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Effects of Organic and Conventional Agricultural Practices on Soil Microbial Communities and Molecular Detection of Soil Borne Disease

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EFFECTS OF ORGANIC AND CONVENTIONAL AGRICULTURAL PRACTICES ON SOIL MICROBIAL COMMUNITIES AND MOLECULAR DETECTION OF SOIL BORNE DISEASE

by

HOLLI K. MILNER

(Under the direction of Tiehang Wu)

ABSTRACT

Agricultural practices affect soil microbial communities and health through the input of pesticides, herbicides, fertilizers, and cycling of crop rotation. By examining the microbial community structure, we analyzed how microbial species respond to the environment that individual farms create. Early detection of soil borne disease is essential for agricultural success. However, monitoring incidence of disease based on plant growth response to pathogenic inoculation may not reveal the amount of pathogenic DNA in soil. A comparative study of tomato production systems was conducted by analysis of soil microbial community structure from four farms in Southeast Georgia for the years 2012 and 2013, and incidence level of disease and plant growth of tomato plants grown in greenhouse soil were measured. The results indicated that the soil fungal, bacterial, and animal communities were unique to each farm (ANOSIM $P<0.0001$) for 2012 and 2013. The soil chemical characteristics were significantly different between each farm (MANOVA, $P<0.0001$) for both years. Calcium base saturation and soil pH were the characteristics that were not significantly different between farming management practice for 2012 and 2013. While plant growth (height and leaf count) was not significantly different ($P=0.5552$ and $P=0.0719$ respectively) between plants grown in soil inoculated with soil borne disease and un-inoculated soil in the greenhouse experiment, there was significantly higher amounts of total fungal and *Sclerotium rolfsii* DNA ($P=0.0454$ and $P=0.0278$

respectively) in the inoculated than un-inoculated soil measured by quantitative polymerase chain reaction (Q-PCR). Fluorescent in situ hybridization (FISH) was used as an alternative for visual detection of *Sclerotium rolfsii* through whole cell hybridization. A higher hybridization signal was detected in soil with high *Sclerotium* DNA (15.55333 pg/µl) than in soil with low *Sclerotium* DNA (0.0155 pg/µl). In conclusion, this study suggested that farming management practices have an effect on the microbial community structure and chemical components of agricultural soil and that plant growth in a greenhouse setting was not a clear representation of the amount of pathogenic DNA in the soil. Molecular detection of pathogenic DNA in soil could provide important information on predicting the potential for disease development in agricultural ecosystems.

Key words: Microbial community structure, Q-PCR, FISH, *Sclerotium rolfsii,* Soil borne disease

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DEDICATION

This thesis is dedicated to my mom and to anyone who has been my teacher in any capacity. I could not have come this far without you.

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Chapter 1

Literature Review

The farm and agriculture related business represents 5% of the gross-domestic product (U.S. USDA. Economic Research Service), and 16% of the employment (2007 Agricultural Census) in the United States. For these agricultural businesses to be successful in the long term, they must balance an emphasis of high crop yields while maintaining soil quality. Farm management is classified as organic or conventional depending on the components of applied chemicals, measures of sustainability, and the planting of genetically modified organisms (GMOs) (U.S. Department of Agriculture 2013, Agricultural Marketing Service 2013). Organic agriculture aims to balance the interest of humans and nature while conventional farming is separated from nature through technological control (Verhoog et al. 2007). Legally certified organic products must follow strict regulations set by the federal government while conventional practices receive more leniencies regarding pesticide and fertilizer use. "Certified Organic" is a trademark that promises production has adhered to the approved methods and regulations that mix cultural, biological, and mechanical practices that foster the cycling of resources, promote ecological balance, and conserve biodiversity. This is achieved through the exclusion of most synthetic substances, certain natural substances (e.g., arsenic), GMOs, ionizing radiation, sewage sludge, or nonagricultural/nonorganic substances used in or on processed products (U.S. Department of Agriculture 2013, Agricultural Marketing Service 2013). In contrast to organic management, conventional management has the freedom to integrate synthetic fertilizers, sewage sludge, irradiation, and GMOs (U.S. Department of Agriculture 2013, Agricultural Marketing Service 2013). Conventional farming products remain the most popular in the United States. However, organic products have begun to gain a more committed following of consumers and

represent a growing proportion of food sales affecting the economy. Organic agricultural cultivation is an important and growing management practice. Between the years 2001-2011, organic food sales have nearly doubled in the United States (Lotter 2003; Osteen et al. 2012).

There is an estimated level of about 50,000 bacteria species in one gram of soil (Roesch et al. 2007). These abundant groups include gram- negative, nitrogen fixing, and parasitic bacteria. The natural, tightly linked processes of the carbon and nitrogen cycles including decomposition of organic matter, and the mineralization and immobilization of nitrogen, is an essential function of soil microorganisms (Bloem et al. 1995; Berthrong et al. 2013). Soil microorganisms can be directly/indirectly beneficial or detrimental to plant health. Soil borne/arbuscular mycorrhizal fungi (AMF), forms a mutualistic relationship with plants by providing access to limited nutrients to enhance plant health (Jefferies et al. 2003; Gosling et al. 2006). AMF can have the same positive relationship with weeds, which compete for space and nutrients with crops (Massenssini et al. 2013). Alleopathic microorganisms also colonize plant roots (Barazani & Friedman 2001; Kremer 2006), and release phytotoxic metabolites, which can inhibit plant growth (Kremer 2013).

PLFA research provides evidence that macro invertebrates in the soil, such as earthworms, will increase the relative abundance of bacteria and decrease the fungi in surface organic soil (Dempsey et al. 2013). Fertilization based with organic manure increases microbial biomass to nearly double than that obtained with chemical fertilizers (Zhang et al. 2012). No tillage management systems support a greater abundance of micro-arthropods, and increase's soil organic matter and bulk density in the top layer of the in comparison to conventional tilling management practices (Sapkota et al. 2012). It has been reported that the yield of organically managed crops showed less fluctuation in inter-annual crop yield over an 11 year time span than

conventional crops (18% compared to 30.4% respectively; Kaffka et al. 2005). Research using T-RFLP shows that the AMF diversity is higher in organic soil than conventional soil (Verbruggen et al. 2010). The crop yield in organic fields was up to 54% lower than in conventional fields for winter wheat cereal (Gabriel et al. 2013). In a greenhouse experiment, tomatoes grown in organic soil showed a 22% increase in above ground plant weight in comparison to tomatoes grown in conventional soils, but had no difference in height or stem diameter (Kokalis-Burelle et al. 2005). In a greenhouse setting, the plant pathogenic fungi *Sclerotium rolfsii*, known as southern blight, developed faster and had a higher level of incidence on tomato plants grown in conventional soils rather than organic soils (Liu et al. 2008).

Tomatoes are a major vegetable product of Georgia and are challenged by soil-borne disease. Common soil-borne fungal diseases of tomatoes include *Fusarium* wilt, *Verticillium*, *Phytophthora*, *Rhizoctonia*, and *Sclerotium*. Infection with bacterial diseases is apparent due to bacterial wilt, bacterial canker, and bacterial speck. Disease caused by micro invertebrate pests includes root knot, caused by the nematode, *Meloidogyne* spp.. Sustainably controlling these diseases is not easy. The current, most sustainable way, to fight against disease is the application of biological control through the use of natural enemies of pathogens to decrease the prevalence of disease, known as antibiosis (Baker 1987). Southern blight can reduce crop yield by more than 50% (Khettabi et al. 2004), and can be biologically controlled by inoculation with the antagonistic fungi, *Trichoderma* sp. for certain types of vegetables (Mukherjee & Raghu 1997). While research supports the biological control of *Sclerotium rolfsii* by *Trichoderma*, it is only effective between the temperatures 25°C-30°C and is therefore, not always effective (Mukherjee & Raghu 1997). Culture methods show that soil bacterium *Pseudomonas* cf. *monteilii* 9, strains were able to produce non-volatile diffusible metabolites and were able to inhibit *Sclerotium*

rolfsii growth 100% (Rakh et al. 2011).The root-knot nematode, *Meloidogyne* spp., causes 5% of worldwide crop losses (Hussey & Janssen 2002) and is considered the most deleterious of the ten important genera of parasitic nematode (Sasser & Freckman 1987). *Fusarium* wilt, *Fusarium oxysporum* f. spp, lacks effective fungicide treatments (Borrero et al. 2004) and there are no commercially acceptable tomato cultivars with adequate resistance (Jones et al. 1991).

A significant amount of time and skill is needed to assess soil microbial communities and disease development results (Doran & Zeiss 2000). Densities of culturable bacteria/fungi are estimated by ten-fold dilutions of soil spread on nutrient agar, followed by incubation and tedious counting of colony forming units (CFUs) (Abrams & Mitchell 1980).

454 pyrosequencing and quantitative real time polymerase chain reaction (Q-PCR) of the 18S ribosomal RNA gene reveal a significant difference between the soil fungal community membership and structure, between organic and conventionally managed potato crops in Colorado. More specifically there was a higher diversity, evenness, and abundance of the fungal pathogen *Pythium ultimum* in organically managed soil eukaryotic communities, and a higher abundance of *Alternaria solani* in the conventionally managed soil (Sugiyama & Vivanco 2010). Denaturing gradient gel electrophoresis (DGGE), an alternative to cloning and sequencing, shows that complex (compost plus dung) organic carbon amendments slightly but significantly delayed disease development of *Fusarium* wilt on flax and directly correlated with higher pH levels when compared to simpler applications (slurry, compost, or slurry plus dung) (Senechkin et al. 2014). When studying soil microbial diversity, sequencing can be very time consuming while DGGE requires highly skilled personnel. By grouping together genetically similar clones, restriction fragment length polymorphism, (RFLP), has increased efficiency by serving as an intermediate technique, lowering the sequencing reactions needed for soil microbial population

assessment (Ramos et al. 2010). Dot blot hybridization and PCR-based assays has provided a highly sensitive and reliable tool for the detection and differentiation of corn smut disease caused either by *Ustilago maydis* or *Sporisorium reiliana* (Xu et al. 1999). Genomic in situ hybridization (GISH), similar to FISH, is a laboratory technique that has allowed scientists to identify parental genomes of intergeneric and interspecific hybrids in *Rhododendrons* (Czernicka et al. 2010), *Brassica* (Snowdon et al. 1997), and *Lilium* (Barba-Gonzalez et al. 2005).

Linkage between soil borne disease and microbial community structure is not completely understood but research supports a connection between management practices and disease incidence (Chellemi et al. 2012; Burton et al. 2010). Microbial inoculants contain a great potential for sustainable management for agricultural pathogens, however, this field of study has not yet been investigated enough to provide any products for wide scale use (Thomashow 1996). Proper manipulation of the microbial community structure can decrease the abundance of plant pathogens through competition for space, nutrients, and metabolic functions (Harrier & Watson 2004; Mazzola 2004). The application of organic manure including the antagonistic microorganisms *Bacillus subtilis, Paenibacillus polymyxa,* and *Trichoderma harzianum* has been shown to suppress *Fusaruim* wilt by 83% in cucumber cultivation (Qiu et al. 2011), and of southern blight disease by 58-73% in tomatoes (Curtis et al. 2010) and this significantly reduced yield losses compared to when an organic fertilizer was used on its own. Compost amendments that cause pH increases are the only consistent factors found in the suppression of some diseases (Noble 2011). The pH of the soil moderates the accessibility of many nutrients including iron, phosphorus, magnesium, manganese, copper, and zinc (Cotxarrera 2002; Alabouvette 1999) and can predict up to 91% of the variation in severity of soil borne diseases (Borrero et al. 2004). The PH level is an important index for soil pathogens infecting tomatoes because these ionic

micronutrients will be unavailable for consumption, and future reproduction of pathogens (Mazzola 2002).

This study hypothesized that there are differences in the microbial community structure between different agricultural soils in southeast Georgia. The objective is to observe the presence/absence of one particular soil-borne pathogen: southern blight (*Sclerotium rolfssi*). Measurements of the abiotic components such as pH, organic matter rate, NO_3- etc., of the soil will be included in analyses between practices. An analysis of relationship between the biotic factors, abiotic factors, and natural development/suppression of particular disease will be linked to management practice. A greenhouse experiment including an inoculation with disease causing microorganisms used molecular techniques and growth measurements to quantify the effects of disease. We hypothesize that farm location and management history will have a significant impact on the microbial and chemical characteristics of the soil. This project is important because it helps fill in gaps in the knowledge of human management impacts on the ecology of agricultural soil and the influence these inputs have on the development of disease and the crop yield of tomatoes. Ecological research focusing on the effect of anthropogenic land management can define sustainable human interaction with nature (Odum 1969).

Chapter 2

Title: Similarities of microbial community structures and chemical components within organic and conventional agricultural soil

Abstract: Agricultural practices affect soil microbial communities and soil health through the input of pesticides, herbicides, and fertilizers. By examining the microbial community structure, we analyzed how different microbial species respond to the environment that organic and conventional farming practices create. A comparative study of individual tomato production systems was conducted by analysis of soil microbial community structure from two organic and two conventional farms in Southeast Georgia for years 2012 and 2013. The molecular method, length heterogeneity polymerase chain reaction (LH-PCR), was applied to analyze soil fungal, bacterial, and animal communities. The results indicated that the fungal, bacterial, and animal communities were unique to the individual farming locations (ANOSIM $P<0.0001$) for 2012 and 2013. The overall chemical characteristics were significantly different between farming locations (MANOVA, $P<0.0001$) for both years. Out of the characteristics showing a significant difference between farm for 2012 and 2013, organic matter and ENR showed the greatest correlation (greater than 30%) to the fungal (2012 & 2013), and bacteria (2013 only) community structures. In conclusion this study suggests that these individual farms had an effect on the microbial community structure and chemical components of agricultural soil.

Key words: Microbial community structure, Length-heterogeneity polymerase chain reaction, organic, conventional

1. Introduction

Agricultural soil supports an abundance of life that is not limited to the crops being harvested. The number of bacteria species per gram of soil has been estimated to be between 2,000 and 8.3 million (Gans et al. 2005; Schloss & Handelsman, 2006; Ingham et al. 1985). The most abundant groups of soil bacteria consist of *Bacteroidetes, Betaproteobacteria,* and *Alphaproteobacteria* (Roesch et al. 2007). The essential functions of the soil microorganisms involve the natural, tightly linked processes of the carbon and nitrogen cycles including, decomposition of organic matter, as well as the mineralization and immobilization of nitrogen (Bloem et al. 1995; Berthrong et al. 2013; Bååth & Anderson, 2003).

There are many ecological factors that alter microbial community structures within the soil substrate. Agricultural management practices have the greatest effect on microbial diversity and community structures in soils (Jangid et al. 2008). According to a redundancy analysis of phospholipid fatty acid (PLFA) patterns, nearly 65% of the variance in microbial communities can be attributed to treatment factors affecting pH, soil organic carbon and total nitrogen levels e.g., farming system (organic/ conventional) and crop rotation patterns used (Esperschutz et al. 2007). Targeting bacterial 16S and fungal 28S rRNA, terminal restriction fragment length polymorphism (T-RFLP) supports that soil management leaves a longer lasting effect on microbial community diversity than substrate addition by allowing greater microbial nitrogen mineralization, and bacteria diversity in organic management when compared to conventional farming (Berthrong et al. 2013). Low input or organic systems support greater soil productivity (Reganold et al. 1987), microbial activity (Reeve et al. 2010), and enhance nutrient cycling by consistently supporting a higher level of genes encoding for the carbon (α-amylase, pullulanase, arabinofuranosidase, xylanase, cellobiase, endochitinase, isocitrate lyase, malate synthase,

limonene-1,2-epoxide hydrolase, vanillin dehydrogenase, *aclB*, CODH, Pcc, and Rubisco genes),

nitrogen (*gdh*, *ureC*, *hzo*, *nirK*, *nirS*, *nrfA*, and *nifH*, genes), phosphorus and sulfur cycles

(*ppx*, *aprA*, *dsrA*, and *sox* genes) than in conventional systems (Xue et al. 2012). Based on the

functional genes listed above, the conventionally managed soils showed a lower proportion of

unique genes (13.6%), compared to the organic (19.1%) (Xue et al. 2012). Organically managed

plots also maintain a more stable microbial C-to-N ratio while conventionally managed plots

undergo more fluctuation (Gunapala & Scow, 1998).

Molecular methods have revealed greater diversity in soil microorganisms than

traditional methods. Culture-dependent methods only represent ~7% of total soil microbial

diversity when compared to molecular techniques such as cloning (Smith et al. 2001). Use of

molecular methods has proven to be an effective tool for assessing the dynamics of soil

microbial communities. Research suggests that Length Heterogeneity Polymerase Chain

Reaction (LH-PCR) is a valuable method for profiling microbial diversity and dynamics (Tiirola

et al. 2003). The LH-PCR molecular method has been used to clearly separate organic soils and

inorganic soils community structures based on genetic differences while sevidence shows that

organic inputs allow the soil to support higher eubacteria community diversity than chemical

fertilization does (Balachandar et al. 2012). LH-PCR has been a successful laboratory method

used to analyze diversity in soil microbial communities affected by cadaver decomposition

(Moreno et al. 2011), and in industrial wastewater (Tiirola et al. 2003). Various limitations

continue to exist in molecular techniques. PCR based approaches house limitations due to

variable efficiencies during DNA extraction and amplification, and due to the specificity/G-C

content of DNA primers (Kelly 2003; Ramos et al. 2010; Smith & Osborn 2009).

The objectives of this project are: to analyze diversity and communities of soil organisms

(bacterial, fungal, and animal community) between organically and conventionally managed soil, to compare soil abiotic characteristics between organically and conventionally managed soils, and to assess the correlation between these abiotic characteristics and the microbial communities. The hypothesis of this project was that there was a significant difference in the microbial community structures and the physical environment in the soil of organic and conventionally managed farms. The similarity of soil microbial community structure between two organic and two conventionally managed farms located in southeast Georgia were analyzed. LH-PCR was used to assess soil bacterial, fungal, and animal communities. The abiotic components such as pH, organic matter rate, NO_3^- etc., of the soil were measured and analyzed between practices and correlated to the microbial communities. This ecological research will study the effects of human management on the environment and could provide insights on how to interact with nature in a more sustainable and symbiotic way (Odum, 1969).

2. Materials & Methods

2.1 Study sites

Study sites consisted of two organic and two conventional farms located south of the Fall line in the coastal plains of Georgia. Each of the four farms sampled were located in the surrounding areas of Statesboro, Georgia. The Berry (32'16"N, 82'30"E) and Acacia (32'35"N, 82' 31"E) farms followed organic management practices, and the Strickland (32'31"N, 81'70"E) and Honeydew (32'18.8"N, 50'0.8"E) farms used the conventional management practices. These four farms were chosen because of their location, management practice, and cultivation of *Solanum lycopersicum* (tomatos) in the fall season of 2012 & 2013. Variables such as soil texture, field location, and growing season can affect interpretation of results. The growing

season was controlled for in sampling. Classification of soil textures was determined by United States Department of Agriculture textural triangle (Brown, 2003). (Table 2.1.)

2.2 Sampling

Soil collected in fall 2012: Soil was collected from the rhizosphere of the tomato crop, up to 20 cm deep and temporarily stored in partially sealed plastic bags on ice packs until permanently stored in a 4˚C cooler. Soil samples were haphazardly collected for the fall of 2012. Approximately 10 grams per sample was collected, 5 samples were collected for each strain of tomato, depending on availability. (Table 2.1).

Soil collected in late summer/early fall 2013: Soil was collected the same way as year 2012 with the following changes: a soil core was used to sample up to depths of 10 cm, 10 subsamples were taken for each sample to ensure that each sample was a good representative of the entire field, the subsamples were mixed together to equal one sample and the samples were randomly chosen by setting up a grid and generating random numbers. (Table 2.1).

2.3 DNA extraction

Microbial DNA was extracted from 0.5 grams of collected soil using a PowerMax® Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) per supplier instructions. To avoid incorrect results due to patchy distribution of organisms, the soil sample was mixed before extraction of a large sample. Extracted DNA was stored in a -20˚C freezer until further use.

2.4 LH-PCR

Microbial communities were then assessed using Length Heterogeneity Polymerase Chain Reaction (LH-PCR). Fragments sizes were measured and relative abundance of each species' particular fragment length was calculated. Fragments of the animalia 18S rRNA gene were amplified using specific primers, which included rotifers (Wu et al. 2009, 2011). A reverse

primer was used to amplify fragments that were less than 500 bp amplicons to ensure accuracy of the LH-PCR analysis. The forward primers 18S 11m (5'-GTCAGAGGTTCGAAGRCG-3') corresponded to positions 1037-1054 of the human sequence (NR_003286 in GenBank) and to a region that is relatively constant among animalia, but had positions that vary in other eukaryotes. The reverse primer 18S0r (5'GGGCATCACAGACCTGTTATTGC-3′) corresponded to positions 1480-1502 of the human sequences. Animalia PCR was ran for 30 cycles. Bacteria 16S rDNA was amplified from extracted DNA using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 355R (5'-GCTGCCTCCCGTAGGAGT-3'). Bacteria PCR was ran for 25 cycles. Fungal DNA was amplified using the primers NSI1 (5'-GATTGAATGGCTTAGTGAGG-3') and 58A2R (5'-CTGCGTTCTTCATCGAT-3'). The forward primer in each category was labeled with 6-FAM fluorescent dye. Fungal PCR was ran for 25 cycles. These location of these replications were chosen based on the conservation of the gene sequence and the existence of natural variation between species (Table 2.2)

2.5 Fragment Analysis

After amplification denatured DNA, formamide, and a 500 bp size standard was combined for the fragment analysis. The fragments were analyzed by the Applied Bio-systems Genetic Analyzer 3500 (Applied Biosystems, Foster City, CA). Relative abundance was then calculated for each sample. Each fragment size was assigned to operational taxonomic units (OTUs) using a $\pm$ 0.5 base pair (bp) criterion which, likely lumped together individual species. Cluster analysis was used to compare microbial bacterial, fungal, and animalia communities in soil samples using PRIMER-E (Plymouth Marine Laboratory, UK) software. Cluster analysis allowed for a visually quantitative diagram for comparison of species between and among management practices.

2.6 Chemical Analysis

The chemical analysis was performed by A&L Plains Agricultural Laboratory, Inc (Lubbock, TX) for years 2012 and 2013. The abiotic components measured were organic matter, estimated nitrogen release (ENR), potassium ppm, magnesium ppm, calcium ppm, soil pH, cation exchange capacity, nitrate, K% base saturation, Mg% base saturation, and Ca% base saturation.

2.7 Statistical analysis of bacteria, fungal, and animal communities for years 2012 & 2013

Statistics analyzed by PRIMERe software

The cluster analysis used to represent the bacteria, fungal, and animal community structures were analyzed using the Analysis of similarities (ANOSIM) to test statistically whether there was a significant difference between the two groups of sampling units (organic and conventional management). Diversity, richness, and evenness of the microbial communities were generated by PrimerE and then analyzed using the ANOVA parametric test. Similarity percentages (SIMPER) were used to break down the contribution of each species to the observed similarity between management types. SIMPER allowed for identification of each particular fragment length (species) that were most important in creating the observed pattern of similarity. BIOENV was used to find the correlation between community similarities and environmental factors.

Statistics analyzed by JMP software

Multivariate analysis of variance (MANOVA) was used to analyze the overview of the chemical characteristics of the soil. This test was used because there were multiple dependent variables. To analyze each individual chemical component a model II ANOVA test was used to get a P value for assessment.

3. Results

3.1 Results for the 2012 bacteria, fungal, and animal community structures

For the soil samples collected in 2012, there was an overall species similarity level of approximately 18% for bacteria. The organic samples showed a higher level of genetic similarity to other organic samples (~50% similarity) while the conventional samples only had a similarity level of ~18%. Overall, there was a lot of overlap in the separation of each farm and management practice. (Figure 2.1).

For the 2012 fungal community, samples from each farm showed a higher level of species similarity to samples from the same farm, Berry (40% similarity), Acacia (30% similarity excluding one outlier), Honeydew (40% similarity excluding 3 samples as outliers), & Strickland (40% similarity) in the cluster analysis. All farms shared a species similarity of 25%. The conventional samples shared a 30% similarity, while the organic samples shared a 28% similarity. (Figure 2.2).

For the 2012 animal cluster analysis there was an overall species similarity level of 5% for all samples. There was only clear separation due to location for the Honeydew farm. (Figure 2.3).

For year 2012, there was a significant difference between farms in richness, for the bacterial and fungal community. There was a significant difference in evenness for the animal community. There was a significant difference in diversity for the animal communities. (Table 2.5). ANOSIM gave a significant value for 2012 bacteria (P<0.0001, R=0.282), fungal (P<0.0001, R=0.658), and animal (P<0.0001, R=0.408) communities. (Table 2.7).

SIMPER analysis showed that the bacteria fragment found at the highest level for all four farms was fragment length 315 base pairs. For the fungal community, the fragment that

existed at the highest contribution for all four farms was at 341 base pairs. Fragment 457 base pairs were found at to have the highest percentage contribution for all four farms. (Table 2.6 A).

3.2 2013 Results for the 2013 bacteria, fungal, and animal community structures

For the 2013 bacteria cluster analysis there was an overall species similarity level of approximately 60% for all samples. The Acacia and Berry farm's showed a higher similarity level to samples taken at the same location (72% & 78% respectively) than to the organic management (60% similarity). The conventional farms showed an overall similarity level of 75%. (Figure 2.4).

For the 2013 fungi cluster analysis, there was an overall similarity level of approximately 40% (ignoring one sample as the outlier). The conventionally managed Strickland farm was the only farm that grouped all 5samples together at a similarity level of 60%. Seven out of ten Honeydew samples grouped together at 60%. The organic samples were scattered and only show a 40% similarity level to samples from the same location, same management, and conventional management. (Figure 2.5).

For the 2013 animal cluster analysis, all samples showed a similarity level of approximately 20%. All samples were scattered and showed no pattern regarding location or management practice. (Figure 2.6).

For year 2013, there was a significant difference between farms in richness and evenness for all three communities. There was a significant difference in diversity for the bacterial and animal communities. (Table 2.5). ANOSIM gave a significant value for 2013 bacteria ($P<0.0001$, $R=0.903$), fungal ($P<0.0001$, $R=0.545$), and animal ($P<0.0001$, $R=0.384$) communities due to farm location.

SIMPER analysis showed that the bacteria fragment found at the highest level for all four farms was fragment length 317 base pairs. For the fungal community, the fragment that existed at the highest contribution for all four farms was at 341 base pairs. Fragment 458 base pairs were found at to have the highest percentage contribution for all four farms. (Table 2.6 B).

3.3 Statistical results for the chemical analysis

The chemical characteristics were significantly different between farm locations for year 2012, MANOVA fit (P<0.0001). (Figure 7A & 7B). According to the Model II ANOVA test, the individual characteristics that were significantly different between management types were organic matter (P<0.0001), estimated nitrogen release (P<0.0001), K parts per million (ppm) (P=0.0002), Mg ppm (P<0.0001), Ca ppm (P<0.0001), cation exchange capacity (P=0.0402), K% base saturation (P<0.0001), Mg% base saturation (P<0.0001), and NO_3^- (P=0.0479). (Table 2.3A).

The chemical characteristics were also significantly different between farm locations for year 2013, MANOVA fit (P<0.0001). (Figure 2.8A & 2.8B). The results of the Model II ANOVA test gave a significant values for organic matter (P<0.0001), estimated nitrogen release (P<0.0001), K ppm (P<0.0001), Mg ppm (P<0.0001), Ca ppm (P<0.0001), cation exchange capacity (P<0.0001), K% base saturation (P<0.0001), Mg% base saturation (P<0.0001), Ca% base saturation (P<0.0001), and NO_3^- (P<0.0001). . (Table 2.3B).

A total of 30 samples from 4 farms in 2012 (increments of 5-10) gave a POWER value of 1.0 (organic matter), 1.0 (estimated nitrogen release) 0.9924 (Potassium PPM), 0.9994 (Magnesium PPM), 0.9998 (Calcium PPM), 0.9999 (soil pH), 0.6685 (cation exchange capacity), 0.6413 (Nitrate), 0.9998 (K% base saturation), 0.9995 (Mg% base saturation), and 0.1771 (Ca% base saturation) for the abiotic characteristics. The power could have been increased by increasing the sample size used in this study. The chemical analysis was performed by A&L Plains Agricultural

Laboratory, Inc (Lubbock, TX).

A total of 25 samples, 300 subsamples, from 4 farms in 2013 gave a POWER value of
0.9999 (organic matter), 0.9999 (estimated nitrogen release), 0.9990 (Potassium PPM), 1.0
(Magnesium PPM), 1.0 (Calcium PPM), 0.1113 (soil pH), 1.0 (cation exchange capacity), 1.0
(Nitrate), 1.0 (K% base saturation), 1.0 (Mg% base saturation), and 1.0 (Ca% base saturation).

3.4 Correlation of microbial community structures and chemical components of soil

Using the ENVIRO-BEST analysis the correlation between microbial community
structure and individual chemical characteristics was analyzed. Estimated nitrogen release and
organic matter were the chemical components of the soil that were significantly different for both
years with the most consistent correlation to the microbial community structures. There were no
correlation values higher than 0.3 for the animal community for either year. (Table 2.4).

4. Discussion

For years 2012 and 2013, the soil bacterial, fungal, and animal community structures were
significantly different for organic and conventional management. This conclusion supports our
hypotheses and is consistent with previous research results that microbial community structure
can be dependent on individual farm management (Balachandar et al. 2012; Liliensiek et al.
2012; Enwall et al. 2007; Jangid et al. 2008). The animal community clusters appear scattered
with no organization due to farm. In support of the organization of the bacterial and fungal
clusters, and with a lack of support for the animal clusters, the analysis of similarities (ANOSIM)
showed significant differences between each management and each farm for the 2012, and 2013
field season, (P<0.001), supporting the hypotheses that management and location of farms can
support different microbial community structures.

Organic matter concentrations were significantly higher in the farms who organically

managed the soil, and highly correlated (greater than 30%) to 2012 bacterial, and 2013 bacterial and fungal community structures. This data suggests that organic production is better at maintaining soil productivity by reducing erosion and stabilizing the topsoil through use of micro biotic crusts, which help retain nutrients in the topsoil (Reganold et al. 1987; Eldridge & Greene, 1994). This conclusion was made because this study in addition to others has concluded that organic farming practices support a higher level of organic matter than conventionally managed soils (Birkhofer et al. 2008). The significant difference in soil pH was not consistent for the two sampling years. The pH of the soil moderates the accessibility of many nutrients including iron, phosphorus, magnesium, manganese, copper, and zinc (Cotxarrera, 2002; Alabouvette, 1999). For this reason, pH is an important index for soil pathogens infecting the tomato since these micronutrients will be unavailable for consumption, and future reproduction of the pathogen (Mazzola, 2002; Borrero et al. 2004). Because the pH was not consistently different, all four farms, organic or conventionally managed, were capable of inducing comparable microbial communities due to availability of nutrients, which could explain why no distinct difference could be observed in the cluster diagrams for the 2013 fungi and the 2012 and 2013 animal community (Bååth & Anderson, 2003).

ENVIRO-BEST analysis revealed a high correlation value of the ENR to the community structure of bacteria (0.3358 and 0.403 correlation value for 2012 & 2013 respectively), and for fungi (0.474 correlation value for 2013 respectively) (Table 2.4). Soil nitrogen is an essential macronutrient important for plant growth and development. These data along with previous studies suggest that soil nitrogen influences fungi and bacteria communities and is possibly directly related to nitrogen inputs through management (Lilleskov et al. 2002; Kennedy et al. 2005; Liliensiek et al. 2012: Balachandar et al. 2012).

Particular species that contributed a significant impact for each community structure was determined by the SIMPER analysis, (Table 2.6A, 2.6B). A high incidence of a single bacteria family (*Enterobacteriaceae*), regardless of management, on edible flowers was detected in a previous study (Wetzel et al. 2010) which is consistent with this study that the same species (fragment length) can contribute to individual farms and both management type species similarity values. It may be possible to explain this phenomenon by considering two factors: temperature condition and indigenous crop specific–microbial relationships (Liliensiek et al. 2012; Wardle et al. 1997) in the samples. All soil samples were taken from the same general geographic area undergoing similar rainfall, temperature, and model organism (tomato crop).

There was a significant difference between individual farms found for bacterial (2012 & 2013), and animal (2012) diversity. The literature gives conflicting ideas about whether soil microbial diversity actually affects soil function. Some studies support no consistent effect to resilience and resistance to carbon cycling during disturbance based on the diversity levels (Degens 1998; Griffiths et al. 2001A; Wertz et al. 2006; 2007) while other studies support a positive correlation for diversity and function (Griffiths et al. 2000; 2001b; Degens et al. 2001; Girvan et al. 2005; Tobor-Kaplon et al. 2005). This particular study cannot support or reject this idea. By sequencing the abundant species in the soil, future scientists could apply this idea to agricultural management to possibly adjust for soil function.

In conclusion, this study suggests that individual farms under organic and conventional farming practices harbor different soil bacterial, fungal, and animal soil communities. The inputs of each management practice significantly affect the abiotic factors of the soil. To further the knowledge that this study provides, we suggest sequencing the fragments that had the highest contribution to each community to better understand what species are present and why. A larger

sample size and incorporating soil from various crops would provide an interesting insight on the interaction of management and crop species dependent microorganisms and how this affects the community structure. Results need to be confirmed in field experiments under a variety of climatic conditions, soil types, and regional locations.

Table 2.1 A.
Summary of farm name, management practice, soil series, location, number of samples collected, and inputs for field experimental sites for 2012 and 2013.

	Acacia	Berry	Honeydew	Strickland
Management[a]	Organic	Organic	Conventional	Conventional
Soil Texture[b]	Loamy sand	Loamy sand	Sand	Sand
Location	Adrian, Ga	Vidalia, Ga	Statesboro, Ga	Brooklet, Ga
# of samples for 2012	5	10	10	5
# of samples for 2013	5	5	10	5
Strain of tomato	Betterboy	Mountain magic	Solid gold	Cristas
Chemicals used	N/A	N/A	Mancozeb + Copper; Chlorothalonil	Bidrin, Baythroid, Dual magnum, Bravo
Organic fertilizers used	Chicken litter	Rock phosphate, Magnesium, tea, fish (organic gem)	N/A	N/A
Years of farming	>5	>5	>5	>5

[a] Management histories were either conventional production systems with synthetic fertilizer and pesticide use, or organic production systems that had at least 3 years of organic amendments and no pesticide use.
[b] Classified according to the University of Florida Institute of Food and Agricultural Sciences (Brown, 2003) and based primarily on soil particle size

Table 2.2.
Summary of community specific primers used for LH-PCR.

Community	Name	Primer sequence	# of PCR cycles
Animal	11MA	5'-GTCAGAGGTTCGAAGRCG-3'	30
	18S0R	5'-GGGCATCACAGACCTGTTATTGC-3'	
Bacteria	27F	5'-AGAGTTTGATCMTGGCTCAG-3'	25
	355R	5'-GCTGCCTCCCGTAGGAGT-3'	
Fungi	NSI1	5'-GATTGAATGGCTTAGTGAGG-3'	25
	58A2R	5'-CTGCGTTCTTCATCGAT-3'	

Table 2.3 A.
Summary of Model II ANOVA test values for the 2012 chemical characteristics.

Chemical	% from farm	% from within	P- Value
Organic matter	77.57	22.43	<.0001*
Estimated nitrogen release	78.21	21.79	<.0001*
K ppm	54.23	45.76	0.0002*
Mg ppm	62.72	37.28	<.0001*
Ca ppm	64.85	35.15	<.0001*
Soil pH	67.9	32.1	<.0001*
Cation exchange capacity	23.28	76.72	0.0402*
K% bs	65.66	34.32	<.0001*
Mg % bs	62.83	37.17	<.0001*
Ca% bs	-4	104.35	0.5615
NO_3^-	21.83	78.17	0.0479*

[a] bs is abbreviation for base saturation
*represents a significant differences between farms

Table 2.3 B.
Summary of Model II ANOVA test values for 2013 chemical characteristics.

Chemical	% from farm	% from within	P- Value
Organic matter	71.63	28.27	<.0001*
Estimated nitrogen release	71.65	28.35	<.0001*
K ppm	66.51	33.49	<.0001*
Mg ppm	81.67	18.33	<.0001*
Ca ppm	94.18	5.82	<.0001*
Soil pH	-11	111	0.7722
Cation exchange capacity	90.94	9.06	<.0001*
K% bs	96.35	3.65	<.0001*
Mg % bs	78.84	21.16	<.0001*
Ca% bs	89.59	10.41	<.0001*
NO_3^-	76.44	23.56	<.0001*

[a] bs is abbreviation for base saturation
*represents a significant differences between management practices

Table 2.4.
Summary of ENVIRO-BEST data for 2012 & 2013 for bacterial, fungal, and animal communities. Included are only environmental factors at a correlation value >0.30.

Year	Community	Variable	Correlation
2012	Bacteria	ENR	0.338
		Organic matter	0.321
	Fungi	-	-
	Animal	-	-
2013	Bacteria	NO_3-	0.571
		Ca ppm	0.57
		C.E.C.	0.565
		Mg ppm	0.428
		ENR	0.403
		Ca% bs	0.392
		Mg% bs	0.386
		Organic matter	0.373
	Fungi	ENR	0.474
		NO_3-	0.456
		Organic matter	0.425
		Ca ppm	0.403
		C.E.C.	0.393
	Animal	-	-

-Represents that none of the chemical characteristics measured had a correlation value of >0.30.

Table 2.5.
Summary of ANOVA P-values used to compare the richness, evenness, and diversity data calculated by PrimerE for 2012 and 2013.

Year	Community	Richness	Evenness	Diversity
2012	Fungi	0.0277*	0.6982	0.234
	Bacteria	0.0005*	0.3138	0.0273*
	Animal	0.7546	0.0468*	0.7237
2013	Fungi	0.0182*	0.0045*	0.1213
	Bacteria	<0.001*	0.0013*	0.0054*
	Animal	0.0006*	<0.001*	<0.001*

*Represents values that are statistically different.

Table 2.6 A.
Summary of the SIMPER analysis analyzing the contribution of each fragment size made to each community structure for 2012. The top 3 contributors from each community from each farm is included in the table to easily see that there is no clear pattern to dominant species for the community or management.

SIMPER					
Community	Species	Acacia 2012	Berry 2012	Honeydew 2012	Strickland 2012
Bacteria	317	15.18	10.99	4.76	6.92
	315	16.56	13.43	6.28	8.04
	329	6.84	3.66	8.37	-
	340	9.11	6.74	2.19	2.06
	341	-	-	9.99	5.71
	360	-	-	10.74	-
	346	3.56	7.71	2.99	7.52
Fungi	341	10.87	4.35	8.28	12
	332	-	-	1.08	7.54
	343	-	5.08	7.63	6.42
	328	4.58	-	1.71	2.33
	357	2.68	7.59	1.64	2.11
	350	3.52	5.75	-	0.58
	356	1.38	5.65	0.92	-
	340	-	-	11.85	-
Animal	460	14.32	15.98	-	32.69
	457	23.07	16.24	28.24	27.47
	455	-	2.79	-	8.96
	456	6.37	10.95	-	3.59
	461	5.28	-	36.08	-
	458	-	22.53	5.71	-

Table 2.6 B.
Summary of the SIMPER analysis analyzing the contribution of each fragment size made to each community structure for 2013. The top 3 contributors from each community from each farm is included in the table to easily see that there is no clear pattern to dominant species for the community or management.

SIMPER					
Community	Species	Acacia 2013	Berry 2013	Honeydew 2013	Strickland 2013
Bacteria	317	15.45	7.6	11.52	12.04
	328	10.94	1.54	5.15	4.29
	341	6.35	-	12.44	12
	315	-	13.17	11.51	11.9
	342	3.34	10.36	-	-
	348	4.77	8.34	2.72	5.61
Fungi	381	2.97	-	0.85	1.68
	388	2.62	1.63	1.48	1.14
	382	2.59	-	0.7	1.72
	331	-	3.34	3.26	3.49
	358	0.73	3.18	1.67	2.34
	306	-	2.88	0.82	-
	341	1.17	1.67	5.61	6.41
	340	0.63	0.88	4.18	2.46
	334	1.14	1.04	3.45	1.9
	327	-	0.91	0.8	5.59
	328	1.83	1.45	-	5.27
Animal	457	17.91	-	9.32	4.91
	461	29.94	-	2.2	-
	458	33.24	12.53	1.3	19.69
	462	2.92	12.4	2.92	1.45
	442	-	3.72	-	2.68
	459	-	-	5.06	9.65
	463	-	-	3.29	2.05
	456	-	3.46	-	15.88

Table 2.7

Summary of the ANOSIM values analyzing each community at each farm.

Year	Community	P value	R value
2012	Bacteria	0.001*	0.282
	Fungi	0.001*	0.658
	Animal	0.001*	0.408
2013	Bacteria	0.001*	0.903
	Fungi	0.001*	0.545
	Animal	0.001*	0.384

*Represents values that are statistically different.

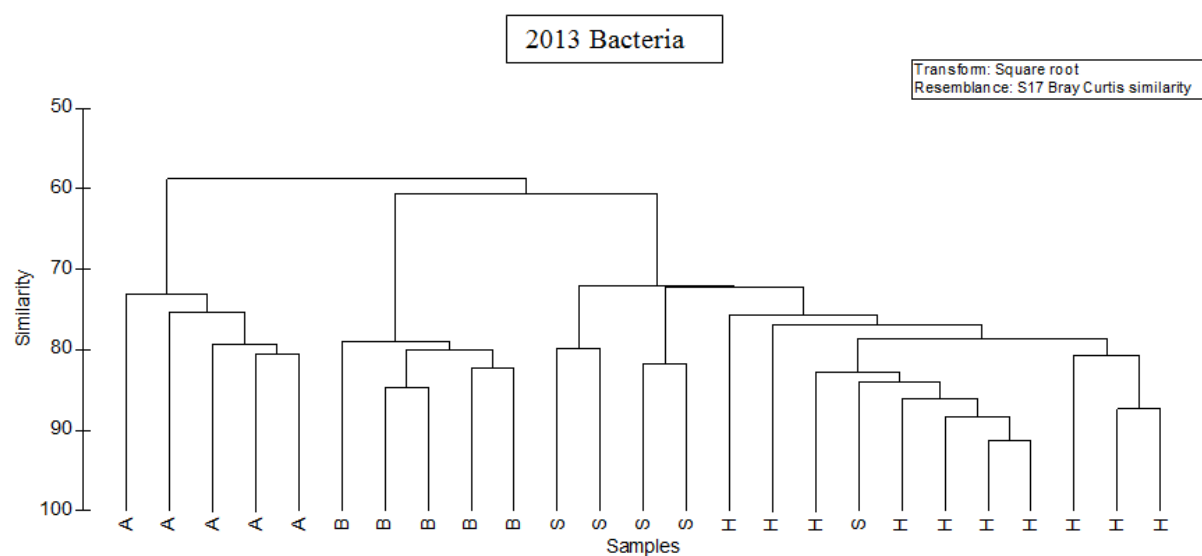**Figure 2.1.**
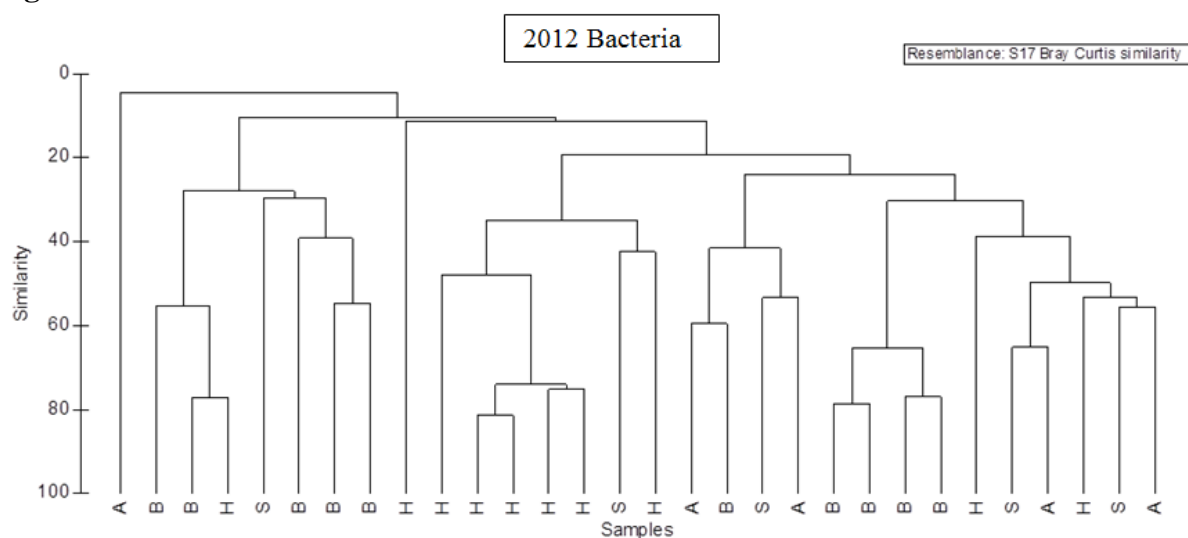
Figure 2.1.
Cluster analysis of 2012 Bacteria community based on similarity levels of LH-PCR. Organic samples are represented by the letters A (Acacia farm), and B (Berry farm). Conventional samples are represented by the letters H (Honeydew farm), & S (Strickland farm). Sample R03 was removed from the analysis due to mechanical/ technical issues during processing. Horizontal lines correspond to the abundance of OTU similarity level shared between samples.

Figure 2.2.

Figure 2.2.
Cluster analysis of 2012 Fungi community based on similarity levels of LH-PCR fragments. Organic samples are represented by the letters A (Acacia farm), B (Berry farm). Conventional samples are represented by the letters H (Honeydew farm), & S (Strickland farm). Sample R03 was removed from the analysis due to mechanical/ technical issues during processing. Horizontal lines correspond to the abundance of OTU similarity level shared between samples.

Figure 2.3.

Figure 2.3.
Cluster analysis of 2012 Animal community based on similarity levels of LH-PCR fragments. Organic samples are represented by the letters A (Acacia farm), B (Berry farm). Conventional samples are represented by the letters H (Honeydew farm), & S (Strickland farm). Sample R03 was removed from the analysis due to mechanical/ technical issues during processing. Horizontal lines correspond to the abundance of OTU similarity level shared between samples. There is no order to the animal community. Organic and conventional communities have not separated completely showing mixed animal communities for each management type.

Figure 2.4.

Figure 2.4.
Cluster analysis of 2013 Bacteria community based on similarity levels of LH-PCR fragments. Organic samples are represented by the letters A (Acacia farm), B, (Berry farm). Conventional samples are represented by the letters H (Honeydew farm), & S (Strickland farm). Horizontal lines correspond to the abundance of OTU similarity level shared between samples.
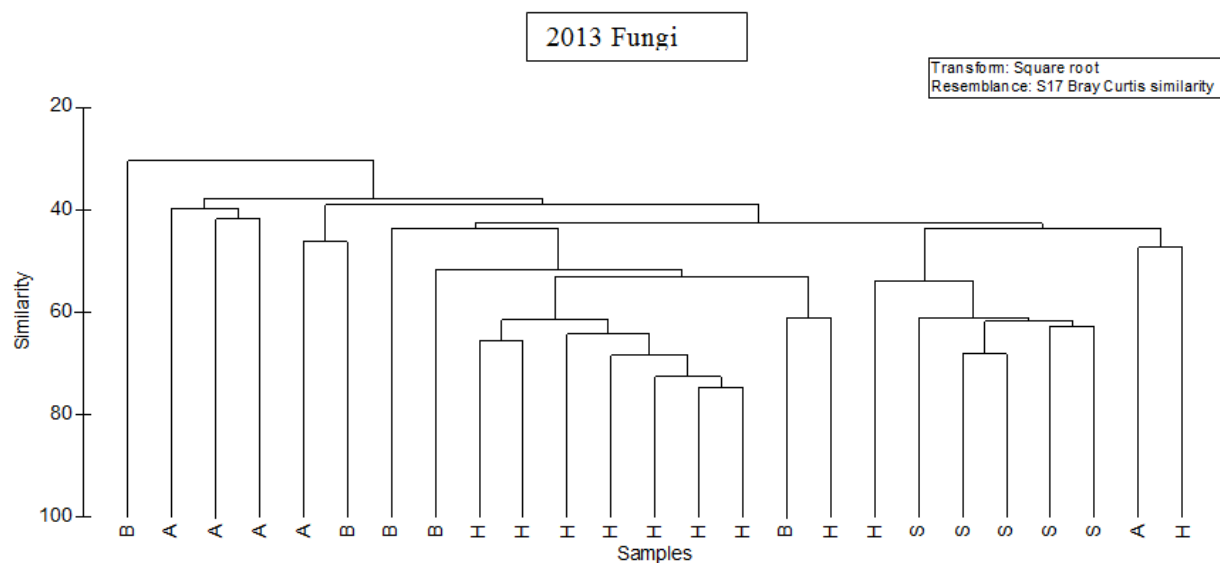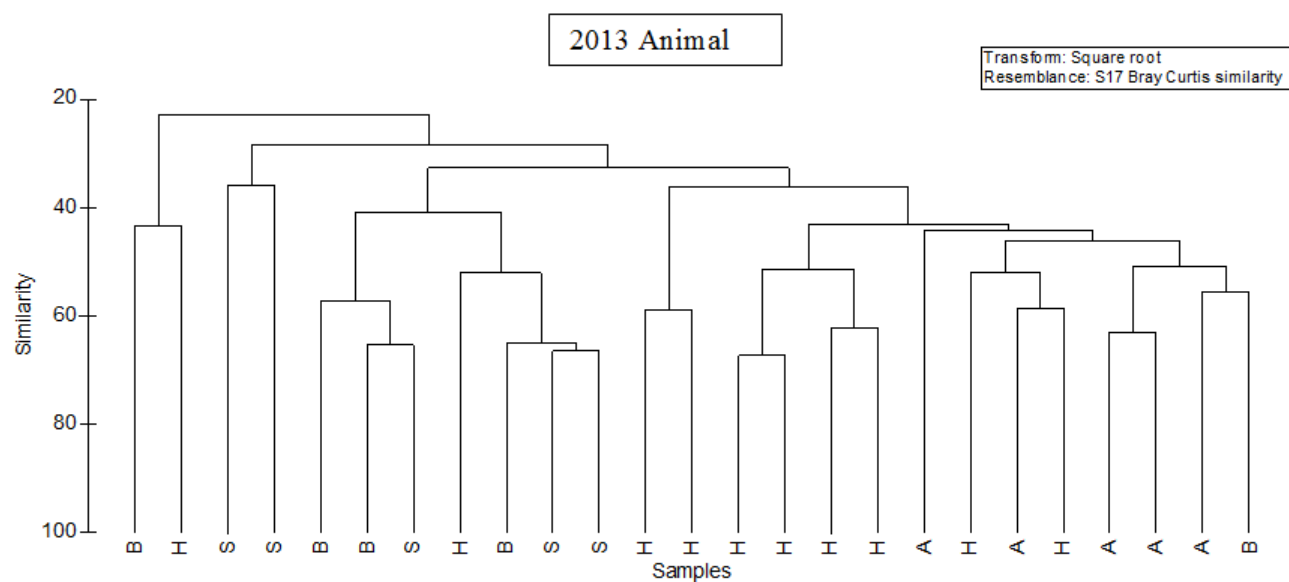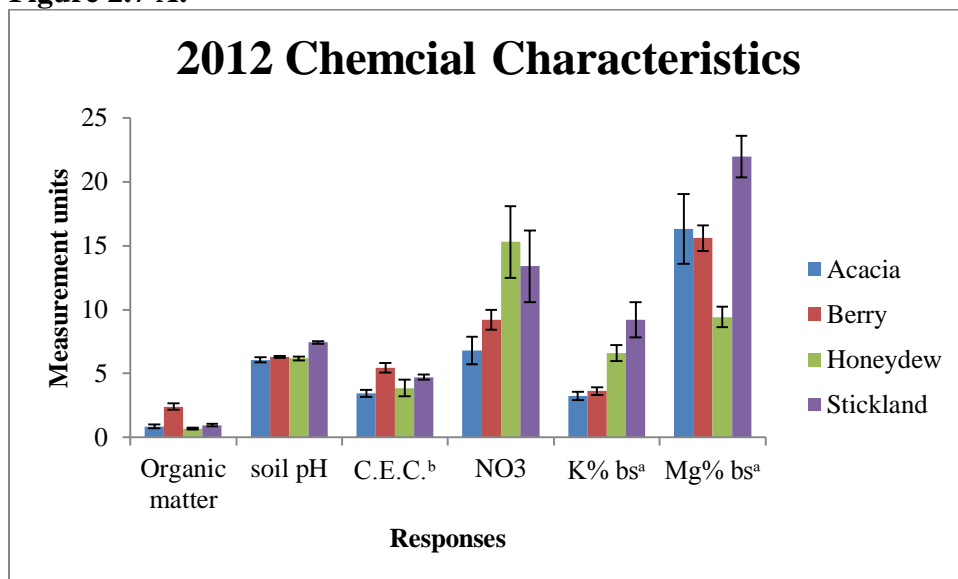
Figure 2.5.

Figure 2.5.
Cluster analysis of 2013 Fungi community based on similarity levels of LH-PCR fragments.
Organic samples are represented by the letters A (Acacia farm), and B (Berry farm).
Conventional samples are represented by the letters H (Honeydew farm), & S (Strickland farm).
Horizontal lines correspond to the abundance of OTU similarity level shared between samples.
The conventional communities have separated from the organic showing a higher similarity level
to their than management practice. All organic samples are scattered and do not show a pattern of
similarity.

Figure 2.6.

Figure 2.6.
Cluster analysis of 2013 Animal community based on similarity levels of LH-PCR fragments. Organic samples are represented by the letters A (Acacia farm), B, (Berry farm). Conventional samples are represented by the letters H (Honeydew farm), & S (Strickland farm). Horizontal lines correspond to the abundance of OTU similarity level shared between samples.
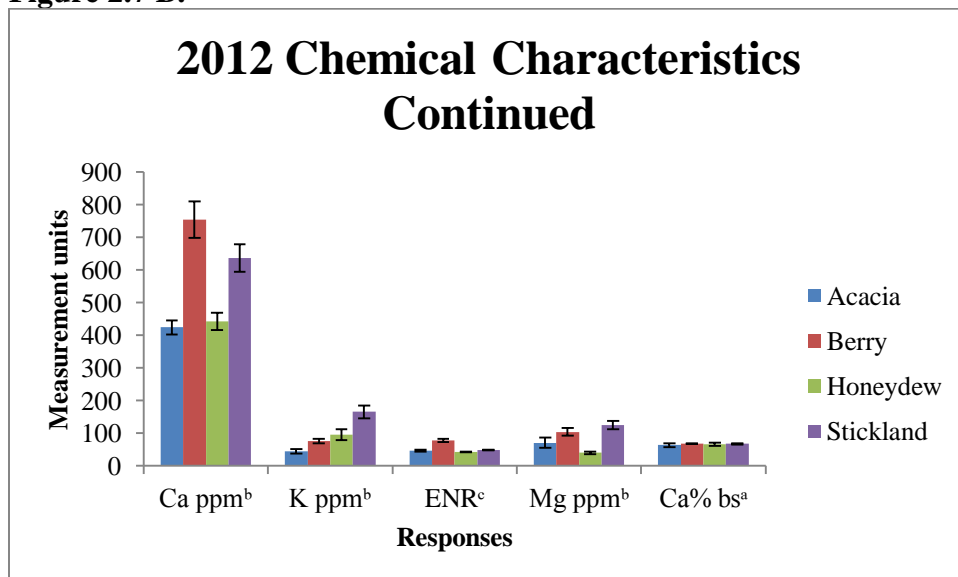
Figure 2.7 A.

2012 Chemcial Characteristics

ᵃ bs is an abbreviation for base saturation

ᵇ C.E.C. is an abbreviation for cation exchange capacity

Figure 2.7 B.

2012 Chemical Characteristics Continued

ᵃ bs is an abbreviation for base saturation

ᵇ ppm is an abbreviation for parts per million

ᶜ ENR is an abbreviation for estimated nitrogen release

Figure 2.7
Summary of chemical analysis of field soil for sampling year 2012.

Figure 2.8 A.

2013 Chemical Characteristics

[a] bs is an abbreviation for base saturation
[b] C.E.C. is an abbreviation for cation exchange capacity

Figure 2.8 B.

2013 Chemical Characteristics Continued

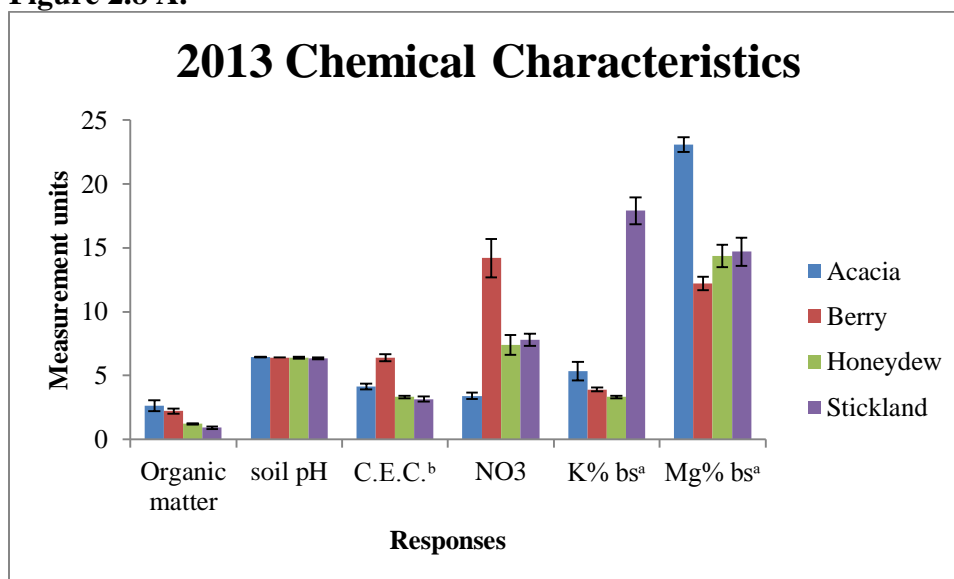[a] bs is an abbreviation for base saturation
[b] ppm is an abbreviation for parts per million
[c] ENR is an abbreviation for estimated nitrogen release

Figure 2.8
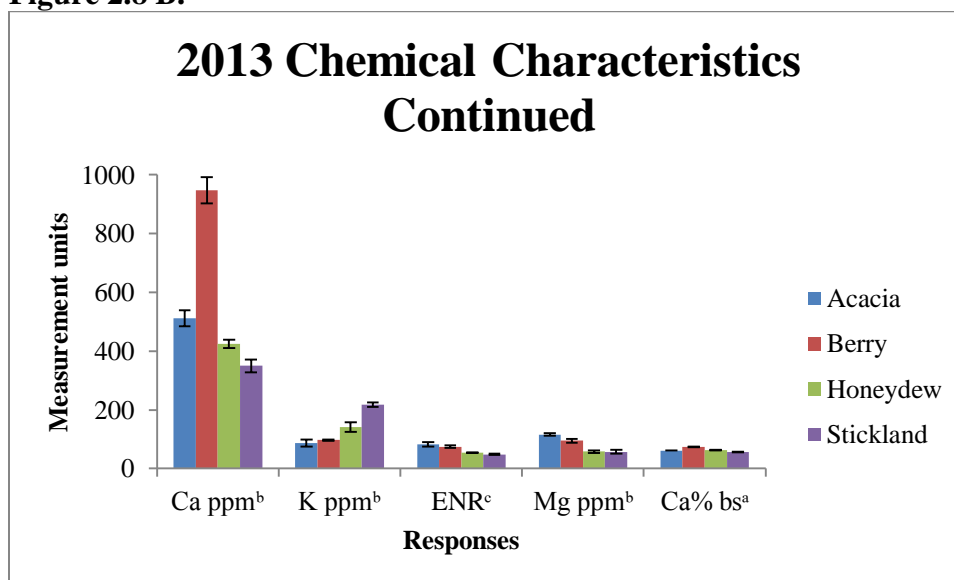Summary of chemical analysis of field soil for sampling year 2013.

Chapter 3

Title: Morphological and molecular detection of soil borne disease *Sclerotium rolfsii*, *Fusarium oxysporum*, and a contrast bacterium, *Escherichia coli*.

Abstract: Early detection of soil borne disease is essential for agricultural success. However, monitoring incidence of disease based on plant growth after pathogenic soil inoculation may not reveal the amount of pathogenic DNA in the soil. Quantitative polymerase chain reaction (Q-PCR) was used to measure the amount of soil general fungal and *Sclerotium rolfsii* DNA. Incidence of disease and growth of tomato plants grown in greenhouse soil were measured. Results indicated that the growth (height and leaf count) was not significantly different (P=0.5552 and P=0.0719 respectively) between pants grown in inoculated and un-inoculated soils, whereas, there was significantly higher amounts of total fungal and *Sclerotium rolfsii* DNA (P=0.0454 and P=0.0278 respectively) in the soil. Fluorescent in situ hybridization (FISH) was used as an alternative for visual detection of *Sclerotium rolfsii, Fusarium oxysporum*, and *Escherichia coli* through whole cell hybridization. FISH lacked the need for expensive equipment and skills needed to preform Q-PCR, such as DNA extraction and isolation. A higher hybridization signal for *Sclerotium rolfsii* were detected in soil with high *Sclerotium* DNA levels than in soil with low *Sclerotium* DNA levels.

Key Terms: Quantitative polymerase chain reaction, Fluorescent in situ hybridization, *Sclerotium rolfsii,* soil borne disease

1. Introduction

Southern blight, *Sclerotium rolfsii*, is a soil borne fungal pathogen whose fungicidal control has a harmful impact on the environment (Curtis et al. 2010). The negative effects of chemically controlling southern blight include technical and toxicological issues such as the contamination of water, the development of pathogen resistance to chemical control, and accidental human ingestion (Curtis et al. 2010). Southern blight can reduce crop yields by more than 50% (Khettabi et al. 2004), and can survive within the soil for up to 20 years, even in absence of host plants (Coley-Smith et al. 1990). The current, most sustainable way, to fight against disease is the application of biological control through the use of the natural enemies of particular pathogens to decrease the prevalence of disease, this isknown as antibiosis (Baker, 1987). Soil inoculation of varying types of bacteria and fungi has led to many discoveries about how the soil microbes affect photosynthetic rates (Zhang et al. 2013), plant growth (Wang et al. 2011), microbial diversity, and soil functioning (Bakhoum et al. 2012). A sustainable option for controlling southern blight is through inoculation of the antagonistic fungi, *Trichoderma* sp. for certain vegetable species (Mukherjee & Raghu, 1997B). Organic production has shown more populations of *Trichoderma* than on conventional farms (Bulluck III et al. 2002), which implies that organic soil might be able to better suppress incidence of southern blight disease. In the greenhouse, the plant pathogenic fungi *Sclerotium rolfsii*, known as southern blight, progressed faster and had a higher level of incidence on tomato plants grown in conventional soils rather than organic soils (Liu et al. 2008). While research supports the biological control of *Sclerotium rolfsii* by *Trichoderma*, it is only effective between the temperatures 25°C-30°C (Mukherjee & Raghu, 1997A). Culture methods show that soil bacterium *Pseudomonas* cf. *monteilii* 9, strains

were able to produce non-volatile diffusible metabolites and were able to inhibit *Sclerotium rolfsii* growth (Rakh et al. 2011).

Use of molecular methods has proven to be an effective means to assessing the dynamics of soil microbial communities. Quantitative polymerase chain reaction (Q-PCR) is a fast and effective technique for detecting and quantifying bacteria, fungi, and monitoring gene expression. Q-PCR has been successfully used in monitoring the amount of probiotic and lactic acid bacteria in dairy products (Boyer & Commbrisson, 2013), and detecting *Flavobacterium psychrophilum* in rainbow trout from naturally occurring bacteria cold water disease (Marancik & Wiens, 2013). 454 pyrosequencing and Q-PCR of the 18S ribosomal RNA gene show prudence of a significant difference between the soil fungal community and structure between organic and conventionally managed potato crops in Colorado, more specifically a higher diversity, evenness, and abundance of the fungal pathogen *Pythium ultimum* in organically managed farm soil eukaryotic communities, and a higher abundance of *Alternaria solani* in the conventionally managed farm soils (Sugiyama & Vivanco, 2010).

Fluorescent in situ Hybridization (FISH) can be used for direct visualization of microorganisms and is one of many nucleic acid techniques used for studying microorganisms in their natural environments. FISH has become a powerful technique through the development of its sensitivity and the speed of the hybridization reaction (Amann et al. 1990). FISH has been used to detect specific DNA segments of probiotic and putative pathogenic bacteria in the gut of tilapia, *Oreochromis niloticus* (Del'Duca et al. 2013), to identify, and locate bacteria in the gastrointestinal tract of young turkeys (Skowrońska et al. 2009), and to detect fluctuation in the level of bacteria in human intestinal ecosystem (Franks et al. 1998). FISH was used successfully to discover the spatial distribution and growth stages of hyphomycetes fungi in aquatic systems

(Baschien et al. 2008), and has provided the first analysis of an active bacteria community in tundra soils (Kobabe et al. 2004).

The objectives of this study were to measure how inoculation will affect physical characteristics such as, height and number of leaves, and amount of *Sclerotium rolfsii* in the soil of greenhouse grown tomato plants. We hypothesize that that inoculation of soil borne disease will increase the amount of *Sclerotium rolfsii* specific DNA in the soil and will have a significant effect of growth. In addition, this study has an objective of optimizing a Fluorescent in situ hybridization (FISH) method for visual detection of *Sclerotium rolfsii, Fusarium oxysporum,* and *Escherichia coli*. We hypothesize that FISH can be directly applied to a soil smear once the stringency is worked out through trial and error. Finally this study aimed to compare the molecular methods Q-PCR and FISH for quantifying soil borne disease in a soil smear.

2. Methods

2.1 Collection of soils & Growing of seedlings

Tomato Celebrity F1 seeds (Lot: 13355, Harris Seeds, New York) were planted in Metro-Mix 360 (Sun-Gro) growth media on Friday August 16, 2013. Seedlings were placed in a greenhouse under equal light and watering conditions. Seedlings were transplanted into pots after 4.5 weeks (9/24/2013) that contained haphazardly collected soil from two established organic (Acacia & Berry) and two established conventional (Honeydew & Strickland) farms located in the surrounding area of Statesboro, Georgia. Once the seedlings were transplanted into the 3L pots (15 pots per farm), they were given equal amounts of sun light and water. At the end of the greenhouse experiment (10 weeks) above ground biomass was measured. Pots were organized randomly in a 60 square foot area of a greenhouse and were moved every week while

measurements were being taken to ensure that the placement in the greenhouse did not cause growth or disease incidence bias.

2.2 Inoculation of Greenhouse Plants

Hard red wheat was soaked in a 1% malt extract solution overnight. The excess water was removed and the wheat was autoclaved before being introduced to pure cultures of *Sclerotium rolfsii* previously isolated from a naturally infected plant found at the Strickland farm. The mycelium was allowed to grow for nearly 2 weeks (10/3/13-10/15/13) at 25°C. Six grains, per pot, of mycelium-covered hard red wheat were placed on the top of the soil in 5 pots from each farm to inoculate the soil. Tomato seedlings were inoculated with the soil-borne pathogen, southern blight (*Sclerotium rolfssi*) on the first day of week 3 after transplantation. The height and leaf count of the plants was recorded weekly throughout the inoculation process. When watering the plants, extra precautions were used to prevent disease pathogens from traveling through the water. At the end of 10 weeks each plant was assessed on a scale of 0-5 of severity of disease. The disease index scale was from 1-5 where 0= no symptoms of basal stem lesions or wilting of leaves; 1=<20% of leaves wilted and/or 20% of basal stem with lesion; 2=20-40% of leaves wilted and/or 20-40% of basal stems with lesions; 3=40-60% of leaves wilted and /or 40-60% basal stem with lesions; 4=60-80% of leaves wilted and/or 60-80% of basal stem with lesions; and 5= >80% of leaves wilted or dead plant.

2.3 Soil Sample Collection & DNA Extraction

After completion of the greenhouse experiment, soil microbial DNA was extracted from soil samples using a PowerMax® Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad,

CA) per supplier instructions. To avoid incorrect results due to patchy distribution of organisms, the soil sample was mixed before extraction of a large soil sample made up of 5 subsamples each. Extracted DNA was stored in a -20°C freezer for up to six months before used.

2.4 Quantitative Polymerase Chain Reaction (Q-PCR)

The reaction was performed using two different primers. One primer was a general fungal primer, which replicated and measured the weight of total fungal DNA in the soil while the other was a species specific primer targeting *Sclerotium rolfsii* (Jeeva et al. 2010) to quantify the presence of the inoculated pathogenic DNA in the soil (Table 3.1). The *Sclerotium rolfsii*-specific forward primer (SCR) and reverse primer (SCR-R) amplified a 540-bp product that contained parts of the internally transcribed spacer 1 and 2 (ITS1, ITS2) and the whole 5.8S rDNA subunit. TAQ Polymerase with SYBR green (dye) was used in the Q-PCR reaction. If the primer annealed and allowed for replication then quantification was recorded in pico grams per microliter. Standards of varying concentration levels of pure *Sclerotium rolfsii* were used in the quantification process of all reactions (general fungi and species specific primers). The DNA for the standards was isolated and extracted from pure colonies grown in the laboratory on malt extract nutrient agar. The DNA used for the standards was also used as controls to test the efficiency of the primers and ensure that they were specific in detecting only *Sclerotium rolfsii*. The Q-PCR data was done in triplicate to control for mechanical and technical error. The mean of all three DNA quantities was used for statistical analysis.

2.5 Fluorescent in situ hybridization (FISH) Protocol

FISH was performed on pure cultures of one species of bacteria, and two species of fungi. Hybridization was carried out using three species specific DNA probes for *Sclerotium rolfsii, Fusarium oxysporum,* and *Escherichia coli*. (Table 3.3). Pure cultures were fixed in freshly prepared 3.7% paraformaldehyde/ phosphate buffered saline (PBS) solution for 4 hours at 4° C after undergoing mild sonication with a FB4418 microprobe attached to a 550 sonic dismembrator at a setting of 2 for 30 seconds. Twenty microliters of the fixed and dispersed samples were spotted on gelatin-coated (2% gelatin) Lab Scientific laminated slides. The samples were allowed to air-dry before the sample was heat fixed to the slide (45° C for 5 minutes), and dehydrated by serial immersion of the slides in 50%, 80%, and 96% ethanol. The FISH method was first used directly on pure cultures because there is much less auto fluorescent interference than when using a soil sample. After highly stringent hybridization temperatures were achieved, this procedure was then applied to a soil smear.

Before the hybridization occurred, the slides were stained with the universal protein stain DTAF. Freshly prepared stain solution consisted of 2mg of DTAF dissolved in 10 mL of phosphate buffer (0.05M Na_2HPO_4 with 85% NaCl, pH 9 (Sherr et al. 1987)). The staining procedure was done as described by Bloem et al. 1995, and Kobabe et al. 2004. About 20μl of the stain solution was placed on the sample and incubated at room temperature for 30 minutes, The slide was then washed three times (20 minutes each) with phosphate buffer (pH9). Finally the slide was passed through four water baths each for a few seconds and then air-dried.

All oligonucleotide probes used in this study were labeled with the cyanine dyes Cy3 or Cy5. The formamide concentration was set at 50% for all hybridizations. To calculate stringency the following formula was used: $T_m = 81.5 + 16.6 (logM) + 0.41 (\%G+C) - 0.72 (\%Formamide)$,

which calculated to be 45.7ºC for the bacterial hybridization, and 45.68ºC for the fungal

hybridizations.

Hybridizations were preformed similarly to other studies (Snaidr et al. 1997; Stahl &

Amann 1991; Kobabe et al. 2004). A 9µl aliquot of hybridization buffer (0.9 M NaCl, 20mM

Tris-HCl; pH 8.0), 1µl 0.02% sodium dodecyl sulfate (SDS), and 11µl formamide was dropped

onto each sample and pre-hybridized at 37ºC for 25 minutes to minimize non-specific binding.

After pre-hybridization 1µl of the corresponding probe at a concentration level of ~30ng/µl was

dropped onto the sample and the slide was incubated at the appropriate hybridization temperature

for 3 hours.

Slides were washed at 40ºC for 10 minutes in washing buffer (20mM Tris-HCL, pH 8.0),

5mM EDTA, 1%SDS, 1% NaCl. Finally, slides were washed in cold distilled water and air-

dried. Finally slides were mounted in Citifluor AF1 antifadent (Plano; Wetzlar, Germany) and

covered with a coverslip. Slides were viewed under a fluorescent LSM710 confocal microscope

(Carl Zeiss MicroImaging, Thornwood, NY 10594).

2.6 Statistical Analysis

Statistics analyzed by VasserStats

The physical measurements of the growth (height, leaf count, and aboveground biomass)

of the tomato plants were analyzed using the non-parametric Mann-Whitney U analysis using

VasserStats software. The non-parametric Mann-Whitney U analysis was used to analyze the Q-

PCR measurements for the *Sclerotium rolfsii* primers.

Statistics analyzed by JMP software

Q-PCR measurements were analyzed using a t-test for the general fungal primers.

 No statistical tests were run for the FISH procedure.

3. Results:

3.1 Greenhouse Growth Results

The measured physical characteristics represented a trend of control plants growing taller, and having more leaves (Figure 3.1), however, the Mann-Whitney U analysis did not give significant differences for height ($P=0.5552$), number of leaves ($P=0.0719$), or the final biomass ($P=0.1236$). Figure 3.6 shows the significant difference ($P=0.0005$) in the final disease incidence observed between the plants grown in inoculated or control soil.

3.2 Q-PCR Results

The inoculated soil harbored a significantly higher level of total fungal DNA ($P=0.0454$, Figure 3.5), and *Sclerotium rolfsii* specific DNA ($P=0.0278$, Figure 3.6) than un-inoculated soil.

3.3 FISH results

Species specific DNA probes used under the appropriate conditions helped to identify and detect *Fusarium oxysporum* (Figure 3.7), *Sclerotium rolfsii* (Figure 3.8), and *Escherichia coli* (Figure 3.9), without the expensive and time consuming process of DNA extraction and amplification. Visual quantification of *Sclerotium rolfsii* in a soil smear gave greater amount of background florescence but allowed for visual differentiation of the amount of DNA (Figure 3.10 & 3.11).

4. Discussion:

Through this study, we have discovered that *Sclerotium rolfsii* grown on hard red wheat is an appropriate way to incorporate a greater amount of specific DNA into the soil substrate. Based on the final growth that occurred in the greenhouse, it is evident that the above ground characteristics, including plant height and the amount of leaves, on a young tomato plant were not directly correlated to amount of pathogenic DNA within the soil. It is not wise to assume that

a healthy plant growing in greenhouse soil is lacking the potential to be infected with microorganisms. This statement is based on the significantly different abundance of DNA with-in the soil from different inoculation conditions, measured by Q-PCR analysis, but no significant difference in growth levels between the inoculated and un-inoculated control plants. Based on the collected greenhouse data, inoculating soil with soil borne fungal pathogen can significantly increase total level of fungal and species specific DNA present in the soil. This study did not find a significant difference in plant growth based on the presence or absence of soil borne disease inoculation. This could have several explanations including the amount of time that the seedlings were allowed to grow before inoculations took place, the length of the experiment, and the virulence of the strain of inoculum used. Inoculated soil in this study possesses a significantly higher level of disease incidence and DNA quantity of *Sclerotium rolfsii*. Therefore, Q-PCR is an effective molecular method for the prediction of inoculum potential and future incidence of disease for *Sclerotium*. This study supports other findings that quantitative polymerase chain reaction is an ideal method for detection of soil borne disease (Taylor et al. 2010: Agust-Brisach et al. 2014; Peng et al. 2013)

FISH procedures using *E. coli* could be completed (start to finish) on slides instead of the previously utilized way of preforming hybridizations in a tube (Yilmaz & Noguera, 2004) or on a membrane (Baudart & Lebaron, 2010). Whole cell hybridization experiments were also successful with the fungal species *Sclerotium rolfsii*, and *Fusarium oxysporum*. Completing hybridizations on a slide minimizes the amount and variety of expensive equipment needed and allows the procedure to be carried out *in situ* without DNA extraction. Biological auto fluorescence can be minimized by using a fluorophore with a near infrared (NIR) emission (similar to Cy 5) and extended camera exposure times (Coleman et al. 2007). While some

research showed increased fluorescent responses when the hybridization time was increased to three days (Yilmaz & Noguera, 2004), this study found that the conventional three hour hybridizations were long enough to produce signals using high stringency temperatures. The fluorescent in situ hybridization using Cy3 and Cy5, in combination with the universal protein stain DTAF is able to emit light simultaneously using the correct wavelengths. By using the protein stain in combination with a probe that emits light at a different wavelength, it is possible to use the interaction of the fluorescence to help differentiate between background fluorescence and the fluorescent signal from successful hybridization of the probe to target DNA.

In conclusion, this study supports inoculation as an effective way to increase amounts of *Sclerotium rolfsii* into the soil of greenhouse tomato plants. Q-PCR is a quick way of quantifying small amounts of DNA in the soil. FISH is an effective method of visually detecting soil microorganisms without the need for DNA extraction or isolation. Molecular detection of soil borne pathogenic fungal DNA provided evidence for predicting the potential of disease development in agricultural ecosystems.

Table 3.1.
Summary of the forward and reverse primer sequences used for replication of general and species specific primers used for Q-PCR.

Target	Name	Primer sequence
Sclerotium rolfsii	SCR	5'-CGTAGGTGAACCTGCGGA-3
	SCR-R	5'-CATACAAGCTAGAATCCC-3'
General Fungi	NSI1	5'-GATTGAATGGCTTAGTGAGG-3'
	58A2R	5'-CTGCGTTCTTCATCGAT-3'

Table 3.2.
Summary of the P-value calculated by the non-parametric Kruskal Wallis test for height, & number of leaves, t-test value for general fungal DNA amount, and the non-parametric Mann-Whitney U test for incidence and *Sclerotium rolfsii* DNA. Significant differences represented by values <0.05.

| Variable	U- Value	P-Value
General Fungi DNA (pg/µl)	[1]	0.0454*
Sclerotium rolfsii specific DNA (pg/µl)	118	0.0278*
Incidence	624	0.0005*

*represents a significant differences between treatments.
[1]No U-value is associated with this statistic because the data fits the assumptions for a normal T-test.

Table 3.3.
Summary of the species specific and group specific fluorescently labeled probes used to detect *Sclerotium rolfsii, Fusarium oxysporum* , and *Escherichia coli* during FISH.

| Name	Target	Dye	Probe sequence
SCR-R	*Sclerotium rolfsii*	Cy3	5'-CATACAAGCTAGAATCCC-3'
ALF968	*Escherichia coli*	Cy3	5'-GGTAAGGTTCTGCGCGTT-3'
CLOX2	*Fusarium oxysporum*	Cy5	5'-CTTGTCAGTAACTGGACGTTGGTAC-3'

Figure 3.1.

Greenhouse Growth Data
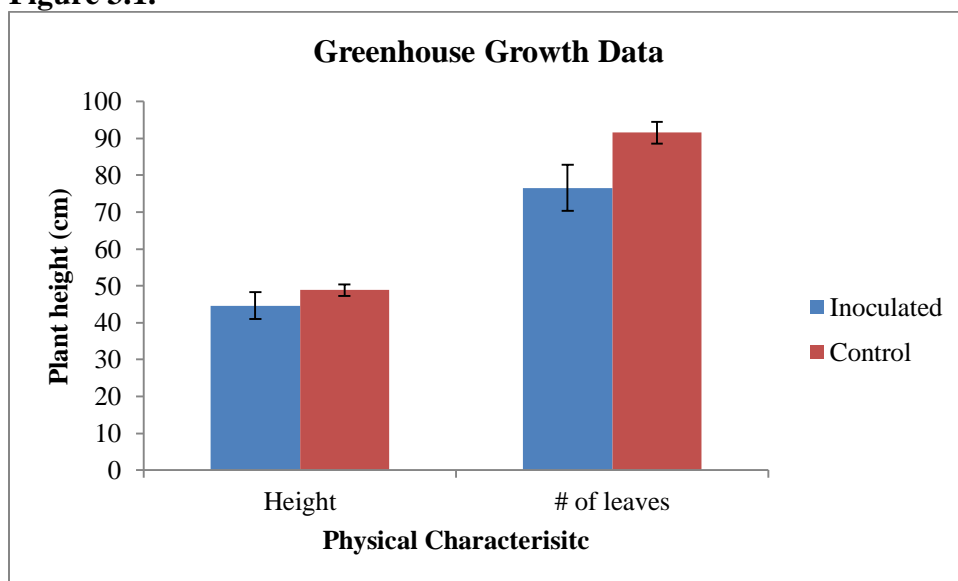
*Based on the mean of 20 total inoculated plants, and 40 total control plants.

Figure 3.1.
Summary of the height (cm), and leaf number observed in tomato plants after 10 weeks in a greenhouse setting. There is not a significant difference in the number of leaves for the inoculated and control plants, P=0.0719, or between height P=0.2108. Bars represent standard error. Data was lumped to give a more accurate representation of what to expect when inoculating soil using this method described.

Figure 3.2 A.
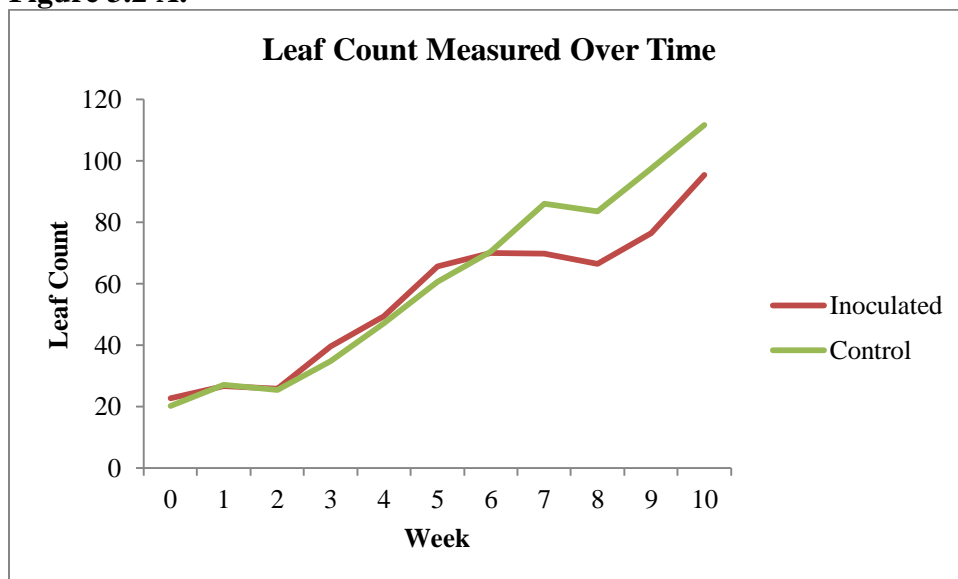
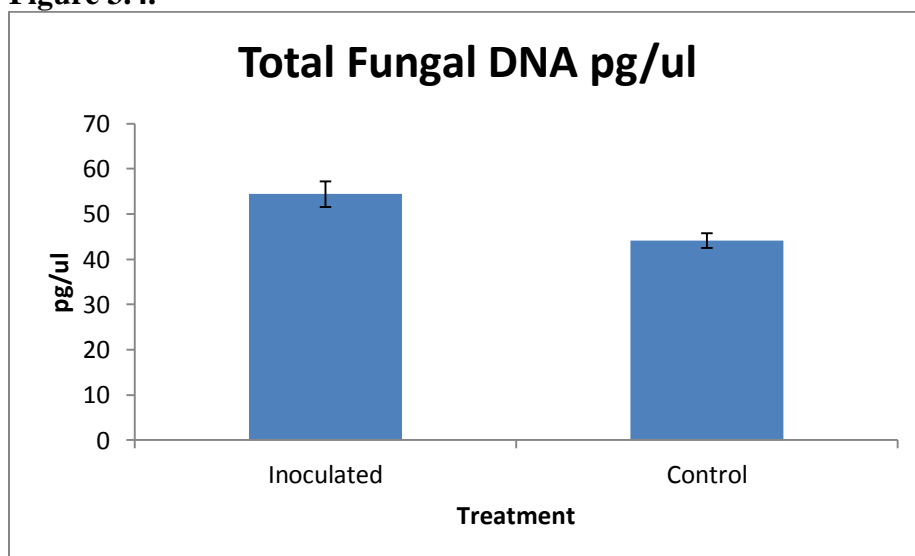**Figure 3.2 B.**
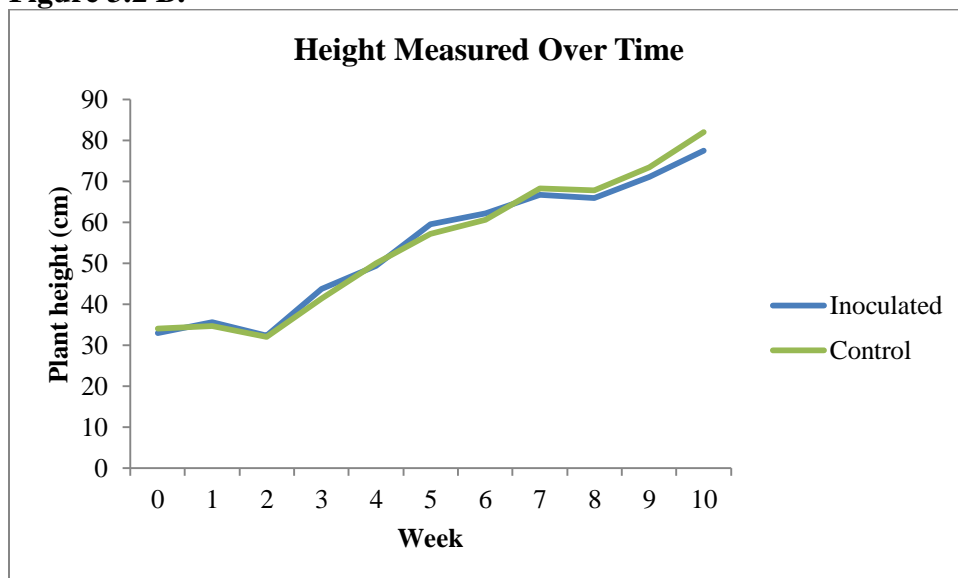
Figure 3.2.
Growth curve of leaf number (A) and height (B) summarizing the mean of the weekly measurements taken during the 10 week greenhouse experiment. Week 0 represents the seedling leaf count and the seedling height during transplantation into a pot.
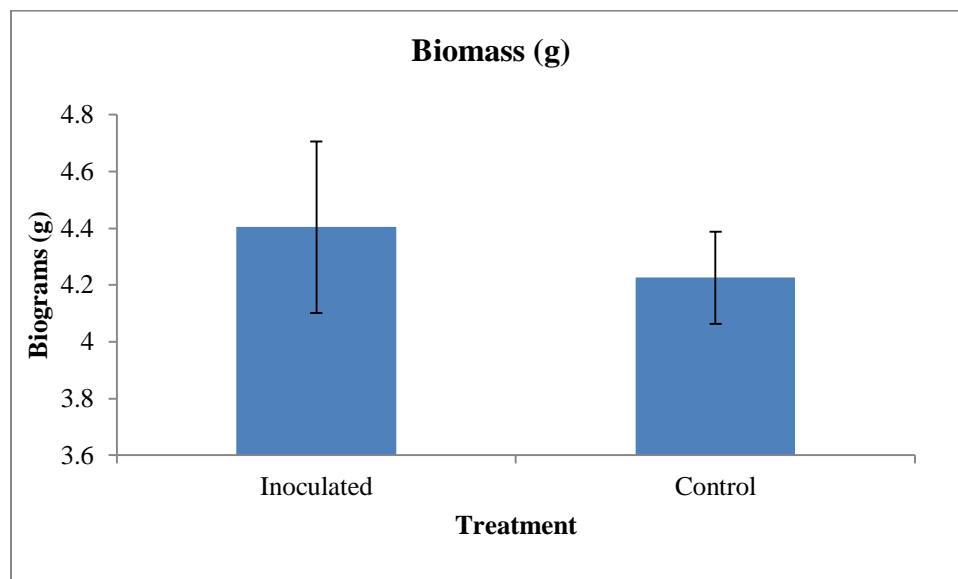
Figure 3.3.

Biomass (g)

*Based on the mean of 20 total inoculated plants, and 40 total control plants.
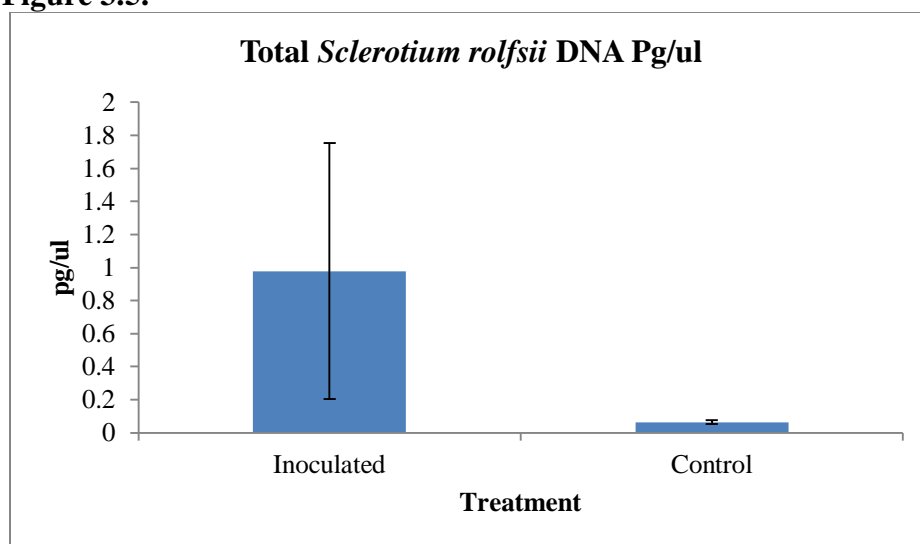
Figure 3.3.
Summary of the average final biomass measured of the inoculated and control plants after 10 weeks of introduction to disease. There is no significant difference between the inoculated and control plants biomass, P=0.3591. Bars represent standard error. Data was lumped to give a more accurate representation of what to expect when inoculating soil using this method described.

Figure 3.4.

Total Fungal DNA pg/ul

*Based on the mean of 20 total inoculated plants, and 20 total control plants.
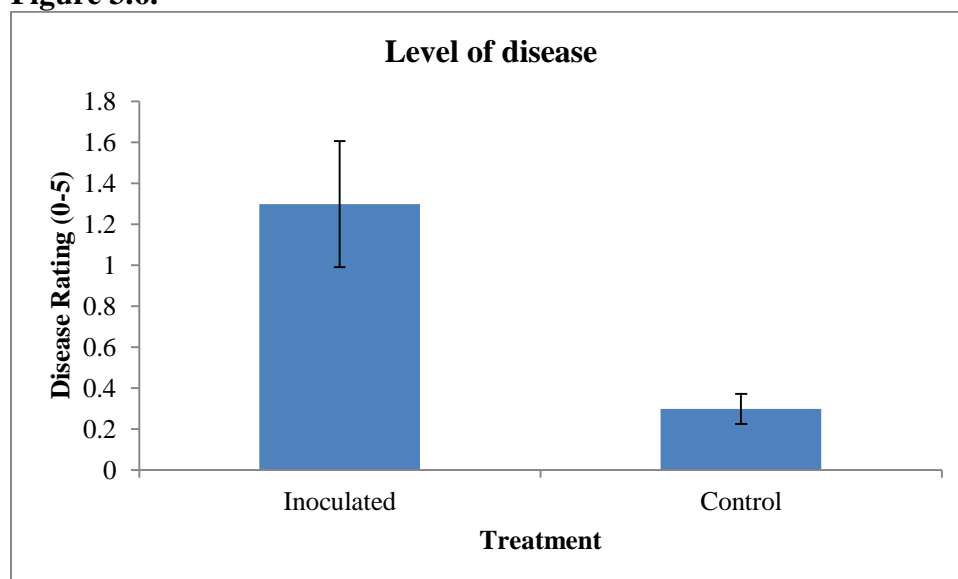
Figure 3.4.
Summary of the average total fungal DNA measured by Q-PCR. There is significantly more general fungi DNA in the inoculated soil than the control soil, P=0.0454. Bars represent standard error. Data was lumped to give a more accurate representation of what to expect when inoculating soil using this method described.

Figure 3.5.

Total *Sclerotium rolfsii* DNA Pg/ul

*Based on the mean of 20 total inoculated plants, and 20 total control plants.
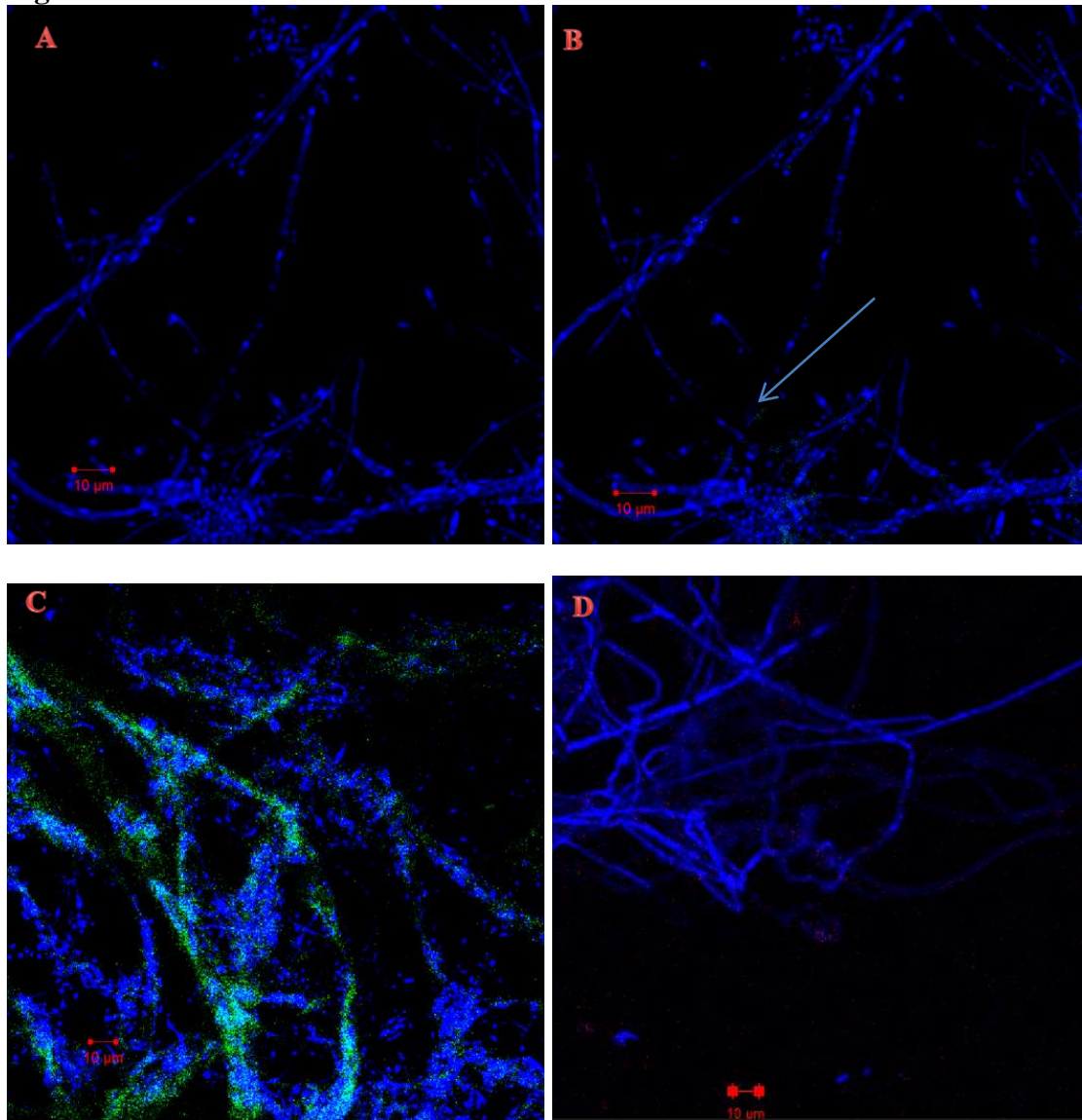
Figure 3.5.
Summary of the average total (pg/µl) amount of species specific (*Sclerotium rolfsii*) fungal DNA measured by Q-PCR. There is significantly more *Sclerotium* DNA in the inoculated soil than the control soil (P=0.0278). Bars represent standard error. Data was lumped to give a more accurate representation of what to expect when inoculating soil using this method described.

Figure 3.6.

Level of disease — bar chart. Y-axis: Disease Rating (0-5), from 0 to 1.8. X-axis: Treatment. Inoculated bar ≈ 1.3; Control bar ≈ 0.3. Bars represent standard error.

*Based on the mean of 20 total inoculated plants, and 40 total control plants.

Figure 3.6.
Summary of the average level of observed incidence of disease at the end of the ten week greenhouse experiment. The inoculated soil harbors plants that have a significantly higher level of disease (P=0.0005). Bars represent standard error. Data was lumped to give a more accurate representation of what to expect when inoculating soil using this method described.
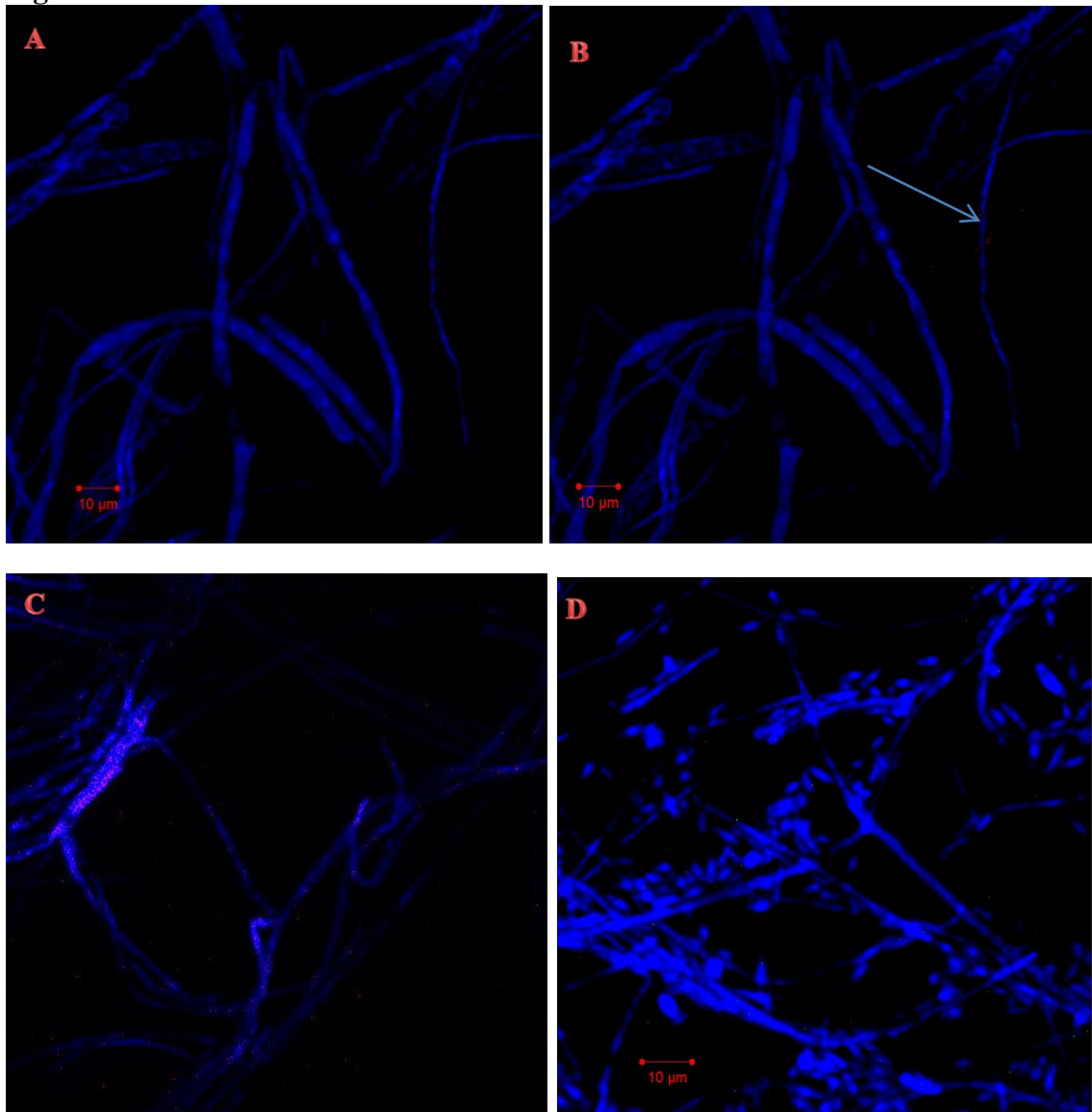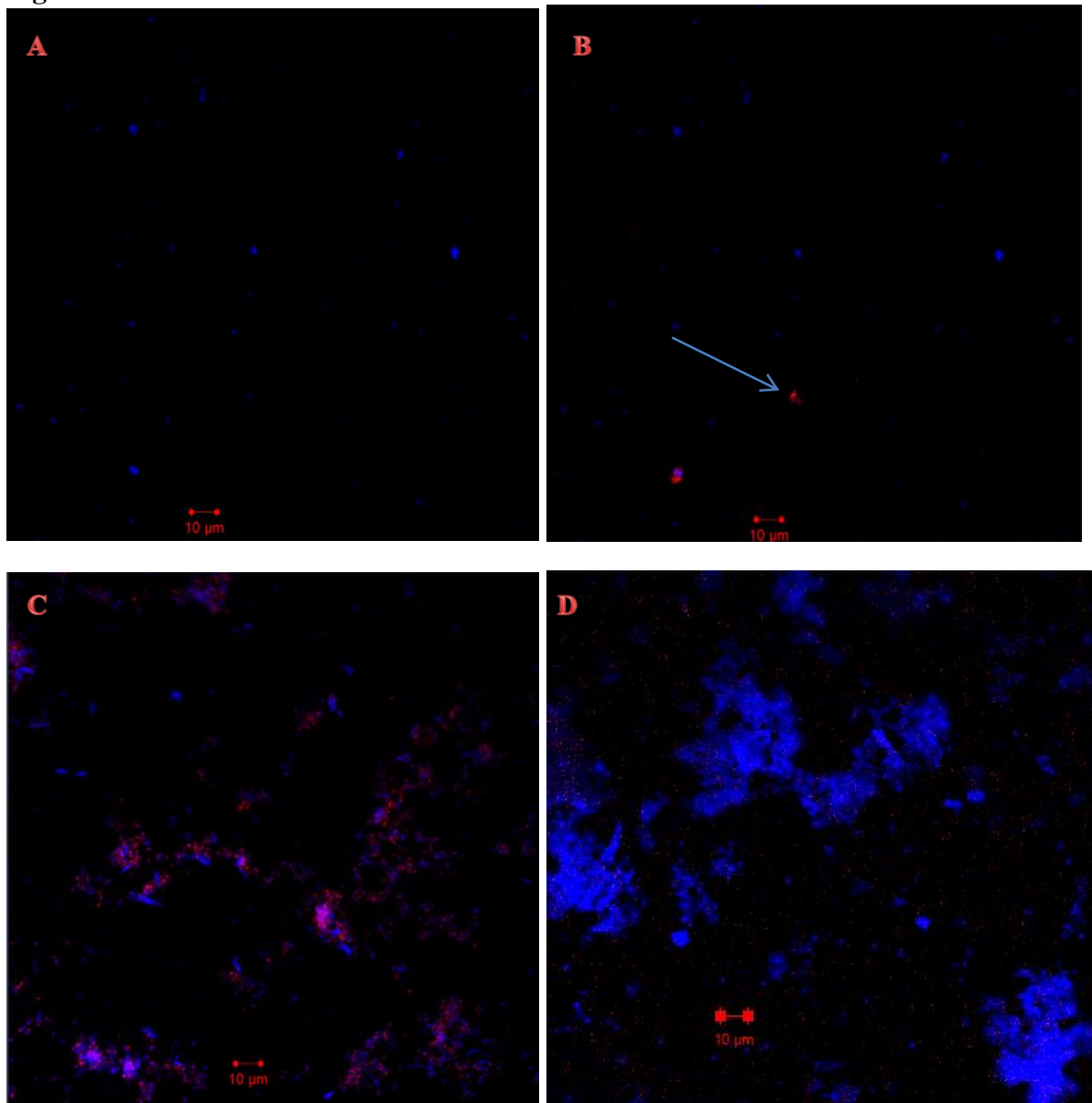
Figure 3.7.

Figure 3.7.
Fluorescent in situ hybridization of *Fusarium oxysporum*. **A**. *Fusarium* hyphae stained with DTAF only, represented by the blue color. **B**. *Fusarium* hyphae stained with DTAF only, veiwed under the wavelength used to detect Cy 5. The green color represents autofluorescence or background. **C**. DTAF is represented by blue, while Cy 5 is represented by green. Where the colors are overlapping each other represents hybridization because 3.7B shows that there is low noise. **D**. *Sclerotium rolfsii* serves as a control to show the amount of non-specific binding that the hybridization technique produce. Non-specific binding is represented by red.
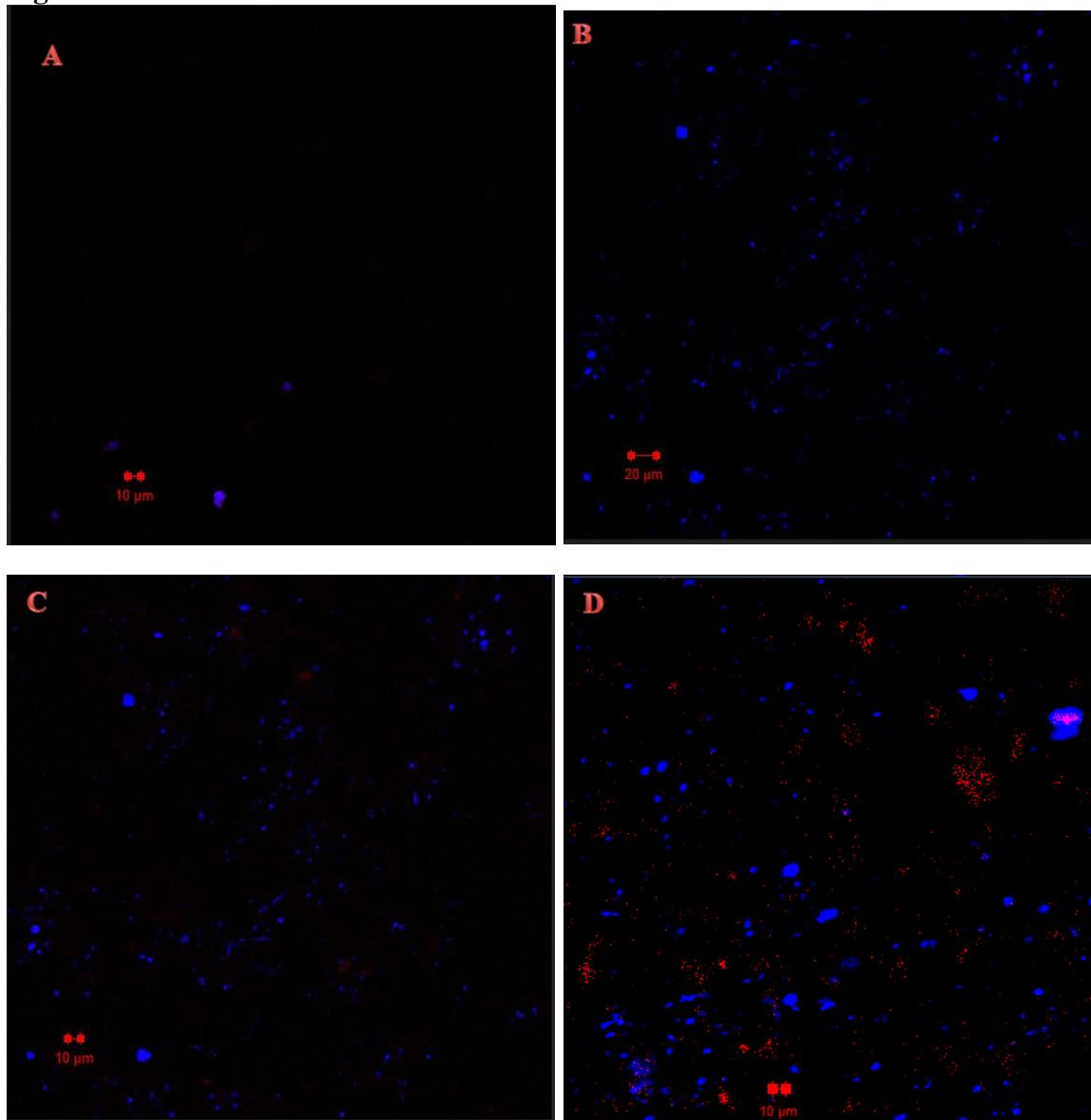
Figure 3.8.

Figure 3.8.
Fluorescent in situ hybridization of *Sclerotium rolfsii*. **A.** *Sclerotium* hyphae stained with DTAF only, represented by the blue color. **B**. *Sclerotium* hyphae stained with DTAF only, veiwed under the wavelength used to detect Cy 3. The red color represents autofluorescence or background noise. **C.** DTAF is represented by blue, while Cy 3 labeled and specific *Sclerotium rolfsii* oligonucleotide probe is represented by red. Where the colors are overlapping eachother represents hybridization since 3.8B shows that there is low noise. **D**. *Fusarium oxysporum* serves as a control to show the amount of non-specific binding that the hybridization technique produces. Non-specific binding is represented by red.
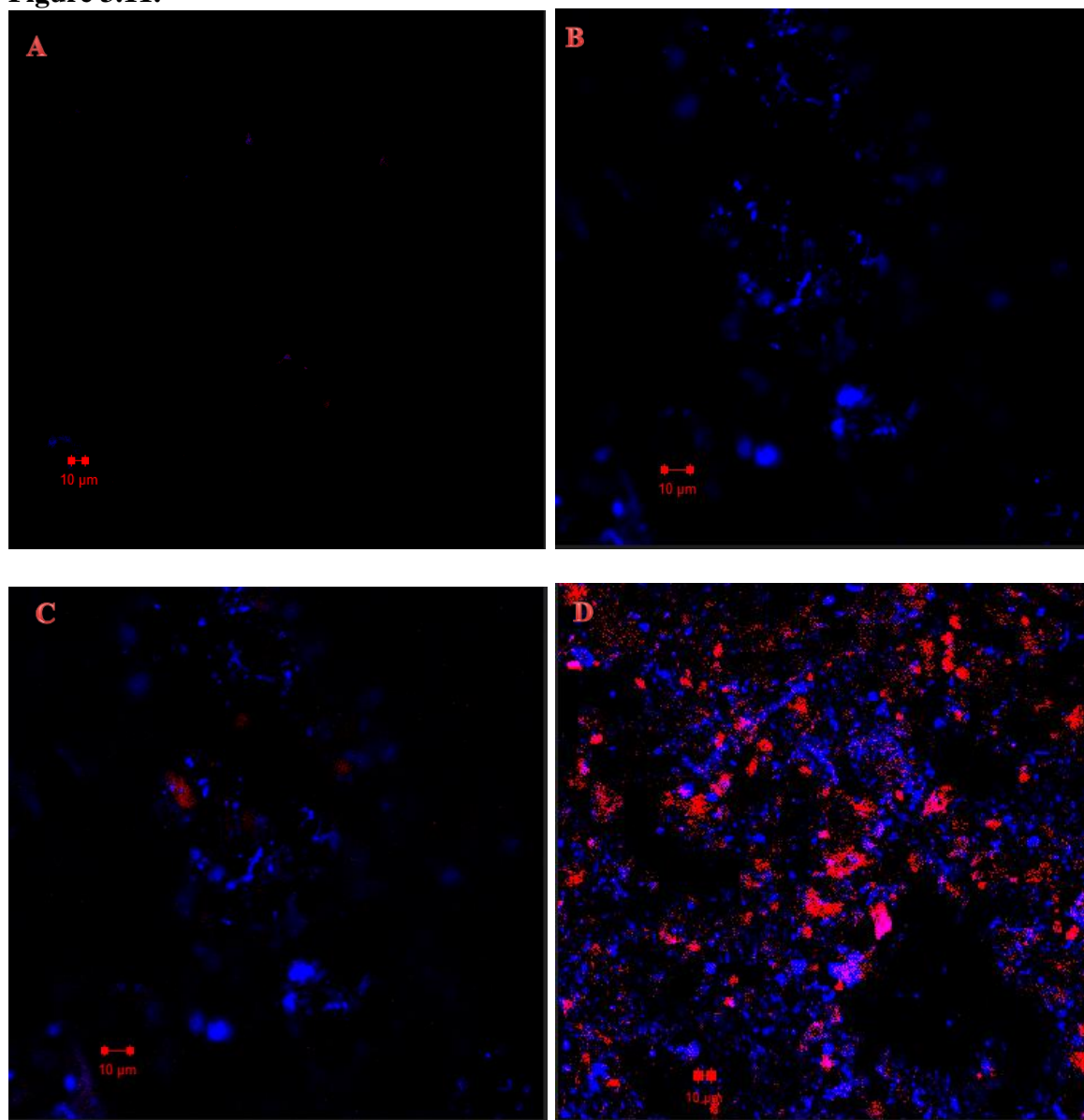
Figure 3.9.

Figure 3.9.
Fluorescent in situ hybridization of Escherichia coli. A. Escherichia coli cells stained with DTAF only, represented by the blue color. B. Escherichia coli cells stained with DTAF only, veiwed under the wavelength used to detect Cy 5. The red color represents autofluorescence or background noise. C. DTAF is represented by blue, while Cy 5 is represented by red. Where the colors are overlapping eachother represents hybridization since 3.9B shows that there is low noise. D. Sclerotium rolfsii serves as a control to show the amount of unspecific binding that the gram negative specific probe is capable of. Non-specific binding is represented in red. There this is more biological nutrient on the control slide 3.9D i.e. DTAF stain.

Figure 3.10.

Figure 3.10.
Fluorescent in situ hybridization in soil smears containing low amounts of *Sclerotium rolfsii* (0.0155 pg/µl). The blue color represents DAPI nuclear stain, and the red color represents the species specific Cy 5 labeled probe used to detect *Sclerotium rollsii*. **A**. Unstained soil smear. Fluorescent signal represents auto fluorescence from the soil under the wavelengths used to detect DTAF and Cy 5. **B**. Soil smear stained with DTAF, represented by the blue color. **C.** Soil smear stained by DTAF, under the wavelength used to detect Cy 5. The blue represents the protein stain and the red represents auto fluorescence that exists when detecting Cy 5. **D.** Soil smear containing low quantities of *Sclerotium* DNA, Q-PCR results give an average of 0.0155 pg/µl of *Sclerotium* DNA in soil taken from the same location.

Figure 3.11.

Figure 3.11.
Fluorescent in situ hybridization in soil smears containing high amounts of *Sclerotium rolfsii* (15.55333 pg/μl). The blue color represents DAPI nuclear stain, and the red color represents the species specific Cy 5 labeled probe used to detect *Sclerotium rolfsii*. **A**. Unstained soil smear. Fluorescent signal represents auto fluorescence from the soil under the wavelengths used to detect DTAF and Cy 5. **B**. Soil smear stained with DTAF, represented by the blue color. **C.** Soil smear stained by DTAF, under the wavelength used to detect Cy 5. The blue represents the protein stain and the red represents auto fluorescence that exists when detecting Cy 5.   **D.** Soil smear containing high quantities of *Sclerotium* DNA, Q-PCR results give an average of 15.5533 pg/μl of *Sclerotium* DNA in soil taken from the same location.

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