups in Bio-inocula						ME of Functional Groups in Soil at 4th Week					
	2015 F-LEM	2016 F-LEM	2017 F-LEM	2015 LEM	2016 LEM	2017 LEM	2015 Control	2016 Control	2015 F-LEM	2016 F-LEM	2015 LEM	2016 LEM
Nitrogen-Fixers	0	0	0	141	145	160	138	132	134	153	248	156
Ammonia Oxidizing Bacteria	0	0	0	182	127	0	109	123	105	200	100	111
Nitrite Oxidizing Bacteria	0	0	0	100	0	0	100	0	0	100	0	100
Nitrite Oxidizing Archaea	0	0	0	100	100	0	100	100	100	100	100	100
Phosphorus-Solubilizing Bacteria	98	100	100	222	229	251	214	107	228	127	274	125
Phototrophic Bacteria	0	0	0	146	145	141	54	68	58	145	50	40
Bio-controller	100	0	0	103	45	95	76	104	75	110	106	85

Note: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Table 3.3. Edamame biomass and yield (kg ha^{-1}) in 2015, 2016 and 2017 at time of harvest.

	Year 2015			Year 2016			Year 2017		
Treatment	**Control**	**F-LEM**	**LEM**	**Control**	**F-LEM**	**LEM**	**Control**	**F-LEM**	**LEM**
Biomass (kg ha^{-1})	1598[a] (108)	1329[a] (175)	1712[a] (102)	2826[a] (151)	3228[a] (436)	2600[a] (98)	3395[a] (166)	3052[a] (209)	2838[a] (517)
Yield (kg ha^{-1})	9129[a] (382)	7398[a] (548)	7867[a] (792)	6443[a] (249)	6040[a] (406)	5785[a] (403)	4568[a] (1573)	5381[a] (1769)	5006[a] (1650)

Lower letters indicate significant differences between treatments (*P <0.05) and mean standard error is given in parenthesis**. Note**: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Table 3.4. Protein, sucrose, fiber, fat and ash content (%) in edamame beans determined by near-infrared spectrometry in 2016 and 2017.

	Year 2016			Year 2017		
	----------%			----------%		
Treatment	**Control**	**F-LEM**	**LEM**	**Control**	**F-LEM**	**LEM**
Protein	43^{a} (1)	43^{a} (1)	42^{a} (1)	43^{a} (1)	42^{a} (1)	42^{a} (1)
Sucrose	10^{b} (1)	10^{b} (1)	12^{a} (1)	11^{b} (1)	11^{b} (1)	13^{a} (1)
Fiber	3^{a} (1)	3^{a} (1)	2^{a} (1)	3^{a} (1)	4^{a} (1)	2^{a} (1)
Fat	15^{a} (1)	15^{a} (1)	16^{a} (1)	15^{a} (1)	15^{a} (1)	16^{a} (1)
Ash	5^{b} (1)	5^{ab} (1)	7^{a} (1)	5^{a} (1)	5^{a} (1)	6^{a} (1)

Lower letters indicate significant differences between treatments (*P <0.05) and mean standard error is given in parenthesis. Note: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Table 3.5. Calcium, magnesium, potassium and zinc density (mg kg^{-1}) in edamame beans in 2015, 2016 and 2017.

	Year 2015			Year 2016			Year 2017		
	----------mg kg^{-1}----------								
Treatment	**Control**	**F-LEM**	**LEM**	**Control**	**F-LEM**	**LEM**	**Control**	**F-LEM**	**LEM**
Ca	583^{b} (29)	675^{b} (40)	847^{a} (50)	1961^{b} (74)	1661^{a} (44.)	2002^{a} (80)	582^{b} (56)	738^{ab} (65)	957^{a} (41)
Mg	1408^{b} (9)	1448^{a} (6)	1474^{a} (6)	1440^{a} (13)	1443^{a} (12)	1415^{a} (12)	3394^{b} (59)	4050^{a} ^{b} (22)	4094^{a} (45)
Zn	15^{c} (2)	21^{b} (2)	30^{a} (2)	48^{b} (1)	49^{b} (1)	56^{a} (1)	47^{b} (1)	49^{b} (1)	58^{a} (1)
K	1436^{a} (70)	1668^{a} (64)	1661^{a} (60)	2336^{a} (96)	2290^{a} (37)	2339^{a} (25)	6044^{a} (42)	6038^{a} (25)	6056^{a} (25)

Lower letters indicate significant differences between treatments (*P <0.05) and mean standard error is given in parenthesis**. Note**: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Appendix 3.1

Making LEM

The LEM was made by collecting the O horizon (loose and actively decomposing leaf litter) from upland, healthy, well established mixed pine and hardwood forests. This is the microbial inoculant. The LEM inoculum was then combined with 23 kg of organic semolina, 250 gm of baker's yeast, 10 kg of crushed natural hardwood charcoal, and 4 L each of molasses, antibiotic-free raw goat milk, and chlorine-free water to make a solid mixture. This solid mixture was then placed into a container and sealed for 6 weeks to allow for anaerobic fermentation. After first solid phase fermentation, a liquid phase fermentation was carried out for at least 2 weeks. Liquid phase fermentation: 3 kg of solid phase material is placed into a porous sack and suspended in a sealed 120 L tank with 60 L of water and 4 L of molasses.

Making False-LEM

Because the growing media (molasses and semolina, yeast and raw milk) contains nutrients and microbes, the False-LEM (F-LEM) was also prepared with the growing media at the same ratio as the LEM but without the forest microbial inoculum.

List of Figures

Figure 3.1. pH of soil (0-15 cm) at different sampling phases in 2015, 2016 and 2017. Different lower-case letters indicate significant difference between treatments on a sampling date (*P<0.05). **Note**: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Figure 3.2. The cumulative NH_3 loss (mg N m^{-2}) from soil in (a) 2015, (b) 2016 and (c) 2017 after treatment application. Different lower-case letters indicate significant difference between treatments on a sampling date (*P <0.05). **Note**: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Figure 3.3. (a) Inorganic Nitrogen (NH_4^+ + NO_3^-; inorganic nitrogen) and (b) Nitrate content of soil at 0-15 cm depth at different soil sampling phases in 2015, 2016 and 2017. Different lower-case letters indicate significant difference between treatments on a sampling date (*P < 0.05). **Note**: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

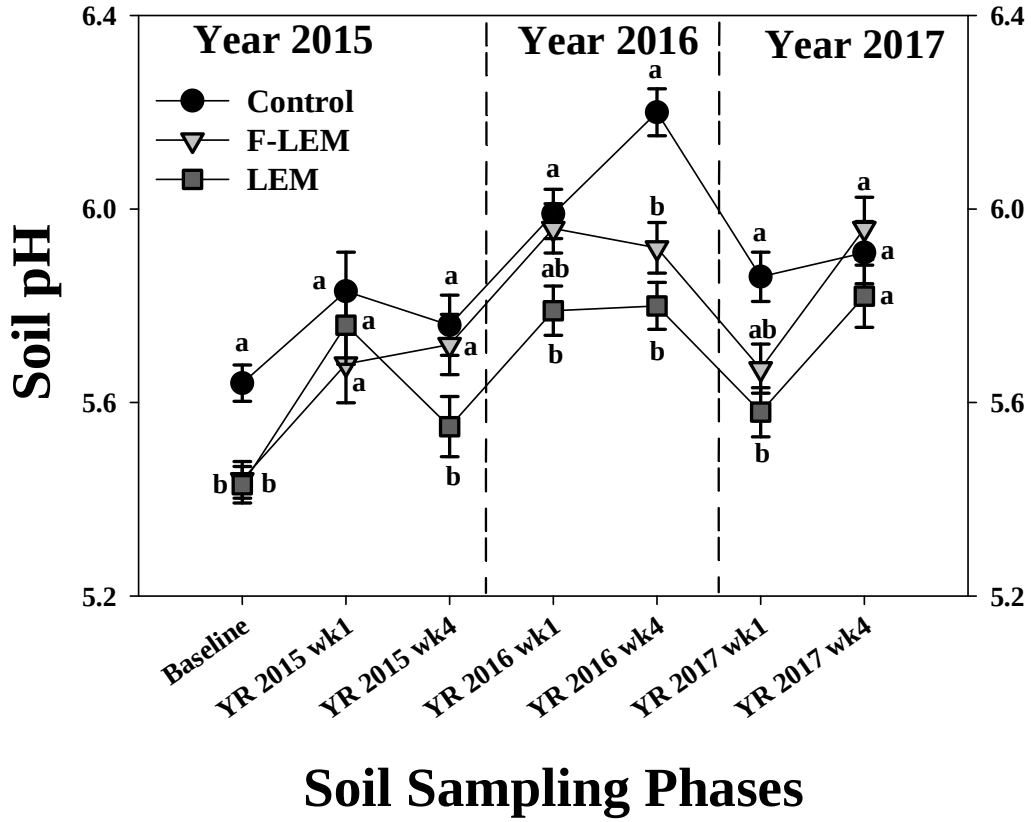

Figure 3.1. pH of soil (0-15 cm) at different sampling phases in 2015, 2016 and 2017. Different lower-case letters indicate significant difference between treatments on a sampling date (*P<0.05). **Note**: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

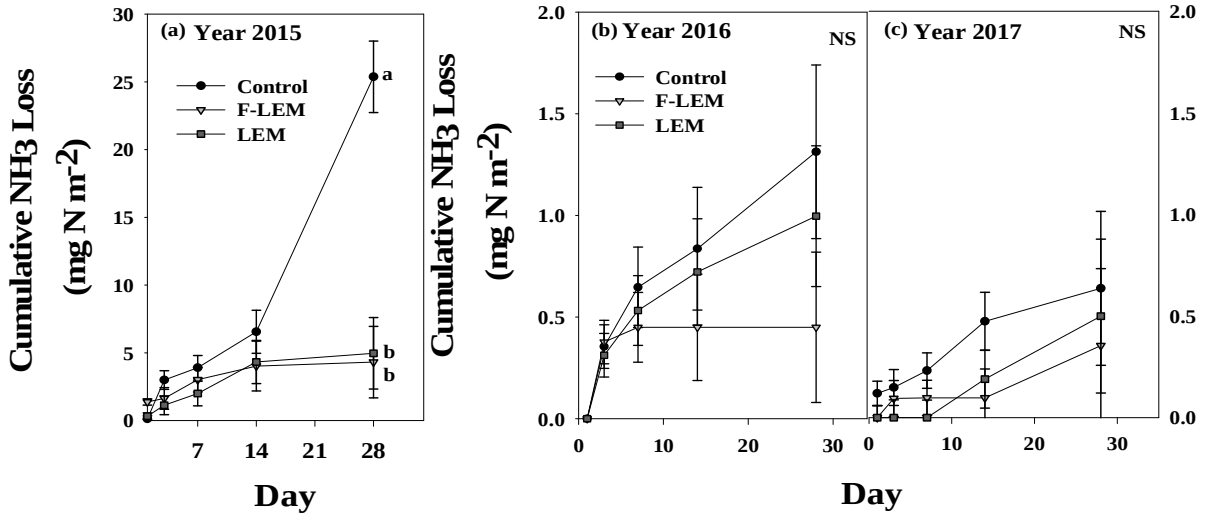

Figure 3.2. The cumulative NH_3 loss (mg N m^{-2}) from soil in (a) 2015, (b) 2016 and (c) 2017 after treatment application. Different lower-case letters indicate significant difference between treatments on a sampling date (*P <0.05). **Note**: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

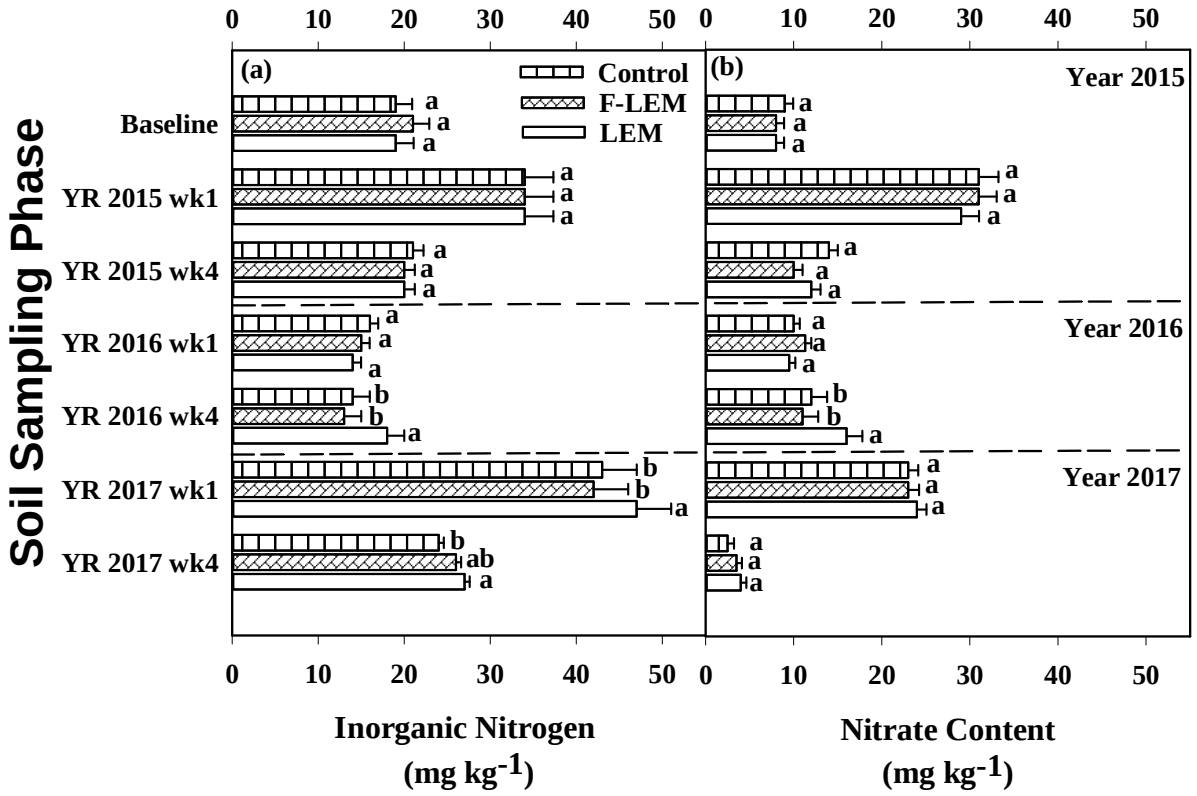

Figure 3.3. (a) Inorganic Nitrogen (NH_4^+ + NO_3^-; inorganic nitrogen) and (b) Nitrate content of soil at 0-15 cm depth at different soil sampling phases in 2015, 2016 and 2017. Different lower-case letters indicate significant difference between treatments on a sampling date (*P < 0.05).

Note: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Supplemental Material 1. Prominent microbial families (%) present in liquid LEM and F-LEM in 2015, 2016 and 2017

Phylum	Order	Family	2015 LEM	2016 LEM	2017 LEM	2015 F-LEM	2016 F-LEM	2017 F-LEM
Proteobacteria	Rhizobiales	*Hyphomicrobiaceae* (Pht)	46 (8)	45 (6)	41 (1)	Abs	Abs	Abs
		Bradyrhizobiaceae (N-fix)	37 (2)	35 (5)	59 (2)	Abs	Abs	Abs
		*Methylobacteriacea*e (PGP)	5 (0.9)	1 (0.2)	Abs	Abs	Abs	Abs
		Rhizobiaceae (PSB) (N-fix)	3 (0.5)	6 (0.9)	Abs	Abs	Abs	Abs
		Xanthobacteraceae (N-fix)	1 (0.2)	4 (0.5)	Abs	Abs	Abs	Abs
	Rhodospirillales	*Rhodospirillaceae* (N-fix)	53 (2)	72 (2)	43 (0.5)	Abs	Abs	Abs
		*Acetobacteracea*e (N-fix) (PSB)	47 (2)	28 (0.9)	57 (0.7)	Abs	Abs	Abs
	Rhodobacterales	*Rhodobacteraceae* (AnPht)	100 (0.1)	100 (0.2)	Abs	Abs	Abs	Abs
	Caulobacterales	*Caulobacteraceae* (Pdtx)	100 (2)	100 (2)	Abs	Abs	Abs	Abs
	Nitrosomonadales	*Nitrosomonadaceae* (AOB)	100 (2)	100 (0.5)	Abs	Abs	Abs	Abs
	Burkholderiales	*Burkholderiaceae* (PSB)	11 (1)	60 (4)	100 (1)	Abs	Abs	Abs
		Comamonadaceae (Dntf)	8 (0.8)	23 (1)	Abs	Abs	Abs	Abs
	Pseudomonadales	*Pseudomonadaceae* (PSB) (BC)	18 (6)	3 (2)	Abs	100	Abs	Abs
	Chromatiales	*Chromatiaceae* (PrSlfB) (AOB)	82 (3)	27 (1)	Abs	Abs	Abs	Abs
		Ectothiorhodospiraceae (PrSlfB)	18 (0.5)	68 (0.5)	Abs	Abs	Abs	Abs
	Desulfobacterales	*Desulfobacteraceae* (SlfRB)	71 (0.2)	93 (0.9)	Abs	Abs	Abs	Abs
		Desulfobulbaceae (SlfRB)	29 (0.09)	7 (0.06)	Abs	Abs	Abs	Abs
Actinobacteria	Actinomycetales	*Frankiaceae* (N-fix)	4 (3)	0.4 (0.2)	Abs	Abs	Abs	Abs
		Streptomycetaceae (BC)	16 (11)	11 (7)	Abs	Abs	Abs	Abs
Bacteroidetes	Sphingobacteriales	*Sphingobacteriaceae* (Biocon)	44 (36)	38 (28)	Abs	Abs	Abs	44 (21)
	Cytophagales	*Cytophagaceae*	100 (15)	78 (10)	Abs	Abs	Abs	Abs
Nitrospirae	Nitrospirales	*Nitrospiraceae* (NOB)	100 (100)	Abs	Abs	Abs	Abs	Abs
Cyanobacteria	Oscillatoriales	*Phormidiaceae* (Pht)	100 (100)	100 (97)	100 (100)	Abs	Abs	Abs
Acidobacter	Acidobacterales	*Acidobacteriaceae* (NitR)	72 (60)	67 (55)	Abs	Abs	Abs	Abs
Firmicutes	Lactobacillales	*Lactobacillaceae* (PSB)	100 (98)	100 (42)	99 (97)	98 (98)	100 (100)	100 (99)
		Bacillalaceae (PSB) (BC)	43 (0.5)	32 (13)	95 (95)	Abs	Abs	Abs
	Clostridiales	*Clostridiaceae* (PP)	25 (0.09)	27 (3)	100 (1)	Abs	Abs	100 (0.8)
		Heliobacteriaceae (UD)	11 (0.02)	Abs	Abs	Abs	Abs	Abs
Crenarchaeota	Nitrososphaerales	*Nitrososphaeraceae* (NOA)	100 (100)	100 (100)	Abs	Abs	Abs	Abs

Abbreviations for bacterial functions within LEM and F-LEM are: AnPhtB, anaerobic photosynthetic bacteria; AOB, Ammonia oxidizing bacteria; BC, bio-controller; DnB, denitrifying bacteria; FBC, fungal bio-controller; FeRB, iron reducing bacteria; N-fix, nitrogen fixing bacteria; NOA, nitrite oxidizing Archaea; NOB, nitrite oxidizing bacteria; NitR, nitrate reducers; ; Pdtx, Phosphate detoxifier; PGP, plant growth promoter; Pht, phototrophic Bacteria; PP, Plant Pathogen; PrSlfB, purple sulfur reducing bacteria; PSB, phosphorus solubilizing bacteria; SlfRB, sulfur reducing bacteria and UD, undetermined. *Abs = Absent. Percent abundance of order is given in parenthesis for respective families. **Note:** LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Supplemental Material 2. Prominent microbial families (%) belonging to different orders in 2015 broiler litter and composted broiler litter

Phylum	Order	Family	2015 Baseline	2015 Control	2015 F-LEM	2015 LEM
Proteobacteria	Rhizobiales	*Hyphomicrobiaceae* (Pht)	64 (7)	49 (4)	Abs	45 (11)
		Bradyrhizobiaceae (N-fix)	19 (2)	16 (1)	Abs	35 (7)
	Rhodospirillales	*Rhodospirillaceae* (N-fix)	100 (5)	92 (3)	100 (3)	72 (4)
		Acetobacteraceae (N-fix) (PSB)	Abs	8 (0.3)	Abs	28 (1)
	Rhodobacterales	*Rhodobacteraceae* (AnPhtB)	100 (0.7)	8 (0.2)	Abs	100 (0.3)
	Caulobacterales	*Caulobacteraceae* (Pdtx)	100 (0.6)	100 (0.7)	100 (0.4)	100 (0.4)
	Nitrosomonadales	*Nitrosomonadaceae* (AOB)	100 (0.6)	100 (1)	Abs	100 (1)
	Burkholderiales	*Burkholderiaceae* (PSB)	12 (0.7)	29 (1)	Abs	60 (1)
		Comamonadaceae (DnB)	3 (0.2)	27 (1)	Abs	23 (2)
	Pseudomonadales	*Pseudomonadaceae* (PSB)	5 (1)	5 (3)	Abs	3 (7)
	Xanthomonadales	*Xanthomonadaceae* (PP)	98 (17)	100 (2)	100 (13)	61 (11)
	Chromatiales	*Ectothiorhodospiraceae* (PrSlfB)	63 (0.4)	53 (0.08)	100 (1)	27 (2)
Actinobacteria	Actinomycetales	*Thermomonosporaceae* (UD)	16 (15)	0.4 (0.4)	Abs	13 (0.09)
		Streptomycetaceae (BC)	12 (11)	4 (4)	Abs	11 (2)
Cyanobacteria	Oscillatoriales	*Phormidiaceae* (Pht)	100 (100)	100 (99)	Abs	100 (98)
Acidobacter	Acidobacterales	*Acidobacteriaceae* (NitR)	35 (35)	61 (47)	Abs	67 (33)
		Koribacteraceae (UD)	65 (65)	39 (30)	Abs	33 (31)
Firmicutes	Lactobacillales	*Lactobacillaceae* (PSB)	85 (8)	11 (1)	18 (0.6)	100 (1)
	Bacillales	*Bacillaceae* (PSB) (BC)	60 (44)	88 (75)	99 (96)	32 (91)
	Clostridiales	*Clostridiaceae* (PP)	43 (7)	18 (0.9)	Abs	27 (0.3)
Crenarchaeota	Nitrososphaerales	*Nitrospiraceae* (NOA)	Abs	Abs	Abs	100 (100)

Abbreviations for bacterial functions within LEM and F-LEM are: AnPhtB, anaerobic photosynthetic bacteria; AOB, Ammonia oxidizing bacteria; BC, bio-controller; DnB, denitrifying bacteria; FBC, fungal bio-controller; FeRB, iron reducing bacteria; N-fix, nitrogen fixing bacteria; NOA, nitrite oxidizing Archaea; NOB, nitrite oxidizing bacteria; NitR, nitrate reducers; ; Pdtx, Phosphate detoxifier; PGP, plant growth promoter; Pht, phototrophic Bacteria; PP, Plant Pathogen; PrSlfB, purple sulfur reducing bacteria; PSB, phosphorus solubilizing bacteria; SlfRB, sulfur reducing bacteria and UD, undetermined. *Abs = Absent. Percent abundance of order is given in parenthesis for respective families.

Supplemental Material 3. Prominent microbial families (%) belonging to different orders in 2016 broiler litter and composted broiler litter

Phylum	Order	Family	2016 Baseline	2016 Control	2016 F-LEM	2016 LEM
Proteobacteria	Rhizobiales	*Hyphomicrobiaceae* (Pht)	45 (4)	16 (33)	100 (0.2)	100 (0.6)
		Bradyrhizobiaceae (N-fix)	39 (4)	Abs	Abs	Abs
		Methylobacteriaceae (PGP)	Abs	79 (2)	Abs	100 (1)
	Rhodospirillales	*Rhodospirillaceae* (N-fix)	94 (3)	100 (7)	100 (1)	100 (1)
		Acetobacteraceae (N-fix) (PSB)	6 (0.2)	Abs	Abs	Abs
	Rhodobacterales	*Rhodobacteraceae* (AnPht)	100 (7)	100 (5)	100 (2)	Abs
	Nitrosomonadales	*Nitrosomonadaceae* (AOB)	100 (0.3)	Abs	Abs	Abs
	Burkholderiales	*Burkholderiaceae* (PSB)	23(1)	Abs	Abs	Abs
		Comamonadaceae (DnB)	15 (0.9)	Abs	Abs	Abs
		Alcaligenaceae (FBC)	44 (3)	100 (1)	100 (1)	100 (0.7)
	Pseudomonadales	*Pseudomonadaceae* (PSB)	20 (9)	75 (4)	100 (60)	100 (53)
	Xanthomonadales	*Xanthomonadaceae* (PP)	89 (6)	100 (8)	100 (1)	100 (2)
	Chromatiales	*Ectothiorhodospiraceae* (UD)	43 (0.9)	72 (0.9)	35 (0.6)	28 (0.2)
		Chromatiaceae (PrSlfB) (AOB)	39 (0.8)	28 (2)	65 (0.2)	72 (0.4)
	Thermoleophilales	*Thermoleophilaceae* (UD)	100 (0.9)	Abs	Abs	Abs
Cyanobacteria	Oscillatoriales	*Phormidiaceae* (Pht)	100 (100)	100 (100)	Abs	Abs
Acidobacter	Acidobacteriales	*Acidobacteriaceae* (NitR)	60 (52)	Abs	Abs	Abs
		Koribacteraceae (UD)	40 (35)	Abs	Abs	Abs
Firmicutes	Lactobacillales	*Lactobacillaceae* (PSB)	14 (5)	Abs	Abs	Abs
		Carnobacteriaceae (UD)	Abs	100 (38)	96 (40)	98 (63)
	Bacillales	*Bacillaceae* (PSB) (BC)	81 (22)	47 (26)	60 (21)	60 (20)
	Clostridiales	*Clostridiaceae* (PP)	16 (6)	8 (0.5)	32 (7)	24 (1)
Crenarchaeota	Nitrososphaerales	*Nitrospiraceae* (NOA)	Abs	Abs	Abs	Abs

Abbreviations for bacterial functions within LEM and F-LEM are: AnPhtB, anaerobic photosynthetic bacteria; AOB, Ammonia oxidizing bacteria; BC, bio-controller; DnB, denitrifying bacteria; FBC, fungal bio-controller; FeRB, iron reducing bacteria; N-fix, nitrogen fixing bacteria; NOA, nitrite oxidizing Archaea; NOB, nitrite oxidizing bacteria; NitR, nitrate reducers; ; Pdtx, Phosphate detoxifier; PGP, plant growth promoter; Pht, phototrophic Bacteria; PP, Plant Pathogen; PrSlfB, purple sulfur reducing bacteria; PSB, phosphorus solubilizing bacteria; SlfRB, sulfur reducing bacteria and UD, undetermined. *Abs = Absent. Percent abundance of order is given in parenthesis for respective families.

Supplemental Material 4. Prominent microbial families (%) present in soil at 4th week of sampling in 2015 and 2016.

Abbreviations for bacterial functions within LEM and F-LEM are: AnPhtB, anaerobic photosynthetic bacteria; AOB, Ammonia oxidizing bacteria; BC, bio-controller; DnB, denitrifying bacteria; FBC, fungal bio-controller; FeRB, iron reducing bacteria; N-fix, nitrogen fixing bacteria; NOA, nitrite oxidizing Archaea; NOB, nitrite oxidizing bacteria; NitR, nitrate reducers; ; Pdtx, Phosphate detoxifier; PGP, plant growth promoter; Pht, phototrophic Bacteria; PP, Plant Pathogen; PrSlfB, purple sulfur reducing bacteria; PSB, phosphorus solubilizing bacteria; SlfRB, sulfur reducing bacteria and UD, undetermined. *Abs = Absent. Percent abundance of order is given in parenthesis for respective families.

Phylum	Order	Family	2015 Control	2015 F-LEM	2015 LEM	2016 Control	2016 F-LEM	2016 LEM
Proteobacteria	Rhizobiales	*Hyphomicrobiaceae* (Pht)	54 (8)	58 (8)	50 (4)	68 (19)	45 (12)	40 (12)
		Bradyrhizobiaceae (N-fix)	27 (4)	31 (4)	44 (3)	27 (7)	45 (12)	48 (15)
		*Methylobacteriacea*e (PGP)	2 (0.3)	2 (0.3)	2 (0.1)	2 (0.5)	8 (2)	4 (1)
		Rhizobiaceae (PSB) (N-fix)	7 (0.6)	1 (0.2)	2 (0.1)	3 (0.7)	Abs	3 (1)
		Xanthobacteraceae (N-fix)	4 (0.6)	2 (0.3)	2 (0.2)	Abs	Abs	1 (0.4)
	Rhodospirillales	*Rhodospirillaceae* (N-fix)	56 (1)	69 (1)	21 (0.1)	100 (6)	100 (7)	90 (6)
		*Acetobacteracea*e (N-fix) (PSB)	44 (0.9)	31 (0.5)	79 (0.4)	Abs	Abs	10 (0.6)
	Sphingomonadales	*Sphingomonadaceae* (Biorem)	100 (35)	99 (22)	100 (14)	100 (13)	100 (7)	100 (7)
	Nitrosomonadales	*Nitrosomonadaceae* (AOB)	100 (0.7)	100 (0.7)	100 (0.1)	100 (4)	100 (2)	100 (1)
	Burkholderiales	*Burkholderiaceae* (PSB)	43 (2)	43 (4)	42 (1)	55 (8)	85 (19)	73 (9)
		Comamonadaceae (Dntf)	7 (0.3)	15 (1)	Abs	Abs	Abs	8 (1)
	Pseudomonadales	*Pseudomonadaceae* (PSB) (BC)	4 (1)	6 (2)	2 (1)	Abs	Abs	Abs
	Xanthomonadales	*Xanthomonadaceae* (PP)	92 (3)	90 (5)	92 (3)	100 (8)	56 (7)	77 (14)
	Chromatiales	*Chromatiaceae* (PrSlfB) (AOB)	9 (0.07)	5 (0.06)	Abs	23 (0.8)	100 (0.9)	11 (0.5)
		Ectothiorhodospiraceae (PrSlfB)	91 (0.7)	90 (1)	100 (0.1)	77 (3)	Abs	89 (4)
	Desulfobacterales	*Desulfobacteraceae* (SlfRB)	100 (0.6)	100 (1)	100 (0.2)	100 (4)	100 (9)	100 (8)
	Desulfuromonadales	*Geobacteraceae* (FeRB)	48 (0.6)	Abs	Abs	43 (0.6)	Abs	100 (0.7)
Actinobacteria	Actinomycetales	*Chitinophagaceae (Celldigst)*	84 (81)	76 (58)	63 (47)	100 (100)	78 (64)	100 (82)
		Catenulisporaceae (Ligdeg)	2 (0.9)	2 (1)	Abs	Abs	Abs	2 (2)
		Streptomycetaceae (BC)	Abs	7 (5)	Abs	35 (26)	50 (37)	21 (19)
		Mycobacteriaceae (BC)	22 (13)	16 (10)	57 (33)	4 (3)	14 (10)	16 (15)
		Pseudonocardiaceae (BC)	4 (2)	5 (3)	Abs	16 (12)	4 (3)	10 (9)
		Thermomonosporaceae (Ligdeg)	3 (2)	4 (2)	Abs	3 (2)	8 (6)	17 (16)
Nitrospirae	Nitrospirales	*Nitrospiraceae* (NOB)	100 (100)	Abs	Abs	Abs	100 (100)	100 (100)
Cyanobacteria	Oscillatoriales	*Phormidiaceae* (Pht)	100 (100)	100 (99)	100 (100)	Abs	100 (100)	Abs
		Synechococcophycideae (Pht)	Abs	Abs	Abs	24 (76)	Abs	Abs
Acidobacter	Acidobacterales	*Acidobacteriaceae* (NitR)	71 (62)	73 (56)	83 (83)	68 (55)	55 (44)	79 (61)
Firmicutes	Lactobacillales	*Lactobacillaceae* (PSB)	70 (3)	100 (1)	100 (11)	Abs	Abs	Abs
	Bacillales	*Bacillalaceae* (PSB) (BC)	46 (36)	47 (32)	49 (21)	49 (29)	42 (32)	38 (18)
Crenarchaeota	Nitrososphaerales	*Nitrososphaeraceae* (NOA)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)

Note: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution

CHAPTER 4

PHOSPHORUS-SOLUBILIZING BACTERIA IN LOCALLY SOURCED EFFECTIVE MICROORGANISMS (LEM) AND THEIR ROLE IN ENHANCING WHEAT GRAIN NUTRIENT DENSITY IN TWO ORGANIC SYSTEMS

Mahmud Kishan, Franklin Dorcas, Ney Laura, Cabrera Miguel, Hancock Dennis, Habteselassie Mussie, Newcomer Quint, Dahal Subash and Subedi Anish. To be submitted in Nutrient Cycling in Agroecosstems.

ABSTRACT

Soil microbial communities play an important role in biofortifying soil and plant dynamics by macro- and micronutrient mineralization and solubilization in the root rhizosphere. This study investigated the effect of two locally sourced bio-inocula; local effective microorganisms (LEM) and false-local effective microorganisms (F-LEM) in solubilizing soil P and facilitating the uptake of calcium, magnesium and zinc in wheat grains in two organic cropping systems. LEM plots showed greater abundance in plant available phosphorus in the system previously fertilized with bio-inoculated composted broiler litter (System-2) ($P < 0.05$). Calcium content of wheat grain was 35% higher in LEM grains compared to Control (water only) in the system previously fertilized with bio-inoculated swine effluent (System-1) and 40% higher compared to F-LEM in System-2 ($P < 0.05$). This study also found, Zn content in grains to be 13% higher in LEM grains compared to F-LEM in System-2 ($P < 0.05$).

Keywords: Microbial communities, local effective microorganisms, phosphorus, wheat, micronutrient

INTRODUCTION

Phosphorus (P), next to nitrogen (N), is the most essential element for plant nutrition (Alori et al. 2017) but its deficiency in soil can be severe and its level of availability can influence plant nutrient uptake of other nutrients such as Zn, Ca, and Mg and vice-versa (Alloway, 2009). In general, only 10 to 25% of the added P is available for plant uptake (Sharma et al. 2013) and due to pH-dependent fixation, the rest is fixed in soil systems as calcium-phosphates (alkaline soils) or iron or aluminum-phosphates (acid soils) making it less available for plants and microbial use (Gichangi et al. 2009,; Meena et al. 2018; Redel et al. 2016). Thus, P bio-availability is very low in soil for optimum crop performance (Jordan-Meille et al. 2012). Worldwide, P deficiency is usually resolved by applying P-fertilizers, but, since much of the added P becomes unavailable to plants, P accumulation in soil can lead to various environmental hazards (Alori et al. 2017).

Historically, agriculture has overlooked the need to formulate strategies to ensure the micro- and macro-nutrient value of crops (nutrient density of crops) by putting yield first, which could thereby create a “Hidden Hunger” (Rana et al. 2012) that is said to be affecting more than 2 billion people worldwide (FAO 2013), especially in developing countries. Intensive cultivation practices have led macronutrient depletion such as calcium (Ca), magnesium (Mg) potassium (K) and zinc (Zn) of soil (Altomare and Tringovska 2011) and subsequently in fruits, seeds or grains (White and Broadley 2011). Deficiency of Ca and Mg in the everyday human diet might lead to osteopenia and/or osteoporosis, especially in rural demographics of developing countries (Reddy and Edwards 2019; Graham et al. 2001), and Zn deficiency is listed as the fifth most important risk factor that can cause illness and death in developing countries (WHO, 2011). Zinc deficiency affects millions of hectares of croplands worldwide and almost one third of human

population is suffering from inadequate daily dietary zinc (Alloway 2009). Analysis of historical data gathered from the Broadbalk Wheat Experiment, Rothamsted, UK, revealed that the Zn content of wheat grain had decreased (Fan et al. 2008). Grain contents of Mg (Rosanoff 2013) and potassium (K) (Ekholm et al. 2007) also declined significantly as did many other nutrients, which coincided with the introduction of cultivars selected for higher yields and rapid growth after 1960s (Davis et al. 2004).

Soil microbial community abundance can also influence micronutrient availability to plants (Alloway 2009; Rengel et al. 1999). Plant growth-promoting rhizobacteria (PGPR) or plant growth-promoting microbes (PGPM) (Kloepper and Schroth1978) are involved in plant growth and development through the release of substances (Kloepper and Schroth 1981) which ensure cycling of essential plant nutrients (Behera et al. 2014). These microbes are in part responsible for improved soil health, increased crop yield (Suslow and Schroth 1982), and improved plant nutrient density (Rengel et al 1999). As an alternative means of synthetically produced agrochemicals, PGPR are increasingly gaining popularity among producers (Shaik and Sharaf 2017). Their function in the rhizosphere include solubilizing, mineralizing, and immobilizing nutrients such as phosphorus (Bhattacharyya and Jha 2012). Some examples of the functions include phosphorus solubilization by bacterial communities (PSBs) such as, Pseudomonas, Bacillus, Rhodococcus, Arthrobacter, Xanthomonas, Enterobacter, Klebsiella (Sharma et al. 2013; Chen et al. 2006; Wani et al. 2005 and De Freitas et al. 1997), direct fixation of nitrogen (Zahran 2001), production of phytohormones (Pérez-Flores et al. 2017), and biofortification of macronutrients such as Ca, K, and Mg contents (Rana et al. 2012), as well as Zn content in cereal crops such as wheat and cereal rye (Alloway 2009). PGPM have also been reported as being responsible for greater productivity in a variety of different crops, vegetables

and fruits, such as, wheat, soybean, rice, tomatoes, lettuce, and apple (Deshmukh et al. 2016; Etesami and Alikhani 2016; Bhattacharyya and Jha 2012). A locally sourced bio-inoculate called organisms of the mountains (MM), a modified version of EM™ (Higa 2000) originated in Japan (Higa and Wididana 1991), is utilized in many parts of Caribbean and Central America. The primary use of MM in Central American farm operations is for suppressing pests and odors of animal waste. It is reported that the EM™ contains specific strains of beneficial and synergistic microorganisms such as ammonia oxidizers, actinomycetes, lactic acid bacteria, yeasts and phototrophic bacteria (Higa 2000; Mupondi et al. 2006). We adapted the MM formulation for temperate climates which resulted in two bio-inoculates that can be grown and utilized by individual producers and have termed them 1) Local Effective Microorganisms (LEM) and 2) False-LEM. The objective of the study was to a) identify phosphorous-solubilizing bacterial communities in LEM and F-LEM inocula and b) investigate the effects of these inocula on soil pH, total phosphorus, organic phosphorus and plant available phosphorus in soil and their effect on the content of macro- and micronutrients, calcium, magnesium and zinc in wheat seeds (Triticum aestivum).

MATERIALS AND METHODS

Site Description

This study was conducted in the southeastern United States at the J. Phil Campbell Research and Education Center in Watkinsville, Georgia, (33°52′ N, 83°27′ W). The soil at the site is a fine kaolinitic, thermic Typic Kanhapludults in the Cecil sandy loam series with a 2–6% slope (Soil Survey Staff 2006.). The region has 123 cm average annual rainfall and an average minimum and maximum temperature of 10.4 °C and 22.5 °C, respectively.

Locally sourced bio-inocula (LEM and F-LEM)

The treatments compared were 1) LEM; 2) F-LEM, and 3) Control (non-chlorinated groundwater from a well). The LEM treatment was made by collecting the O horizon (actively decomposing leaf litter) from upland, healthy, well established mixed pine and hardwood forests, whereas the False-LEM was made with the same growing media as LEM but without the O-horizon. The detailed process of making LEM and F-LEM is described in Appendix 4.1.

Experimental Design and System Layout

In our study, we had two different organic cropping systems (one fertilized with swine effluent, and one fertilized with composted poultry litter) and three inoculum treatments (LEM, F-LEM, Control) that generated 6 treatments arranged in a completely randomized design with four replications (24 plots, each 3 x 6 m). System 1 had annual ryegrass (*Lolium multiflorum*) in winter-spring of 2015 and was fallow during summer 2016. Inoculum treatments mixed with swine effluent (SE) were applied on 11 November 2015 and 18 February 2016 (Table 4.1). System 2 had cereal rye (*Secale cereal*) in winter-spring of 2015 and butterbean edamame (*Glycine max*) in summer 2016. Inoculum treatments and composted broiler litter were applied on 8 June 2016 during edamame summer 2016. In early December 2016, all plots were first tilled with a BCS (BCS America, 14151 Fir Street, Oregon City, Oregon, United States of America) walk behind tiller to a depth of 10 cm and smoothened and planted with organic Hard Red Turkey Winter wheat (*Triticum aestivum*) on December 10, 2016 with a Jang planter (Mechanical Transplanter Company, Holland, Michigan, USA) at a depth of 2.5 cm and a rate of 112 kg ha^{-1}. At the end of the experiment, on May 5, 2017, wheat was harvested using a

Wintersteiger/Hege 125C (Wintersteiger Inc. 4705 W. Amelia Earhart Drive, Salt Lake City, Utah, United States of America)

Soil

Baseline soil sampling was done prior to planting of wheat on 5 Dec 2016. A second soil sampling was conducted one week after planting (17 Dec 2016) and at 4 weeks after planting (9 Jan 2017) using a 2.5-cm diameter soil auger at a depth of 0 – 15 cm. Soils were kept refrigerated at 4 ºC until they were air-dried, ground and passed through a 2-mm sieve for further chemical analysis. For microbial analysis, soil samples were separated and kept at -200C until the soil genomic DNA was extracted.

Soil pH, Total Kjeldahl Phosphorus (TKP), Plant Available Phosphorus (M1 P) and Organic Phosphorus (OP)

Soil pH was determined in water at a ratio of 1:5 (soil: water). Phosphorus fractions total Kjeldahl P, organic P and Mehlich-1 soil P were analyzed on Baseline and 4th week soil samples. Total phosphorus was determined by the Kjeldahl method (Taylor 2000). Plant available phosphorus, Mehlich-1 extraction was carried out according to Mehlich (1953), and organic phosphorus was determined by ashing soil samples at 550oC for an hour followed by sulfuric acid (0.5 M) extraction (Sparks et. al., 1996). The P from each of the extractions was determined by a modified single solution blue dye method (Murphy and Riley. 1962) using Tecan Infinite® 200 PRO Series, 2014 (Tecan Trading AG, Switzerland).

16sRNA analysis of liquid LEM and F-LEM inoculums applied and Soil Samples at 4th week

Microbial DNA were extracted from liquid LEM, F-LEM inoculum in 2015 and from soil at 4th week of planting in 2016 by QIAGEN DNeasy PowerSoil Kit (DNeasy PowerSoil Kit Handbook, May 2017, Qiagen, Valencia, California, United States). Extracted DNA was further

amplified by Ion 16S Metagenomics™ kit to amplify the V4 and V5 hyper variable regions of bacterial 16S rRNA. Bacterial universal primers 515F (5/-GTGYCAGCMGCCGCGGTAA-3/) and 806R (5/-GGACTACNVGGGTWTCTAAT-3/) were combined with barcoded adapter sequences. The sample libraries were prepared by Fusion Method as described in Ion Amplicon Library Preparation (Ion Torrent Systems Incorporated, Guilford, Connecticut, United States of America). Enriched products were loaded on the 316™-chip using the Ion PGM™ Hi-Q™ sequencing kit and sequenced in the Ion PGM™ sequencer (Ion Torrent Systems Incorporated, Guilford, Connecticut, United States of America). Sequence reads were analyzed by Ion Reporter™ software with the default parameter setting by MicroSEQTM(R) 16S Reference Library (v2013.1) and Greengenes (v13.5) as reference database. The sequencing was done in Coastal Plains Soil, Water, and Plant Research Center, United State Department of Agriculture Agricultural Research Service, Florence South Carolina.

Nutrient Density of Wheat

Wheat grains were first ground using mortar pestle followed by a concentrated acid digestion (sulfuric acid) by the Kjeldahl digestion method (Taylor 2000). After digestion, the digestates were analyzed for Ca, Mg and Zn using atomic absorption spectrometer, model AAnalyst200 (Perkin Elmer, 11695 Johns Creek Parkway, Suite 150, Johns Creek, Georgia, 30097-1881, United States of America).

Statistical Analysis

Analysis of variance, JMP PRO 13 software (SAS Institute, 2013), was used to determine differences between treatments by each soil sampling stage for pH, total Kjeldahl P, plant available P, organic P, and nutrient analysis ($*P < 0.05$). Comparisons between multiple means was determined with Tukey's HSD ($*P < 0.05$).

RESULTS AND DISCUSSION

Soil pH

In both systems, there was no significant difference in Baseline soil pH between inoculum treatments (Figure 4.1a and 4.1b). In System-1 (Sys-1) and System-2 (Sys-2), 4th week LEM soils had significantly lower pH than Baseline soils (*$P < 0.05$), and LEM treatments had lower pH than the Control (*$P < 0.05$; Figure 4.1). PSBs solubilize soil P through the production and release of organic acids which can lower soil pH (Maliha et al. 2004).

Microbial Communities in Bio-inocula, in Soil at 4th Week of Sampling and Phosphorus Dynamics in Soil in System-1(Sys-1) and System-2 (Sys-2)

The bacterial 16S rRNA gene sequencing of liquid LEM and F-LEM, revealed that LEM had more diverse microbial communities or greater functional diversity for both PSBs and nitrogen cycling microbial families than FLEM (Figure 4.2). The LEM inoculum had 7 PSB and 7 nitrogen cycling microbial families while F-LEM had only one PSB, *Lactobacillaceae* and no nitrogen cycling microbial families (Figure 4.2). Among PSBs found in LEM and within each of the orders (for example *Lactobacillaceae* within the lactobacillales order) *Lactobacillaceae*, was present in highest percentage (100%) in lactobacillales order followed by *Burkholderiaceae* (60% of burkholderiales), *Bradyrhizobiaceae* (35% of rhizobiales) and *Acetobacteraceae* (28% of rhodospirillales).

Among the nitrogen cycling microbial families in LEM, the most dominating N-utilizer family was *Nitrosomonadaceae* (100% of nitrosomonadales), *Rhodospirillaceae* (72% of rhodospirillales) and *Bradyrhizobiaceae* (35% of rhizobiales) (Figure 2b). F-LEM liquid had none of nitrogen cycling microbial families.

In soil at the fourth week of sampling, both systems were dominated by proteobacteria (Table 4.2). In addition to proteobacteria, lignin-degrading actinobacteria, and phosphorus-solubilizing *Bacillalaceae* and *Lactobacillaceae* from firmicutes phylum were identified (Table 4.2). In both Sys-1 and Sys-2, the total PSB family abundance was greater in LEM treatment at 4 weeks after application, where *Lactobacillaceae* was the dominant family (Figure 4.3) while in the Control and F-LEM *Pseudomonadaceae* was the dominant PSB family. In both Sys-1 and Sys-2, TKP, M1 P and OP were not significantly different between treatments at Baseline. At 4th week, however, differences between inoculum treatments were evident where Control soil TKP and OP were significantly greater in F-LEM and LEM soils (*$P < 0.05$). In Sys-2, at 4th week, M1 was significantly lower in the FLEM soil than in either the Control or LEM. PSBs plays a crucial role in solubilizing fixed P to plant available P forms. In both systems, the significant reduction in TKP at 4th week from Baseline is suggestive of P mineralization and solubilization by PSBs (Figure 4.4a and 4.4b). Solubilized P can be fixed again, part of M1, part of the soil biomass (part of OP) or be taken up by plants. In Sys-1, at 4th week, M1 P content was not significantly less than Baseline nor is it significantly different from the other inoculum treatments.

As mentioned above, the abundance of PSBs (*Lactobacillaceae* as prominent PSB) was higher in LEM and soil pH was significantly lower in LEM (Figure 4.1a and 4.3a) and LEM had greater OP (compared to Control) which could imply incorporation of mineralized P into soil microbial biomass as microbial OP which possibly have a potential to be released slowly as bio-available P over a longer period of time (Turner and Haygarth 2001; Bonkowski 2004). In Sys-2, significantly higher M1 P in LEM compared to F-LEM could be a combined function of higher

percentage of PSBs (*Lactobacillaceae* and *Bacillalaceae* as prominent PSBs) and the resulting lower soil pH (Figure 4.1b and 4.3b).

Biomass Yield and Nutrient Density of Wheat Grain

Wheat biomass presented is lower than what was in the field, but collection was consistent enough to compare between treatments. Biomass was not different between inoculum treatments (Figure 4.5a and 4.5b). Due to poor weather conditions and multiple breakdowns of harvesting equipment (shattering of heads and blower malfunctions) grain yield data was not reliable. Biomass data was more consistent but much of the biomass was left chopped on the plots.

Wheat grown with LEM consistently had one of the highest calcium densities regardless of system. However, depending on system, F-LEM or Control had similar Ca density to LEM (Figure 4.6). In a review by Marles (2017) Ca density in Wheat ranged globally from 80 to 800 $mgkg^{-1}$. In Sys-1 and Sys-2, Ca grain density in LEM plots (434 mg kg^{-1} and 375 mg kg^{-1}, respectively) was 35% higher ($*P < 0.05$) compared to Control (282 mg kg^{-1}) and 40% higher compared to F-LEM (223 mg kg^{-1}), respectively (Figure 4.6a and 4.6b). Increased Ca content in grain, in Sys-1, for LEM and F-LEM treatments could be explained by a combined effect of greater abundance of PSBs releasing organic acids (Goldstein 1995; Deubel et al. 2000) and lowering pH (Marra et al. 2011), thereby, solubilizing Ca-P complex in soil by H^+ substitution for Ca^{2+} (Goldstein, 1994; Villegas and Fortin 2002). In Sys-2, we found that Ca content in grain was lowest in F-LEM where lower abundance of PSBs might have led to less Ca-P solubilization. The Mg content of wheat grain was not influenced by either system or inoculum treatment. Zn content in LEM (39 mg kg^{-1}) was 12% ($*P < 0.05$) higher compared to F-LEM (34 mg kg^{-1}) in Sys-2, however, no significance was found in Sys-1 between treatments.

Pseudomonas spp are one of the major Zn solubilizers (Naz et al. 2016). In Sys-2 LEM had the greatest abundance of *Pseudomonadaceae* family. A greater abundance of this family alone was possibly not enough to facilitate higher Zn uptake. A combination of other microbial consortium including PSBs and Zn-solubilizers might have enhanced Zn uptake in wheat grain in Sys-2 LEM (Mäder et al. 2011).

CONCLUSIONS

LEM showed greater PSBs abundance in soil at 4th week in both systems. M1 P was similar between treatments in Sys-1 but in Sys-2, LEM had higher M1 P compared to F-LEM. A greater abundance of PSBs in soil may have been responsible for the increased calcium content in wheat grains from LEM treatments in both systems. Bio-inoculates performed differently depending on cropping system. However, regardless of cropping system, bio-inoculum amended systems had greater nutrient density of Ca and/or Zn than when no bio-inoculum was applied. Turkey red wheat grain grown after Sys-1 with LEM (annual rye amended with LEM Inoculum mixed with swine effluent) were 35% greater in Ca content than Control grains, while, the same grain was grown after Sys-2 (cereal rye/soybean amended with broiler litter inoculated with F-LEM) were 40% greater in Ca content and 12 % greater in Zinc content when LEM was the bio-inocula compared to F-LEM grains. Our research strongly suggests that the addition of inoculum LEM or F-LEM can improve nutritional value of wheat in Southeastern, USA iron rich soils.

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TABLES AND FIGURES

Table 4.1 Nitrogen (N) and phosphorus (P) applied (kg ha^{-1}) during in Sys-1 (winter-spring of 2015-2016) and in Sys-2 (summer 2016).

	Sys-1					**Sys-2**				
Treatments	N from SE (kg ha^{-1})	P from SE (kg ha^{-1})	Ca from SE (kg ha^{-1})	Mg from SE (kg ha^{-1})	Zn from SE (kg ha^{-1})	N from CBL (kg ha^{-1})	P CBL (kg ha^{-1})	Ca from CBL (kg ha^{-1})	Mg from CBL (kg ha^{-1})	Zn from CBL (kg ha^{-1})
Control	27	7	7	4	0.24	40	25	31	12	1
F-LEM	27	7	7	4	0.24	37	25	36	14	1
LEM	27	7	7	4	0.24	38	25	34	14	1

Note: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution. SE; Swine Effluent and CBL; Composted Broiler Litter

Table 4.2 Prominent microbial families (%) present in soil at 4th week of sampling in Sys-1 and Sys-2. Percent proportion of families belonging to their respective phyla is given in parenthesis

Phylum	Order	Family	Sys-1 Control	Sys-1 F-LEM	Sys-1 LEM	Sys-2 Control	Sys-2 F-LEM	Sys-2 LEM
Proteobacteria	Rhizobiales	*Hyphomicrobiaceae* (Pht)	52 (13)	50 (15)	69 (11)	68 (9)	56 (8)	59 (9)
		Bradyrhizobiaceae (N-fix) (PSB)	42 (11)	44 (13)	24 (4)	24 (3)	35 (5)	32 (5)
		*Methylobacteriacea*e (PGP)	2 (0.6)	2 (0.6)	1 (0.2)	2 (0.3)	1 (0.2)	Abs
		Rhizobiaceae (PSB) (N-fix)	Abs	Abs	1 (0.2)	3 (0.3)	4 (0.5)	4 (0.6)
		Xanthobacteraceae (N-fix)	Abs	Abs	1 (0.1)	0.3 (0.04)	0.7 (0.09)	2 (0.3)
	Rhodospirillales	*Rhodospirillaceae* (N-fix)	15 (0.4)	100 (2)	76 (3)	68 (2)	82 (2)	74 (3)
		*Acetobacteracea*e (N-fix) (PSB)	Abs	Abs	24 (0.8)	32 (0.9)	18 (0.4)	26 (1)
	Sphingomonadales	*Sphingomonadaceae* (BioR)	100 (23)	100 (31)	100 (39)	99 (23)	100 (21)	100 (24)
	Caulobacterales	*Caulobacteraceae* (Pdtx)	100 (0.41)	Abs	100 (0.7)	100 (0.7)	100 (0.9)	100 (1)
	Nitrosomonadales	*Nitrosomonadaceae* (AOB)	100 (2)	100 (0.5)	100 (2)	100 (2)	100 (1)	100 (2)
	Burkholderiales	*Burkholderiaceae* (PSB)	27 (3)	56 (4)	18 (3)	10 (1)	5 (1)	12 (2)
		Comamonadaceae (Dntf)	4 (0.4)	Abs	9 (1)	2 (0.2)	6 (2)	9 (10
	Pseudomonadales	*Pseudomonadaceae* (PSB) (BC)	100 (11)	100 (11)	73 (1)	5 (1)	12 (1)	13 (1)
	Xanthomonadales	*Xanthomonadaceae* (PP)	96 (12)	100 (8)	85 (6)	89 (5)	88 (8)	82 (8)
	Chromatiales	*Chromatiaceae* (PrSlfB) (AOB)	28 (0.8)	Abs	59 (1)	51 (1)	44 (0.8)	22 (0.5)
		Ectothiorhodospiraceae (PrSlfB)	73 (2)	100 (1)	41 (1)	47 (0.9)	49 (0.9)	68 (1)
	Desulfobacterales	*Desulfobacteraceae* (SlfRB)	88 (3)	72 (5)	86 (1)	96 (2)	96 (4)	80 (0.9)
		Desulfobulbaceae (SlfRB)	12 (0.4)	Abs	14 (0.2)	4 (0.09)	4 (0.03)	20 (0.2)
	Desulfuromonadales	*Geobacteraceae* (FeRB)	Abs	Abs	33 (0.07)	Abs	100 (0.1)	25 (0.1)
Bacteroidetes	Sphingobacteriales	*Sphingobacteriaceae (*BC*)*	48 (36)	68 (39)	44 (22)	33 (14)	29 (15)	32 (24)
		Chitinophagaceae (Cldg)	52 (39)	32 (19)	56 (28)	66 (28)	70 (36)	68 (50)
	Flavobacteriales	*Flavobacteriaceae* (Ptdg)	100 (7)	100 (10)	100 (37)	96 (35)	98 (27)	79 (8)
Actinobacteria	Actinomycetales	*Catenulisporaceae* (Lgdg)	21 (19)	Abs	Abs	2 (0.9)	Abs	Abs
		Streptomycetaceae (BC)	9 (8)	56 (47)	4 (2)	7 (3)	4 (2)	2 (1)
		Micromonosporaceae (BC)	24 (21)	10 (9)	6 (3)	10 (5)	7 (4)	10 (0.6)
		Frankiaceae (N-fix)	Abs	Abs	10 (5)	9 (4)	5 (3)	5 (3)
		Thermomonosporaceae (Lgdg)	8 (7)	Abs	15 (7)	5 (2)	8 (4)	12 (7)
Nitrospirae	Nitrospirales	*Nitrospiraceae* (NOB)	Abs	Abs	100 (100)	100 (100)	100 (100)	100 (100)
Cyanobacteria	Oscillatoriales	*Phormidiaceae* (Pht)	Abs	Abs	Abs	100 (100)	100 (94)	100 (98)
Acidobacter	Acidobacterales	*Acidobacteriaceae* (NitR)	83 (76)	80 (80)	77 (67)	66 (55)	72 (61)	64 (57)
		Koribacteraceae (UD)	17 (15)	20 (20)	23 (21)	33 (27)	28 (24)	36 (31)
Firmicutes	Lactobacillales	*Lactobacillaceae* (PSB)	Abs	Abs	2 (1)	2 (0.06)	Abs	0.07 (0.02)
	Bacillales	*Bacillalaceae* (PSB) (BC)	65 (27)	53 (33)	57 (42)	53 (34)	55 (33)	51 (31)
	Erysipelotrichales	*Erysipelotrichaceae* (UD)	Abs	Abs	Abs	100 (2)	100 (1)	Abs
	Clostridiales	*Clostridiaceae* (PP)	32 (19)	27 (8)	30 (6)	15 (3)	20 (5)	23 (7)
Crenarchaeota	Nitrososphaerales	*Nitrososphaeraceae* (NOA)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)	100 (100)

Note: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution. AOB, Ammonia oxidizing bacteria; BC, bio-controller; BioR, bio-remediation; Cldg, cellulose digestion; Dntf, denitrifoer; FeRB, iron reducing bacteria; Lgdg, lignin degradation; N-fix, nitrogen fixing bacteria; NOA, nitrite oxidizing Archaea; NOB, nitrite oxidizing bacteria; NitR, nitrate reducers; ; Pdtx, Phosphate detoxifier; Pht, phototrophic Bacteria; PP, Ptdg, protein digestion; plant pathogen; PrSlfB, purple sulfur reducing bacteria; PSB, phosphorus solubilizing bacteria; SlfRB, sulfur reducing bacteria and UD, undetermined. *Abs = Absent.

Appendix 4.1

Making LEM

The LEM was made by collecting the O horizon (loose and actively decomposing leaf litter) from upland, healthy, well established mixed pine and hardwood forests. This is the microbial inoculant. The LEM inoculum was then combined with 23 kg of organic semolina, 250 gm of baker's yeast, 10 kg of crushed natural hardwood charcoal, and 4 L each of molasses, antibiotic-free raw goat milk, and chlorine-free water to make a solid mixture. This solid mixture was then placed into a container and sealed for 6 weeks to allow for anaerobic fermentation. After first solid phase fermentation, a liquid phase fermentation was carried out for at least 2 weeks. Liquid phase fermentation: 3 kg of solid phase material is placed into a porous sack and suspended in a sealed 120 L tank with 60 L of water and 4 L of molasses.

Making False-LEM

Because the growing media (molasses and semolina, yeast and raw milk) contains nutrients and microbes, the False-LEM (F-LEM) was also prepared with the growing media at the same ratio as the LEM but without the forest microbial inoculum.

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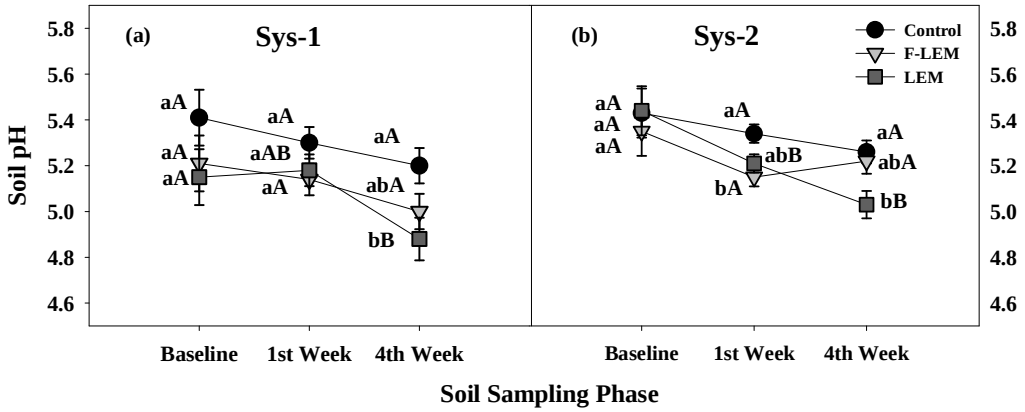

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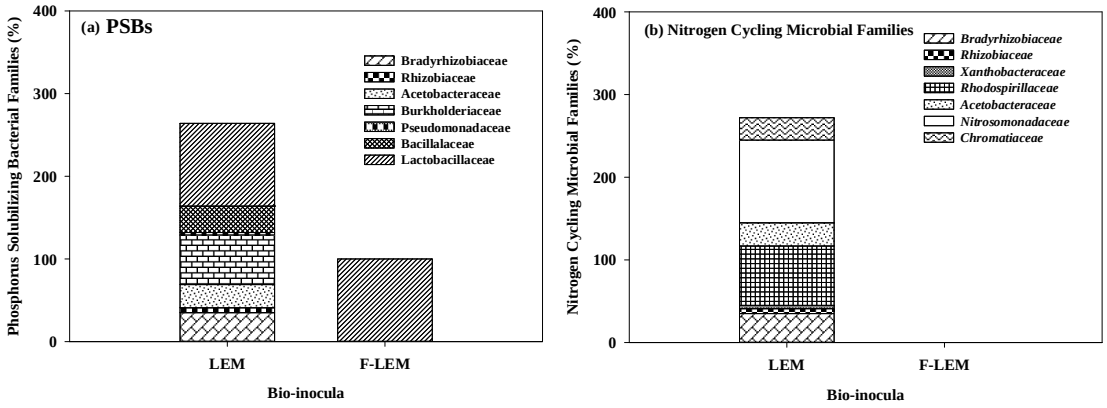

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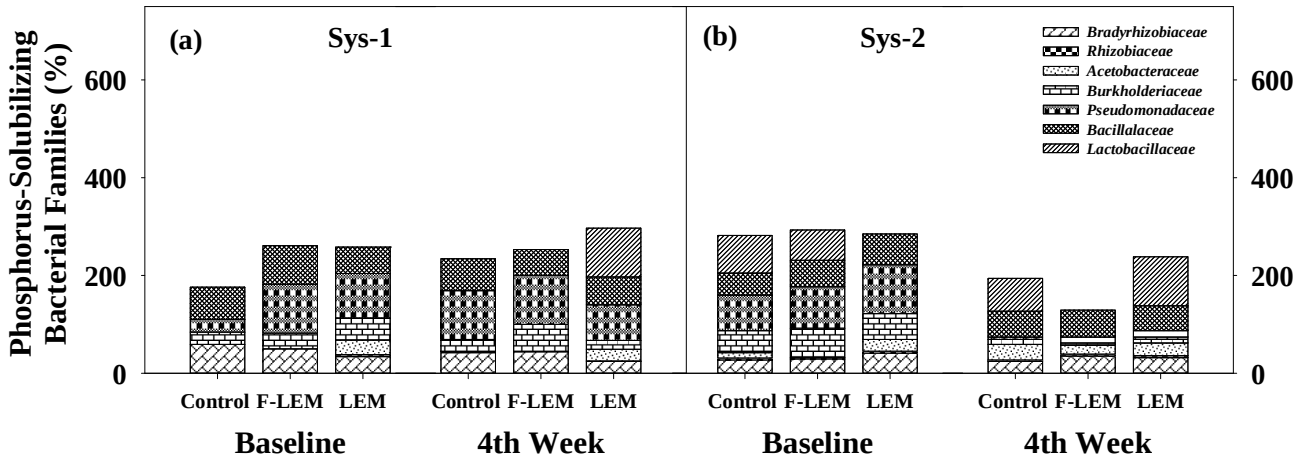

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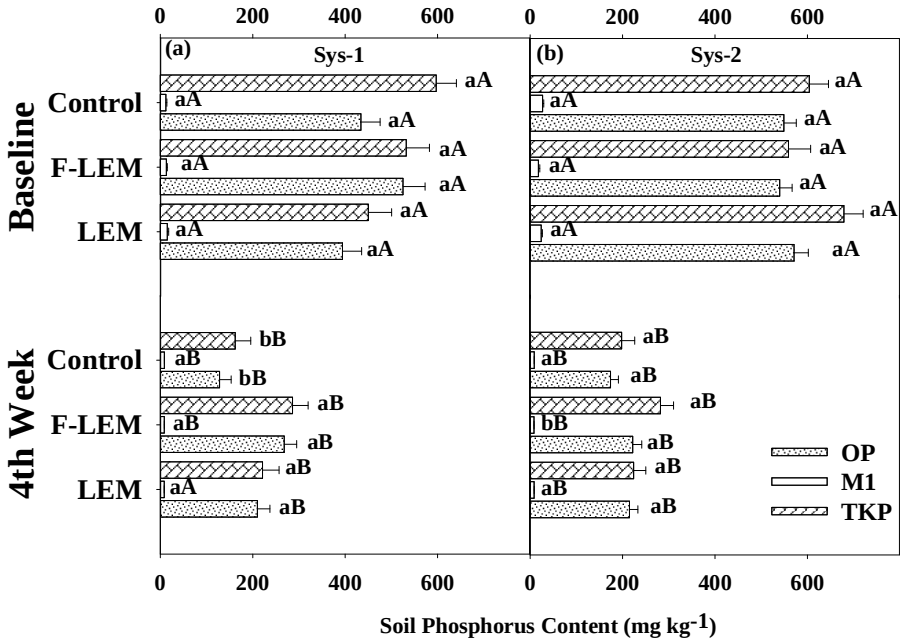

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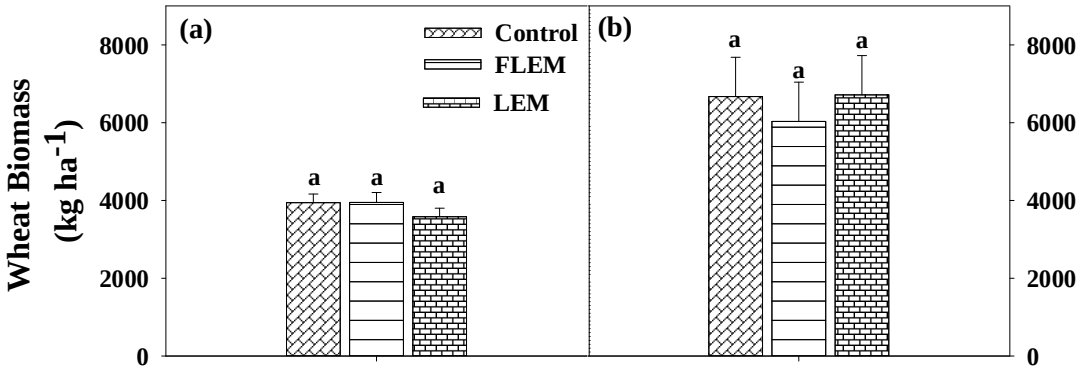

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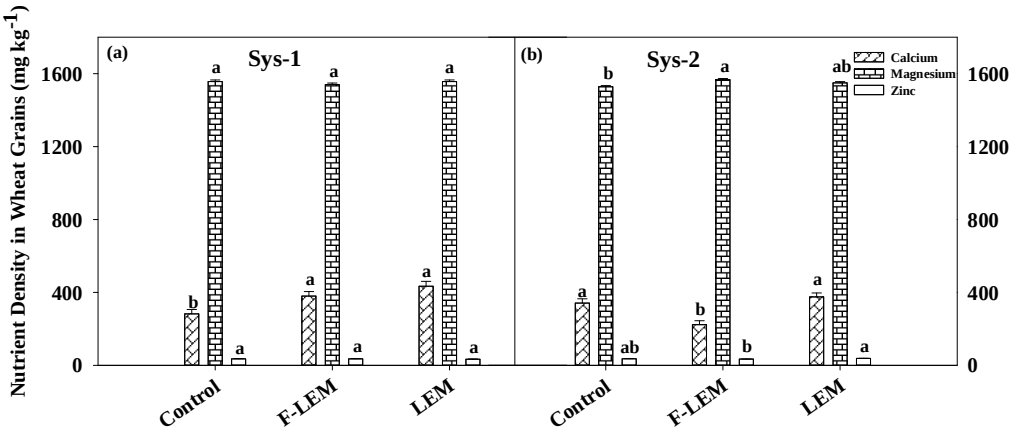

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CHAPTER 5

NITROGEN AND PHOSPHORUS DYNAMICS OF SOIL AS IMPACTED BY TWO LOCALLY SOURCED BIO-INOCULA GROWN WITH EDAMAME AND PUMPKIN

Mahmud Kishan, Franklin Dorcas, Cabrera Miguel, Ney Laura, Dahal Subash and Subedi Anish. Submitted to Nutrient Cycling in Agroecosystems, July 18, 2019.

ABSTRACT

Soil macro and micronutrient mineralization, solubilization and their uptake by plants are critically reliant upon an active presence of soil microbiome. This study investigated the effect of two locally sourced bio-inocula; local effective microorganisms (LEM) and false-local effective microorganisms (F-LEM) in mineralizing N and P in soil and facilitating the uptake of Ca, Mg, K and Zn in edamame (*Glycine max*) and pumpkin (*Cucurbita maxima*) grown in Summer 2017 and 2018, respectively. LEM treated plots showed higher plant available N during the first week (edamame season) and fourth week (pumpkin season) of treatment application ($P < 0.05$). Ammonia volatilization was also significantly lower in LEM plots during pumpkin season ($P < 0.05$). Post-treatment plant available P was greater in both summers in LEM plots ($P < 0.05$). Ca, Mg, K and Zn content in beans were 19%, 3%, 5% and 16% higher in LEM plots compared to Control ($P < 0.05$), respectively. Pumpkin pulp and seeds were also greater in cumulative Ca and Zn content compared to Control ($P < 0.05$). Cumulative Ca content in LEM treated pulp and seeds was 27% and 34% higher and cumulative Zn content was 33% and 44% greater compared to Control. LEM performed significantly better than F-LEM in terms of cumulative Mg and K content. Cumulative Mg content was 42 % and 37% higher and cumulative K content was 31% and 45% greater compared to F-LEM, in pumpkin pulp and seeds, respectively.

Keywords: Soil microbial communities, local effective microorganisms, nitrogen mineralization, phosphorus, micronutrient density, pumpkin

INTRODUCTION

Primary plant production in most agroecosystems is regulated by nitrogen (N) (Lambers et al., 1998) and net N mineralized largely depends on the nature of organic amendments used in agricultural management practices (Franklin et al. 2015). Next to N, phosphorus (P) is the most essential macronutrient (Hamim et al. 2019) and growth and development of all crops are contingent upon availability of P in soil (Wyngaard et al. 2016). Worldwide, soil P is highly deficient across many agroecosystems (Zhu et al. 2018). One reason is that P is readily complexed and occluded by Fe and Al at lower pH and Ca at alkaline conditions and can become less accessible for plant uptake (Kruse et al. 2015). In addition, plant nutrient contents may be declining in several food crops (Davis, 2009) and may result in reduced human nutrition if abundant food supplies are not available (Marles, 2017). Global nutrient deficiencies of great concern for adequate human nutrition are iron (Fe), zinc (Zn), iodine (I) (Horton et al. 2008) and of additional concern are calcium (Ca) and selenium (Se) (Black et al. 2008). Nutrient deficiencies affect close to 2 billion people worldwide (FAO 2013), most of them in developing countries. In the USA, nutrient deficiencies of most concern are Ca, Mg, and K (USDA and USDHHS 2015). Shortfalls in crop nutrient densities indicate a need to formulate strategies to conserve and make macronutrients and micronutrients more bioavailable.

In sustainable agricultural practices, efficient use of soil amendments, such as animal manures or organic wastes serve as important nutrient sources for crop productivity. The cycling and plant availability of macro and micronutrients relies upon an abundant and diverse, and active suite of soil microbial communities. As the need to implement strategies to improve nutrient use efficiencies grows the importance to better understand and improve soil health grows as well. Better understanding of the intricacies of soil microbial functional groups in

providing macro and micro nutrients in various agroecosystems may help us to reduce the amount of fertilization (Bargaz, et al. 2018) and thereby conserve global resources while also restraining the potential for environmental degradation (Fu et al. 2010 and Sun et al. 2012).

Various rhizosphere-dwelling microorganisms known as plant growth-promoting microorganisms (Kloepper and Schroth 1978) (PGPM) promote plant growth and health by fixing N, solubilizing P, mineralizing macro/micro elements (calcium, magnesium, potassium and zinc), excreting phytohormones (directly influence plant growth and metabolisum) and resisting pathogens (Lynch, 1990; Meeting, 1992; Bonkowski et al., 2009; Buée et al., 2009; Raaijmakers et al., 2009 and Mendes et.al., 2013). Due to their mutualistic interactions within the root rhizosphere and their positive effects on fortifying the soil microbiome, farmers, industry and researchers have given considerable attention to the formation and application of exogenous microorganisms.

Many farmers are using commercial bio-additives, for instance, Effective Micro-organisms® (EM^{TM}), Euro Mest-mix® (Mx), and Agrimest® (Am) (Van et. al., 2007). EM^{TM} advertises to have specific strains of beneficial and synergistic microorganisms such as ammonia oxidizers, actinomycetes, lactic acid bacteria, yeasts and phototrophic bacteria (Higa, 2000; Mupondi, Mnkeni and Brutsch, 2006). Throughout Central and South America a locally sourced bio-inoculate called microorganisms of the mountains (MM), a localized version of EM^{TM}, is made and utilized to suppress pests and odors associated with manures and composts. Research on the efficacy of EM^{TM} has had contrasting results. Javaid and Bajwa (2010) found significant yield increases in mung amended with EM^{TM} while others found no beneficial effects in crop production (Mayer et al., 2010 and Vilet et. al., 2006). With widespread use and few published results, more information is needed to understand edaphic and agronomic impact of locally

produced microbial inoculum. In our study, we adapted the MM formulas for temperate climates which resulted in two bio-inoculates that can be grown and utilized by individual producers. We have designated them as: 1) Local Effective Microorganisms (LEM) and 2) False-LEM. The LEM bio-inoculum is gathered from actively decomposing upland forest litter, combined with carbohydrate-rich growing media and is anaerobically fermented. LEM contains a wide range of functional microbial groups typical of EM^{TM}, except LEM is collected from the local environment and can be cultured on farm; therefore, easily adapts to the immediate environment (Mahmud et. al., 2019, in review, Ney et al. 2018). F-LEM is the same growing media used in LEM, fermented as was LEM, but without forest floor bio-inoculum. The objective of this study was to investigate the effects of bio- inocula: LEM or F-LEM on a) plant available nitrogen and nitrate-nitrogen content of soil; b) total phosphorus, organic phosphorus and plant available phosphorus fractions in soil and c) on the uptake of phosphorus, calcium, magnesium, potassium and zinc in edamame (Glycine max) bean and pumpkin (Cucurbita maxima) pulp and seed.

MATERIALS AND METHODS

Site Description

This study was conducted in the southeastern United States at the J. Phil Campbell Research and Education Center in Watkinsville, Georgia, (33°52′ N, 83°27′ W). The soil at the site is a fine kaolinitic, thermic Typic Kanhapludults in the Cecil sandy loam series with a 2–6% slope (Soil Survey Staff. 2006.). The region has 123 cm average annual rainfall and an average minimum and maximum temperature of 10.4°C and 22.5°C, respectively.

Bio- inocula: LEM or F-LEM

The microbial inoculant, LEM, was made by collecting the O horizon (actively decaying forest floor material) from healthy, well established mixed pine and hardwood forests. The LEM inoculum (30 kg bag by volume of O horizon material) was then combined with 23 kg of organic semolina, 250 g of baker's yeast, 10 kg of crushed natural hardwood charcoal, and 4 L each of molasses, antibiotic-free raw goat milk, and chlorine-free water to make a solid mixture. This solid mixture was then placed into a food grade plastic container and sealed for 6 weeks to allow for anaerobic fermentation. After this first solid phase fermentation, a liquid phase fermentation was carried out for at least 2 weeks. For that purpose, 3 kg of solid phase material was placed into a porous sack and suspended in a sealed 120-L tank with 60 L of water and 4 L of molasses. False-LEM (F-LEM), was prepared without the forest microbial inoculate but with the growing media at the same ratio as the LEM.

Experimental Design and Treatments

Plots (6 m × 6 m) were arranged in a completely randomized design (CRD) with four replications of each the following three treatments: 1) LEM, 2) F-LEM and 3) Control. When the first two treatments were applied in liquid form, an equal amount of untreated water was applied to the Control plots. The experiment started in early July for two consecutive summers in 2017 and 2018. In summer 2017 we grew edamame (*Glycine max L.*) and in 2018 we grew pumpkin (*Cucurbita maxima Duchesne*). In summer 2017, bio-inocula treatments were surface applied directly below edamame plants, three weeks after crop emergence at 2 L liquid m^{-2}. In summer 2018, broiler litter composted with each of the three treatments was applied to provide 50 kg P ha^{-1} and tilled. After pumpkin emergence (~ 3 weeks), bio-inocula treatments were surface

applied (2 L liquid m^{-2}) directly below pumpkin plants at the contact point of the stem and the soil. Nutrient application rates are detailed in Table 5.1.

In Summer 2017, after tilling with a BCS rototiller to a depth of 15 cm (BCS America, 14151 Fir Street, Oregon City, Oregon, United States of America) and smoothening, edamame was planted (8 July 2017) with a Jang planter (Mechanical Transplanter Company, Holland, Michigan, USA). at a depth of 2.5cm at 38-cm spacing. During Summer 2018, after tilling and smoothing, two mounds (6 m x 0.5 m x 0.1 m) in each plot were made with the help of Tillie (Carts and Tools, Corvallis, Oregon, USA) and 18 seeds were planted in each mound and thinned to 9 plants.

Soil Sampling

Baseline soil sampling was done prior to treatment application in both summers. Soils were sampled at a depth of 0-15 cm using 2.5-cm diameter soil augers (8 to 10 soil cores). In addition, soil samples were taken after the 1st and 4th week of treatment application in each year. The samples were kept refrigerated at 4ºC until they were air-dried, ground and passed through a 2-mm sieve for further chemical analysis. For microbial analysis, soil samples were separated and kept at -20oC until the soil genomic DNA was extracted.

Microbial Community Analysis

Microbial DNA was extracted from liquid LEM and F-LEM inoculum in Summer 2017 and from inoculated composts applied in 2018. Resources did not allow us to do microbial DNA analysis of 2018 bio-inocula. Samples were extracted by QIAGEN DNeasy PowerSoil Kit (DNeasy PowerSoil Kit Handbook, May 2017, Qiagen, Valencia, California, United States). Extracted DNA was further amplified by Ion 16S Metagenomics™ kit to amplify the V4 and V5 hyper variable regions of bacterial 16S rRNA. Bacterial universal primers 515F (5/-

GTGYCAGCMGCCGCGGTAA-3/) and 806R (5/-GGACTACNVGGGTWTCTAAT-3/) were combined with barcoded adapter sequences. Fusion Method as described in Ion Amplicon Library Preparation (Ion Torrent Systems Incorporated, Guilford, Connecticut, United States of America) to prepare sample library. Ion PGM™ Hi-Q™ sequencing kit was used to load the enriched samples on 316™-chip and sequenced in the Ion PGM™ sequencer (Ion Torrent Systems Incorporated, Guilford, Connecticut, United States of America). Sequence reads were analyzed by Ion Reporter™ against two reference database, MicroSEQTM(R) 16S Reference Library (v2013.1) and Greengenes (v13.5). The sequencing was done in Coastal Palins Soil, Water, and Plant Research Center, United State Department of Agriculture Agricultural Research Service, Florence South Carolina. Major phosphorus-solubilizing and nitrogen-utilizing bacterial families found are listed in Table 5.2.

Soil respiration (CO_2) and Ammonia (NH_3) Volatilization

CO_2 respiration and NH_3 volatilization were measured from each plot in Summer 2017 and 2018. Measurements were made on days 0 (day before application), 1(immediately following application), 3, 7, 14 and 28 after each treatment application. In each plot, two static gas trap chambers (20-cm ID PVC pipe open at the bottom containing two metal rings inside with airtight removable lid), were utilized on each sampling date. Within each of the chambers, the two metal rings held glass jars, one with alkali (100 mL 2 M NaOH) to trap CO_2 and one with acid (100 mL 1 M H_3BO_3) to trap NH_3. The chambers were inserted 5 cm into soil, alkali and acid traps were placed into the metal rings and the lids were closed for 24 hours. After, 24 hours the jars were collected and brought back to the lab for chemical analysis. CO_2 was measured by placing a 10-mL aliquot from the glass jars into centrifuge tubes and adding 8 ml $BaCl_2$ followed by centrifugation (1500 rpm for 3 minutes) and subsequent back titrating the

aliquot with 1M HCl (standardized) (Yiqi and Zhou 2010). NH_3 was measured by the change in pH of the H_3BO_3 in the glass jars followed by back titration with a weak HCl (0.0025M HCl) to initial pH of 1 M H_3BO_3 (4.8 to 5.0) (Bremner and Mulvaney 1982).

Soil Analysis

Soil pH was determined in water in the ratio of 1:5 (soil: water) for all soil sampling phases. Baseline soil samples along with 1st week and 4th week soil samples after treatment applications were extracted for plant available nitrogen (PAN) and nitrate content by using cold KCl (2M) extraction method (Maynard and Kalra 1993). All extractants were frozen until they could be analyzed spectrophotometrically on a Tecan Infinite® 200 PRO Series (Tecan Trading AG Switzerland). The ammonium (NH_4^+) content was determined by using salicylic acid method (Kempers and Zweers 1986) and the nitrate (NO_3^-) content was determined by vanadium (III) chloride method (Doane et al. 2003). Plant available nitrogen (PAN) was obtained by adding NH_4^+ and NO_3^- fractions. Phosphorus fractions, total Kjeldahl P, organic P and Mehlich-1 soil P were analyzed on Baseline and 4th week soil samples. Total phosphorus was determined by Kjeldahl method (Taylor 2000). Plant available phosphorus, Mehlich-1 extraction was carried out according to Mehlich (1953), and organic phosphorus was determined by ashing soil samples at 550^0C for 1 hours followed by sulfuric acid (0.5 M) extraction (Sparks et al. 1996). The P from each of the extractions was determined by a modified single solution blue dye method (Murphy and Riley 1962) using Tecan Infinite® 200 PRO Series, 2014 (Tecan Trading AG Switzerland).

Edamame and Pumpkin Productivity

Edamame was hand harvested from the inside eight rows and fresh bean and pod weight were recorded. Edamame biomass was also weighed and dried at 60°C for 48 h. The stabilized

dry weights were recorded and used in dry matter (DM) correction of fresh weights. Pumpkins were harvested for a total of 4 consecutive weeks. Individual pumpkin were carefully hand harvested based on their maturity and ripening. Pumpkin weight, circumference, and stem length were recorded each week immediately after each harvest. From each harvest, two pumpkins were randomly selected for each treatment for elemental analysis.

Nutritional Value of Edamame and Pumpkin

Edamame beans from Summer 2017 were freeze dried and then ground using mortar pestle. Pumpkins were cut into eight individual pieces, seeds removed and stored at -20°C until analysis. Two of the eight pieces were digested for elemental content of pulp. Pulp was ground with a Ninja Professional 1000W blender (SharkNinja Operating LLC, Massachusetts, USA) and stored at -20°C until analysis. Prior to elemental analysis, pulp and seed were oven dried at 65oC. Seeds were ground with a mortar pestle. Ground edamame bean, pumpkin pulp and seed were digested by the Kjeldahl digestion method (Taylor 2000). The digestants were analyzed for Ca, K, Mg and Zn using atomic absorption spectrometer, model AAnalyst200 (Perkin Elmer, 11695 Johns Creek Parkway, Suite 150, Johns Creek, Georgia, 30097-188.1, United States of America) at 423 nm, 766 nm, 285 nm and 214 nm, respectively. Total P of digestants were determined spectrophotometrically for each treatment with Tecan Infinite® 200 PRO Series (Tecan Trading AG Switzerland) by following modified single solution blue dye method (Murphy and Riley 1962)

Statistical Analysis

Analysis of variance with JMP PRO 13 software (SAS Institute, 2013),was used to determine differences between treatments by each soil sampling stage for pH, plant available nitrogen, nitrate content, total P, available P and Organic P ($P < 0.05$). Analysis of variance was

also used to determine the differences between treatments for nutrient content of edamame bean and pumpkin pulp and seed were analyzed by using ($P < 0.05$). Comparisons between multiple means of different N and P fractions, and the nutrient density of plants were determined with Tukey's HSD ($P < 0.05$).

RESULTS AND DISCUSSIONS

Soil pH

In both growing seasons, at 1st week, F-LEM and LEM had significantly lower soil pH compared to Control ($*P < 0.05$) followed by an increase at 4th week, however, no significant differences between treatments were observed (Figure 5.1a and 5.1b) at 4th week. The significantly higher soil pH at 1st week in Control than in F-LEM and LEM could possibly due to the lack of PSBs' abundance in Control treatments. Both LEM and F-LEM treatments had greater PSBs (*Acetobacteraceae*, *Burkholderiaceae* and *Lactobacillaceae* in edamame season; *Pseudomonadaceae* and *Bacillalaceae* in pumpkin season) (Figure 5.1a and 5.1b; Table 5.2) which have been reported to produce organic acids (Sánchez et al. 2017 and Wickramatilake et al. 2011).

NH_3 Volatilization and CO_2 respiration from soil in Edamame- Summer 2017 and Pumpkin-Summer 2018

In both growing seasons, cumulative loss of NH_3 was significantly greater from Control plots compared to both F-LEM and LEM ($*P < 0.10$; Figure 5.2a and $*P < 0.05$; Figure 5.2b) at Day 28. In edamame plots, lower cumulative loss of ammonia from both F-LEM and LEM might have facilitated higher plant available nitrogen (PAN) at the initial stages of the field experiment (discussed in the following section), however, this was not the case for the pumpkin season. In

both growing seasons, overall, cumulative loss of ammonia was very low and ranged from 1 to 5 mg N m^{-2}, which is likely due in large part to the overall lower pH of soil (Rochette et al. 2009). Another factor that may have also influenced the lower ammonia volatilization in LEM, in Pumpkin- Summer 2018, is the abundance of Rhodospirillaceae and Chromatiaceae in plots where LEM-inoculated composts were applied. These bacteria are described as ammonia consuming bacteria (Bast, 1977; Pfenning and Trouper, 1992) and in our earlier research these ammonia consumers were also found to be present when ammonia volatilization was significantly lower (Mahmud et al 2019; in review). Cumulative soil respiration (kg CO_2 ha^{-1}) in both seasons was not statistically significant between treatments (data not shown).

Nitrogen Fractions of Soil

In edamame-summer 2017, 1st week soil samples from LEM plots had significantly greater amounts of N mineralized (greater PAN in LEM) compared to both Control and F-LEM, LEM plots (*$P < 0.05$) (Figure 5.3a). Microbially driven mineralization of organic forms of N to ammonium (NH_4^+) is of major significance to N availability in soil for plant uptake (Richardson et al., 2013). In a recent incubation study by Ney et al. (2018), similar results were observed where LEM soils compared to F-LEM and Control soils, showed greater N mineralization within the first week of incubation. At 4th week, PAN decreased significantly from 1st week and there was no significant difference in PAN between treatments. Nitrate at 4th week was also significantly lower from both Baseline and 1st week, although no sampling phase showed differences between treatments (Figure 5.3b). During pumpkin-summer 2018, soil PAN also lowered significantly from Baseline to both 1st and 4th week (Figure 5.3c). In the 4th week PAN was significantly greater when in LEM plots than in either Control or F-LEM plots (Figure 5.3c).

Phosphorous Fractions of Soil

PSBs plays a crucial role in solubilizing fixed P into plant available P forms. In both summers, soil TKP and OP content decreased significantly (*P < 0.05) by 4th week from Baseline for all treatments (Figure 5.4). In edamame summer, soil M1 P at Baseline was not significantly different between treatments, however, LEM inoculum had significantly greater M1 P compared to either Control or F-LEM at 4th week, possibly due to the influx of PSBs (prominent PSBs are *Bacillaceae* and *Lactobacillaceae*; Table 5.2) (Shrestha et. al., 2014 and Giassi et. al., 2016). At Baseline significantly higher TKP in Control compared to F-LEM and LEM was evident (*P < 0.05). At 4th week, F-LEM had significantly lower TKP than either Control or LEM (*P < 0.05). In edamame summer, the significant decrease in TKP from all treatments from Baseline to 4th week is suggestive of P mineralization and solubilization by PSBs (Figure 5.4a). During pumpkin season, M1 P increased significantly from Baseline to 4th week for all treatments and at 4th week, both LEM and F-LEM inoculum demonstrated significantly greater M1 P compared to Control (*P < 0.05) (Figure 5.4b). Increased M1 P, in this case, could be a contribution of greater abundance of PSB families in LEM inoculated compost (prominent PSBs are *Pseudomonadaceae* and *Bacillalaceae*; Table 5.2) compared to Control (Table 5.2). In addition, OP was higher in both F-LEM and LEM applied plots than Control which might act as a potential P source for next crop rotation (Sharpley, 1995).

Edamame and Pumpkin Productivity; Nutritional Value of Butterbean Edamame and Pumpkin

This study did not find any significant difference between treatments either in cumulative pumpkin productivity nor in edamame biomass and yield (Table 5.3). We did however find significant differences in both bean and pumpkin nutrient densities of either Ca, Mg, K, or Zn, depending on crop, both years. In Edamame-Summer 2017, nutrient density was consistently

higher in LEM-inoculated edamame bean compared to Control edamame bean. Density of Ca (19%), Mg (13%), K (5%) and Zn (16%) were significantly higher in LEM edamame (*P < 0.05) compared to Control edamame (Table 5.4). Except for Ca density, LEM inoculum also performed significantly better compared to F-LEM inoculum (Table 5.4). P content of edamame was not significantly different between treatments.

In Pumpkin pulp and seed in the summer of 2018, LEM inoculation also facilitated increased uptake of Ca, Mg, K, P and Zn. Ca and Zn density in LEM inoculated pumpkin pulp was 27% and 33% greater (*P < 0.05) than Control (Table 5.4). Pumpkin seeds also accumulated significantly greater Ca (34%) and Zn (44%) in LEM inoculated plots (*P< 0.05) than Control (Table 5.4). Significantly greater density of Mg and K in pumpkin pulp and seeds was observed in LEM inoculum compared to F-LEM inoculum (Table 5.4). Pumpkin pulp also had greater cumulative Mg (856 mg/100 g) and K (820 mg/100 g) content when grown with LEM compared to F-LEM (494 and 563 mg/100 g, respectively). Cumulative Mg and K were also significantly higher (P<0.05) in LEM seeds (854 and 1077 mg/100 g, respectively) than F-LEM (535 and 591 mg/100 g, respectively) (Table 5.4). One of the most important traits of plant growth promoting microbial consortia is mineral solubilization and increased nutrient availability for plant uptake (Rokhbakhsh-Zamin et al. 2011; Colla et. al. 2015 and Lamont et. al. 2017). We also found that the P content was significantly higher in pumpkin pulp and seed in LEM inoculated plots compared to Control which can partly be explained by greater M1 P in LEM plots by 4th week (Figure 5.4). In both summers, greater macro and micro-nutrient density (Ca, Mg, K and Zn) in LEM plots may have been a contribution of higher abundance and diversity of PSBs applied in the LEM inoculum. Addition of F-LEM which was predominately lactobacillus or bacillus also helped improve Ca density in edamame compared to the Control. These groups of microbiome

have the potential to release organic acids in soil such as succinic acid or indole acetic acid (Mohite e. al. 2013; Shrestha et al. 2014, Ramesh et al. 2014and Kang et al. 2015) and lower soil pH (Fankem e. al. 2006; Li et al. 2010 and Marra et al. 2011 and Gonita-Mishra et al. 2017), thereby, releasing complexed macro and micro nutrients for plant use.

CONCLUSION

The use of LEM as a plant growth promoting microbial consortium has the potential to facilitate mineralization of N and P to plant available forms. The application of LEM as bio-inoculum rapidly mineralized N (within 1st week of bio-inoculum application) as evident in edamame-summer. During both summers and likely regulated by greater abundance and diversity of PSBs, LEM performed significantly better in releasing plant available P (M1) although the greater plant available P only resulted in greater P content in pumpkin seed and pulp in summer, 2018. The greater abundance and diversity of PSBs applied may have also been responsible for the increased Ca, Mg, K, and Zn density in edamame beans, and Ca, and Zn density in pumpkin pulp and seeds compared to Control. While regenerating soil health by amending with an abundant and diverse bi-inocula such as LEM did not increase yields, it can help improve nutrient density of edamame and pumpkin grown in iron rich soils while maintaining plant productivity.

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TABLES AND FIGURES

Table 5.1. Nitrogen, phosphorus, calcium, magnesium, potassium and zinc applied from treatments in Edamame- Summer 2017 (Only liquid inoculant applied) and Pumpkin- Summer 2018 (bio-inoculated compost applied, and liquid inoculant applied)

Note: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

	Summer 2017		**Summer 2018**					
	N (kg ha^{-1})	P (kg ha^{-1})	N from compost (kg ha^{-1})	P from compost (kg ha^{-1})	Ca from compost (kg ha^{-1})	Mg from compost (kg ha^{-1})	K from compost (kg ha^{-1})	Zn from compost (kg ha^{-1})
Control	0	0	80	50	62	24	112	2
F-LEM	0	0	74	50	72	28	132	2
LEM	0	0	76	50	68	28	126	2

Table 5.2. Phosphorus-solubilizing and Nitrogen-utilizing microbial communities in applied liquid LEM and F-LEM inoculum in Summer 2017; LEM, F-LEM and Control (non-chlorinated water) treated composts in Summer 2018

Abbreviations for bacterial functions within LEM and F-LEM are: AOB, ammonia-oxidizing bacteria; BC, bio-controller; N-fix, nitrogen fixing bacteria; PrslfB, purple sulfur bacteria; PSB, phosphorus-solubilizing bacteria. Percent abundance of order is given in parenthesis for respective families. **Note**: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Phylum	**Class**	**Order**	**Family**	**F-LEM (2017)**	**LEM (2017)**	**Control Compost (2018)**	**F-LEM Compost (2018)**	**LEM Compost (2018)**
Proteobacteria	α-Proteobacteria	Rhizobiales	*Hyphomicrobiaceae* (Pht)	Abs	41 (1)	16 (33)	100 (0.2)	100 (0.6)
			Bradyrhizobiaceae (N-fix)	Abs	59 (2)	Abs	Abs	Abs
		Rhodospirillales	*Rhodospirillaceae* (N-fix)	Abs	43 (0.5)	100 (5)	100 (2)	100 (1)
			*Acetobacteracea*e (N-fix) (PSB)	Abs	57 (0.7)	Abs	Abs	Abs
	β-Proteobacteria	Burkholderiales	*Burkholderiaceae* (PSB)	Abs	100 (1)	Abs	Abs	Abs
	γ - Proteobacteria	Pseudomonadales	*Pseudomonadaceae* (PSB) (BC)	Abs	Abs	75 (4)	100 (60)	100 (53)
			Chromatiaceae (PrSlfB) (AOB)	Abs	Abs	28 (2)	65 (0.2)	72 (0.4)
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae* (PSB)	100 (99)	99 (97)	Abs	Abs	Abs
		Bacillales	*Bacillalaceae* (PSB) (BC)	Abs	95 (95)	47 (26)	60 (21)	60 (20)

Table 5.3. Cumulative yield (yield was recorded for 4 consecutive weeks during growth season) of Pumpkin- Summer 2018 and biomass and yield (kg ha^{-1}) of edamame in Edamame- Summer 2017 and in Pumpkin- Summer 2018.

Lower letters indicate significant differences between treatments (*P < 0.05) and mean standard error is given in parenthesis**.** LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Treatment	**Cumulative Pumpkin Yield (kg ha^{-1})**	**Edamame Biomass (kg $ha^{-1)}$**	**Edamame Yield (kg $ha^{-1)}$**
Control	2455^{a} (274)	3395^{a} (166)	4568^{a} (1573)
F-LEM	2035^{a} (336)	3052^{a} (209)	5381^{a} (1769)
LEM	2026^{a} (243)	2838^{a} (517)	5006^{a} (1650)

Table 5.4. Phosphorus, calcium, magnesium, potassium and zinc content of butterbean edamame and pumpkin pulp and seeds (mg per 100 gm dry weight).

Lower letters indicate significant differences between treatments (*P < 0.05) and mean standard error is given in parenthesis**.** LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

	---------- Edamame ---------					**---------- Pumpkin Pulp ---------- (Cumulative of 4 weeks)**					**----------Pumpkin Seeds ------- (Cumulative of 4 weeks)**				
Treatment	P	Ca	Mg	K	Zn	P	Ca	Mg	K	Zn	P	Ca	Mg	K	Zn
Control	289[a] (13)	44[b] (3)	156 b (2)	562[b] (9)	5[b] (1)	13.3b (1)	140[b] (7)	650[ab] (38)	692[ab] (41)	2[b] (1)	273b (2)	462[b] (38)	683[ab] (55)	828[ab] (595)	15[b] (1)
F-LEM	283[a] (11)	47[ab] (2)	157 b (1)	525[c] (5)	5[b] (1)	9.2b (3)	139[b] (9)	494[b] (81)	563[b] (94)	2[b] (3)	270b (21)	543[b] (155)	535[b] (84)	591[b] (822)	18[b] (1)
LEM	266[a] (17)	54[a] (2)	161[a] (1)	591[a] (5)	6[a] (1)	25a (5)	193[a] (18)	856[a] (60)	820[a] (45)	3[a] (2)	342a (21)	700[a] (204)	854[a] (60)	1077[a] (82)	27[a] (3)

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Figure 5.1. Soil pH (0-15 cm) in different soil sampling phase in (a) Edamame-Summer 2017 and (b) Pumpkin- Summer 2018. Different lower-case letters indicate significant difference (*P < 0.05) between treatments on a sampling date and different capital letters indicate significant difference (*P < 0.05) between soil sampling dates. **Note:** LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Figure 5.2. Cumulative ammonia loss (mg N m^{-2}) from soil in (a) Edamame- Summer 2017 and (b) Pumpkin- Summer 2018. Different lower-case letters indicate significant difference at *P < 0.05 between treatments on a sampling date and Different lower-case letters with † indicate significant difference at **P < 0.1 between treatments on a sampling date. **Note:** LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Figure 5.3. (a, c) plant available nitrogen (PAN) and (b, d) nitrate content in soil (0-15 cm) in edamame- Summer 2017 and in pumpkin- Summer 2018, respectively. Different lower-case letters indicate significant difference (*P < 0.05) between treatments on a sampling date and different capital letters indicate significant difference (*P < 0.05) between soil sampling dates. **Note:** LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

Figure 5.4. Total Kjeldahl phosphorus (TKP), plant available phosphorus (M1 P) and organic phosphorus (OP) in soil (0-15 cm) during edamame- Summer 2017. Different lower-case letters indicate significant difference (*P < 0.05) between treatments on a sampling date and different capital letters indicate significant difference (*P < 0.05) between soil sampling dates. **Note:** LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

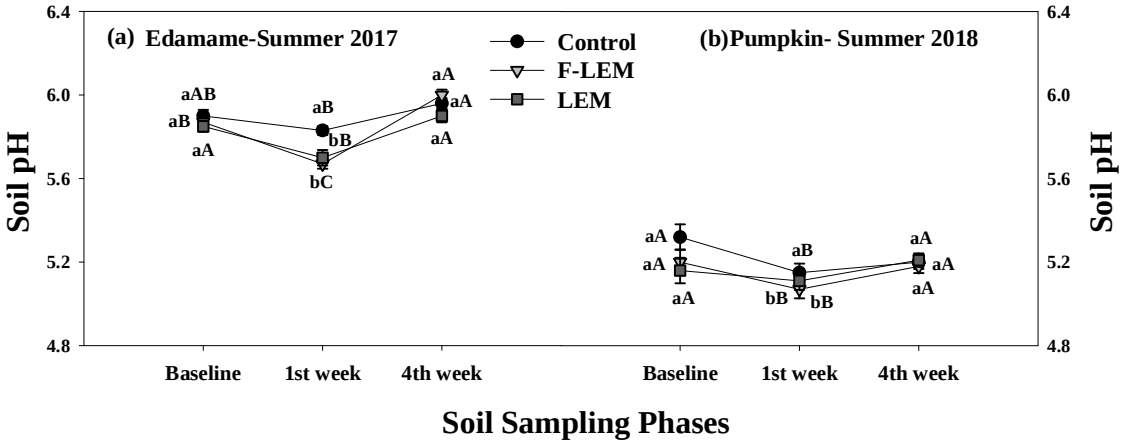

Figure 5.1. Soil pH (0-15 cm) in different soil sampling phase in (a) edamame-Summer 2017 and (b) pumpkin- Summer 2018. Different lower-case letters indicate significant difference (*P < 0.05) between treatments on a sampling date and different capital letters indicate significant difference (*P < 0.05) between soil sampling dates. **Note:** LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

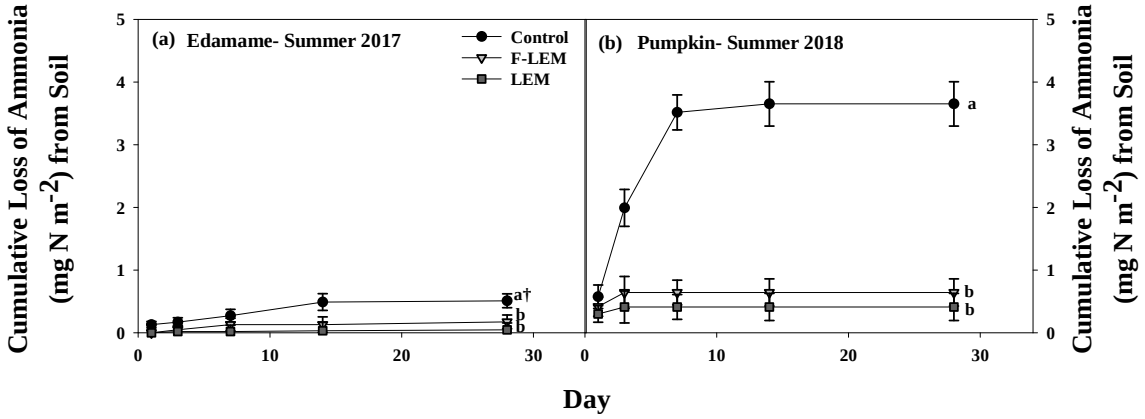

Figure 5.2. Cumulative ammonia loss (mg N m^{-2}) from soil in (a) Edamame- Summer 2017 and (b) Pumpkin- Summer 2018. Different lower-case letters indicate significant difference at *P < 0.05 between treatments on a sampling date and Different lower-case letters with † indicate significant difference at **P < 0.1 between treatments on a sampling date. **Note:** LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

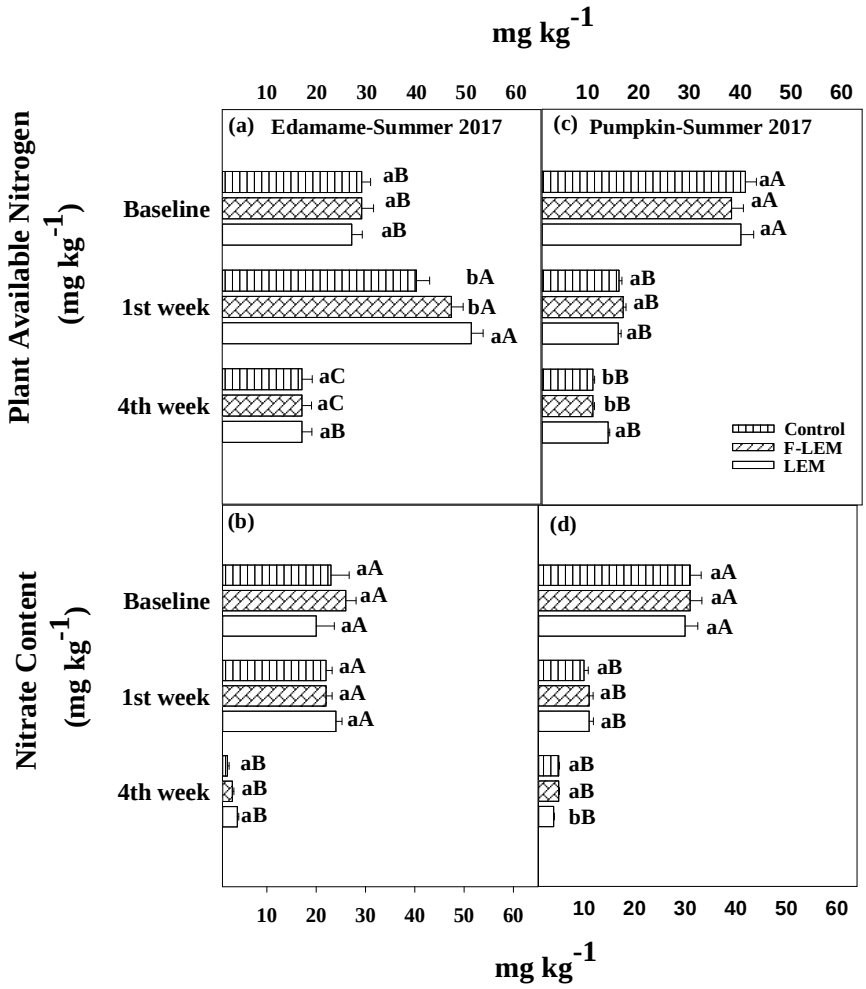

Figure 5.3. (a, c) plant available nitrogen (PAN) and (b, d) nitrate content in soil (0-15 cm) in edamame- Summer 2017 and in pumpkin- Summer 2018, respectively. Different lower-case letters indicate significant difference (*P < 0.05) between treatments on a sampling date and different capital letters indicate significant difference (*P < 0.05) between soil sampling dates.

Note: LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

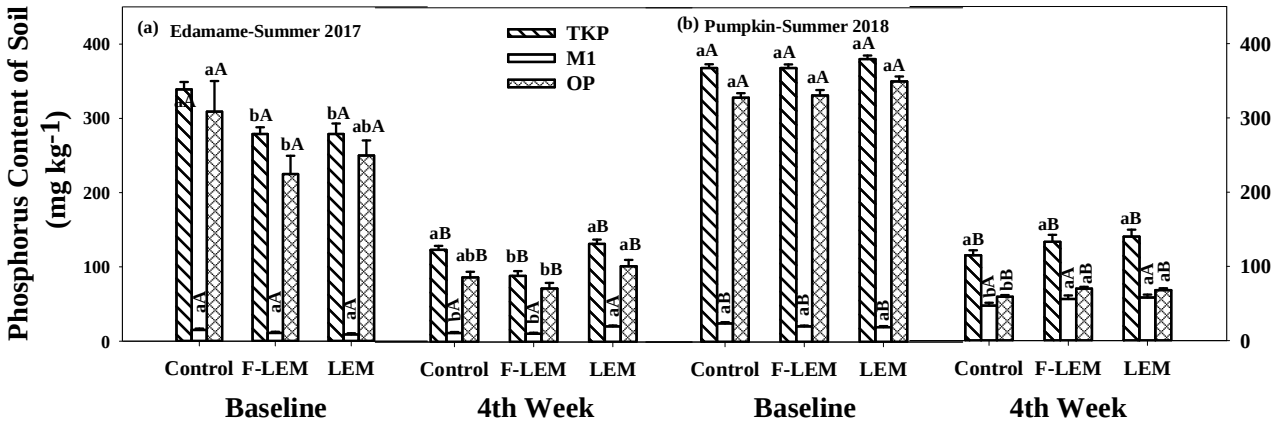

Figure 5.4. Total Kjeldahl phosphorus (TKP), plant available phosphorus (M1 P) and organic phosphorus (OP) in soil (0-15 cm) during (a) edamame- Summer 2017 and (b) pumpkin Summer 2018. Different lower-case letters indicate significant difference (*P < 0.05) between treatments on a sampling date and different capital letters indicate significant difference (*P < 0.05) between soil sampling dates. **Note:** LEM, local effective microorganisms is locally sourced bio-inoculate for the temperate region and is fermented mixture of actively decomposing leaf litter and a carbohydrate rich solution; F-LEM, is a fermented mixture of only the carbohydrate rich solution.

CHAPTER 6

CONCLUSION

In this doctorial research several positive aspects of locally effective Microorganisms (LEM) and of False-LEM (a derivative of LEM production) have been found. These ranged from greater redundancy of function in LEM to greater nutrient density in wheat grain, edamame bean, and in pumpkin pulp and seed in both.

MICROBIAL REDUNDANCY

Soil nutrient cycling is dependent on the presence, abundance and diversity of microbial communities in soil. Redundancy in function provides an ecosystem with the ability to more competently respond under varied stressed conditions. Presence of functional redundancy in bio-inoculum that is intended to be used in agricultural practices, is critical if we are to improve resistance and resilience to environmental stresses such as extreme heat, cold, dryness, or wetness. In this doctorial work a broader range of microbial functional groups and a broader range of microbial families within a functional group were identified in the LEM bio-inoculum compared to F-LEM bio-inoculum in each of the materials: liquid inoculum, compost, and soil for most years. These different functional groups, such as ammonia oxidizers, nitrogen-fixers, nitrogen mineralizers, phosphorus-solubilizers, bio-controllers in LEM makes the inoculum more robust and effective as compared to commercially available inocula which typically contain some specific bacterial strains. Since, LEM is locally sourced, it has the potential to adapt to its immediate environment and interact mutualistically with the indigenous microbial communities in that ecosystem.

WASTE MANAGEMENT

On farm generated waste can be a good source of nutrients as soil amendments. However, nutrient release from these wastes will depend upon the mineralizing and solubilizing capabilities of microbial communities present in them or in the soil once they are applied. Nitrogen, a vital crop nutrient can be lost as ammonia via volatilization from organic wastes such as poultry or broiler litter. This loss of nitrogen as ammonia may lead to a nutrient poor waste for optimum crop growth. Use of bio-inoculum are gaining popularity among different farming communities around the world. Some farmers resort to commercial inoculum or use an inoculum that is formulated on farm. In our study, we observed the loss of nitrogen as ammonia was significantly lower from LEM inoculated composts in both years compared to either the Control or the F-LEM composts. This lower degree of ammonia volatilization was the combined function of 1) lower thermophilic index during composting, 2) lower pH and 3) greater abundance of ammonia oxidizers in LEM inoculated composts. An acceptable percent germination index was attained for cucumber seeds in LEM treated composts. Our efforts to investigate the organic waste characteristics (in our case it is composted broiler litter) has led us to a few conclusions. The use of LEM might retain more nitrogen within the compost as indicated by lower ammonia loss. However, the scope of this study did not include the measurements of nitrate leaching from compost or emission of nitrous oxide. Further studies are required to investigate the use of LEM for utilizing the nitrate in compost and its impact on the generation of nitrous oxide.

LEM IN SOIL NUTRIENT CYCLING

LEM in Nitrogen Cycling

The use of LEM as a plant growth promoting microbial consortium has the potential to facilitate mineralization of organic forms of nitrogen and phosphorous to plant available forms,

although, much more research is needed to understand best application rates and resulting mineralization rates. We found significantly greater plant available nitrogen in the second and third year of the study in soils that received LEM as treatment. In addition, we found enhanced nitrogen mineralization at 4th week soil samples in 2016 edamame summer and rapid nitrogen mineralization within the 1st week of LEM application in 2017 edamame summer where we had added a direct application of LEM after plant emergence. As a result of our research, a direct application of LEM after plant emergence can be recommended for enhanced N mineralization.

LEM in Phosphorus Cycling

Liquid LEM bio-inoculum showed greater phosphorus solubilizing community abundance and diversity compared to F-LEM inoculum. Higher PSBs were found in soil at 4th week during wheat growing season. LEM demonstrated higher plant available phosphorous compared to F-LEM bi-inoculum studied in this research. In 2018, pumpkin grown with LEM which also had greater PSB diversity compared to F-LEM likely also had significantly better performance in releasing plant available phosphorous and nutrient densities of both pumpkin pulp and seed. In the summer (2017) edamame study (Chapter 4) we only applied a direct application of LEM 3 weeks after plant emergence. We postulate this to be because of a build-up of biodiversity after several applications.

LEM IN ENHANCING CROP NUTRIENT DENSITY

Current agricultural practices for increased crop yields will not be enough to feed the world population by 2050 (Godfray et al., 2010). An estimated 925 million people are food insecure and suffer from chronic hunger (FAO, IFAD, UNICEF, 2010). While food security remains a pressing global concern, nutritional security should also be viewed as an equally important issue. Food insecurity is often regarded as a threat to developing countries but,

inadequate nutritional provision is a problem that pervades even into the most advanced economic areas (Finn, 2014). Our research did not find any significant increase in edamame, wheat or pumpkin productivity. Nor did LEM treated crops show significant decline in crop productivity compared to other treatments. However, the nutrient density of the crops painted a more promising picture. Nutrient density of Mg, K and Zn in edamame bean was significantly greater. Recent reviews by have indicated that Zn deficiencies in soybeans are becoming more pronounced as atmospheric CO2 increases (Myers et al. 2014). As Zn is already one of the minerals that is in great shortfall in global human nutrition, this result gives pronounced importance to these findings and hope that we may find somewhat simple ways to adapt to climate change. Nutrient density of wheat grain, edamame bean and pumpkin pulp and seed showed promising results in response to applications of LEM. Compared to the Control for years 2015, 2016, and 2017, edamame bean, density of Ca (31%, 20% and 19%, respective of year) and Zn (50%, 15% and 16%, respective of year) were greater in LEM bio-inoculum receiving plots. Compared to Control, density of Mg was greater in LEM bean in summer 2015 (5% greater) and in summer 2017 (3% greater). In Red Turkey winter wheat grain grown in the winter of 2016, LEM grain had significantly greater Ca (35%) density compared to Control grain. Additionally, LEM grain had significantly greater Ca (40%) and Zn (13%) density compared to F-LEM grain as well. Similar results were found in pumpkin pulp and seed, compared to Control, density of Ca (27% and 34%), and Zn (33% and 44%) in pumpkin pulp and seeds, respectively, were significantly greater in LEM treatments.

LEM, as a bio-inoculum might be a solution to increase the macro/micronutrient density in crops which is often over looked in everyday human diets. This is particularly true for low income families, where the staple food is a high-calorific food such as wheat, rice or edamame.

LEM may secure higher nutrient density into those crops and provide with a macro/micronutrient enriched diet.

MONETARY TRANSLATION OF USING LEM

Since, LEM is locally sourced, a farmer can easily make it with ingredients found on or near the farm regardless of farm size. This will encourage a farmer not to obtain a commercially available bio-inoculum which might involve a long journey to the retail shop causing transportation cost. Use of LEM will also give the grower a freedom to make it and use it as per need basis.

The major obstacle LEM might face among farmers could be the lack of response in yield. As stated above, LEM treated crops did not show significant increase in productivity, this might discourage a grower to adopt LEM as a bio-fertilizer. However, since the crops are biofortified with macro/micronutrients, the monetary value could be higher compared to conventionally grown crops if labelled as such. Globally but more often in more affluent societies, there is a high demand of naturally grown/organically grown crop products and consumers are often willing to pay more from naturally/organically grown farms. This consumers could also be more willing to pay more for a more nutritionally rich crop. Enriched nutrition in crops such as edamame and pumpkin, could therefore add income to farms that grow them.

F-LEM AS A BIO-INOCULUM

Of additional importance are our results on F-LEM. On a few occasions, F-LEM also showed similar ability to improve crop nutritional value. F-LEM inoculum was primarily the Lactobacilliceae family as is also found in LEM. The lactobacillus bacteria originated from the raw milk which is a part of the growing media and has the potential to solubilize mineral-

complexes and release minerals (Lamont et al. 2017). Use of F-LEM could also be advocated in regions where undisturbed and healthy forest litter is hard to obtain for the local producers.

FINAL REMARKS

Future use of LEM will have to address a few issues. We have observed a buildup of biodiversity, especially PSBs, however, we might just be scratching the surface. The build up of microbial diversity through LEM application might take more than 3 years, which might be reflected in future crop yield. Repeated application of LEM for 5 to 7 years might result in greater build up of ammonia oxidizers, nitrogen fixers, actinomycetes in soil. The amount of time required to fortify the soil biome may dependent on mode, rate, time, and number of applications as well as what fertilization is paired with LEM or F-LEM.

In addition, the rates at which a farmer should apply LEM and how often the application would be recommended will vary depending on the region it is being used, soil condition, types of organic wastes, and crops grown. Further research is needed to understand these aspects.

Liquid inoculum, from two different years showed different phyla abundance (although similar diversity). Depending on the region from where forest litter is collected and the environmental condition during the formulation process of LEM, different phyla domination may be observed. This may cause buildup of one functional group compared to others. However, since LEM provides greater diversity in functional groups as compared to commercially available bio-inocula, LEM will likely have better chance in surviving in soil and continue to provide several ecosystem services.

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