ABSTRACT

SHIRAISHI, KATIE. Bio-Mitigation Strategies to Reduce the Survival and Persistence of *Salmonella enterica* in Soils within Vegetable Production Systems. (Under the direction of Eduardo Gutierrez-Rodriguez).

Due to the lack of cost sensitive and sustainable remediation techniques for enteric pathogen contaminated produce production soils, crop and field abandonment with extended periods without the production of high value crops are common practice within the United States. In this study, the efficacy of short-term cover cropping (mustard greens, buckwheat, and sunn hemp) coupled with solarization was tested on the bio-remediation of persistent *Salmonella enterica* serovar Typhimurium contamination in agricultural soils within a typical 3-month cropping cycle. This was accomplished through three experiments of increasing scale from in vitro plant inhibition tests, to microcosm studies, to field trials using an avirulent *Salmonella* Typhimurium (AST) strain.

Plant inhibition tests using slurries produced from the leaves/stems and roots of mustard greens, buckwheat, and sunn hemp were established to determine the influence of secondary plant metabolites on the survival of AST. AST was inoculated at a concentration of log 2 and log 4 CFU/ml and the population was evaluated for 24 hours during incubation at 37 °C. Mustard greens leaves/stems slurries significantly reduced the population of AST by an average population of log 2 CFU/ml after 24 hours incubation at 37 °C compared to all other cover crops ($P<0.05$). Roots from mustard greens prevented growth of AST, while root slurries from buckwheat and sunn hemp promoted growth of AST over the same time-temperature incubation ($P<0.05$).

In soil microcosm studies, AST was inoculated into raw layer-hen manure (80 kg N/0.4 ha) and added to clay and loam soil for a final nominal concentration of log 5 CFU/g of soil. Soil moisture content was adjusted to field capacity and 25% above and below this water content. Microcosms were incubated at temperatures ranging from 4°C to 37°C, for a total of 4 weeks and population levels were determined every 7 days. Storage temperature significantly ($P<0.05$) decreased the survival of AST, irrespective of soil type and moisture content. In clay soil only, raw layer-hen manure decreased the survival of AST at storage temperature between 20 and 30°C ($P<0.05$). The addition of raw layer-hen manure did not increase the persistence of AST ($P>0.05$) at soil moisture contents at or 25% below field capacity, regardless of soil type and storage temperature. Greater persistence ($P<0.05$) of AST (28 d vs. 21 d) was observed at temperatures ≤ 20 °C compared to 30 or 37°C, irrespective of soil moisture content, soil type, and manure availability.

Within field studies, raw layer-hen manure (80 kg N/0.4 ha) and the AST strain was incorporated into fields by tilling for a final nominal concentration of log 5 and log 8 CFU/g for clay loam and sandy loam trials, respectively. Within clay loam soil, cover crops were sown and grown for 40 days, then disked into soil, followed by a solarization step using clear polyethylene plastic for one month. Within sandy loam soil, survival of AST was assessed during the growth of spinach until the four true leaf stage and after disking the crop into soil. Time to non-detection of AST varied between soil type (clay loam 22 days vs. sandy loam 145 days) and growing season. AST was not detected after growing any of the selected short-term cover crops and after solarization (2-months).

Soil temperature and moisture content appear to be the most important factors controlling the persistence and survival of AST. The use of cover cropping with mustard

greens coupled with solarization seems to be associated with a significant reduction in the survival and persistence of AST; however, further elucidation of the mechanisms governing these proposed bio-mitigation techniques will enable the effective and consistent implementation to expedite replanting of produce.

Bio-Mitigation Strategies to Reduce the Survival and Persistence of *Salmonella enterica* in Soils within Vegetable Production Systems

by
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DEDICATION

Dedicated my wonderful parents Mary and Steven Shiraishi for their continuous love and support.

BIOGRAPHY

Katie Shiraishi was born in Sterling, Virginia on August 30, 1991. She graduated from Potomac Falls High School in 2009 and attended Virginia Tech for her undergraduate degree. She obtained her Bachelor of Science degree in Biology, while minoring in Food Science and Technology in 2013. Katie attended North Carolina State University for her Master's degree in Food Science under the direction of Dr. Gutierrez-Rodriguez.

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CHAPTER ONE – Literature Review

Introduction

Foodborne outbreaks within the United States, associated with the consumption of fresh produce, have increased significantly since the 1970's (Sivapalasingam et al., 2004). In 2013, the source of 16% of the total number of outbreaks within the United States was identified as fresh produce items (CDC, 2015). This increase in reported outbreaks could be attributed to a variety of factors including the increase in consumption of fruits and vegetables as part of a healthy diet (GAO, 2002), advanced epidemiological surveillance methods, and globalization of the food supply chain (Sivapalasingam et al., 2004). Additionally, *Salmonella enterica* was responsible for a large majority of illnesses, hospitalizations, and deaths associated with fresh produce outbreaks in 2013 (CDC, 2015). In the past, a majority of fresh produce outbreaks caused by *Salmonella* were associated with the consumption of salad or leafy greens (Sivapalasingam et al., 2004), but in recent reports, outbreaks implicating seeded vegetables (solanaceous seeded vegetables, vine grown seeded vegetables, and legumes) and sprouts were most common (CDC, 2015). Salmonellosis can cause a range of gastrointestinal symptoms, which include diarrhea, abdominal cramps, vomiting, and fever. Symptoms typically begin 8 to 72 hours after the consumption of contaminated food, but are usually self-limiting lasting a couple of days to a week in duration. Infected people often recover without medical intervention, but some populations

are more at risk than others and include young children, elderly individuals, and those with compromised immune systems (CDC, 2012). Long-term complications can occur, although they are rare, and include the development of Reiter's syndrome. Reiter's syndrome is a disease characterized by joint pain, irritation of the eyes, and painful urination, which could also lead to chronic arthritis in rare cases (USDA-FSIS, 2011).

Pathogen contamination of fresh produce can occur at any time during the production chain, although the likelihood of contamination is elevated during field operations such as cultivation and harvest (Lynch et al., 2009). The introduction of human pathogens during production can involve many routes including the use of contaminated irrigation water (Strawn et al., 2013) or improperly composted manure amendments (Islam et al., 2004a, 2004b). Once pathogens are introduced into the farm environment, they are able to survive and persist for long periods of time within soil (Baloda et al., 2001), which can cause subsequent spread to other areas of the fruit and vegetable production environment or even result in produce contamination. Due to the lack of a lethality treatment during the minimal processing of fresh produce, preharvest strategies are imperative to reduce the potential of human pathogen contamination.

This review assesses the ability of human pathogens, specifically *Salmonella enterica*, to survive within compost/manure and soils in vegetable production systems, which could result in the transfer of those pathogens to produce during production and harvest. An analysis of two bioremediation techniques, i.e. utilization of short-term cover cropping

(mustard greens, buckwheat, and sunn hemp) and solarization, used in soils contaminated with human pathogens are discussed. These low residue cover crops produce distinct classes of secondary plant metabolites, which have been shown to exhibit bactericidal properties against human pathogens (Kyung et al., 1997; Wittstock et al., 2003; Lou et al., 2011) or to display suppression of weed growth through allelopathic effects (Adler and Chase, 2007). The secondary metabolites produced by these plants can be utilized through the maceration and incorporation of tissue into contaminated produce production soils to potentially reduce the survival and persistence of *Salmonella enterica* over time. While the utilization of cover crops is focused on active secondary plant metabolites, and their subsequent allelopathic characteristics, solarization is focused on harnessing solar energy to heat soil, covered in clear polyethylene plastic, to temperatures up to 60°C as a pathogen management strategy (Cohen and Rubin, 2007; Rubin and Benjamin, 1984). During a month long implementation, solarization temperatures can reach up to 40-60°C within the top 5 cm of the moist soil profile, subsequently displaying profound decreases in the mesophilic bacterial groups (Stapleton and DeVay, 1984; Gamliel et al., 2000).

Salmonella **Survival and Persistence: Mechanisms and Adaptations**

Salmonella enterica is a gram-negative, rod-shaped microorganism within the *Enterobacteriaceae* family (FDA, 2012). Members of this genus are generally characterized as motile, although non-motile variants occur, facultative anaerobes with

chemoorganotrophic metabolisms (FDA, 2012). *Salmonella* serovars display remarkable adaptability allowing the survival and persistence in a wide range of extreme and unfavorable environments (Humphrey, 2004). The employment of several stress response mechanisms produces various genomic and physiological changes of *Salmonella* in an effort to overcome hostile surroundings, which may include starvation, thermal, and desiccation stresses (Spector and Kenyon, 2012). Many of these genetic response mechanisms display overlapping resistance characteristics to more than one stress factor, enabling *Salmonella* to persist for prolonged periods when combating multiple stresses at one time (Spector et al., 1999; Wesche et al., 2005; Alvarez-Odronez et al., 2008; Shah et al., 2013; Koseki et al., 2015). Not only does *Salmonella* display mechanisms to respond to general environmental stressors, other physical and genetic survival strategies are employed to overcome heavy metal accumulation, to utilize peptidoglycan as an energy source, and to use cellular hydrophobicity to adhere more closely to soil particles (Stenstrom, 1989; Groisman et al., 1992; Hilbert et al., 1999).

When *Salmonella* encounters an environment depleted of carbon, nitrogen, or phosphate sources, a starvation-stress response (SSR) is induced, which allows for continued survival and also results in increased resistance to other stress factors as well (Kenyon et al., 2002). During the SSR, a series of genetic and physiological changes occur starting with the up-regulation of genes that focus on the utilization of alternative energy sources, and if starvation continues, the physical properties of the cell are transformed to be more

metabolically efficient (Spector and Kenyon, 2012). The up-regulation of genes for alternative energy source utilization includes the development of higher affinity transport systems and the production of enzymes that can metabolize unusual carbon sources (Kenyon et al., 2005). Interestingly, the production of these transport systems and enzymes is induced prior to encountering the substrate within the environment, thus enabling *Salmonella* to take advantage of these unusual carbon sources immediately when found (Spector and Kenyon, 2012). Changes in cell physiology occur with the continued depletion of nutrient sources, which can include the digestion of dispensable components, such as certain phospholipids and proteins within the cell membrane, and the modification of the lipopolysaccharide layer to create a smaller, more coccoid shaped cell with greater desiccation resilience (Kenyon et al, 2007; Spector and Kenyon, 2012). Not only does the starvation-stress response produce greater viability within nutrient scarce environments, but it also allows for improved resistance to high temperatures and acid pH (Spector et al., 1999; Wesche et al., 2005; Alvarez-Odronez et al., 2008).

Drastic increases in temperature initiates a heat shock response (HSR) in *Salmonella*, in order to protect the integrity of the cytoplasmic membrane, maintain protein homeostasis, and reduce DNA and RNA damage in the cell (Spector and Kenyon, 2012). The HSR produces an up-regulation of heat shock proteins that are encoded by the *rpoH* gene (Guisbert et al., 2008). The translation, rather than the transcription, of this gene is temperature mediated, so once temperatures reach above 42°C, one RNA hairpin melts to

allow ribosomal binding and subsequent translation of these heat shock proteins (Annika et al., 2013). Numerous chaperones and proteases are produced to aid in the folding and degradation of misfolded proteins, allowing protein homeostasis within the cell (Guisbert et al., 2008). Various enzymes are also encoded by the *rpoH* gene that aid in maintaining genomic integrity by repairing mismatched DNA or double stranded breaks, and by modifying RNA to enable greater temperature stability (Nonaka et al., 2006).

Salmonella can overcome desiccation stress through certain cellular components that abate the drying process, through the protection of proteins within the cell, and by osmoregulation (Spector and Kenyon, 2012; Finn et al., 2013). *Salmonella* Typhimurium has been known to produce a "rdar" (red, dry, and rough) morphotype, which is a multicellular phenotype, characterized by an extracellular matrix composed of fimbriae (curli) and cellulose (White et al., 2006). These extracellular matrix components have been shown to increase *Salmonella* survival upon desiccation through aiding cell aggregation and forming a protective barrier, encasing individual cells in protein and sugar polymers (Barnhart and Chapman, 2006; White et al., 2006). It has also been hypothesized that under suboptimal conditions, the molecule trehalose can aid in maintaining protein function and stability, although the exact mechanism of action is still under investigation (Crowe et al., 2001; Elbein et al., 2003). Osmoregulation of the cell is another important factor when reducing stress experienced from desiccation (Finn et al., 2013). To avoid further water loss, *Salmonella* concentrates osmoprotectants in the cell to maintain the internal osmolarity to

that of the external environment through the up-regulation of certain transporter systems (Li et al., 2012; Finn et al., 2013).

Salmonella has been shown to display several other physical and genetic strategies that aid in environmental survival through adherence to soil particles, the detoxification of heavy metals, and utilizing peptidoglycan as an energy source (Stenstrom, 1989; Groisman et al., 1992; Hilbert et al., 1999). The cell surface of *Salmonella* Typhimurium displays high hydrophobicity, which has been shown to increase the adhesion of the cell to clay particles, prolonging survival through decreased predation (Vargas and Hattori, 1986; Stenstrom, 1989; Huysman and Verstraete, 1993). Genetic traits, such as the *pcgL* gene, aid *Salmonella* survival within non-host environments through the utilization of peptidoglycan when other nutrients are limited (Hilbert et al., 1999; Mouslim et al., 2002). Another gene, *phoN*, is a phosphatase enzyme that aids survival in soil systems by the detoxification of heavy metals, and although this mechanism of action has been reported in *Citrobacter* spp., *Salmonella* Typhimurium has a homologous enzyme suggesting the same overall function (Groisman et al., 1992; Montgomery et al., 1995).

Survival and Persistence of Human Pathogens in Compost/Manure

The application of raw or composted animal manure to vegetable production fields is a common practice particularly by the organic agricultural industry (USDA, 2010). Manure addition provides soil with many nutrients including nitrogen, phosphorus, and potassium as

well as soil organic matter, which increases water holding capacity in sandy soils and drainage in clay soils (Rosen and Bierman, 2005). Despite the increase in soil water holing capacity and vegetable production due to added nutrients, manure application can also result in the inadvertent incorporation of human pathogens (FDA, 2001; Solomon et al., 2002; Islam et al., 2004b). Proper management of manure, before being used as an agricultural fertilizer, particularly composting, can notably reduce the likelihood of pathogen contamination (FDA, 2001). Therefore, persistence and survival rates of human pathogens in manure depend on the manure condition: un-composted manure, composted manure, or a slurry form (Nicholson et al., 2005; You et al., 2006; Mannion et al., 2007) and the animal origin (Erickson et al., 2014).

The physico-chemical properties of manure can vary significantly between cow, poultry, and swine sources, which can lead to differences between pathogen inactivation times (Combs et al., 2001; Erickson et al., 2014). Even though nitrogen can be present in multiple forms such as nitrate, nitrite, ammonium, and ammonia, it is the ammonium content, and subsequent production of ammonia, that has been shown to affect the survival of human pathogens in manure over time (Erickson et al., 2014). Ammonium (NH_4^+) is converted to ammonia (NH_3^-) at pH values greater than 6.5, which is facilitated through various microbial activities within manure (Chastain et al., 2003). Erickson et al. (2014) reported that ammonia production and the generation of cumulative heat by exothermic microbial activity were

significant variables increasing the die-off rate of *Salmonella* Enteriditis, *Salmonella* Newport, and *Listeria monocytogenes* in poultry and cow manures.

Un-composted animal manure has been known to harbor human pathogens for a significant amount of time with persistence depending on location of the pathogens, pathogen fitness, and the manure source (Himathongkham et al., 1999; You et al., 2006; Erickson et al., 2014). *Escherichia coli* O157:H7 can persist between 47 days and 120 days in aerated bovine and ovine manure piles, respectively, being held under fluctuating environmental conditions (Kudva et al., 1998), while *Salmonella* Newport has been shown to persist for 184 days after inoculation into cow manure held at ambient temperatures (You et al., 2006). Himathongkham et al. (1999) assessed the survival of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in cattle manure held at various temperatures and at various depths. Both pathogens had greater survival at 20°C compared to 4°C and 37°C when the top layers of cow manure were tested, although when the middle and bottom layers were evaluated, both pathogens had lower die-off rates at 20°C and 37°C compared to 4°C. Decimal reduction times (D-value) were less than 25 days for all treatments in this study. In a similar study, Himathongkham et al. (2000) evaluated the persistence of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in poultry manure held at various depths and temperatures. Both pathogens had greater persistence at 4°C compared to 20°C and 37°C; however, depth had no significant effect on the decimal reduction time at 20°C and 37°C. At 4°C this pattern was not observed, instead the top layers had shorter decimal reduction times

for both pathogens compared to middle and bottom layers. Decimal reduction times were less than 17 days for all poultry related treatments; however, for cattle manure, decimal reduction times were 25 days. Persistence of *Salmonella* Newport and *Listeria monocytogenes* is reduced to a greater extent over time in poultry manure compared to cattle and swine manures maintained at 40°C (Erickson et al., 2014). The rapid decline in the survival of both pathogens in poultry manure was correlated with an increase in ammonia concentration and cumulative heat.

Manure effluents from dairy and swine operations are normally stored in oxidation ponds located in close proximity to animal quarters (FDA, 2001). These effluents are sprayed in agricultural fields used to produce row or forage crops to enhance N, P, and K content (FDA, 2001). The ability of human pathogens to persist in a slurry manure matrix is dependent upon the pathogen being examined, dry matter content of the slurry, and animal source. Nicholson et al. (2005) examined the survival periods of *E. coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Campylobacter jejuni* in dairy effluents with two different dry matter percentages. Persistence of *E. coli* O157:H7 and *S.* Typhimurium varied between 32 and 93 days in dairy slurries with 7% and 2% dry matter content, respectively. The dry matter content did not have an effect on the persistence of *Campylobacter jejuni* and *Listeria monocytogenes*. Survival periods of 32 days for *Campylobacter* and 185 days *Listeria* were observed in both dairy effluents. Mannion et al.

(2007) reported the persistence of *Salmonella* Typhimurium and *Salmonella* Derby in swine effluents with 2% dry matter content as totaling 34 and 23 days, respectively.

In order to reduce the human pathogen load in manure sources, various composting processes can be employed, including static composting or turned composting (FDA, 2001). Static and turned composting methods are both aerobic processes in which the minimum temperature achieved must be 55°C at the center of the pile (21 C.F.R. § 16 and 112). Static composting must maintain this internal temperature for 3 days and is followed by a curing process, while in turned composting, the manure is turned a minimum of five times during a period of 15 days (21 C.F.R. § 16 and 112). Both composting methods have been shown to significantly reduce pathogen survival in manure, although relatively long persistence times can still be observed. Nicholson et al. (2005) reported the survival times of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Campylobacter jejuni* in dairy cow and swine manure using static and turned composting processes. Survival times for all pathogens were generally less than a week for both composting processes and both manure types; however, when using a turned composting method in swine manure, *Salmonella* Typhimurium and *Escherichia coli* O157:H7 persisted for a longer period (16 and 32 days, respectively). Grewal et al. (2005) reported longer persistence times for *Listeria monocytogenes* and *Salmonella* Typhimurium in swine manure using a thermophilic composting method that reached temperatures of 55°C. By day 3, dramatic decreases in the population of *Salmonella* Typhimurium and *Listeria monocytogenes* of around log 3-3.5

CFU/g of manure were seen, although extremely low levels of each inoculum were able to be detected for over 56 days.

Currently in the United States, the FDA is trying to promote the use of composted manure for soil amendments over the use of raw manure, due to the increased safety associated with the composting treatment. The final Produce Safety rule, of the Food Safety Modernization Act, has removed the time interval between the application of composted manure and the harvesting of fresh produce and has deferred in the decision, for five years, for the time interval between application of raw manure and fresh produce harvest (21 C.F.R. § 16 and 112). Even though there is no time interval implemented between composted manure application and harvest, composted manure amendments must still meet microbial standards set forth by the FDA and must be treated using a validated process (21 C.F.R. § 16 and 112).

Survival and Persistence of Human Pathogens in Soils

The survival of pathogens in soil, especially *Salmonella*, is extremely dependent on the environmental conditions of the area, but have been reported to persist in soil for over 300 days (Baloda et al., 2001). Temperature (Holley at al., 2006; Garcia et al., 2010; Abd-Allel and Maysa, 2015), soil moisture content (Chandler and Craven, 1980; Cools et al., 2001; Holley et al., 2006), soil physico-chemical properties (Wessendorf and Lingens, 1989; Cools et al., 2001; Lau and Ingham, 2001), soil C:N ratio (van Elsas et al., 2011), microbial

community composition (Franz et al., 2005), and UV light exposure (Turpin et al., 1993) are all factors that influence the ability of pathogens to survive in soil.

One of the most influential elements affecting the persistence of *Salmonella* in soil is ambient temperature (Holley at al., 2006; Garcia et al., 2010; Abd-Allel and Maysa, 2015), which can be compounded by exposure to UV light associated with the top layer of soil (Turpin et al., 1993). Numerous studies have shown that there is a direct correlation between temperature and recovery of inoculated bacteria, including the recovery of *Salmonella* spp., with lower temperatures generally increasing survival (Holley at al., 2006; Garcia et al., 2010; Abd-Allel and Maysa, 2015). In a study performed by Holley et al. (2006), survival of various *Salmonella* serovars was higher at 4°C compared to 25°C throughout the study time course, while Abd-Allel and Maysa (2015) and Garcia et al. (2010) found that there was longer recovery and longer survival of *Salmonella* Typhimurium specifically at 5°C compared to higher evaluated temperatures. Pathogens located within the top surface layer of soil can be exposed to UV rays produced by the sun and may be consequently inactivated (Turpin et al., 1993) through the interruption of DNA replication within the cell (Sizer and Balasubramaniam, 1999).

The soil moisture content plays a major role in the survival of bacteria, including human pathogens, with higher moisture contents typically increasing survival in soil over time (Chandler and Craven, 1980; Cools et al., 2001; Holley et al., 2006). Many studies base soil moisture content values as a percentage of the field capacity of the soil, which is defined

as “the amount of water held in the soil after the excess gravitational water has drained away and after the rate of downward movement of water has materially decreased” (Veihmeyer and Hendrickson, 1931). Texture of each soil type is directly related to the field capacity of that soil, with coarser soil textures like sand having lower field capacities compared to finer soil textures, like clay, due to the increased moisture retention associated with finer particles (Assouline and Or, 2014). Cools et al. (2001) proposed that since bacterial growth occurred within the areas of liquid and soil blending, increased survival would occur in soils with higher moisture contents. This is supported through the longer persistence times observed for *Escherichia coli* and *Salmonella* Typhimurium when held within soils at higher moisture contents (Chandler and Craven, 1980). Longer bacterial persistence times were also observed for *E. coli* and *Enterococcus* spp. in soils at 100% field capacity, compared to lower moisture content values of 60% and 80% of field capacity (Cools et al., 2001). In the same study, higher moisture contents still played a significant role in the greater recovery of human pathogens over time even when soil was amended with manure. Holley et al. (2006) reported that the persistence of multiple *Salmonella* serovars was extended in manure-amended soils at 80% field capacity compared to 60%.

Soil physical properties, like soil type and texture, affect microorganisms in their ability to form microhabitats, and thus their survival over time (Wessendorf and Lingens, 1989; Cools et al., 2001; Lau and Ingham, 2001). It is hypothesized that desiccation rates of cells will vary based on the physical characteristics of soil that are linked to moisture

holding, with lower desiccation rates seen with soil types that have a higher tension rate, holding moisture to individual soil particles (Chandler and Craven, 1980). Interestingly, Bushby and Marshall (1977) postulated that increased bacterial desiccation tolerances could be associated with the reduced internal water content within cells in the presence of clay particles, since clay particles have a higher affinity for water compared to bacterial cells. Denser soil types, with higher water holding capacities, can also facilitate the longer persistence of microorganisms through the aggregation of moisture in certain areas of the soil (Wessendorf and Lingens, 1989; Lau and Ingham, 2001).

Protection against predation is another characteristic that denser soil types may provide, which increases pathogen persistence times (Cools et al., 2001). However, in some studies, no significant difference in the persistence of human pathogens was seen when evaluating various soil types (Natvig et al., 2002: Danyluk et al., 2008), which may be due to the broad range of differences between experimental designs or differences between other features of the soil not evaluated. For instance, higher soil organic matter contents have been associated with an increase in the survival and persistence of human pathogens compared to soil type and texture characteristics individually (Mallmann and Litsky, 1951; Cools et al., 2001).

Nutrient content of soil, such as the ratio of readily available C and N sources, appears to be one of the most important predictors of the survival and persistence rates seen for some human pathogens in agricultural soils (van Elsas et al., 2011). Soil systems that are

generally characterized as oligotrophic, or having low nutrient availability, are viewed as control mechanisms by reducing enteric pathogen survival (Franz and van Bruggen, 2008). Franz et al. (2008) reported that the addition of manure with high C:N ratios, from animals fed a high fiber diet, vegetable production systems can achieve a lower state of nutrient availability for enteric pathogens, which is characterized by a slower nutrient release rate. This type of system has been shown to significantly decrease the survival rate and persistence of *Escherichia coli* O157:H7 in manure amended soils, due to the addition of C and N in forms that are not readily available for microbial use (Franz et al., 2008). It is hypothesized that microorganisms with faster growth rates, like *Escherichia coli*, are outcompeted by microorganisms with slower growth rates in these types of systems, since greater nutrient availability is needed in shorter periods of time for bacteria with faster growth rates (Franz et al., 2008). Similarly, high levels of dissolved organic carbon and dissolved organic nitrogen, which are more readily available for bacterial utilization, and microbial biomass C, have been associated with increased persistence of *E. coli* O157:H7 in soils with and without manure amendments (Franz et al., 2008; Ma et al., 2013; Wang et al., 2014).

Soil ecosystems are highly competitive environments with extensive biodiversity and have even been known to harbor millions of distinct genomes within a single gram of soil (Gans et al., 2005; Urich et al., 2008). Due to the considerable size of the endogenous soil microbial community, the survival and persistence of introduced pathogens are highly influenced by a range of microorganisms through competition for nutrient sources (Franz et

al., 2005), or even by predation from certain protozoan species (Recorbet et al., 1992; Garcia et al., 2010). Certain groups of naturally occurring bacteria, such as *Proteobacteria* and *Bacteroidetes* in soil, have been known to decrease the persistence of *Escherichia coli* O157:H7, which may be due to the competition between these microorganisms for nutrients, as reported by Ma et al. (2013). Contrastingly, a positive correlation was seen between the presence of *Actinobacteria* and *Acidobacteria* and the survival of *E. coli* O157:H7 (Ma et al., 2013; Wang et al., 2014). In this case, it was hypothesized that bacterial agents within these two Phyla have the ability to break down C compounds that are of high molecular weight and thus improve the soil nutrient availability for human pathogens (Ma et al., 2013). When introduced into soil through a manure source, pathogens compete with naturally occurring microflora for nutrients, which may compromise their survival (Franz et al., 2005). The application of manure into soil systems has been shown to significantly reduce the survival of *Salmonella* Typhimurium over time compared to non-manure amended soils (Garcia et al., 2010).

The animal source can also have an effect on the survival of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in manure-amended soils, with cattle manure reducing the persistence of both pathogens to a greater extent compared to poultry manure (Nyberg et al., 2010), which may be partially influenced by the variations between the microbial diversity of manure sources. Protozoan predators have also been shown to be a contributing factor, as they influence the ability of pathogens to survive in soil (Recorbet et

al., 1992; Garcia et al., 2010). Garcia et al. (2010) reported a decrease in populations of *Salmonella* Typhimurium associated with the simultaneous increase in protozoa counts. Similarly, Recorbet et al. (1992) reported protozoan predation upon *E. coli* populations, thus reducing their survival over time.

Transfer of Human Pathogens from Soil to Crops

Transfer of human pathogens to fresh produce can take place at any time during the production chain from seedling growth to postharvest grading and handling and can also occur in a multitude of ways ranging from contaminated irrigation water to wildlife intrusion (Steele and Odumeru, 2004; Strawn et al., 2013; Jay et al., 2007; Gardner et al., 2011). The proximity of produce to contaminated soil and the concentration of pathogens within the soil are two risk factors contributing to the probability of produce contamination during field production (Jacobsen and Bech, 2012). Generally, produce that is lower to the ground, like root crops or leafy greens, have a higher likelihood of contamination compared to produce that develop further above the soil surface, like tomatoes (Doyle and Erickson, 2008).

The applications of poor quality irrigation water and manure addition are thought to be the most likely sources of pathogen contamination in vegetable production fields (Strawn et al., 2013). Groundwater sources are generally of the highest microbial quality, unless contaminated by the percolation of surface runoff through the soil profile, and are frequently used for agricultural purposes (Steele and Odumeru, 2004). Surface water, like ponds and

streams, has a wider range of quality and levels of pathogen contamination, which may be linked to proximity to land used for livestock and agricultural production (Haley et al., 2009). Water from various sources have been shown to harbor a diverse array of pathogens and frequently *Salmonella* (Lou et al., 2015) because of its ubiquitous nature and its ability to persist for long periods within the aquatic environment (Wright, 1989). Irrigating fields close to harvest has been shown to increase the probability of pathogen detection on produce (Strawn et al., 2013), with spray irrigation increasing the risk of transmission of pathogens to plant surfaces compared to drip irrigation (Steele and Odumeru, 2004). Not only can irrigation water contaminate fresh produce directly, but it can also contaminate the soil used for vegetable production, which can subsequently lead to an increased risk of contamination due to the ability of certain pathogens to persist for prolonged periods within soil. Interestingly, Islam et al. (2004b) reported that pathogen persistence rates in soil were similar, regardless if introduced through an irrigation source or contaminated compost, with pathogens having the ability to survive even after crop rotations. It is hypothesized that pathogens attached to soil particles can contaminate fresh produce through the splashing that occurs during rainfall (Jacobsen and Bech, 2012), with a higher likelihood of contamination resulting from better adherence properties associated with soils that have a high clay content (Natvig et al., 2002). Amending soil with manure from various animal sources has been associated with the introduction of a range of human pathogens, with their exceptional persistence characteristics within soil contributing to the potential transfer to crops over time

(Solomon et al., 2002; Islam et al., 2004b). The use of manure amendments, within a year of time, was identified as one of the top risk factors influencing the isolation of *Salmonella enterica* and *Listeria monocytogenes* from commercial produce production fields, emphasizing the importance of proper manure management prior to field application (Strawn et al., 2013).

Transmission of human pathogens can occur during the germination of seeds or after the transplantation of seedlings in contaminated soil (Barak and Liang, 2008; Islam et al., 2004a, 2004b). Seedling colonization by *Salmonella enterica* is influenced by many genes, including those responsible for swarming and biofilm formation, (Barak et al., 2009) and are generally not those involved with the colonization of animal cells (Noel et al., 2010). Barak and Liang (2008) reported that, prior to the sowing of tomato seeds, *S. enterica* inoculated into fields through the use of contaminated irrigation water was able to persist within soil for weeks. These long persistence times resulted in the contamination of the phyllosphere and rhizosphere after tomato seeds were sown and the crop was grown. The spinach rhizosphere was also shown to support and even increase the population of *S. enterica* serovar Weltevreden when inoculated into vegetable production soil, while populations persisted on leaf samples for 21 days post inoculation (Arthurson et al., 2010). Longer persistence times for two pathogens within soil and on plant leaves were reported by Islam et al. (2004a, 2004b). In these studies, seedlings of lettuce and parsley were transplanted into soil, which was previously amended with manure containing *Salmonella* Typhimurium or *Escherichia*

coli O157:H7, and the persistence was monitored over time in the soil and on plant leaves. Persistence times for *Salmonella* Typhimurium were longer for parsley and parsley soil each at 231 days, compared to lettuce and lettuce soil with 63 and 161 days, respectively. *E. coli* O157:H7 persisted in manure amended soils for up to 217 days and was detected on lettuce and parsley for up to 77 and 177 days, respectively.

The internalization of human pathogens, specifically enteric pathogens, through root uptake has been a point of debate due to the inconsistencies among numerous scientific studies. Generally, internalization of human pathogens has been reported at a greater extent when plants were grown in hydroponic systems within high levels of inoculum (Warriner et al., 2003; Bernstein et al., 2007; Franz et al., 2007; Sharma et al., 2009), compared to contaminated soils (Warriner et al., 2003; Franz et al., 2007; Erickson et al., 2010). Warriner et al. (2003) hypothesized that, due to the lack of competitive natural microflora within hydroponic solutions, when being compared to soil systems, root colonization is more easily achieved, which can lead to the later colonization of inner portions of the plant. In contrast, Franz et al. (2007) hypothesized that the higher densities of internalized enteric pathogens within lettuce plants grown in contaminated soils could be due to the wounding of root surfaces imparted by soil particulates. The high variability between research methods and protocols may be partially responsible for the inconsistent results obtained in multiple studies evaluating the internalization of enteric pathogens into plant tissues (Hirneisen et al., 2012).

Other potential risk factors associated with the transfer of human pathogens to fresh produce during pre-harvest activities such as wildlife intrusion, insect presence, field cultivation, and harvesting with contaminated equipment (Alam and Zurek, 2004; Strawn et al., 2013; FDA, 2001). Wild animals have been known to harbor pathogenic microorganisms and thus could potentially cause the contamination of produce and produce fields from the dissemination of those pathogens through fecal material (Beuchat, 2006). Numerous studies have reported the isolation of pathogenic *Salmonella* serovars including *Salmonella* Typhimurium and *Escherichia coli* O157:H7 from collected fecal samples of various birds, including feral pigeons, gulls, birds of prey, and crows (Wallace et al., 1997; Schmidt et al., 2000; Refsum et al., 2002). Wildlife intrusion has also been noted as a potential risk factor associated with several fresh produce outbreaks, including the presence of Sandhill cranes (*Grus canadensis*) with *Campylobacter jejuni* contamination on peas (Gardner et al., 2011) and feral pig (*Scus scrofa*) sightings with *E. coli* O157 contamination on spinach (Jay et al., 2007). The transfer of pathogenic bacteria to fresh produce could also be associated with insect and nematode vectors, with several studies implicating flies, slugs, and *Caenorhabditis elegans* as carriers of *Escherichia coli* O157 and *Salmonella* (Alam and Zurek, 2004; Sproston et al., 2006; Kenney et al., 2006). Field cultivation was also reported as a risk factor associated with the presence of *Salmonella enterica* and *Listeria monocytogenes* within produce fields (Strawn et al., 2013). Strawn et al. (2013) hypothesized that soil cultivation exposed human pathogens that subsequently inhabited deeper soil layers, and thus

were more easily detected, although it was also noted that pathogen presence would eventually decrease due to the harsh environmental conditions experienced within the top layers of soil (UV light). The harvesting equipment used can also be a potential source of produce contamination, with the FDA (2001) stating that contaminated soil, encrusted on harvesting equipment, may accidentally come into contact with fresh fruits and vegetables during harvest.

Soil Bioremediation Methods - Cover Crops

Mustard greens, *Brassica juncea* L.

Brassica juncea L., also known as brown mustard, Chinese mustard or India mustard, is a type of mustard plant with an unmistakable horseradish-mustard flavor (UW-Madison, 2015). The cultivation of this crop is mainly done for human consumption, since the leaves and stem are edible, and for the production of mustard oil and condiments derived from the seeds (Duke, 1983), although there has been increasing interest in using these plants as biofumigants and as cover crops (Motisi et al., 2009). *Brassica juncea* belongs to the Brassicaceae family (formerly known as Cruciferae), which comprises nearly 350 genera and 3,000 species of cruciferous plants (Beilstein et al., 2006).

All species within the Brassicaceae family are known to produce glucosinolates, which are the compounds responsible for their unique and sharp flavor attributes, and are also used as a plant defense mechanism against insect predation (Wittstock et al., 2003).

Although over 120 different glucosinolates have been identified within sixteen plant families (Brassicaceae being one of the major producers), closely related taxonomic groups of plants tend to only produce a few each (Verkerk et al., 2009). These secondary plant metabolites are β-thioglucoside, *N*-hydroxysulfates and are formed from different amino acids (Halkier and Gershenzon, 2006). Dependent on which amino acid the glucosinolate was originally derived from, they can be further subdivided into three separate groups: aromatic (phenylalanine or tyrosine), indolyl (tryptophan) and aliphatic (aliphatic amino acids such as methionine) (Halkier and Du, 1997).

Glucosinolates are water-soluble compounds that are resistant to degradation until they come into contact with the enzyme myrosinase, which then hydrolyzes them into isothiocyanates, nitriles, and other products (Halkier and Gershenzon, 2006). The hydrolysis products produced by this reaction are the main compounds responsible for the biological inhibition of certain bacteria and plants (Wittstock et al., 2003). This enzyme is not only sequestered within plant material, but also present in certain fungal systems (Sakorn et al., 2002), cruciferous aphids (Francis et al., 2002), and in some bacterial species (Rask et al., 2000). Myrosinase and glucosinolates are located within different parts of the plant and the enzyme only comes into contact with glucosinolates after tissue damage such as freeze thawing, mastication, or bruising of the plant (Bones and Rossiter, 1996). The exact location of myrosinase enzymes is still under debate, although research suggests that myrosinase

enzymes are sequestered in the cytoplasm of specialized cells called myrosin cells or within aqueous vacuoles (Bones and Iversen, 1985).

Mustard, *Brassica juncea*, has been shown to produce a variety of glucosinolates, with various studies identifying many compounds in all plant organs. The glucosinolate 2-propenyl, or sinigrin, is the predominant glucosinolate identified for *Brassica juncea* and has been identified in the leaves and roots (Tong et al., 2014; et al., 2005; Gimsing and Kirkegaard, 2006). Other, less prevalent glucosinolates such as 4-pentenyl, 3-butenyl, 3-indolylmethyl, 4-methoxy-3-indolylmethyl, have been identified in the leaves as well (Yang et al, 2014; Tong et al., 2014; Gimsing et al., 2005). Some glucosinolates have been found only in the root system in some studies, while the glucosinolates 4-hydroxy-3-indolylymethyl, 1-methoxy-3-indolylymethyl and 2-phenylethyl can be found in both the upper and lower components of the root system (Tong et al., 2014).

The total concentration of each glucosinolate in *Brassica juncea* varies widely between different plant organs, and concentration is affected by factors such as age of the plant, species variations, and environmental conditions. Seedlings often contain the largest quantity of glucosinolates by weight, with the relative amount by weight decreasing over time with aging of the plant (Verkerk et al., 2009). Environmental factors such as nutrient content of the soil may also have a significant effect on the distribution of glucosinolates in various plant organs and the total concentration produced (Fahey et al., 2001; Tong et al., 2014).

Due to the factors mentioned previously, large variations are reported in the literature when investigating the glucosinolates in *Brassica juncea* and the relative amount in each plant organ. Gimsing and Kirkegaard (2006) reported Brown mustard containing relatively high amounts of glucosinolates, including 28.6 μmol of 2-propenyl (sinigrin) per gram of plant biomass in the shoots during flowering and 8.0 μmol of 2-propenyl per gram of plant biomass in the roots. Glucosinolates present in the highest amounts within the shoots were 4-pentenyl and 2-hydroxy-2-phenylethyl with levels of 0.9 and 0.8 μmol/g of plant biomass, respectively. Other glucosinolates such as 3-butenyl, 2-phenylethyl, 3-indolylmethyl, 4-methoxy-3-indolylmethyl and 1-metho such as 3-butenyl, 2-phenylethyl, 3-indolylmethyl, 4-methoxy-3-indolylmethyl and 1-methoxy-3-indolylmethyl have also been detected in the shoots at a concentration between 0.1 and 0.3 μmol/g of plant biomass. The second and third highest glucosinolates present in the roots were 4-pentenyl and 2-hydroxy-2-phenylethyl at 2.0 and 1.7 μmol/g of plant material, respectively.

The hydrolysis products produced when parent glucosinolates come into contact with myrosinase are known to display inhibitory characteristics against a range of human pathogens. The production of these compounds is influenced by environmental conditions such as the soil pH and the presence of ferrous ions (Bones and Rossiter, 1996). Under neutral soil pH conditions, sinigrin (2-propenyl), the glucosinolate present in the highest amounts in mustard species is hydrolyzed into allyl isothiocyanate, which is highly volatile (Dai et al., 2014). In numerous studies, allyl isothiocyanate, both in the gaseous and liquid

state, has been shown to exhibit bacteriostatic and bactericidal properties. Allyl isothiocyanate, in a liquid form, displays a minimum inhibitory concentration of 100 ppm for *Salmonella* Typhimurium, 50 ppm for *Escherichia coli*, and 100-200 ppm for *Listeria monocytogenes* (Kyung et al., 1997). This is compared to sinigrin, for which concentrations up to 1000 ppm showed no bacterial growth inhibition suggesting the parent glucosinolate is not responsible for the antimicrobial activity (Kyung et al., 1997). Delaquis et al. (1997) reported an inhibitory concentration of allyl isothiocyanate of 1,000 μg/L of air for the growth of *Salmonella* Typhimurium, *Listeria monocytogenes* and *Escherichia coli* at an inoculum level of log 6 CFU/cm^2, although exposure was not lethal to all cells. Tang (1974) hypothesized that the sulfur groups of isothiocyanates react with cellular proteins or enzymes and reduce cellular metabolism to achieve antimicrobial properties. Lin et al. (2000) reported that the bactericidal properties of allyl isothiocyanate were very similar to polymixin B, which induces cellular leakage by alteration of the bacterial cellular membrane, enabling greater permeability.

Use of *Brassica* spp., especially *Brassica juncea*, as a biofumigant has been a topic of interest in recent studies to control soil borne plant pathogens. Motisi et al. (2009a) reported a reduction in disease incidence of sugar beet root rot, caused by *Rhizoctonia solani,* when *Brassica juncea* was grown as a cover crop and then incorporated into soil. It was also shown that yearly use of *Brassica juncea,* as a cover crop incorporated into soil, displayed a continued effect with greater efficacy when reducing the prevalence of disease year after

year. Motisi et al. (2009b) reported on the efficacy of *Brassica juncea* used as above ground plant parts (leaves and shoots) and below ground plant parts (roots) for pathogen suppression of *Rhizoctonia solani* and *Gaeumannaomyces graminis* var. *tritici* in soil. The two treatments of above ground plant parts alone and a combination of above and below ground plant parts, were shown to significantly suppress the growth of both plant pathogens compared to controls. Below ground plant parts, when incorporated into soil, also suppressed the growth of these plant pathogens, but not nearly to the extent of above ground plant parts. Within this study, suppression of these two plant pathogens was accomplished over a 13-day period following the incorporation of *Brassica juncea* tissue into the soil.

A decreased amount of glucosinolate hydrolysis products within soil has been shown after the incorporation of *Brassica* spp. relative to the levels of their parent glucosinolate compound within the original plant biomass. This is likely because much of the glucosinolate in the plant never comes into contact with myronsinase, reducing various hydrolysis product production, during the incorporation of the plant into the soil when used as a biofumigant. Gimsing and Kirkegaard (2006) reported that about 50% of the 2-propenyl glucosinolate content of the original plant biomass was recovered as isothiocyanate products in the soil after incorporation. These results suggest that increased production efficiency of hydrolysis products from plant material can be achieved by increasing the maceration of the plant, and subsequent contact between myrosinase enzymes and glucosinolates, before or during incorporation into the soil.

Buckwheat, Fagopyrum esculentum L.

Fagopyrum esculentum, also known as Buckwheat is in the Polygonaceae family, is a type of pseudo-cereal grain that is cultivated mainly for seed harvesting and to make flour (Wijngaard et al., 2006). It is considered a food for the health conscious due to the content of protein, vitamins, minerals, and dietary fibers in the seeds and plant material (Kim et al., 2004). Buckwheat also contains a variety of polyphenols, phenolic acids, and flavonoids, and is especially recognized for its rutin content (Kim et al., 2004). Besides its recent interest and popularity as a health food, buckwheat is also commonly used as a cover crop in diverse agricultural crop rotations. Buckwheat is an easily managed cover crop and is highly regarded for its quick growth and subsequent weed suppression (Kumar et al., 2009).

An assortment of phenolic compounds are produced in buckwheat and vary in concentration and location in the plant organs. Buckwheat has been shown to produce gallic acid, (-)-epigallocatechin, chlorogenic acid, (-)-catechin hydrate, caffeic acid, (-)-epicatechin, quercetin, ferulic acid, and rutin in the leaves, stems, flowers and roots of the plant (Li et al., 2010). Rutin is by far the most abundant phenolic compound, found in the flowers, leaves, and stems at concentrations of 6,809, 5,524, and 294 mg/100g dry weight of buckwheat, respectively. The phenolic compound produced in the highest concentration in roots is (-)-catechin hydrate at 21.07 mg/100mg of dry weight of buckwheat (Li et al., 2010). The vast majority of phenolic compounds are found within the above ground biomass, rather than the roots. This is due to the fact that these phenolic compounds have antioxidant activity. For

instance, rutin protects against UV-B radiation and is more highly produced in plant organs exposed to solar radiation (Kim et al., 2008).

In buckwheat, some phenolic compounds, such as the flavonoids, are produced through the phenylpropanoid pathway. The phenylpropanoid pathway is a biosynthesis pathway that initially starts with the amino acid phenylalanine and requires numerous enzymes to create various intermediate and final end-products (Li et al., 2010). Chlorogenic acid is produced through the esterification of cinnamic acid, which is an intermediate in the biosynthesis pathway that produces rutin (Schijlen et al., 2004). Eventually there is a split in the pathway between the production of anthocyanins, pigment molecules, and quercetin (Schijlen et al., 2004). Rutin is then produced by the glycosylation of quercetin.

Production and subsequent concentration of phenolic compounds in buckwheat can be influenced by environmental factors, age of the plant, and species variety. Environmental stressors like exposure to elevated nickel concentrations have been shown to the increase the levels of chlorogenic acid in buckwheat (Sytar et al., 2013), while salinity stress increases the concentration of rutin in seedlings (Li et al., 2012). Age can also determine the concentration of phenolic compounds. For example, an increase in the concentration of rutin, quericitrin (an aglycone of quercetin), and chlorogenic acid was observed in seedlings as compared to their seeds (Kim et al., 2004). Seedlings seven to eight days after sowing displayed concentrations of rutin and quericitrin of 2,236 and 2,312 mg/100 g dry weight of plant material (Kim et al., 2004). Li et al. (2010) found concentrations of rutin around 5,000

mg/100 g dry weight of leaves comparable to the concentrations mentioned in the previous study in flowered buckwheat. Phenolic compounds in buckwheat have a variety of functions and thus concentrations vary throughout the lifetime of the plant.

Phenolic compounds in buckwheat have been shown to display some inhibitory activities against certain bacterial pathogens under laboratory conditions. Arima et al. (2002) investigated the potential inhibition of quercetin, found only in the flowers and roots of buckwheat, and rutin, which is found in all plant parts in fairly high concentrations, using a paper disk method. Quercetin displayed inhibition of both *Salmonella* Enteritidis and *Bacillus cereus* at 100 μg/disk, while rutin did not display any inhibition at concentrations of 400 μg/disk. The minimum inhibitory concentrations were also tested in the same study in a liquid medium. Rutin did not show any inhibition at 1,000 μg/ml, but when combined with quercetin a synergistic effect was seen against *Salmonella* Enteritidis. Both compounds combined produced a minimum inhibitory concentration of 100 μg/ml, while quercetin alone displayed a minimum inhibitory concentration of 250 μg/ml against *Salmonella* Enteritidis. Cetin-Karaca et al. (2015) found similar minimum inhibitory concentrations when testing rutin, quercetin, and other phenolic compounds against *Salmonella* Enteritidis and *Escherichia coli* O157:H7. Minimum inhibitory concentrations found for rutin, quercetin, and chlorogenic acid were over 20 ppm for each compound for both pathogens. A further investigation into the inhibitory properties of chlorogenic acid was evaluated independently on a range of human pathogens, and was shown to have a minimum inhibitory concentration

of 80, 40, and 40 ppm for *Escherichia coli*, *Salmonella* Typhimurium, and *Staphyloccocus aureus,* respectively (Lou et al., 2011). Chlorogenic acid increases the permeability of the outer membrane of bacteria, and thus allows the leakage of intracellular components and eventually leads to cell death (Lou et al., 2011).

The incorporation of buckwheat tissue into soil has displayed some allelopathic characteristics for the control of certain weeds. Haramoto and Gallandt (2005) and Kumar et al. (2009b) reported delayed emergence of redroot pigweed and lambsquarters and Powell amaranth, associated with the incorporation of buckwheat plant residues into soil. In these studies, no weed growth suppression was reported when growing buckwheat in the soil, suggesting that allelochemical exudates were not released through the root system during growth.

Sunn hemp, *Crotalaria juncea*

Crotalaria juncea, also known as sunn hemp, is part of the Fabaceae family and is commonly cultivated in India for its fiber production and as a green manure crop in temperate, tropic, and subtropic regions of the world (Cook and White, 1996). Beneficial properties of sunn hemp include drought tolerance, ability to fix nitrogen, and improvement of soil quality, while reducing soil erosion and conserving soil moisture (Sheahan, 2012). Sunn hemp is also known to suppress root-knot nematodes (Rotar and Joy, 1983) and can be used as a trap crop for soybean cyst nematodes (Kushida et al., 2003).

Crotalaria juncea produces a class of compounds called pyrrolizidine alkaloids, concentrated particularly in the seeds, which can be toxic to poultry and livestock when consumed (Sheahan, 2012). These nitrogen-containing compounds are formed from the intermediate ornithine during arginine biosynthesis (Mosjidis et al., 2012). Pyrrolizidine alkaloids are converted in the liver to toxic derivatives that interact with cellular DNA (Mattocks, 1978) and can cause damage to the liver, lungs, and heart (Yan et al., 1995). To remediate this threat, in 1982 the Natural Resources Conservation Service (NRCS), in coordination with the College of Tropical Agriculture & Human Resources at the University of Hawaii, released a cultivar of sunn hemp called 'Tropic Sun' which had reduced amounts of pyrrolizidine alkaloids (USDA-NRCS, 2009). This variety was deemed non-toxic to poultry and livestock, but only produces seeds south of the 28°N latitude (Treadwell and Alligood, 2008). Seed production in the United States is limited to the deep south, and therefore seed prices are extremely high (USDA, 1999).

Few studies have been performed to quantify the concentration of dehydropyrrolizidine alkaloids in sunn hemp seeds, with even less isolating and quantifying them from the whole plant biomass. Colegate et al. (2012) found several dehydropyrrolizidine alkaloids in the roots, stems, leaves, and seeds of the Tropic sun cultivar of sunn hemp. Concentrations of junceine, trichodesmine, isohemijunceine, and acetylisohemijunceine were quantified. Junceine had the highest concentrations, with the roots and leaves having 0.021 and 0.03 g/100g dry weight, respectively.

Acetylisohemijunceine was the most highly concentrated compound in the stems at 0.44 g/100g dry weight, and isohemijunceine was found in the greatest quantities in seeds at 0.07 g/100g dry weight.

Sunn hemp has been shown to exhibit allelopathic properties against other plant species during direct competition (Collins et al., 2008) and during the incorporation of sunn hemp residue into soil (Adler and Chase, 2007). Through direct competition, sunn hemp suppressed production of smooth pigweed (*Amaranthus hybridus*) biomass by 51% when grown in fields together at high sowing rates of 100 plants / m^2 (Collins et al., 2008). Sunn hemp has also displayed allelopathy against the germination of other plants when residues were incorporated into soil. Adler and Chase (2007) used sunn hemp extracts incorporated into soil at different rates of application, which delayed the germination of livid amaranth and bell pepper plants. Further studies need to be performed to investigate the mode of action for germination delays when sunn hemp extracts are incorporated into soils.

Soil Bioremediation Methods - Solarization

Soil solarization is a non-chemical and sustainable farming technique that utilizes solar energy to heat soil covered in plastic sheets that are commonly made of clear polyethylene (Stapleton, 2000). This approach of heating soil by trapping solar radiation has been known for centuries to control weeds, pests, and plant pathogens, although the employment of polyethylene plastic is a relatively new technique developed by Katan et al.

(1976). Soil solarization reduces the survival and persistence of mesophilic microorganisms, plant pathogens, and the weed seed bank within the first 4 to 5 cm of the soil profile, by raising the temperature of the soil to 40-55°C (Sofi et al., 2013). Critical to achieving these temperatures is the application of water to the soil profile prior to covering with polyethylene plastic, and the use of this technique in the summer months when longer photoperiods and higher temperatures increase the efficiency of the process (Cohen and Rubin, 2007; Rubin and Benjamin, 1984; Gamliel et al., 2000). Soil solarization typically requires 4 to 5 weeks at 40-55°C to effectively reduce plant pathogens via a greenhouse-like mechanism, which is produced underneath the plastic by trapping solar energy, moisture, and CO_2, thus increasing soil temperatures for prolonged periods of time (Sofi et al., 2013; Gamliel et al., 2000).

Additional factors that positively impact solarization efficiency are proper field preparations that increase aeration and conductivity in the soil, including ripping, tilling, organic matter incorporation, and irrigation events (Cohen and Rubin, 2007). The wetting of soil in order to increase conductivity is an extremely important factor when implementing solarization because it enables deeper soil layers to attain the desired increase in temperature (Mahrer et al., 1984). Smooth soil surfaces are also crucial to reduce the potential of plastic tearing which releases trapped moisture, subsequently reducing conductivity and maximum temperatures attained throughout the process (Gamliel, 1993). The addition of organic amendments such as incorporation of cover crops and animal manure can also increase the

effectiveness of solar heating by raising soil temperatures by 1-3°C via mechanisms of exothermic microbial activity and thermal conductivity (Gamliel, 1993).

The selection of the appropriate polyethylene plastic is an important factor when implementing soil solarization. The ability of the plastic to trap solar energy, volatiles, and retain moisture impacts the efficiency of the heating process (Cohen and Rubin, 2007). Clear polyethylene plastic, compared to black, is preferred due to its ability to allow the passage of solar radiation, while trapping high amounts of moisture and volatiles in the soil. Contrary to clear plastic, black polyethylene plastic absorbs most of the solar radiation, and thus depletes the amount of energy that reaches the soil (Rubin and Benjamin, 1984).

The process of soil solarization is not suitable for all climates or seasons, and therefore must be implemented in regions and times when appropriate. Solarization is commonly practiced around the world in zones with long photoperiods and high ambient temperatures (>30°C). Areas around the Mediterranean (Stapleton, 2000) and certain regions of the United States can use this technique for the control of plant pathogens due to their characteristically hot summers with long photoperiods (> 11 h), high UV indexes, and infrequent rain and extreme wind events (Egley, 1983, Standifer et al., 1984, Chellemi et al., 1997). These climactic advantages allow more solar energy to penetrate the polyethylene covers, thus enabling the soil to reach higher temperatures compared to other seasons or regions.

A successful solarization process can achieve many things besides the reduction of plant pathogens, pests, and weeds. Positive outcomes include increased plant growth responses (Gamliel, 1993), increased levels of some soil minerals (Adams et al., 2010), and population changes in a wide range of soil bacteria and fungi (Stapleton and DeVay, 1984). These changes are quite complex, having different outcomes depending on soil type, incorporation of organic amendments, and region/time of the solarization process. Changes in microbial community structure and composition of previously solarized soil are characterized by a decrease in fungal and many mesophilic bacterial species including *Escherichia coli* (Wu et al., 2009). Wu et al. (2009) reported a significant log 3 CFU/g decrease in the population of *E. coli* in soil after one week of solarization, with complete elimination, by the end of four weeks. Some thermophilic bacterial species from the genera *Bacillus*, survive this thermal process and thus play a role in the re-colonization and restructuring of the soil microbial communities. The improvements of overall plant growth, health, and yields have been associated with this re-colonization and redistribution of the soil microbial community post solarization, even after numerous crop cycles (Gamliel, 1993; Stapleton, 2000; Ibarra-Jimenez et al., 2012; Lombardo et al., 2012; Sofi et al., 2013). One example of this enhanced plant growth was reported by Ibarra-Jimenez et al. (2012), who found that the total yield of dry beans increased by over 1.5 t/ha when grown in solarized soils compared to non-solarized soils. The rise in total yield of dry beans was attributed to various factors including an increase in nutrient availability (organic carbon, nitrogen, and

potassium), suppression of plant pathogens, and an increase in the populations of beneficial microorganisms compared to non-solarized soils (Ibarra-Jimenez et al., 2012; Sofi et al., 2013). Lombardo et al. (2012) also reported higher plant growth and yields in tomato plants grown in solarized soil during the first cropping season; however in subsequent seasons, growth yield decreased slightly although it was still significantly higher than yields associated with plants grown in non-solarized soil. Overall, soil solarization has the potential not only to control plant diseases and weed populations, which are both detrimental to crop yields, but also to potentially serve in reducing the survival and persistence of human pathogens in agricultural soils.

Bioremediation Potential of Cover Cropping (mustard greens, buckwheat, and sunn hemp) and Solarization to Treat *Salmonella* Contamination in Soils

To date, there is a clear absence of sustainable mitigation techniques that farmers can implement when facing a soil contamination event by any human pathogen that allows the replanting of fields within a relatively short timeframe. Currently, when field contamination is identified, the industry's best practice is to abandon these fields and move farming practices to other growing regions, using time as the remediation strategy (Suslow, 2014). Human pathogens, specifically *Salmonella enterica,* have shown incredible persistence within certain soil systems, effectively increasing abandonment times of fields used to produce high value cash crops and capital investment losses. Consequently, it is vital to

develop and implement soil remediation practices that reduce or eliminate human pathogen contamination for the long-term sustainability and stability of the produce industry. Bio-mitigation techniques that follow sound and sustainable farming practices are potential alternatives to chemical based remediation methods that could eventually hinder soil health and plentiful crop production. Short-term cover cropping and solarization have been proposed as potential alternatives to reduce human pathogen contamination in soils and also have the ability to be implemented across a wide range of cropping systems. The objective of this proposed study is to evaluate the efficacy of these practices on reducing enteric pathogen contamination in soils by various mechanisms including the allelopathic characteristics associated with mustard greens (*Brassica juncea*), buckwheat (*Fagopyrum esculentum*), and sunn hemp (*Crotalaria juncea*) and prolonged soil heating from solarization techniques.

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CHAPTER TWO – Bio-mitigation Strategies to Reduce the Survival and Persistence of *Salmonella enterica* in Vegetable Production Soils

Abstract

The lack of economically realistic, sustainable, and practical soil remediation techniques to reduce or eliminate the survival and persistence of enteric pathogens in vegetable production soils, commonly leads to crop and field abandonment with extended periods between replanting. In this study, short-term cover cropping (mustard greens, buckwheat, and sunn hemp) coupled with solarization was assessed as a potential bio-remediation tool to reduce the survival and persistence of *Salmonella* Typhimurium (ST) in agricultural soils in a typical 3-month cropping cycle. A three-tier approach was followed to assess the survival and persistence of avirulent *Salmonella* Typhimurium (AST), which included *in-vitro*, microcosm, and field studies. *In-vitro* plant inhibition tests, using slurries from mustard greens leaves/stems, significantly reduced the population of AST by an average of log 2 CFU/ml after 24 hours incubation at 37°C ($P<0.05$). Roots from mustard greens prevented growth of AST, while root slurries from buckwheat and sunn hemp promoted growth of AST over the same time-temperature incubation ($P<0.05$). In microcosm studies, higher storage temperature significantly ($P<0.05$) decreased the survival of AST, irrespective of soil type and moisture content. In clay soil only, the application of raw layer-hen manure (80 kg of N/0.4 ha) significantly decreased the survival of AST at storage temperature between 20 and 30°C. Persistence of AST was extended by 1 week

when stored at temperatures ≤30°C with soil moisture contents 25% above field capacity, irrespective of soil type ($P<0.05$). The addition of raw layer-hen manure did not increase the persistence of AST ($P>0.05$) at soil moisture contents at or below field capacity, regardless of soil type and storage temperature. Greater persistence ($P<0.05$) of AST (28 d vs. 21 d) was observed at temperatures ≤ 20 °C compared to 30 or 37°C, irrespective of soil moisture content, soil type, and manure availability. The persistence of AST in clay soil was 14 d longer than in sandy loam soil at moisture contents 25% below field capacity and storage temperatures greater than 30°C. In field trials, time to non-detection of AST varied between soil type (clay loam 22 days vs. sandy loam 145 days) and growing season. AST was not detected after growing any of the selected short-term cover crops and after solarization (2-months). The use of cover cropping with mustard greens coupled with solarization seems to be associated with a significant reduction in the survival and persistence of AST; however, further studies are needed to describe the potential interactions governing these bio-mitigation strategies.

Keywords: *Salmonella* Typhimurium, soil, remediation, cover crops, solarization, persistence

Introduction

Salmonella enterica has been identified as the bacterial agent causing the largest number of foodborne illnesses and fresh produce outbreaks (> 70) from 2004 to 2012 (Sivapalasingam et al., 2004; Callejon et al., 2015; CDC, 2015). Due to the lack of a lethality treatment during the minimal processing of fresh fruits and vegetables, pre-harvest control measures are extremely important to reduce pathogen contamination and prevent disease. To date, farmers facing a soil contamination event with any human pathogen have no tangible and effective remediation strategies that will allow them to replant their fields within a short period of time. Currently the industry best practice is to abandon contaminated fields and move farming practices to other growing regions. Consequently, it is imperative for the sustainability of the agricultural industry within the U.S. to test and implement practical soil remediation techniques that can reduce or eliminate enteric pathogens contaminating agricultural soils. It is crucial that these strategies are time and cost effective for farmers, regardless of production scale, farm practices, and crop type, to expedite planting or replanting of these fields and to minimize financial losses.

In this study, short-term cover cropping (mustard greens, buckwheat, and sunn hemp) coupled with solarization were tested to assess their bio-remediation efficacy in reducing the survival and persistence of an avirulent *Salmonella* Typhimurium (AST) strain in agricultural soils during a 3-month period. Short-term cover cropping and solarization have been used to control plant pathogens and reduce the weed seed bank and infield weed pressure,

presumably through a combination of allelopathic and thermal inactivation processes, respectively. Despite their promise, there is a relative lack of data on the effectiveness of these practices as management prospects.

The short-term cover crops mustard greens, buckwheat, and sunn hemp each produce a distinct class of secondary plant metabolites that are known to exhibit allelopathic properties against plant pathogens and weeds (Haramoto and Gallandt, 2005; Adler and Chase, 2007; Kumar et al., 2009b; Motisi et al., 2009a; Motisi et al., 2009b, Collins et al., 2008) and bacterial inhibition against human pathogens when in purified form (Kyung et al., 1997, Wittstock et al., 2003, Lou et al., 2011). While the utilization of cover crops focuses on the potential bactericidal properties of secondary plant metabolites, solarization focuses on bacterial inactivation through heat exposure over time (Cohen and Rubin, 2007; Rubin and Benjamin, 1984). The top 5 cm of the soil profile can reach temperatures of 40-60°C as solar energy is captured by clear polyethylene plastic coverings (Stapleton, 2000). The efficacy and length (4-5 weeks) of this non-chemical, sustainable farming technique is also dependent on the wetting of soil prior to covering (Gamliel et al., 2000). The solarization process changes the microbial community of treated soils, which is characterized by a decrease in many mesophilic bacterial groups (Stapleton and DeVay, 1984).

Brassica juncea (mustard greens) produces various glucosinolates, which are sulfur-containing water soluble compounds found in plant vacuoles. Glucosinolates are hydrolyzed into isothiocyanates, and other secondary products, when in direct contact with the enzyme

myrosinase, sequestered in separate plant organelles (Halkier and Gershenzon, 2006). These hydrolysis products are the compounds mainly responsible for the biological activity of glucosinolate producing plants (Wittstock et al., 2003). Sinigrin in the predominant glucosinolate identified for mustard greens (Tong et al., 2014; Gimsing et al., 2005; Gimsing and Kirkegaard, 2006), and is hydrolyzed by myrosinase into allyl isothiocyanate in macerated tissue (Dai et al., 2014). Allyl isothiocyanate, in a liquid form, has shown inhibitory properties against *Escherichia coli, Salmonella* Typhimurium and *Listeria monocytogenes* in concentrations ranging from 50 to 200 ppm (Kyung et al., 1997). It is hypothesized that the bactericidal properties of allyl isothiocyanate are caused by cellular leakage by alteration in the cellular membrane, enabling greater permeability (Lin et al., 2000).

Fagopyrum esculentum (buckwheat) produces a variety of phenolic compounds in the roots, stems, leaves, and flowers, with rutin being the highest produced phenolic compound found in the above ground parts of the plant (Li et al., 2010). In numerous studies, pure rutin alone has not displayed inhibitory properties against human bacterial pathogens (Arima et al., 2002, Cetin-Karaca et al., 2015), although when combined with other phenolic compounds produced by buckwheat, including quercetin, a synergistic inhibitory effect has been observed at a concentration of 100 μg/ml (Arima et al., 2002). Chlorogenic acid is another compound found in relatively high concentrations in the flowers, leaves, and stems of buckwheat. Research has shown that pure chlorogenic acid inhibits the growth of

Escherichia coli, *Salmonella* Typhimurium, and *Staphylococcus aureus* at concentrations of 40-80 ppm by potentially increasing outer membrane permeability (Lou et al., 2011).

Crotalaria juncea (sunn hemp) is cultivated mainly for fiber production, but is also commonly used as a weed suppressant in vegetable and row crop production systems (Cook and White, 1996). Sunn hemp produces a class of compounds called pyrrolizidine alkaloids, which are nitrogen-containing compounds that can be toxic to animals after consumption (Sheahan, 2012). However, no reports to date have linked growth inhibition of human pathogens to exposure to pyrrolizidine alkaloids.

The aim of this study was to determine whether any of the short-term cover crops listed above paired with solarization could expedite *Salmonella* Typhimurium die-off, in organic agricultural soils amended with raw layer-hen manure, to allow the rapid replanting of spinach within a 3-month window. A three-tier approach was followed to assess the survival and persistence of avirulent *Salmonella* serovar Typhimurium (AST), which included *in-vitro*, soil microcosm, and field studies. *In-vitro* plant inhibition tests evaluated the potential bactericidal properties of mustard greens, buckwheat, and sunn hemp as a whole against AST, soil microcosm studies evaluated the survival and persistence characteristics of AST at various moisture contents, soil types, temperatures, and with raw layer-hen manure over time, while field studies evaluated cover cropping paired with solarization on the survival and persistence of AST.

Materials and Methods

Culture Preparation

An avirulent strain of *Salmonella enterica* serovar Typhimurium χ3985 (AST), resistant to rifampicin (rif) (80μg/ml**)**, was used in all our *in-vitro*, microcosm, and field inoculum studies. This strain was chosen to as to differentiate it from competitive environmental strains, to be able to use it in open field environments, and because it has shown similar persistence to pathogenic strains of *Salmonella* in controlled laboratory studies (data not published).

Inoculum Preparation

The selected strain was prepared for *in-vitro*, microcosm, and field inoculation studies following a similar approach to the procedure described by Gutierrez-Rodriguez et al. (2011) and Lopez-Velasco et al. (2012). In brief, AST was first grown overnight at 37°C in trypticase soy broth (Becton, Dickinson and Company, Sparks, MD) amended with rifampicin (50 μg/ml) (Tokyo Chemical Industry Company (TCI), Philadelphia, PA). A 500 μl aliquot of this overnight culture was spread plated onto trypticase soy agar (Becton-Dickinson) amended with pentachloronitrobenzene (PCNB; Sigma Aldrich, St. Louis, MO) (5 mg/ml), pyruvic acid (Sigma Aldrich) (1 ml/L), and rifampicin (50 μg/ml) (TSA-PPR). The TSA-PPR plates were then incubated at 37°C for 24 hours. Bacterial growth was removed by flooding the plate with 1 ml of Butterfield's phosphate buffer (BPB) followed by

removal with a spreader. The bacterial slurry was then pipetted into a 2 ml Eppendorf tube and centrifuged for 2 minutes at 14,000 rpm (Eppendorf, Hauppauge, NY). This latter process was repeated three times, while decanting the supernatant and adding new BPB every time. The cleaned pellet was then re-suspended in 1ml of BPB and the optical density (OD), at 600 nm, of the solution was adjusted to 0.745 using a spectrophotometer (Biomate^{TM} 3S, Thermo Fisher Scientific, Waltham, MA). This OD represented a cell density of log 9 CFU/ml, which constituted the working stock. This was diluted to prepare inoculum concentrations of log 4, 5, or 8 CFU/ml depending on the targeted experiment. The actual final concentrations were confirmed by plating serial dilutions of the prepared inocula on duplicate TSA-PPR plates.

Plant Inhibition Tests

Mustard greens (*Brasicca juncea)*, buckwheat (*Fagopyrum esculentum),* and sunn hemp (*Crotalaria juncea*) were grown under greenhouse or field conditions for 35 to 45 days. Plant material for each inhibition test was collected with sterile clippers and stored at 4°C before initiation of each test within 24 hours of collection. The leaves/stems portion of each crop was kept separate from the roots and excess soil was removed before plant maceration.

The leaves plus stems of each cover crop were then combined with sterile HPLC grade water to achieve a 1:1 ratio (w:v) and then blended using a professional blender

(Model BL660, SharkNinja Operating LLC, Newton, MA) for 5 minutes. The roots of each cover crop were also combined with sterile HPLC grade water, but at a 1:3.25 ratio (w:v) and blended as described above. The slurries produced from the leaves/stems and roots were divided into triplicate 5 ml aliquots for each time point tested and inoculated with AST at a final nominal concentration of log 2 and log 4 CFU/ml of slurry. The surviving population of AST was determined at 1 h intervals for the first 8 h and there after at 12 and 24 h of incubation at 37°C. Bacterial enumeration was determined by 10-fold dilutions using BFB followed by plating onto duplicate TSA-PPR plates. Bacterial enumeration occurred during a period of 24 hours to reduce the potential degradation of secondary plant metabolites during the experimental period.

Soil Processing and Bacterial Enumeration

Soil samples collected from microcosm or field experiments were kept at 4°C for 24 h before processing. To evaluate survival of AST, recovery from a mixed composite sample was achieved using the bacterial recovery method described by Gutierrez-Rodriguez et al. (2011). In brief, a soil subsample was mixed with 0.02 M sodium phosphate buffer + 0.05% Tween 20 (Fisher Scientific, Pittsburgh, PD) at a 1:1 ratio (soil/buffer). Tenfold serial dilutions using BPB were carried out to enumerate AST populations onto duplicate TSA-PPR plates. To evaluate the persistence of AST, recovery below the limit of detection was determined through a sequential enrichment process consisting of a 1:1 ratio of 2x

concentration of Universal Pre-enrichment broth (UPB) (Becton-Dickinson) and soil slurry incubated at 35°C overnight, followed by enrichment of a 10 ml aliquot coming from the UPB mixture with 90 ml of Tetrathionate Base broth (Becton-Dickinson) + Iodine-Potassium Iodide solution (20ml/L) for 6-8 h at 42°C. A final enrichment consisting of a 20 ml aliquot of the TTB enrichment into 180 ml of M-Broth (Becton-Dickinson) amended with and without rif (50 µg/ml) for the isolation of AST and naturally occurring *Salmonella* spp. potentially coming from raw layer-hen manure, respectively. The M-Broth enrichment was finally streaked onto Salmonella Shigella agar (Becton-Dickinson) amended with rif (50 µg/ml) for recovery of AST or XLT4 without rif for naturally occurring *Salmonella* spp. Molecular confirmation of AST or naturally occurring *Salmonella* spp. was performed via probe-based traditional PCR targeting *invA* and *spvC* genes using primers described by Chiu and Ou (1996) and Agron et al. (2001), respectively (Table 1). The 25-µl PCR mixture consisted of 5 µl 5x Green Reaction Buffer (Promega, Fitchburg, WI), 2 mM $MgCl_2$, 0.2 mM deoxyribonucleoside trisphophates combined, 0.1 mM primer pairs, 0.25 µl Go Taq polymerase (Promega), 1 µl bacterial culture, 2.5 µl 10% DMSO, and 11.25 µl sterile HPLC grade water. The thermocycler (Eppendorf, Hamburg, Germany) parameters used for the amplification of the targeted DNA sequences were the following: 45 s at 94°C, 60 s at 56°C, and 2 m at 72°C for a total of 30 cycles followed by a final extension cycle set at 72°C for 10 m. Repetitive element PCR (REP-PCR) was used to determine the recovery efficiency of the inoculated AST and to determine if the same naturally occurring *Salmonella* spp. were

present along different sampling events and experimental conditions (Bech et al. 2014). Serotyping of naturally occurring *Salmonella* spp. was performed at the University of California, San Bernadino, California Animal Health and Food Safety Laboratory (CAHFS).

Raw layer-hen manure Application rates and Bacterial Recovery

Composite samples from raw layer-hen manure were collected from a birdhouse holding 5,000 layer-hens located within the Piedmont Research Station in Salisbury, NC. During each sampling event, a sterile 4 oz scoop and Whirk-pak bag (Nasco, Fort Atkinson, WI) were used to collect approximately 100 g of raw manure. Manure samples were processed within 24 hours of collection and were kept at 4°C prior to processing. The Nitrogen (N) content of this manure was also analyzed at the North Carolina Department of Agriculture and Consumer Services laboratory (Raleigh, NC). This information was used to determine the amount of raw layer-hen manure needed to deliver a final N concentration of 80 kg N/0.4 ha in microcosm and field trials.

Recovery of naturally occurring *Salmonella* spp. from composite raw layer-hen manure samples was accomplished first by placing 25 g of raw manure into a sterile filter bag and mixing the manure with 225 ml of UPB. Each sample was massaged for 1 minute and later incubated at 35°C overnight. After incubation, a two-step enrichment process was followed. First, a 10 ml aliquot of the UPB enrichment was transferred to 90 ml lactose broth, 2x concentration, (Becton-Dickinson) and incubated at 35°C for 24 hours. After

lactose enrichment, a 10 ml aliquot was added to 90 ml of buffered peptone water, 2x concentration, (Becton-Dickinson) and incubated at 35°C for 24 hours. This enrichment was streaked onto XLT4 plates and incubated at 35°C for 24-36 hours. Five colonies per plate were isolated and further purified by streaking prior to molecular confirmation of each strain via probe-based traditional PCR using the method described above. Repetitive element PCR (REP-PCR) was used to determine the recovery efficiency of the inoculated AST and to determine if the same naturally occurring *Salmonella* spp. were present along different sampling events and experimental conditions (Bech et al. 2014). Serotyping of naturally occurring *Salmonella* spp. was performed, as described above, by CAHFS.

Soil Microcosms

Soil was collected from two different fields located within the Piedmont Research Station in Salisbury, North Carolina. The soil collected was kept refrigerated at 4°C until processed. Testing for naturally occurring *Salmonella* spp., as described above, was performed before microcosm establishment. Sandy loam (Warme Loam and Dorian Find Sandy mix) and clay loam (Mecklenburg clay) soils were used in all microcosm and field studies. Prior to the preparation of each soil microcosm, the collected soil was sieved through a 4 mm mesh to remove rocks and other debris. The moisture content of the soil was determined using the method described by Gutierrez-Rodriguez et al. (2011). In brief, a representative sample of 100 g of soil was oven-dried at 70°C in a pre-weighed vessel. The

moisture content was then calculated on a percent basis. The moisture content of all microcosm studies was adjusted to three different levels; field capacity, 25% above, and 25% below field capacity by adding the AST inoculum (log 5 CFU/g of soil) into six different containers (each with 8 to 10 kg of soil) (2 containers for each moisture content). Clay soil moisture contents were 21, 28, and 35%, while loam soil moisture contents were 13.5, 18, and 22.5%. Raw layer-hen manure was then added to one of the duplicate containers and thoroughly mixed with a sterile scoop to achieve a homogenous distribution of the inoculum and manure before the preparation of each 400 g microcosm. Each of the six containers of soil was then further subdivided into 15 different GA-7 Plant Culture boxes (Sigma Aldrich) (3 in x 3 in x 3.8 in), with punctures through the lid to allow proper soil aeration within the 400 g soil microcosm. Three replicates per treatment combination were then stored at five different temperatures (4°C, 10°C, 20°C, 30°C, and 37°C) (total of 90 microcosms). The population of AST for each GA-7 box was determined every 7 days for a total of 4 weeks using the method described above.

Field Trials

Clay Loam Trials

A clay loam field located in the Piedmont Research Station in Salisbury, NC was first plowed, then tilled using a John Deere Rotary Tiller RT11 (Deere & Company, Moline, IL) carrying a 6 m x 1.5 m discs and left fallow for 2 weeks before 10x10x10 fertilizer was

broadcast at a rate of 50 kg/ha. The field was sprinkler irrigated every 3-4 days dependent on weather conditions throughout the entire experiment. Experimental conditions started in summer and ended in late fall 2014.

A total of 16 plots, each 6 m x 6 m, were established in the selected field and raw layer-hen manure was spread through the plots at a rate of 80 kg of Nitrogen per 0.4 hectare and then tilled into the soil. The presence of naturally occurring *Salmonella* spp. was determined from this manure prior to field application as previously described. Once manure was applied, the AST inoculum at a final nominal concentration of log 5 CFU/g of soil was incorporated into each 6 m x 6 m plot at dusk using a Craftsman Pro Series 4 gallon Backpack Sprayer (Sears Brands, LLC, Hoffman Estates, IL). After inoculation, three short-term cover crops (mustard greens (*Brasicca juncea*), buckwheat (*Fagopyrum esculentum*), and sunn hemp (*Crotalaria juncea*) (Harris Seeds, Rochester, NY)) were seed broadcasted onto 4 replicated plots, leaving 4 plots fallow (control). Cover crops were seeded at a rate of 107, 90, and 67 kg/ha, respectively. Cover crops were grown for 35-45 days followed by a second AST inoculation set at final nominal concentration of log 5 CFU/g of soil, with a backpack sprayer followed by tilling.

After tilling, each plot was sprinkler irrigated for 4 hours at a rate of 2.2 gpm with a 25 psi regulator and then covered with clear polyethylene plastic sheets, 0.2 mm in thickness, for a period of one month for the solarization process. Soil samples, for AST enumeration, were recovered prior to covering the soil with plastic and after one month of solarization.

The temperature within each solarized plot was monitored over one month using Watch-Dog B-Series Button Data Loggers (Spectrum Technologies, Aurora, IL). These temperature monitors were placed 30 cm below the soil surface and the temperature was recorded every 3 hours for the duration of the solarization experiment. Soil temperatures at a depth of 10 cm were recorded by the SALI weather station located in the Piedmont Research Station, to compare to solarized soil temperatures.

Sandy Loam Trials

A sandy loam field located in the Piedmont Research Station in Salisbury, NC was first plowed, tilled, and fertilized following the same procedure described above for the clay loam trials. However, at this location a total of 12 beds (4.572 m by 0.762 m, all with raw layer-hen manure applications at a rate of 80 kg N/0.4 ha) were used to assess the survival and persistence of AST inoculated to the soil profile at log 8 CFU/g of soil in the presence of raw layer-hen manure and spinach plants. Spinach (cv. Whale, Harris Seeds) seeding rate was 3 million seeds per 0.4 hectares. This approach was performed during fall 2014 through winter 2014. After 35 days of spinach cultivation, plants, inoculum, and raw layer-hen manure were incorporated into the soil profile using a Husqvarna FT900 205cc 26-in Front-Tine Tiller (Husqvarna Group, Stockholm, Sweden). This tilling procedure allowed the manure and the crop residue to remain within the top 10 cm of the soil profile. The survival

and persistence of AST was determined during a period of 145 days. Soil sampling and recovery of AST followed the same procedures described above.

Statistical Analysis

A randomized complete block design was used in both clay loam and sandy loam field trials. Statistical analysis was performed by one-way analysis of variance (ANOVA) with means separation by the Tukey test using SAS 9.2 (SAS Institute, Cary, NC). A *P* value of <0.05 was considered to be statistically significant for all experiments. Associations of AST persistence with soil type, moisture content, and temperature were determined using a chi-square test using SAS 9.2.

Results

A three-tier approach was followed to evaluate the survival and persistence of AST, i.e., *in-vitro* plant inhibition experiments, microcosm experiments, and field studies.

Plant Inhibition

Plant inhibition tests using slurries produced from the leaves/stems and roots of mustard greens, buckwheat, and sunn hemp were established to determine the influence of secondary plant metabolites (glucosinolates, phenolic compounds, and pyrrolizidine alkaloids, respectively) on the survival of AST. AST was inoculated into leaves/stems and

root slurries at log 2 and log 4 CFU/ml, which resulted in very similar population patterns throughout the entire 24 h incubation period (log 4 data not shown). The population of AST dropped by 1.84 log CFU/ml in the presence of mustard greens leaves/stems after 24 h incubation at 37°C. This was statistically significant. This reduction in AST populations was observed between 8 and 24 h of incubation (Figure 1). Similar population growth patterns were observed for slurries of both buckwheat and sunn hemp leaves/stems. A continuous gradual increase in the population of AST was observed in the presence of buckwheat and sunn hemp slurries from 3 h to 12 h of incubation. The average increase in the population of AST was log 1.7 and 2.38 CFU/ml for buckwheat and sunn hemp, respectively, over a 24 h period. Mustard greens root slurries did not have a significant influence on the population of AST over 24 hours incubation at 37°C ($P<0.05$), but rather produced a latent state in which no significant increase or decrease in the AST population was observed ($P>0.05$)(Figure 2). The AST population increased by 1.1 and 1.3 log CFU/ml in the presence of sunn hemp and buckwheat root slurries, respectively, after 24 h incubation.

Soil Microcosms

Microcosm experiments were established using both clay loam (Mecklenburg clay) and sandy loam (Warme Loam and Dorian Find Sandy mix) soils at varying moisture contents and storage temperatures. Overall, higher storage temperatures were associated with a significant decrease in the survival of AST, irrespective of soil moisture content and

soil type ($P<0.05$) (Table 2). The application of raw layer-hen manure to sandy and clay loam soils did not decrease die-off of AST at all temperatures, except generally for clay loam soils stored between 20 and 30°C. Moisture content and soil type did not change the log reduction of AST for all storage temperatures with or without the addition of raw layer-hen manure.

No significant differences ($P<0.05$) between the populations of AST at various moisture contents were observed during 4 weeks of storage at 4, 20, and 30°C. Storage temperatures below 20°C resulted in prolonged survival of AST over four weeks storage, irrespective of manure addition and soil type ($P<0.05$). Addition of raw layer-hen manure did not have any significant effects on the population of AST at all temperatures within both soil types, except for clay loam soils stored at 20-30°C ($P<0.05$) (Figures 3 and 4).

Longer persistence (28 d vs. 21 d) of AST was observed at temperatures ≤ 20°C, regardless of soil moisture content, soil type, and manure availability (Table 4a and 4b). Irrespective of storage temperature and soil type, the addition of raw layer-hen manure did not change the persistence of AST in soils set at field capacity or 25% below this moisture content. Soil type affected the persistence of AST (clay loam 28 d and sandy loam 14-21 d) only at storage temperatures above 30°C and with soil moisture contents 25% below field capacity.

Field Trials

Clay Loam Trials

The survival and persistence of AST was negatively impacted by the inoculation and tilling methods. The initial population of AST for all plots was on average log 3.76 CFU/g of soil before tilling and cover crop incorporation. Three days after tilling and cover crop incorporation were implemented, the population of AST was on average reduced by log 2.31 CFU/g of soil for all treatments; however, the residual population of AST from the control and mustard greens plots were on average log 0.83 CFU/g lower than that for buckwheat and sunn hemp plots. After 5 days of inoculation and cover crop incorporation, no detectable AST was observed for the control plot compared to an average residual population of log 1.23 CFU/g of soil for the cover crop treatments. No significant difference between the survival of AST was observed after 15 days of inoculation and cover crop incorporation when comparing the control plots to those containing each cover crop ($P>0.05$). Each plot showed an average decrease in the population of AST of log 3.13 CFU/g of soil after 15 days of inoculation, while time to non-detection for all treatments was 22 days (Figure 7). Die-off rates ranged from log 0.13 to 0.18 CFU/g per day for all plots within this field trial. During solarization, the average soil temperature 30 cm below the soil surface was 5.5 °C higher than non-solarized soil temperatures at a depth of 10 cm, as recorded by the SALI weather station in Salisbury, NC (Figure 8). After 31 days of solarization, AST could not be recovered from the control and cover crop plots.

Sandy Loam Trials

The average inoculum dose of AST for plots 1 and 2 was log 7.86 CFU/g of soil. During the spinach cropping cycle, a continuous decline in the population of AST was observed. After 53 days, an average log reduction of 4.86 CFU/g was observed for each plot. Spinach plants were tilled into each plot at day 53 and the survival of AST continued to decrease over the next 50 days to a final average population of log 0.33 CFU/g soil for plot 1. The persistence of AST varied by 44 days between plots with an overall persistence of up to 101 days for plot 1 and 145 days for plot 2 (Figure 9). The die-off rates of AST for plot 1 and 2 were log 0.05 and 0.08 CFU/g of soil per day, respectively.

Discussion

Salmonella enterica is consistently reported as the bacterial agent causing the greatest number of illnesses, hospitalizations, and deaths associated with foodborne outbreaks (Sivapalasingam et al., 2004; Jacobsen and Bech, 2012; CDC, 2015). Due to the lack of a lethality treatment during the minimal processing of fresh fruits and vegetables, pre-harvest control measures are extremely important in order to reduce the likelihood of pathogen contamination across the production chain. To date, farmers facing a soil contamination event with any human pathogen have no practical and effective remediation strategies that will allow the replanting of fields within a short time. Currently the industry best practice is to abandon contaminated fields and move farming practices to other growing regions when

facing a contamination event (Suslow, 2014). Consequently, it is imperative for the long sustainability of the U.S. agricultural industry to test and implement practical soil remediation techniques that can reduce or eliminate human pathogen contamination and are time and cost effective to farmers. In this study, short-term cover cropping (mustard greens, buckwheat, and sunn hemp) coupled with solarization was tested to assess bio-remediation efficacy in reducing the survival and persistence of AST in agricultural soils during a 3-month period.

Within *in-vitro* studies, slurries produced from the leaves and stems of mustard greens were associated with significant reductions in the population of *Salmonella* Typhimurium, which is consistent with various studies evaluating the inhibitory properties of secondary metabolites produced by mustard against various human pathogens (Kyung et al., 1997; Delaquis et al., 1997). In these previous studies, the inhibition of *Salmonella* Typhimurium was evaluated when subjected to allyl isothiocyanate, which is a hydrolysis product of sinigrin, a common glucosinolate produced by mustard greens (Gimsing and Kirkegaard, 2006). Upon tissue damage, sinigrin is hydrolyzed into allyl isothiocyanate through contact with the enzyme myrosinase (Bones and Rossiter, 1996; Halkier and Gershenzon, 2006). In these *in-vitro* studies, plant material was macerated and then mixed with HPLC grade sterile water to achieve the production of allyl isothiocyanate. The high amount of reported sinigrin within the leaves and stems of mustard (Gimsing and Kirkegaard, 2006), and subsequent hydrolysis to allyl isothiocyanate, could be the cause of AST

population reduction. The root slurries from mustard greens had no negative or positive effect on the population of inoculated AST. It has been determined that leaves and stems from mustard greens tend to sequester higher concentrations of sinigrin compared to the roots (Gimsing and Kirkegaard, 2006), explaining the observed difference in the survival of AST between these two different mustard greens slurries. This observation could also be affected by differences in the tissue to water ratio for leaves/stems and roots, since a higher volume of water was needed to prepare root slurries.

Buckwheat leaves/stems and root slurries did not exhibit bactericidal properties against AST. These results differ from previous reports by Lou et al. (2011) and Cetin-Karaca et al. (2015), who found that phenolic compounds from this plant exhibited inhibition against various human pathogens. The different observations reported here could be attributed to lower concentrations of the active phenolic compounds produced by the buckwheat plant, compared to previously described minimum inhibitory concentrations of the purified compounds in the literature (Li et al., 2010; Lou et al., 2011; Cetin-Karaca et al., 2015). The lack of bactericidal activity could also be associated with lower phenolic concentrations in both plant tissues due to differences in growing conditions or plant physiological maturity at harvest (Li et al., 2010; Li et al., 2012; Kim et al., 2004).

To our knowledge, the pyrrolizidine alkaloids present in sunn hemp plants have not been evaluated for their potential inhibitory properties against human pathogen growth, survival, and persistence. Sunn hemp plant slurries did not inhibit growth of AST *in-vitro*.

These observations could be attributed to differences in the mechanism of action between allelopathic inhibition of weeds and plant pathogens (Adler and Chase, 2007) and bacterial inhibition of human enteric pathogens.

Human enteric pathogens, specifically *Salmonella*, display exceptional persistence in soil (Baloda et al., 2001) contributing to the need for prolonged periods of time in which fruit and vegetable fields must remain fallow before replanting. During these prolonged periods of farm inactivity, soils are without any green cover, leading to broad and constant fluctuations in soil moisture content and temperature. These factors have been suggested to increase human pathogen die-off in soil (Chandler and Craven, 1980; Cools et al., 2001; Holley at al., 2006; Garcia et al., 2010; Abd-Allel and Maysa, 2015). In soil Microcosm studies, higher storage temperatures decreased the survival of AST regardless of soil moisture content and soil type, with lower temperatures extending survival through the entire experimental period. This finding is supported by numerous studies investigating the relationship between temperature and survival rate of *Salmonella* in soil systems (Holley et al., 2006; Garcia et al., 2010; Abd-Allel and Maysa, 2015). Higher temperatures increase the microbial activity of endogenous microorganisms and competition for nutrients, thus reducing survival rates of the transient colonizers of the rhizosphere including human pathogens (Cools et al., 2001; Jiang et al., 2002).

The survival of AST was not significantly influenced by soil type, which is supported by studies from Natvig et al. (2002) and Danyluk et al. (2007). However, other studies

including those of Holley et al. (2006), Wang et al. (2014), Ma et al. (2013), and van Elsas et al. (2001) suggest that finer-textured soil types (clay) with higher organic matter content aid in the persistence of microorganisms by promoting the aggregation of water in the soil (Wessendorf and Lingens, 1989; Lau and Ingham, 2001). In our study, soil moisture content was kept constant in a closed system and thus no moisture loss occurred through evaporation or percolation of water, which is common with coarser textured soils. These factors could have contributed to the lack of significant differences in AST survival between soil types over time at most storage temperatures. The addition of raw layer-hen manure only decreased the survival of AST for clay loam soils at temperatures between 20 and 30°C. These findings differ from with previous ones by Abd-Elall and Maysa (2015) and Garcia et al. (2010) who reported that manure addition significantly decreased the survival of *Salmonella* Typhimurium at a range of temperatures from 5-25°C. Within our study, it is possible that the decrease in microbial activity associated with lower storage temperatures (<20°C) attributed to no significant decrease in the population of AST with manure addition. Inversely, higher temperatures (>30°C) may have increased the microbial metabolic rate of both AST and endogenous soil microorganisms to such an extent that the relationship between manure addition and AST survival was not evident, due to the extensive log reduction seen by week 1. Additionally, in soil microcosm study, soil was not sterilized prior to inoculation, so one could assume that naturally occurring microflora in the clay loam soil contributed to the survival dynamics seen of the AST strain at 20 and 30°C. Further, the

physicochemical properties and nutrient availability of sterile soil are quite different from natural soil, which could explain the differences between results when comparing across studies.

In this study, the higher microbial diversity of in clay soil was not associated with reduced survival and persistence of the artificially inoculated AST compared to sandy loam soil that had lower microbial diversity. These result are not in agreement with Brandl (2006), Quilliam et al. (2012), Bourn et al. (2002), and van Elsas et al. (2011) who reported greater die-off of *E. coli* O157:H7 and other enteric pathogens in soils with greater microbial diversity possibly due to increased nutrient accessibility, competition, and predation.

Salmonella enterica has been known to enter a viable but non-culturable (VBNC) state under extreme environmental conditions, in which recovery by direct plating methods fail, but these VBNC *Salmonella* can still be recovered through slow and gradual nutrient supplementation (Roszak et al., 1984; Reissbrod et al., 2002; Oliver, 2009). In this study, the persistence of AST was evaluated at temperatures at the low (4°C) and high end (37°C) of metabolic activity, resembling soil temperature fluctuations in Salisbury, NC. Only at 37°C did we observe a significant reduction in the persistence of this strain, despite following a slow and gradual three-step enrichment process to potentially resuscitate VBNC AST cells. Roszak et al. (1984) suggested that bacterial recovery when under the VBNC phase could only be obtained until a certain point in time (21 d) when held at 25°C. This observation is supported by our microcosm studies, since the ability to recover AST clearly changed based

on incubation temperature and storage time suggesting that this threshold was reached at the latter sampling times and temperatures above 30°C. Differences in the recovery efficiency of artificially inoculated AST could also be associated with genetic variations between *Salmonella* serovars, which influence the time for induction into the VBNC state (Kusumoto et al., 2012) or the expression of resuscitation promoting factors (Panutdaporn et al., 2006).

In a field trial using clay loam soil, no significant difference in the die-off of AST over time was observed between the selected cover crops and the control treatment. This observation could be partially explained by a decrease in the concentration of glucosinolate hydrolysis products in soil compared to in-planta concentrations, as reported by Gimsing et al. (2006). This reduction in the concentration of glucosinolate hydrolysis compounds has been attributed to lack of contact between myronsinase and glucosinolates upon the incorporation of the plant into soil (Gimsing et al., 2006). Increased production efficiency of hydrolysis products from plant material could be achieved by increasing the maceration of the plant before or during incorporation into the soil.

As discussed in the previous plant inhibition section, the active phenolic compounds in buckwheat are not present at high enough concentrations of 80, 40, and 40 ppm for inactivation of *Escherichia coli*, *Salmonella* Typhimurium, and *Staphyloccocus aureus,* respectively (Lou et al., 2011). Lack of thorough maceration and incorporation in the first 5-10 cm of the soil profile coupled with low plant biomass (perhaps due to the short growing

cycle of each cover crop) may have resulted in reduced levels of active compounds per gram of soil within the system.

Time to non-detection of AST was 22 days for each plot. This relatively poor persistence of AST could be explained by the high soil temperatures experienced during the summer months in Salisbury, NC, where temperatures within the first 10 cm of the soil averaged 25°C (State Climate Office of North Carolina, 2015). These results are in agreement with our microcosm studies and with reports by Holley et al. (2006), Garcia et al. (2010), Abd-Allel and Maysa (2015), specific to the survival and persistence of *Salmonella* in soil. Furthermore, strain fitness coupled with environmental pressures for nutrient acquisition and microbial predation in this clay soil could potentially be negatively impacting the survival and persistence of this AST strain. Holley et al. (2006) reported wide variations in fitness between certain *Salmonella* serovars at different temperatures, soil types, and with manure addition. Despite previously performing various environmental fitness tests, comparing this AST strain with pathogenic *Salmonella* strains in different soil types and growing conditions prior to the utilization of the AST strain in our studies, poor strain fitness within our field trial could have lead to the low survival and persistence observed.

Field solarization significantly increased soil temperatures within the first 30 cm of the soil profile. Temperatures averaging around 35°C have been reported at a depth of 30 cm upon solarization (Rubin and Benjamin, 1984). Trials were performed during the summer-fall transition, with temperatures within this system reaching a maximum of 33°C at 30 cm

from the soil surface, while non-solarized soil reached a maximum temperature of 22 °C at 10 cm from the soil surface. This significant difference in soil temperature, between solarized and non-solarized soil, coupled with the environmental fitness of AST, and reports from Roszak et al. (1984) suggesting that bacterial recovery in the VBNC state is influenced by time/temperature factors (21 d / 25°C), could explain why AST was not recovered after 4 weeks of solarization.

Time to non-detection of AST in a sandy loam soil and in the presence of spinach varied between 101 and 145 days. This significant difference between plots could be attributed to variations in the VBNC state of AST within each plot, as suggested by Roszak et al. (1984), or to differences in sampling efficiency between locations. Furthermore, different tilling equipment from clay field trials produced shallower incorporation (top 15 cm of the soil profile) of raw layer-hen manure and spinach inoculated with AST that could have increased the levels of the AST strain per gram of soil within each spinach bed, thus aiding in the recovery upon each sampling point. Holley et al. (2006), Garcia et al. (2010), and Abd-Allel and Maysa (2015), reported longer survival of human pathogens in soil held at temperatures below 15°C. The cooler temperatures of the fall and winters months in North Carolina may have been a factor prolonging the survival of the inoculated strain in this field trial.

Human pathogen persistence in manure-amended soils held at similar temperatures yielded overall shorter times to non-detection compared to times observed in this trial

(Garcia et al., 2010; Abd-Allel and Maysa, 2015). This difference in persistence could be attributed to many components including vast differences between field and laboratory conditions, manure composition, and soil physiochemical properties. The prolonged survival and persistence of this AST strain in this trial could also be attributed to reduced metabolic activity of naturally occurring microorganisms at lower temperatures (Holley et al, 2006), resulting in lower microbial competition for nutrients or lower predation (Barker et al, 1999; Garcia et al., 2010).

Conclusion

Overall our results suggest that soil type and/or nutrient availability from raw layer-hen manure did not appear to be the main factor(s) controlling increased persistence and survival of AST, since soil temperature followed by moisture content appeared to be more important than the other factors previously mentioned. The use of cover cropping with mustard greens coupled with solarization seems to be associated with a significant reduction in the survival and persistence of AST; however, further studies are needed to describe the potential interactions governing these bio-mitigation strategies. These potential interactions are of crucial importance at the macro scale level where other abiotic factors or a combination of them, along with the environmental fitness of the pathogen, dictate the fate of transient rhizosphere colonizers.

TABLES

Table 1. Oligonucleotide sequences used as primers for PCR

Primer	Sequence	Gene	Reference
INVA-1	ACAGTGCTCGTTTACGACCTGAAT	*invA*	Chiu and Ou (1996)
INVA-2	AGACGACTGGTACTGATCGATAAT	*invA*	Chiu and Ou (1996)
SPVC-1	CTCTGCATTTCACCACCATCACG	*spvC*	Agron et al. (2001)
SPVC-2	CTTGCACAACCAAATGCGGAAGAT	*spvC*	Agron et al. (2001)

Table 2. Die-off rates of avirulent *Salmonella* Typhimurium in soil (clay loam and sandy loam) at three different moisture contents with and without the application of raw layer-hen manure during incubation at 4, 10, 20, 30, 37°C over four weeks.

Soil Type	Moisture Content (%)	Manure Presence	AST Die-off Rates (log CFU/g soil * day)				
			4 (°C)	10 (°C)	20 (°C)	30 (°C)	37(°C)
Sandy loam	22.5	M	0.02	0.04	0.12	0.18	0.35
		NM	0.01	0.03	0.11	0.17	0.35
	18	M	0.01	0.03	0.13	0.24	0.35
		NM	0.004	0.02	0.09	0.23	0.35
	13.5	M	0.02	0.03	0.15	0.23	0.35
		NM	0.02	0.04	0.17	0.23	0.69
Clay loam	35	M	0.02	0.03	0.10	0.15	0.34
		NM	0.01	0.01	0.07	0.17	0.33
	28	M	0.02	0.04	0.09	0.17	0.33
		NM	0.003	0.02	0.06	0.22	0.33
	21	M	0.02	0.04	0.11	0.35	0.35
		NM	0.01	0.02	0.08	0.24	0.35

* M = manure application, NM = no manure application, AST = avirulent *Salmonella* Typhimurium, Data represent average die-off rates from n=6 samples per treatment

Table 3. One-way ANOVA analysis for soil microcosms displaying significance of soil type, week, temperature, moisture content, and raw layer-hen manure availability. A *P* value of <0.05 is statistically significant.

Source	df	Sum of Squares	Mean Square	F value	pr>F
Soil Type	1	2.271851	2.271851	0.55	0.4598
Error	1798	7473.059158	4.156318		
Total	1799	7475.331009			
Week	4	1711.448505	427.862126	133.25	<0.0001
Error	1795	5763.882505	3.211077		
Total	1799	7475.331009			
Temperature	4	3861.752495	965.438124	479.57	<0.0001
Error	1795	3613.578514	2.013136		
Total	1799	7475.331009			
Moisture Content	2	12.678872	6.339436	1.53	0.2176
Error	1797	7462.652138	4.152839		
Total	1799	7475.331009			
Manure	1	12.13291	12.13291	2.92	0.0875
Error	1798	7463.198099	4.150833		
Total	1799	7475.331009			

FIGURES

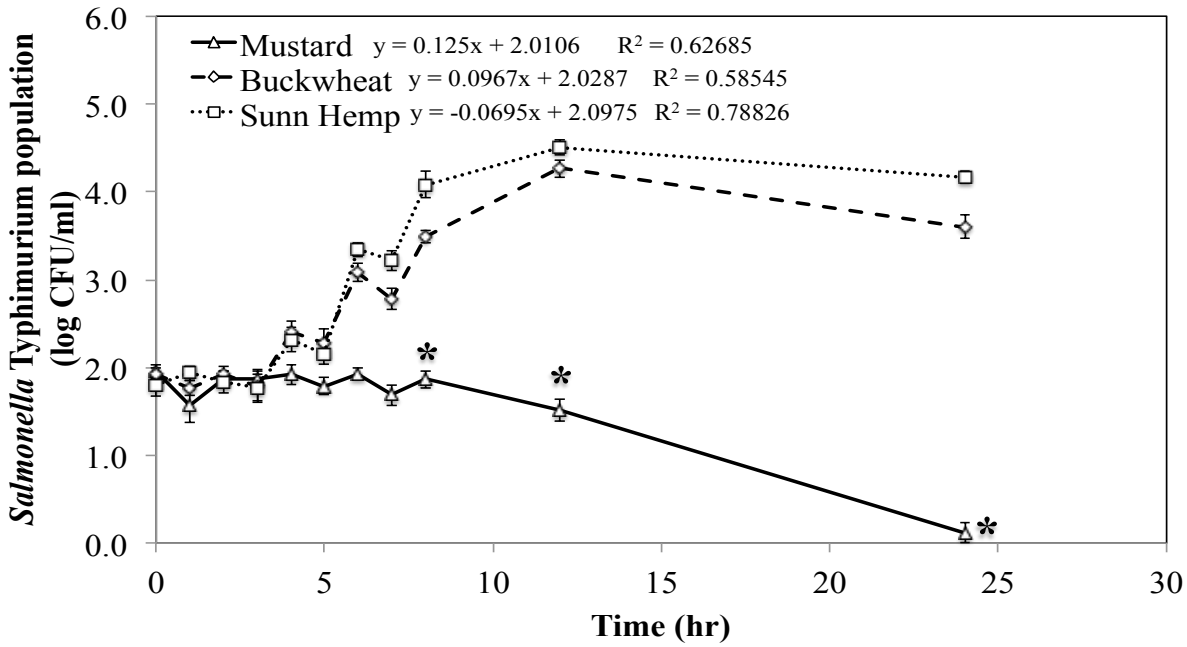

Figure 1. Influence of slurries made from the leaves/stems of mustard greens, buckwheat, and sunn hemp on the population of avirulent *Salmonella* Typhimurium over 24 hours incubated at 37°C. Error bars represent standard errors of the means. Asterisks represent mustard green values significantly different (p <0.05) from both buckwheat and sunn hemp.

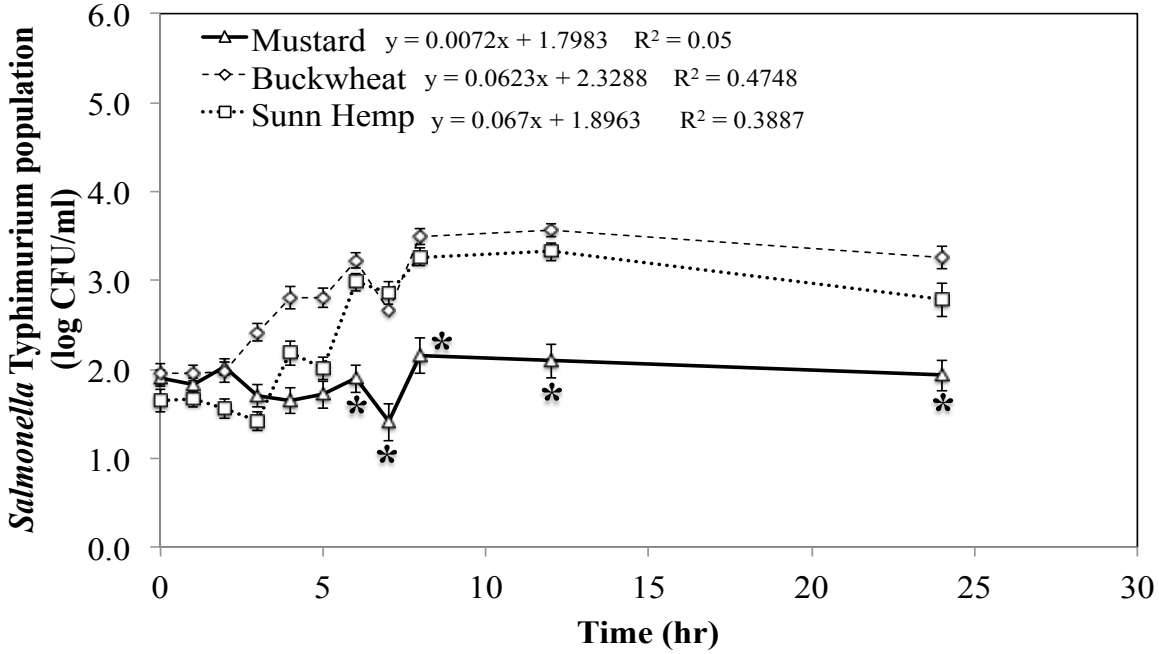

Figure 2. Influence of slurries made from the roots of mustard greens, buckwheat, and sunn hemp on the population if avirulent *Salmonella* Typhimurium over 24 hours incubated at 37°C. Error bars represent standard errors of the means. Asterisks represent mustard green values significantly different (p <0.05) from both buckwheat and sunn hemp.

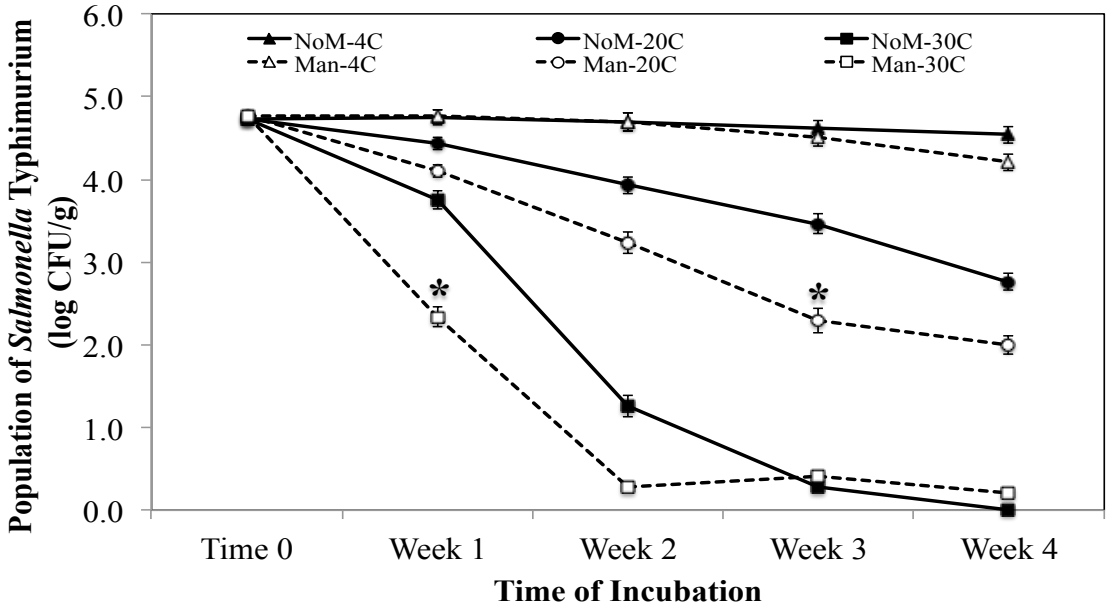

Figure 3a. Population of avirulent *Salmonella* Typhimurium at three different incubation temperatures (4, 20, and 30°C) with (Man) or without (NoM) the addition of raw layer-hen manure over four weeks of storage during Microcosm experiments with clay loam soil. Error bars represent standard errors of the means. Asterisks represent significantly (p <0.05) different values with raw-layer hen manure from those without raw-layer hen manure at the same temperature.

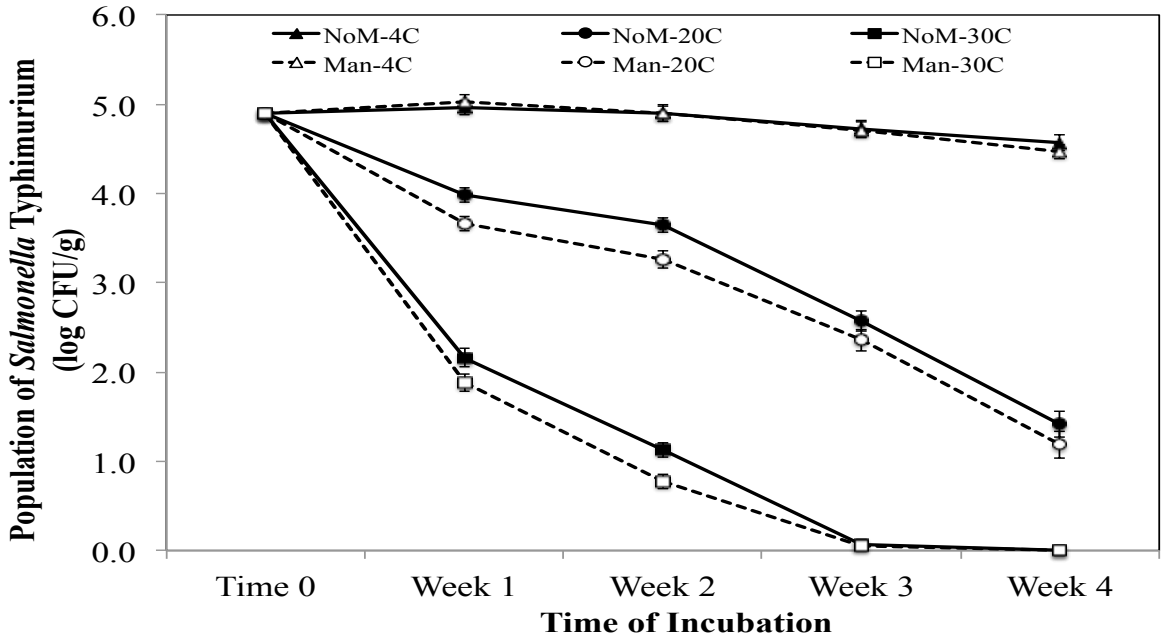

Figure 3b. Population of avirulent *Salmonella* Typhimurium at three different incubation temperatures (4, 20, and 30°C) with (Man) or without (NoM) the addition of raw layer-hen manure over four weeks of storage during Microcosm experiments with sandy loam soil. Error bars represent standard errors of the means. Asterisks represent significantly (p <0.05) different values with raw-layer hen manure from those without raw-layer hen manure at the same temperature.

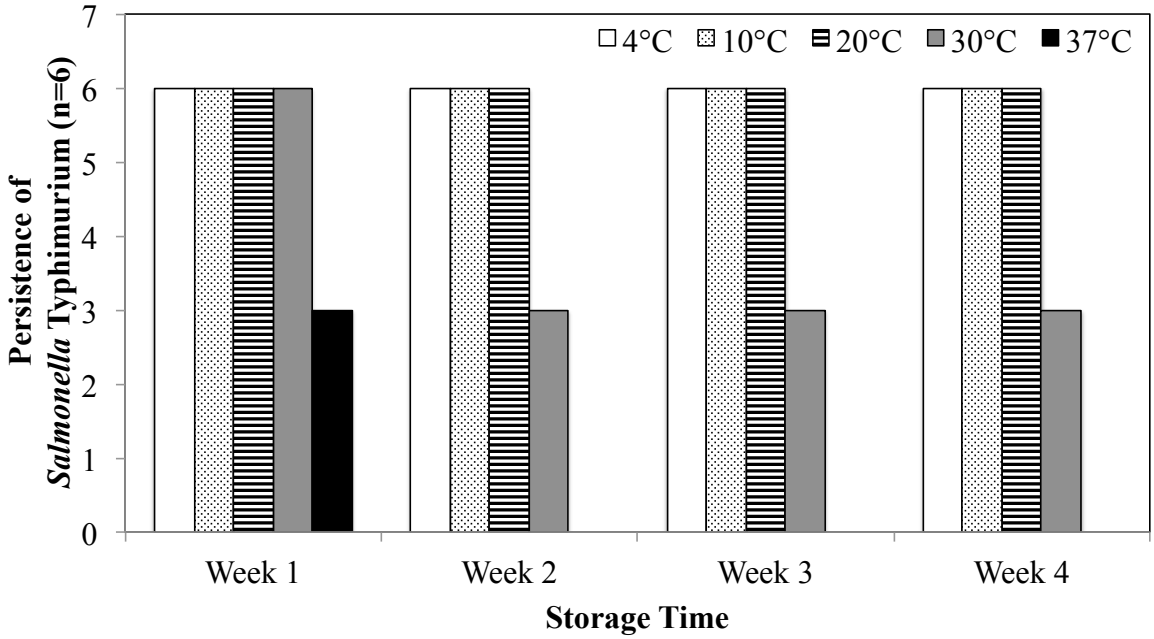

Figure 4a. Persistence of avirulent *Salmonella* Typhimurium over four weeks of storage at five different temperatures (4, 10, 20, 30, and 37°C) in clay soil with raw layer-hen manure at a moisture content 25% above field capacity. Repetitions per each treatment n=6.

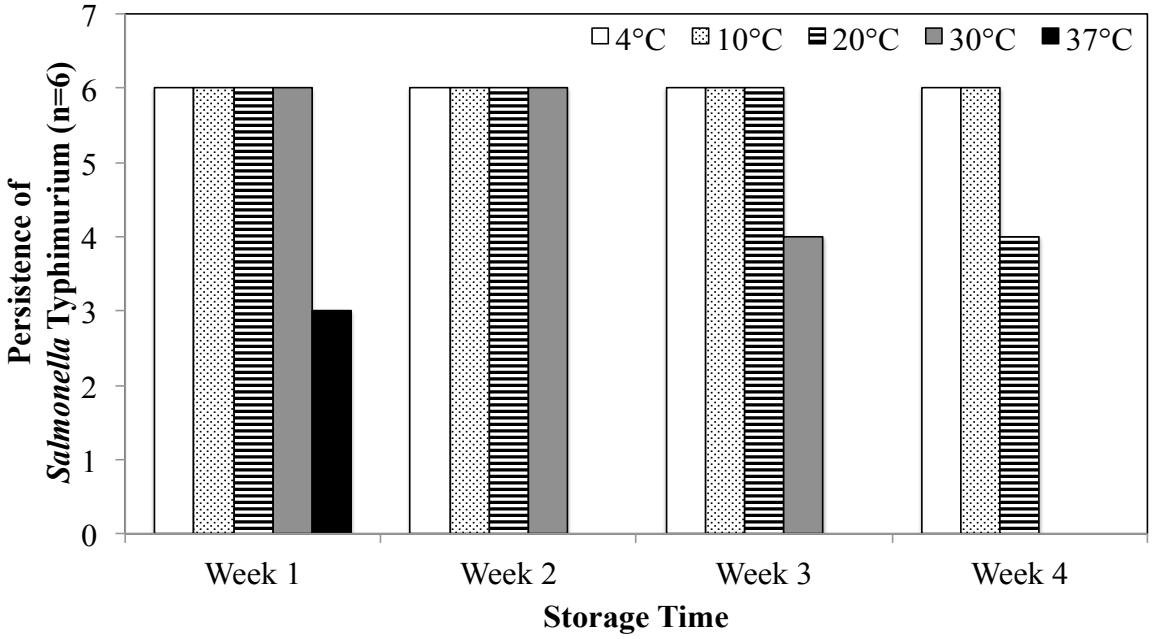

Figure 4b. Persistence of avirulent *Salmonella* Typhimurium over four weeks of storage at five different temperatures (4, 10, 20, 30, and 37°C) in clay soil without raw layer-hen manure at a moisture content 25% above field capacity. Repetitions per each treatment n=6.

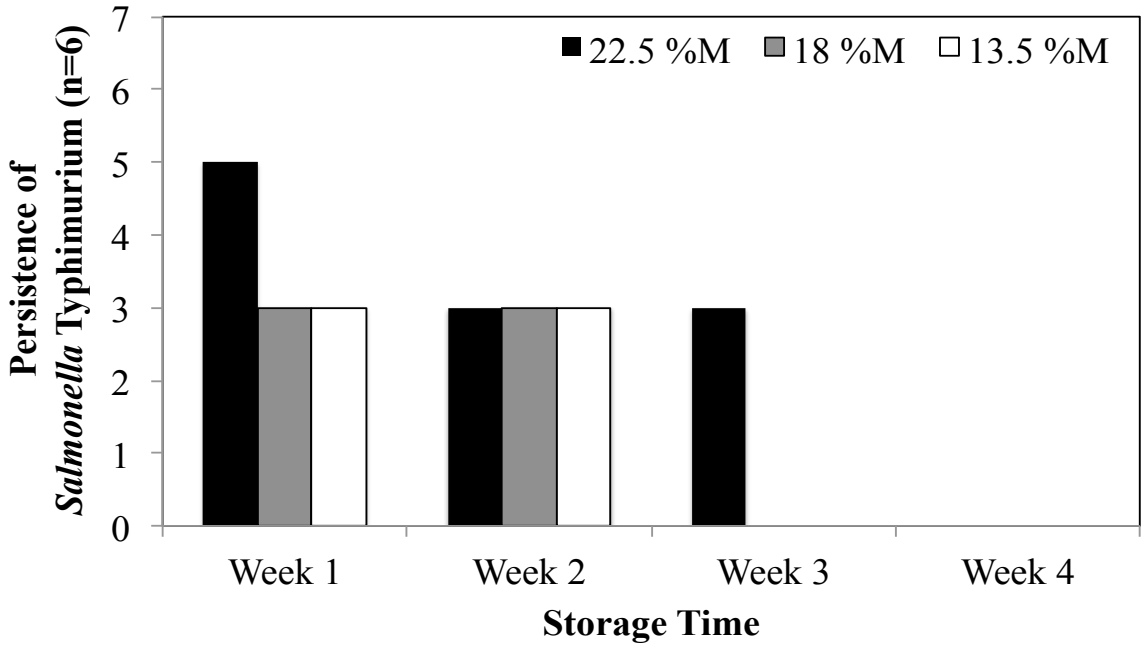

Figure 5a. Persistence of avirulent *Salmonella* Typhimurium over four weeks of storage at field capacity (18 %M), 25% above (22.5 %M) and below (13.5 %M) field capacity in loam soil with raw layer-hen manure at 30°C. Repetitions per each treatment n=6.

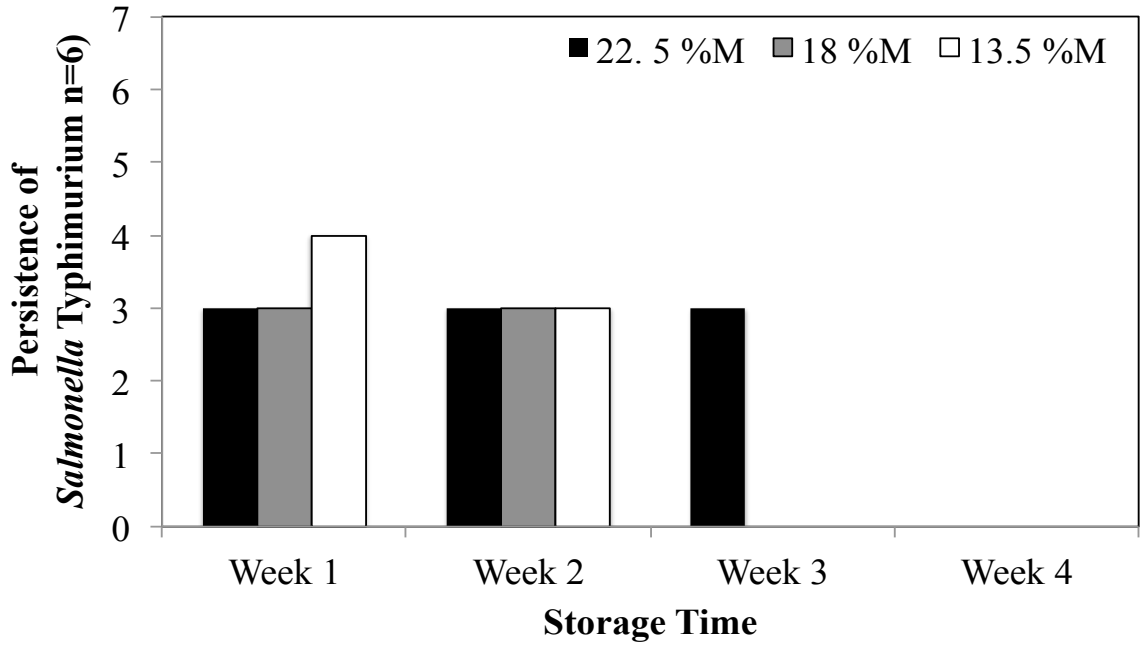

Figure 5b. Persistence of avirulent *Salmonella* Typhimurium over four weeks of storage at field capacity (18 %M), 25% above (22.5 %M) and below (13.5 %M) field capacity in loam soil without raw layer-hen manure at 30°C. Repetitions per each treatment n=6.

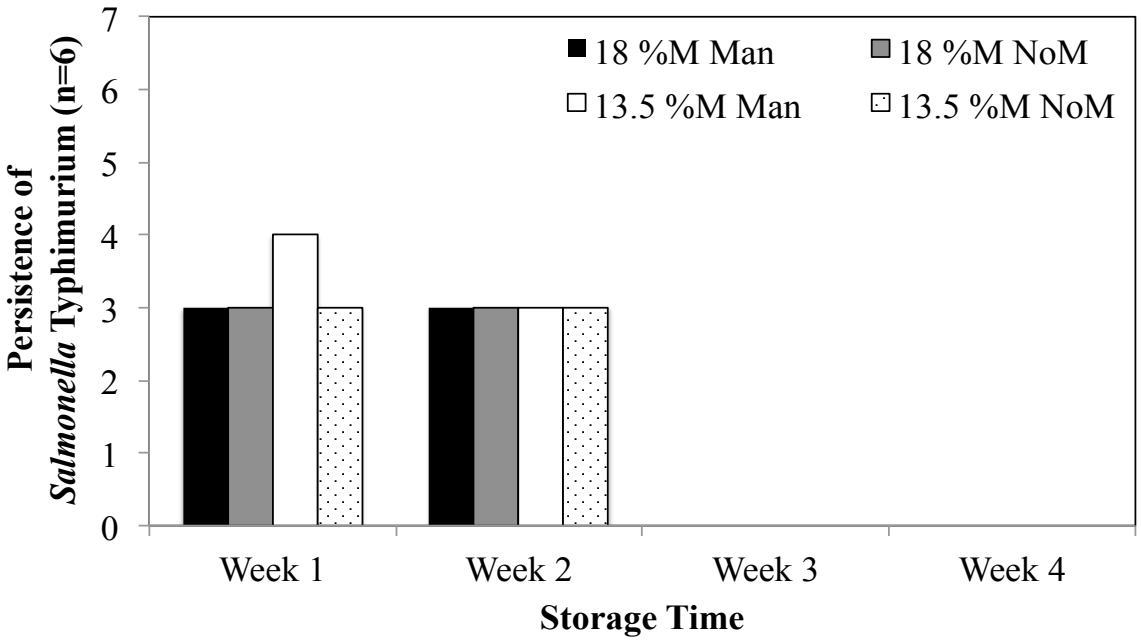

Figure 6. Persistence of avirulent *Salmonella* Typhimurium over four weeks of storage at field capacity (18 %M) and 25% below field capacity (13.5 %M) in loam soil with (Man) and without (NoM) raw layer-hen manure at 30°C. Repetitions per each treatment n=6.

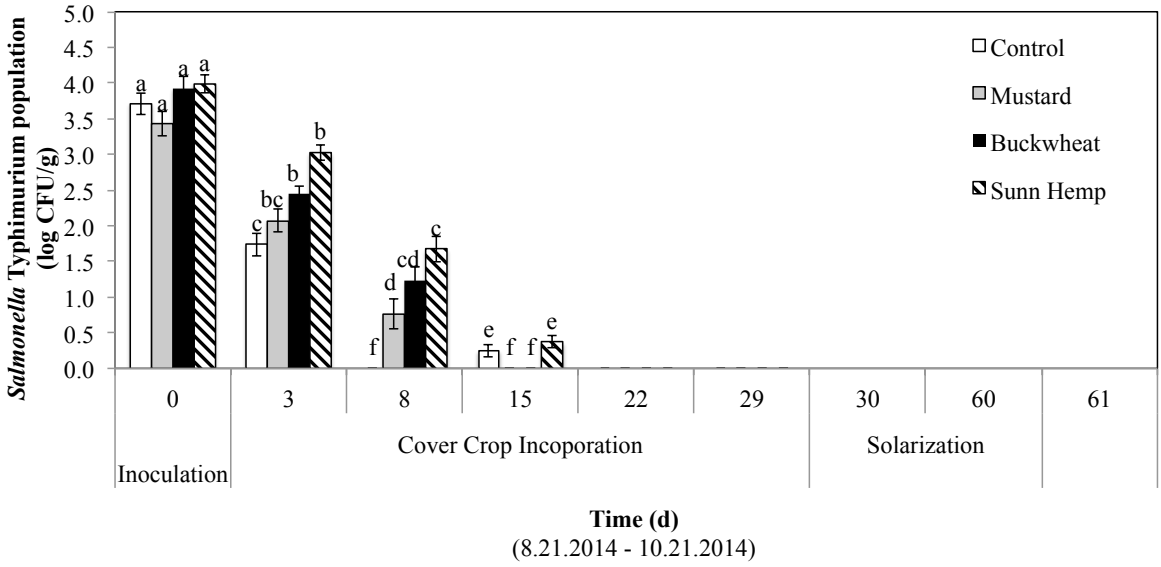

Figure 7. Decrease in the avirulent *Salmonella* Typhimurium population over time for control, mustard greens, buckwheat, and sunn hemp plots in clay loam field trials. Different letters represent statistically significant differences ($P<0.05$) in the population of *Salmonella* Typhimurium between plots. Error bars represent standard errors of the means.

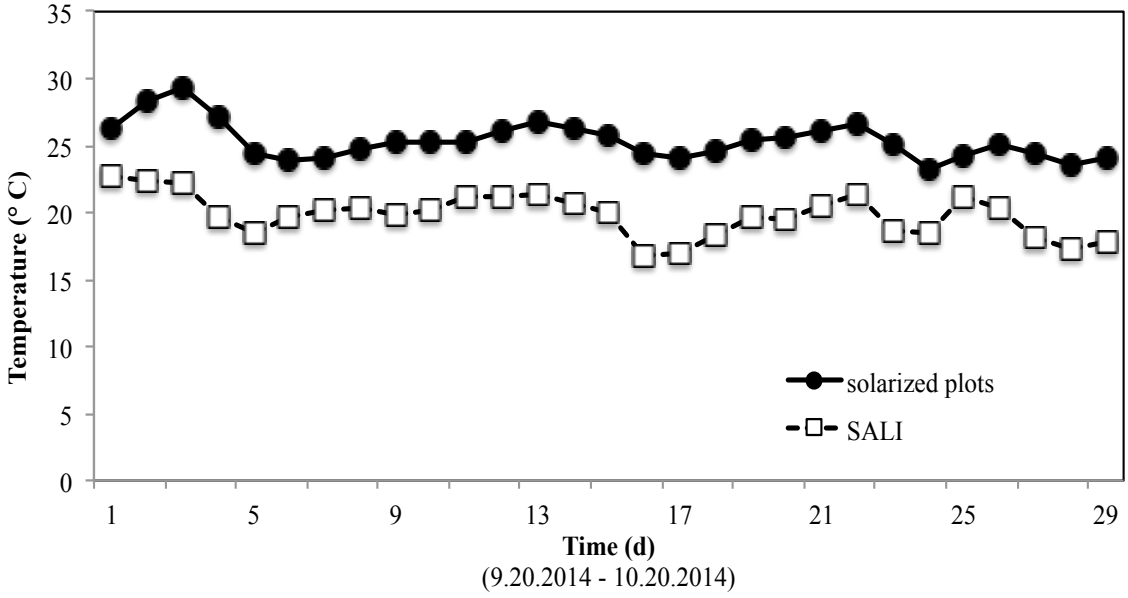

Figure 8. Solarization temperatures within clay loam soils at a depth of 30 cm compared to the average soil temperatures recorded by the weather station located in Salisbury, NC (SALI) at a depth of 10 cm.

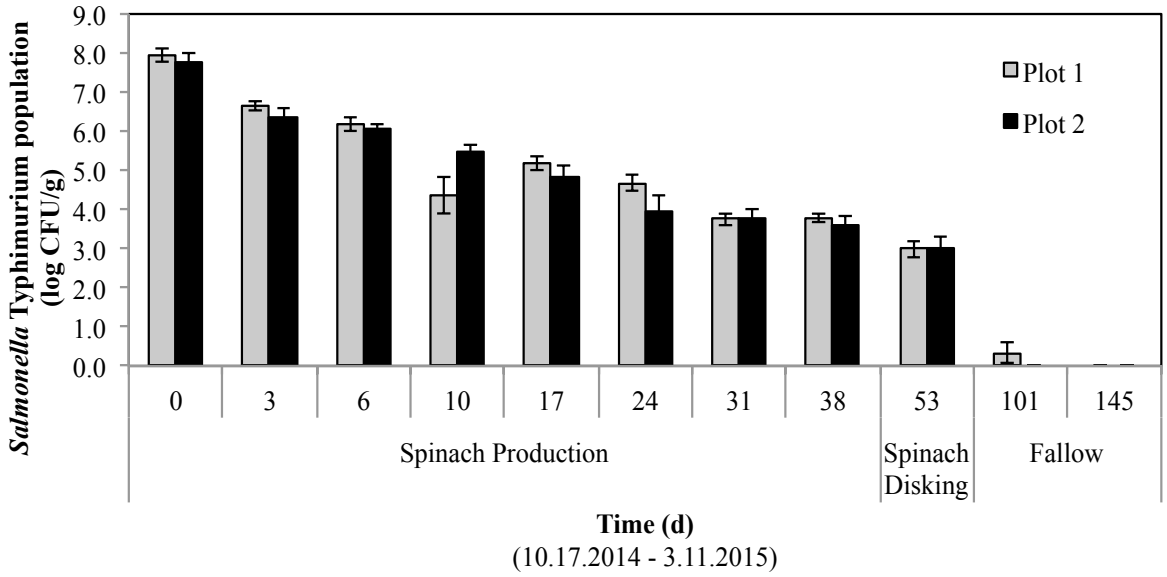

Figure 9. Decrease in the avirulent *Salmonella* Typhimurium population over time for Plot 1 and Plot 2 within sandy loam field trials. Error bars represent standard errors of the means.

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APPENDIX

Appendix A – *Persistence rates of avirulent Salmonella Typhimurium within Soil Microcosms*

Table 4a. Persistence of avirulent *Salmonella* Typhimurium in sandy loam soil over four weeks of storage at three moisture contents and five storage temperatures with and without the application of raw layer-hen manure.

Moisture Content (%)	Temperature (°C)	Manure* Presence	Persistence of AST			
			Week 1	Week 2	Week 3	Week 4
22.5	4	M	100	100	100	100
		NM	100	100	100	100
	10	M	100	100	100	100
		NM	100	100	100	100
	20	M	100	100	100	100
		NM	100	100	100	67
	30	M	50	50	50 (P<0.05)	0 (P<0.05)
		NM	83	50	50 (P<0.05)	0 (P<0.05)
	37	M	17	17 (P<0.05)	0 (P<0.05)	0
		NM	50 (P<0.05)	0 (P<0.05)	0	0
18	4	M	100	100	100	100
		NM	100	100	100	100
	10	M	100	100	100	100
		NM	100	100	100	100
	20	M	100	100	100	67
		NM	100	100	100	100
	30	M	50	50 (P<0.05)	0 (P<0.05)	0
		NM	50	50 (P<0.05)	0 (P<0.05)	0
	37	M	0	0	0	0
		NM	67 (P<0.05)	0 (P<0.05)	0	0
13.5	4	M	100	100	100	100
		NM	100	100	100	100
	10	M	100	100	100	100
		NM	100	100	100	100
	20	M	100	100	100	50
		NM	100	100	100	50
	30	M	67	50 (P<0.05)	0 (P<0.05)	0
		NM	50	50 (P<0.05)	0 (P<0.05)	0
	37	M	0	0	0	0
		NM	0	0	0	0

* M = manure application, NM = no manure application, AST = avirulent *Salmonella* Typhimurium, Data represent percent of positive samples per treatment, n=24 (storage temperature and manure application for four weeks), chi-square analysis

Table 4b. Persistence of avirulent *Salmonella* Typhimurium in clay loam soil over four weeks of storage at three moisture contents and five storage temperatures with and without the application of raw layer-hen manure.

Moisture Content (%)	Temperature (°C)	Manure* Presence	Persistence of AST			
			Week 1	Week 2	Week 3	Week 4
35	4	M	100	100	100	100
		NM	100	100	100	100
	10	M	100	100	100	100
		NM	100	100	100	100
	20	M	100	100	100	100
		NM	100	100	100	67
	30	M	100	50	50 (P<0.05)	0 (P<0.05)
		NM	100	100	67 (P<0.05)	0 (P<0.05)
	37	M	50 (P<0.05)	0 (P<0.05)	0	0
		NM	50 (P<0.05)	0 (P<0.05)	0	0
28	4	M	100	100	100	100
		NM	100	100	100	100
	10	M	100	100	100	100
		NM	100	100	100	100
	20	M	100	100	100	100
		NM	100	100	100	100
	30	M	100	17	50 (P<0.05)	0
		NM	100	83	0 (P<0.05)	0
	37	M	50 (P<0.05)	0 (P<0.05)	0	0
		NM	50 (P<0.05)	0 (P<0.05)	0	0
21	4	M	100	100	100	100
		NM	100	100	100	100
	10	M	100	100	100	100
		NM	100	100	100	100
	20	M	100	100	100	50
		NM	100	100	100	50
	30	M	100	0	0	0
		NM	100	83 (P<0.05)	0 (P<0.05)	0
	37	M	0	0	0	0
		NM	50 (P<0.05)	0 (P<0.05)	0	0

* M = manure application, NM = no manure application, AST = avirulent *Salmonella* Typhimurium, Data represent percent of positive samples per treatment, n=24 (storage temperature and manure application for four weeks), chi-square analysis