Potential interactions between *Salmonella enterica* and *Ralstonia solanacearum* in tomato (*Solanum lycopersicum*) plants and the effects of bacterial wilt on tomato fruit susceptibility to *S. enterica* Newport

Stephanie Kay Pollard

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Steven L. Rideout, Chair
Jeri D. Barak
Renee R. Boyer
Elizabeth A. Grabau
Mark S. Reiter

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Abstract

Over the past decade, the Eastern Shore of Virginia (ESV) has been implicated in at least four outbreaks of Salmonellosis associated with tomato all originating from the same strain, *Salmonella enterica* serovar Newport. In addition to *S.* Newport contamination, the devastating plant disease, bacterial wilt, caused by the phytopathogen *Ralstonia solanacearum* threatens the sustainability of ESV tomato production. Bacterial wilt is present in most ESV tomato fields and causes devastating yield losses each year. Due to the ESV's endemic population of *R. solanacearum* and *S.* Newport, the relationship between the two pathogens is of interest and has never been investigated. Two separate studies were conducted to assess the relationship between these two bacteria. One study consisted of a series of greenhouse trials that involved root-dip inoculations of tomato plants with one of four treatments: 1) *S.* Newport, 2) *R. solanacearum*, 3) a co-inoculation of *S.* Newport + *R. solanacearum*, and 4) a control group with no inoculation. Leaf, stem, and fruit samples were collected from the plants and *S. enterica* presence from the internal tissues was observed. *S. enterica* was recovered from a low percentage of fruit and leaf samples. There were significantly more stem samples from plants co-inoculated with *S.* Newport + *R. solanacearum* positive for *S. enterica* (17.46%) than from other treatments. Another study examined the relationship between the two bacteria via vacuum infiltration inoculations of tomato fruit collected from commercial production fields on the ESV with *S.* Newport. Tomato

fruit were collected from plants expressing symptoms of bacterial wilt (symptomatic) and plants not expressing bacterial wilt symptoms (asymptomatic). After fruit infiltration with *S.* Newport, recovery concentration of *S. enterica* from internal tissues was measured. *S. enterica* populations were greater in fruit originating from asymptomatic (5.20 log CFU/ml) versus symptomatic (5.11 log CFU/ml) plants across five studies. Fruit collected from asymptomatic plants had a significantly higher internal pH (4.60) than fruit collected from symptomatic plants (4.37). These results suggest that *R. solanacearum* can influence *S. enterica* survival and transportation throughout the internal tissues of tomato plants as well as the influence internal tomato fruit pH, which could potentially impact *S.* Newport survival in the fruit.

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Chapter 1: Introduction and Justification

Salmonella enterica

The Center for Disease Control and Prevention (CDC) estimates that approximately 1 out of 6 Americans (~48 million people) become ill, 128,000 are hospitalized, and 3,000 die from food-borne illnesses annually. The CDC released an article in January 2011 stating that non-typhoidal *S. enterica* is the second leading cause of foodborne illnesses annually (approximately 1,027,561 cases accounting for 11% of all foodborne illnesses). Nontyphoidal *S. enterica* is also the leading cause of hospitalizations and deaths associated with food-borne illnesses with 19,336 and 378 reported cases, respectively (Scallan *et al.*, 2011).

Recently, fresh produce gained notoriety as a vehicle of human salmonellosis. Between 1990 and 2005, contaminated produce caused the greatest number of food-borne cases annually and the second highest number of outbreaks (seafood being the highest) (CSPI, 2007). The FDA announced in early 2010 that work has begun on proposed rules for the safe production, harvesting and packaging of fresh produce and is currently in the process of publishing new guidelines for public comment (FDA, 2011). The safety of fresh-market tomato (*Solanum lycopersicum*) and its potential to cause food-borne illness is of growing concern. From 1998 to 2006, 71 produce related outbreaks were reported, of which 12 were linked to tomato (CSPI, 2007; Berstein, 2007). Of the tomato-related *S. enterica* outbreaks, four originated in Virginia, which were all from the same strain of *S. enterica* (serovar Newport PFGE pattern 0061) (Table 1.1).

S. enterica annually causes an estimated 1.4 million human cases of gastroenteritis, approximately 40,000 are culture-confirmed cases reported to CDC, resulting in 600 deaths in

the United States (Scallan *et al.,* 2011). *S. enterica* can infect all age groups, although, groups at greatest risk for severe disease include infants, the elderly, and persons with compromised immune systems (CDC, 2010). To cause disease, *S. enterica* must be present at a sufficient concentration to overcome immune system defenses. The concentration required for disease is variable and is affected by the viability of the bacteria and the degree of the host's immunity (Heringa *et al.*, 2010). Symptoms of salmonellosis include nausea, vomiting, abdominal cramps, diarrhea, and headaches within 6 to 48 hours of ingesting *S. enterica*-contaminated food. Most people recover without treatment; however, in some cases the diarrhea may be so severe that the patient becomes dangerously dehydrated and must be hospitalized (CDC, 2010). Although the disease normally resolves without intervention in 2 to 3 days, patients can shed *S. enterica* in feces for several weeks (Michael *et al.*, 2006). The economic impact of foodborne illnesses can be tremendous. In the United States, diseases caused by the major human pathogens alone are estimated to cost up to $51 billion annually in medical costs and lost productivity (Scharff, 2012).

S. enterica Biology

Representatives of *S. enterica* are Gram-negative, facultatively anaerobic, rod-shaped bacteria that belong to the family *Enterobacteriaceae* and are capable of causing salmonellosis in humans (Michael *et al.*, 2006; Montville *et al.*, 2008). *S. enterica* are motile via peritrichous flagella, although there are a few nonflagellated variants such as *S. enterica* serovars Pullorum and Gallinarum (Montville *et al.*, 2008). The flagella allow for the *S. enterica* to be motile, especially in water, thus making contamination easier through bacterial movement to plant openings (stomata, wounds, etc.), or movement within the body to the intestine.

S. enterica infections begin with consumption of contaminated food. Most bacteria are killed by the naturally acidic conditions in the human stomach; however, a few bacteria may survive and become transported to the intestine. Once in the intestine, *S. enterica* competes with other bacteria that are naturally present in the gut flora. The intestinal wall consists mainly of epithelial cells that protect it from harmful bacteria. *S. enterica* will use its flagella to swim towards the surface of the epithelial cells where it will attach and enter. *S. enterica* enters the cells by using a type III secretion system. The type III secretion system is a needle-like organelle that allows the bacteria to puncture the surface of the epithelial cells to deliver its toxins directly into the intestinal host cells. The toxins, called effector proteins, enter host cells and interfere with proteins and lipids, thus altering their function. The *S. enterica* effectors activate specific host proteins that cause the exoskeleton of the cells to protrude outward allowing for the engulfment of the *Se* bacteria into the host cell. Once inside the host cell, *S. enterica* utilizes a different type III secretion system that releases different effector proteins into the host cell that interfere with host proteins producing a layer around the *S. enterica* that protects it from host defenses. Once the protective layer is formed around the *S. enterica*, it can then begin to replicate and establish an infection. *S. enterica* infection leads to an inflammatory response which causes the typical symptoms of salmonellosis (Michael *et al.*, 2006; Montville *et al.*, 2008).

S. enterica Contamination Routes and Survival

Foods of animal origin, such as poultry, eggs, meat, and dairy products, were historically recognized as vehicles of *S. enterica*. However, salmonellosis has also been associated with consumption of tomato fruit, seed sprouts, watermelon (*Citrullus lanatus*), cantaloupe (*Cucumis melo*), unpasteurized apple cider and orange juice, lettuce (*Lactuca sativa*) and other leafy

greens, and green onion (*Allium* spp.) (Montville *et al.*, 2008). In the farm-to-fork production, during processing, and throughout the distribution chain, there are various possible contamination points of fruits and vegetables with disease-causing microorganisms. *S. enterica* are resilient and can adapt to extreme environmental conditions, making it difficult to determine the exact contamination route of produce (Montville *et al.*, 2008). Potential contamination routes include irrigation water, wash water, fertilizer, handling by workers, and contact with contaminated surfaces (Guo *et al.*, 2002).

Soil

Animal wastes are largely recycled to agricultural land as the most economical and environmentally sustainable means of treatment and reuse. Animal wastes, such as manures, have beneficial attributes that help maintain soil quality and fertility. However, animal manures often contain enteric pathogens, such as *S. enterica*, that lead to pathogen entry into the food chain (Islam *et al.*, 2004; Klerks *et al.*, 2007).

Several studies have shown that *S. enterica* is capable of surviving and multiplying in manure and manure-amended soils. You *et al.* (2006) showed that *S. enterica* concentrations can increase up to 400% in the first 1 to 3 days following inoculation into dairy manure. After the initial increase, *S. enterica* concentrations steadily declined; however, the organism persisted for 184, 332, and 405 days in raw manure, raw manure-amended nonsterilized soil, and manure-amended sterilized soil, respectively (You *et al.*, 2006). Islam *et al.* (2004) analyzed *S. enterica* survival in poultry litter compost, dairy cattle manure compost, and alkaline-pH-stabilized dairy cattle manure compost. They discovered that *S.* Typhimurium survived in soil samples for 203 and 231 days on *Raphanus sativus* (radish) and *Daucus carota* subsp. *sativus* (carrot),

respectively, and was detected after seeds were sown for 84 and 203 days on radish and carrot, respectively. *S. enterica* survival was found to be the greatest in soil amended with poultry litter compost and least in soil containing alkaline-pH-stabilized dairy cattle manure compost (Islam *et al.*, 2004)

Barak *et al.* (2008) studied the role of soil, crop debris, and a plant pathogen have in *S. enterica* contamination of tomato plants. *S. enterica* was capable of surviving in soil and contaminating the phyllosphere of tomato plants six weeks after *S. enterica* was inoculated in the soil. After 24 hours of soil contact, *S.enterica* was visible in the stem scar and subsurface tissue of tomato fruits. It was also determined that contaminated plant debris can serve as *S. enterica* inoculum from one crop to the next. The researchers also studied interactions between a plant pathogen, *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot, and *S. enterica*. It was determined that in the absence of plant disease, presence of *X. campestris* pv. *vesicatoria* was beneficial to *S. enterica*, allowing for multiplication of the human pathogen population. At the three to five leaves and pre-bloom stages, *S. enterica* populations were significantly higher on plants which were co-colonized by *X. campestris* pv. *vesicatoria* as compared to plants inoculated with *S. enterica* alone. It is hypothesized that the plant pathogen could weaken the overall health and immune response, increasing the chance of *S. enterica* infection (Barak *et al.*, 2008).

Semenov *et al.* (2009) also examined the percolation and survival of *E. coli* O157:H7 and *S.* Typhimurium in soil amended with contaminated dairy manure or slurry. The study involved four treatments with *E. coli* O157:H7 and *S.* Typhimurium inoculations including 1) inoculated manure spread on the soil surface, 2) inoculated manure mixed in the top 10 cm of soil, 3) inoculated slurry on the soil surface, and 4) inoculated slurry mixed in the top 10 cm of soil. It

was discovered that the pathogens percolated to greater depths after slurry application compared to manure application for both surface and mixture applications. Distributions of *E. coli* and *S.* Typhimurium were affected by the type of the substrate which was added (manure or slurry), by the method of its application (spread on soil surface or incorporation into the soil), and by the presence of lettuce (*Lactuca sativa*) roots. The survival of *E. coli* was on average 1.39 ± 0.12 times shorter than survival of *S.* Typhimurium for all treatments (Semenov *et al.*, 2009). Similarly, another study conducted by Bech *et al.* (2010) looked at the transport and distribution of *S.* Typhimurium applied in liquid swine manure in loamy and sandy soil monoliths. It was found that *S.* Typhimurium could be transported to a 1 m depth in loamy soil at concentrations reaching 1.3 x 10^5 CFU/ml in leachate. The authors used real-time PCR to target *inv*A DNA (the invasion gene of *S. enterica*) which revealed a clear correspondence between the total and culturable numbers of cells in the leachate, indicating that most cells leached were viable. The highest recovery rate, ranging from 1.5% to 3.8% of the total applied inoculums, was found in the top 0.2 m of the soil.

A similar study was done by Bernstein *et al.* (2007) to assess the contamination potential of lettuce (*Lactuca sativa*) by *Salmonella enterica* serovar Newport. Contamination was evaluated at two bacterial concentrations, 10^6 or 10^8 CFU/g, when the plants were 17 and 33 days old, and the effect of root wounding on bacterial concentration was evaluated. No contamination was detected in the 17 day old plants, however, in the older plants the pathogen was recovered from the above-ground tissue 2 days after inoculation. In plants with intact and damaged roots, the leaves contained *S. enterica* at 500 ± 120 and 5,130 ± 440 CFU/g, respectively. These findings suggest that *S.* Newport can translocate from contaminated roots

(particularly if they are damaged) to the aerial parts of lettuce seedlings and propose that the process is dependent on the developmental stage of the plant (Bernstein *et al.*, 2007).

Irrigation

Irrigation water is a major potential source of *Se* contamination. *S. enterica* is most commonly introduced into a water source through contaminated manure. Once *S. enterica* enters a water supply, it is capable of survival in stream bottom sediments more than surface water (Humphrey, 2005). *S. enterica* can also be introduced into a water supply through runoff from fields that contain manure (Gagliardi and Karns, 2002). Once the pathogen enters irrigation water, produce contamination may occur. In the study conducted by Islam *et al* (2004) contaminated irrigation water was used to study *S. enterica* contamination on carrot and radish. Islam and colleagues found that carrots irrigated with contaminated water contained the pathogen for up to 210 days (Islam *et al.*, 2004).

Studies have also been conducted to determine if *S. enterica* can become internalized within fruit. Hintz *et al.* (2010) conducted a study to determine if *S.* Newport-contaminated irrigation water could result in internal tomato plant contamination under controlled environmental conditions. The control group was irrigated with distilled water at 7 day intervals while the experimental group was irrigated with *S.* Newport-contaminated water (10^7 CFU/ml) at 7 day intervals. Tomato plants were randomly sampled for the presence of *S.* Newport at various growth stages, with the roots, stems, leaves, and fruit being analyzed. It was found that 65% of the roots, 40% of the stems, 10% of the leaves, and 6% of the fruit were confirmed to be contaminated by *S.* Newport after irrigation for the *S.* Newport treatments. This proves that *S.*

Newport may be capable of contaminating aerial tomato plant tissues when consistently high levels are applied at the root site (Hintz, 2010).

In another study conducted by Haley *et al.* (2009), the distribution, diversity, and seasonality of waterborne *Se* in a rural watershed in Georgia (United States) was analyzed. Monthly water samples were collected from six stations in the Little River (Upper Suwannee Basin) for 12 months. *S. enterica* was detected in 57 of the 72 samples collected (79.2%). It was found that concentrations were significantly higher in the summer months compared to other seasons. Levels of *S. enterica* recovered positively correlated with average daily watershed rainfall for the 1 and 2 days preceding each sample collection. Water temperature was also positively correlated with total *S. enterica* levels. In total, 13 *S. enterica* serotypes were identified among 197 *S. enterica* isolates collected. The study suggested that *S. enterica* abundance and diversity in the environment vary temporally and are strongly influenced by seasonal precipitation and water temperature.

S. enterica Internalization in Plants

S. enterica is capable of internalizing and growing in certain plant tissues. Guo *et al.* (2001) determined the fate of *S. enterica* applied to tomato plants. Five *S. enterica* serotypes (Montevideo, Michigan, Poona, Hartford, and Enteritidis) were used to inoculate tomato plants before and after fruit set, either by injecting stems or brushing flowers with inoculum. It was found that of thirty tomato fruit harvested from inoculated plants, 11 (37%) were positive for *S. enterica*. Of the *S. enterica*-positive tomato fruit, 43% were from plants receiving stem inoculation before flower set whereas 40% were from plants receiving stem inoculation after fruit set. Plants that were inoculated with *S. enterica* by flower brush resulted in 25% of fruit

samples contaminated with *S. enterica*. A higher percentage of surface (82%) and stem scar tissue (73%) samples, compared to pulp of *S. enterica*-positive tomato fruit (55%), harbored the pathogen (Guo *et al.*, 2001).

Klerks *et al.* (2007) analyzed the physiological and molecular responses of lettuce to colonization by *S.* Enteritidis. The association of *S.* Enteritidis inside the plant was supported by significant residual concentrations after highly efficient surface disinfection (99.81%) and fluorescence microscopy of *S.* Enteritidis in cross sections of the lettuce at the root-shoot transition region. Also, colonization with *S.* Enteritidis significantly reduced plant biomass compared to that of noncolonized plants. Furthermore, functional grouping of the expressed genes indicated a positive correlation between colonization of the plants and an increase in expressed pathogenicity-related genes. The study indicates that lettuce plants respond to the presence of *S.* Enteritidis at physiological and molecular levels, as shown by the reduction in growth and the concurrent expression of pathogenicity-related genes. It was also confirmed that *S. enterica* can colonize the interior of lettuce plants, thus potentially imposing a human health risk when processed and consumed (Klerks *et al.*, 2007).

The factors that induce *S. enterica* internalization were studied by Kroupitski *et al.* (2009) by incubating *gfp*-tagged *S. enterica* with iceberg lettuce leaves in the light and in the dark. It was found that leaves incubated in the light resulted in aggregation of bacteria near open stomata and invasion into the inner leaf tissue. In contrast, incubation in the dark resulted in a scattered attachment pattern and very poor stomatal internalization. These results imply that the pathogen is attracted to nutrients produced by photosynthetically active cells. Mutations affecting *S. enterica* motility and chemotaxis significantly inhibited bacterial internalization. These findings suggest a mechanistic account for entry of *S. enterica* into the plant's apoplast

and imply that either *S. enterica* antigens are not well recognized by the stoma-based innate immunity or that this pathogen has evolved means to evade detection (Kroupitski *et al.*, 2009)

S. enterica Control

There are several government agencies that are involved in food safety in the United States. The Food and Drug Administration (FDA) inspects imported foods and milk pasteurization plants, promotes better food preparation techniques in restaurants and food processing plants, and regulates the sale of turtles. The U.S. Department of Agriculture (USDA) monitors the health of food animals, inspects egg pasteurization plants, and is responsible for the quality of slaughtered and processed meat. The U.S. Environmental Protection Agency (EPA) regulates and monitors the safety of drinking water (Montville and Matthews, 2008). Thus, efforts are being made to control *S. enterica* outbreaks; however, more research needs to be done to establish specific guidelines and control measures in place in every step of the farm-to-fork process.

National surveillance is conducted through public health laboratories for culture-confirmed cases and through the National Notifiable Diseases Surveillance System (NNDSS). There are many challenges associated with the control and prevention of a salmonellosis outbreak. Some of those challenges include identifying unrecognized major sources of *S. enterica*, determining the sources of *S. enterica* infections in infants, preventing contamination of vegetables with manure from concentrated animal feeding operations, preventing further emergence of highly antibiotic-resistant strains, and developing effective education methods and materials to prevent reptile-associated salmonellosis (CDC, 2010).

When developing preventative methods for *S. enterica* infections, the identification of science based, cost effective strategies to protect all parts of the food chain is necessary (Humphrey, 2005). Tomato fruits are generally consumed raw and can become contaminated anywhere from farm to table. Cooked or canned foods that are contaminated with *S. enterica* can support the survival of the bacteria if the foods are held for long periods of time without heating or refrigeration. Cooked foods heated to 70˚C for at least 10 minutes are considered safe if consumed immediately or if held at 50˚C or stored at 10˚C or lower (Michael *et al.*, 2006).

Numerous methods have been studied to control/prevent *S. enterica* outbreaks. Heringa *et al.* (2010) studied the effect of bacteriophages on *S. enterica.* Bacteriophages specific to *S. enterica* were isolated from sewage effluent and characterized and a five-strain bacteriophage mixture was used to apply to dairy manure compost inoculated with *S. enterica*. The co-inoculated compost yielded greater than a 2-log-unit reduction of *S. enterica* within four hours at all moisture levels as compared to the controls. However, the bacteriophages could not completely eliminate *S. enterica* from compost (Heringa *et al.*, 2010). There is still some controversy surrounding the safety of applying bacteriophages in food and agricultural industries (Garcia *et al.*, 2008; Goodridge *et al.*, 2003; Greer, 2005; Hagens and Loessner, 2007; Hudson *et al.*, 2005).

Biological control has also been investigated as a potential control strategy for *S. enterica*. Leverentz *et al.* (2006) studied the efficacy using 17 different antagonists originally selected for their ability to inhibit fungal postharvest decay on fruit. Apple fruit (*Malus domestica*) contaminated with either *Listeria monocytogenes* or *S. enterica* were treated with one of the 17 antagonists. It was found that most of them increased the growth of the foodborne pathogens. However, three antagonists including *Gluconobacter asaii* (T1- D1), *Discosphaerina*

fagi (ST1 - C9), and *Metschnikowia pulcherrima* (T1 - E2) prevented growth or survival of *S. enterica* on fresh-cut apples. The inhibitory effect of antagonists on foodborne pathogens was not instantaneous, and became only apparent after 2 or 5 days of storage. The investigators suggest combining these antagonists with other agents such as bacteriophage mixtures for greater effect. The bacteriophage treatments have an instantaneous inhibitory effect which could be used to control the food-borne pathogen quickly and the antagonists could control the pathogen populations over time (Leverentz *et al.*, 2006; Leverentz *et al.*, 2001).

The efficacy of chlorine and heat treatment in killing *S. enterica* has also been studied. Jaquette *et al.* (1996) conducted experiments with different concentrations and treatment intervals of chlorine and heat (hot water) on *S. enterica*-contaminated alfalfa (*Medicago sativa*) seeds. Treatment of seeds containing 10^2 to 10^3 CFU/g *S. enterica* submerged in 100 µg/ml active chlorine solution for 5 or 10 min caused a significant further reduction in population, and treatment in 290 µg/ml chlorine solution resulted even greater reduction. However, concentrations of chlorine of up to 1,010 µg/ml failed to result in further significant reductions compared to 290 µg/ml. Treatment of seeds containing 10^1 to 10^2 CFU of *S. enterica* per g for 5 min in a solution containing 2,040 µg/ml chlorine reduced the population to undetectable levels (<1 CFU/g). Seed viability for the chlorine treatments was not evaluated. Treatment of seeds submerged in water for 5 or 10 min at 54˚C caused a significant reduction in *S. enterica* populations, and treatment at ≥ 57˚C reduced populations to ≤ 1 CFU/g. However, treatment at ≥ 54˚C for 10 min caused a substantial reduction in viability of the seeds. Treatment at 57 or 60˚C for 5 min appeared to be effective in killing *S. enterica* without substantially decreasing germinability of seeds. This study proved that treatment of alfalfa seed contaminated with *S. enterica* using chlorine concentrations of up to 1,040 µg/ml is not effective in eliminating the

pathogen, although significant reduction in numbers of viable cells can be achieved. Treatment in hot water is lethal to *S. enterica* but may also decrease germinability of seeds and thus would not be practical. The researchers suggest a 2,000 to 4,000 µg/ml chlorine soak treatment of alfalfa seeds before germination as a method to greatly reduce populations of *S. enterica* while not adversely affecting germination (Jaquette *et al.*, 1996). More research is needed regarding whether these methods can be applied to other crops.

Ralstonia solanacearum

Tomato Production on ESV

Fresh market tomato is the most valuable and widely cultivated vegetable crop on the Eastern Shore of Virginia. Approximately 80% of Virginia's tomato production comes from two counties that comprise the ESV (Accomack and Northampton) (NASS, 2009). Standard production practices for plasticulture are utilized for tomato production on the ESV. The plasticulture cultural methods include silver polyethylene mulch that is placed over raised beds with drip tape irrigation tubing laid within the beds. The plants are staked and strung throughout the growing season using the string-weave method (Gao *et al.*, 2010).

Bacterial wilt is caused by members of the *Ralstonia solanacearum* species complex. Bacterial wilt is a devastating disease worldwide of many solanaceous crops grown in temperate regions and the most costly disease of tomato on the Eastern Shore of Virginia (ESV). Land is limited on the ESV which in turn limits crop rotation options. Because crop rotation on the ESV is not economically feasible, this monoculture production system is perfect for persistent soilborne pathogens such as *R. solanacearum* to become problematic. Bacterial wilt causes

devastating yield losses, which can cause the farmer to forfeit the field altogether. This event is not uncommon on the ESV, where complete crop losses can occur in infested fields.

Bacterial wilt is a vascular disease that causes wilting of the leaves and stem leading to eventual plant death (Agrios, 2005). The initial symptom of bacterial wilt on tomato is the loss of turgidity in one or two upper leaves (McCarter, 1993). Optimal environmental conditions can cause the entire infected plant to rapidly wilt within two to three days of the initial symptoms (McCarter, 1993). In the early stages of the disease, the vascular tissue becomes yellow, eventually turning dark brown. In advanced stages of the disease, the pith and cortex also become brown (McCarter, 1993). When diagnosing a tomato plant with bacterial wilt, it can usually be distinguished by cross sectioning the stem and partially submerging it into water, causing bacterial exudate to stream from the cut ends of the stems.

To successfully manage a soilborne disease such as tomato bacterial wilt, an integrated pest management (IPM) strategy is most effective. Previously on the ESV, bacterial wilt was suppressed primarily by methyl bromide (MeBr) fumigation; however, this fumigant has been phased out due to its harmful environmental impact causing ozone depletion (EPA, 2012). Fumigant alternatives are being examined for weed, nematode, and disease control, however their impact on bacterial wilt is variable and relatively ineffective (Fennimore *et al.*, 2008; Gilreath *et al.*, 2006). Resistant cultivars have also been used to control bacterial wilt, however, these cultivars also have questionable fruit quality producing smaller and less flavorful fruit than traditional cultivars causing low grower acceptance (Wimer, 2009). In addition, resistance and tolerance of plants can also be neutralized by the actions of other pests, namely nematodes. Biological control has also been examined using non pathogenic strains of bacterial organisms and viruses (bacteriophages) (Furuya *et al.*, 1997; Jackson *et al.*, 2005; Minku and Bora, 2000;

Phae *et al.*, 1992). However, the species ecology of *Rs* is highly diverse and as a consequence, no universal control measures exist. Research suggests that the most sustainable and effective management practice to control bacterial wilt is crop rotation (Keshwal and Khare, 2000; Marouelli *et al.*, 2005; Okayama *et al.*, 2003; Yuan *et al.*, 1992). Crop rotation can be particularly effective when attempting to control pathogenic races that exhibit a narrow host range. Three year rotations with *Vicia faba* (faba bean), *Allium sativum* (garlic), *Zea mays* (corn), *Glycine max* (soybean), or *Triticum* spp. (wheat) have been proven effective at reducing disease incidence in tomato (Agrios, 2005).

Tomato Vascular Wilts

Tomato bacterial wilt is a vascular disease that causes wilting and drooping of the stems and leaves. In vascular wilts, the bacteria enter, multiply in, and move through the xylem vessels of the host plants. In the process, bacteria interfere with the translocation of water and nutrients, and this results in the wilting and death of the aboveground parts of the plant. In this manner, bacterial wilts are similar to the fungal vascular wilts caused by species of *Ceratocytis*, *Fusarium*, and *Verticillium*. However, with bacterial wilt, the bacteria often destroy (dissolve) parts of cell walls of xylem vessels or cause them to rupture quite early in disease development. Subsequently, they spread and multiply in adjacent parenchyma tissues, at various points along the vessels and cause the formation of pockets or cavities full of bacteria, gums, and cellular debris (Agrios, 2005).

During infection, the bacteria become motile and travel throughout the xylem of the plant. As *R. solanacearum* cell concentration increases, virulence genes are expressed and the cells become nonmotile and secrete enzymes leading to the death of the plant (Clough *et al.*,

1997; Saile *et al.*, 1997). The bacteria secrete enzymes such as pectinases and cellulases that break down cell wall substances and that, when carried in the transpiration stream, collect at vessel ends, form gels and gums that clog the vessel pores, and block movement of water. These enzymes also cause softening and weakening of the cell walls, which then collapse, causing tissues to droop and wilt. Penoloxidases secreted by the bacteria or released by the disrupted plant cells cause oxidation of phenolics to quinines, which then polymerize to form melanoid substances. The melanoid substances impart a brown coloration to any cell wall or substance to which they become absorbed (Agrios, 2005). In tomato, excessive development of adventitious roots may also take place. Injured or decaying infected tissues release bacteria in the soil.

R. solanacearum can overwinter in plant debris in the soil, in the seed, or in vegetative propagative material. The bacteria enter plants through wounds that expose open vascular elements and multiply and spread throughout the tissue. *R. solanacearum* can be spread from plant to plant through the soil, through handling and tools, or through direct contact of plants. Nematode infections have been proven to facilitate *R. solanacearum* infection through root injury (Agrios, 2005).

Host Range

R. solanacearum can infect more than 200 plant species representing 44 families (Hayward, 1991; Hayward, 1964; McCarter, 1993; McCarter *et al.*, 1971). *R. solanacearum* is classified into five biovars and five races based on the bacterium's metabolism of select disaccharides and hexose alcohols and organism host range (Buddenhagen and Kelman, 1964). *R. solanacearum* causes bacterial wilt of solanaceous plants and Moko disease of *Musa* spp. (banana). It is also frequently severe on *Nicotiana tabacum* (tobacco), *Solanum tuberosum*

(potato), and *Solanum melongena* (eggplant) in the southeastern United States. Other hosts such as *Arachis hypogaea* (peanut), *Glycine max* (soybean), *Musa acuminata* (plantain), and wild herbaceous plants are affected by the bacterium to a lesser extent. Control of bacterial wilt of solanaceous plants and other crops depends mostly on the use of resistant varieties, when available, and proper crop rotation or fallow (Agrios, 2005).

***R. solanacearum* Survival**

R. solanacearum can survive for extended periods of time in plant material, soil, and irrigation among other reservoirs. Because of its ability to survive and persist for long time periods, crop rotation of two to five years is recommended to effectively reduce disease (French, 1994; Yen, 1997). In addition, survival of *R. solanacearum* is strongly dependent on temperature irrespective of inoculum density and physiological state (Agrios, 2005). Long-term survival of *R. solanacearum* in the soil is believed by many researchers to reflect the ability of the organism to infect the roots of susceptible or latent plant hosts, or to colonize the rhizosphere of non-hosts. Soil moisture content, temperature, and soil type can all play a critical role in the survival of *R. solanacearum* in this habitat. The extent to which these factors affect survival can vary and the ultimate effect depends on the physiological and physical requirements of *R. solanacearum* as well as the interactions between these factors (Akiew, 1994; Graham *et al.*, 1979; Granada, 1983; Nesmith and Jenkins, 1983; Val Elsas *et al.*, 2000).

Plant Material:

R. solanacearum can also survive on plant material for long periods of time. It has been detected in *Pelargonium hortorum* (geranium) cuttings imported into Belgium, Germany, the Netherlands, and the United States from Kenya. This highlights the importance of enforcing

strict quarantine regulations related to the movement of plant material over international borders (Janse *et al.*, 2004 and Martins *et al.*, 2005). There are some reports on the survival of *R. solanacearum* on or in true seed from infected plants, but the evidence is conflicting. Seed from naturally-infected tomato plants appears to be pathogen-free. There are also a variety of weed hosts, in which the bacterium can survive and overwinter (Janse *et al.*, 2004 and Martins *et al.*, 2005).

Irrigation

Prolonged survival and even growth has been shown to take place in sterile water under appropriate environmental conditions. Temperature, pH, salt level, surfaces provided by particulate matter, and the presence of competing, antagonistic or parasitic organisms are the key factors influencing the bacterium's ability to survive in aquatic habitats. Both incident light and seawater salts have proved to be detrimental to survival of the bacterium. It has also been determined that *R. solanacearum* can persist in canal sediment saturated with drainage water, but populations decrease when the sediment is dry (Val Elsas *et al.*, 2001).

Management of Bacterial Wilt

Because the *R. solanacearum* species ecology is highly diverse, no universal control measures exist (French, 1994; Yen, 1997). Research has been conducted implementing resistant and grafted cultivars, cultural control, biological control, and chemical and soil treatments. Resistant tomato cultivars do exist; however, they produce questionable fruit quality which are smaller and less flavorful than those from traditional cultivars (Wimer, 2009). Furthermore, the resistant cultivars are only resistant to bacterial wilt when disease pressure is low. Because the bacterial wilt disease pressure on the ESV is high, resistant cultivars are still prone to disease

development. Other disease management methods need to be implemented to fully control bacterial wilt on the ESV.

Cultural Control

Cultural control, primarily crop rotation, is most widely used to control bacterial wilt. Crop rotation can be particularly effective when attempting to control pathogenic races that exhibit a narrow host range (Lemaga *et al.*, 2001). Three year rotations with *Vicia faba* (faba beans), *Allium sativum* (garlic), *Zea mays* (corn), *Glycine max* (soybean), or *Triticum* spp. (wheat) have been proven effective at reducing disease incidence in solanaceous crops (Adhikari and Basnyat, 1998). Rotation length varies, but short-term rotations involving one intermediary crop appear to be ineffective and significant disease reduction can only be achieved using rotations of two to five years (Arthy and Akiew, 1999). However, controlling the actions of pathogenic races that exhibit a wider host range through crop rotation is more problematic (Michel and Mew, 1998). This is especially true of tomato bacterial wilt.

Pathogenic populations of *R. solanacearum* were found to decline somewhat after the soil was left fallow, indicating that a suitable host plant is required to maintain pathogen numbers. Monitoring the population of *R. solanacearum* in soil used for tomato cultivation would suggest that a rotation involving greater than two non-host crops is effective at reducing incidence. In conjunction with crop rotation, weed control can be effective at reducing disease incidence (Adhikari and Basnyat 1998; Arthy and Akiew, 1999; Michel and Mew, 1998).

Chemicals and Soil Treatment

In addition to cultural methods, chemicals and other forms of treatments have been investigated as a means of controlling bacterial wilt disease. Application of stable bleaching

powder gave disease suppression of 70-89% in greenhouse and field trials in Nepal (Dhital *et al.*, 1997). Similar results were obtained in a study in India when applied in conjunction with deep plowing (Kishore *et al.*, 1996). However, contrasting results would suggest that effects can be soil dependent and that soil disinfection is not universally applicable (Michel and Mew, 1998). It has been suggested that control of bacterial wilt of tomato plants may be possible by supplying acidified nutrient solution (Yi and Sul, 1998). Soil amendments, similar to S-H mixture (containing bagasse, rice husk powder, oyster shell powder, urea, etc.) have been used to reduce disease incidence by more than 60% in naturally infested fields of tobacco and tomato. Solarization has also been studied and research has suggested a decline in the number of *R. solanacearum* cells in soil as a result (Pradhanang *et al.*, 2000; Yao *et al.*, 1994;).

Biological control

Biological control methods have been proven to suppress *R. solanacearum* in controlled environments, however, the results have failed to be replicated in field studies. A number of bacteria antagonistic towards *R. solanacearum* have been isolated from various sources and evaluated as control agents. All of these studies have shown encouraging results in the greenhouse or in strictly controlled field tests. None, however, have managed to progress beyond this evaluation phase. The use of such antagonists is thought to exploit conditions which favor the pathogen and, that once established, may be able to persist and thereby provide continuous protection. Using avirulent *R. solanacearum* strains, some of which also produced bacteriocins, protection has been achieved in tobacco, tomato, and potato (McLaughlin and Sequeira, 1988; Ren *et al.*, 1988; Tanaka *et al.*, 1990). Mutants that did not produce bacteriocins were only partially effective at halting the development of disease symptoms and protection was heavily dependent on environmental conditions, amongst other things (Chen and Echandi, 1984).

In most cases, field experiments were too limited and protection was insufficient to warrant commercial development (Luo and Wang, 1983).

Infection and Virulence

Certain qualities and adaptations can affect the virulence of the *R. solanacearum* bacterium. Yao and Allen (2006) conducted qualitative and quantitative chemotaxis assays to reveal that the bacterium is attracted to diverse amino acids and organic acids, and especially to root exudates from the host plant tomato. Exudates from *Oryza sativa* (rice), a nonhost plant, was found to be less attractive. Eight different strains were used and each varied significantly in their attraction to a panel of carbohydrate stimuli, raising the possibility that chemotactic responses may be differentially selected traits that confer adaptation to various hosts or ecological conditions. Previous studies found that an aflagellate mutant lacking swimming motility is significantly reduced in virulence, but the role of directed motility mediated by the chemotaxis system was not known. However, nontactic strains were as virulent as the wild-type strain when the bacteria were introduced directly into the plant stem through a cut petiole, indicating that taxis makes its contribution to virulence in the early stages of host invasion and colonization. It was also found that when tomato plants were coinoculated with a 1:1 mixture of a nontactic mutant and its wild-type parent, the wild-type strain outcompeted both nontactic mutants by 100-fold. These results indicate that chemotaxis is an important trait for virulence and pathogenic fitness in this plant pathogen (Yao and Allen, 2006).

Tans-Kersten *et al.* (2001) have also studied the importance of motility in *R. solanacearum* for invasive virulence on tomato. By examining bacteria from the xylem vessels of infected plants, it was found that the bacterium is essentially nonmotile *in planta*, although it

can be highly motile in culture. Two mutants were made, one lacking a flagella altogether, and the other mutant was usually aflagellate, but about 10% of cells had abnormal truncated flagella. In a soil-soak inoculation virulence assay, both nonmotile mutants were significantly reduced in the ability to cause disease on tomato plants. However, the mutant that lacked the flagella altogether had wild-type virulence when it was inoculated into the plant directly. These results also suggest that swimming motility makes its most important contribution to bacterial wilt virulence in the early stages of host plant invasion and colonization (Tans-Kersten *et al.*, 2001).

Nonetheless, many questions still remain about the role of taxis and motility in *R. solanacearum*-host interactions. However, with the knowledge of motility and chemotaxis playing a major role in initial infection of the host, many more doors are opened for future research to determine the exact interactions taking place.

Connecting R. solanacearum and S. enterica

Bacterial wilt is the number one problem on tomato on the ESV causing devastating yield and economic losses each year. In severe instances, growers have had to forfeit harvesting a field because the bacterial wilt infestation was so severe that it was not possible to make a profit from the harvestable fruit. Good Agricultural Practices (GAPs) urge growers not to harvest fruits from diseased plants in fear that the plant's compromised immune system would make it more susceptible to human pathogens such as *S. enterica* (Anonymous, 2012; Rangarajan *et al.*, 2000). These recommendations are made without the support of scientific evidence. There is a need for science-based research to support these recommendations or to negate them. Although the growers are advised not to harvest fruit from diseased plants, these recommendations are not enforced by law and fruit from health-compromised plants do inevitably end up in our food

system. In addition to the ESV tomato industry's bacterial wilt problem, the ESV has also been implicated in at least four tomato-related Salmonellosis outbreaks causing over 690 illnesses and 47 hospitalizations. Because of the ESV's endemic population of *R. solanacearum* and its associated with *S. enterica*, the relationship between these two pathogens was of interest and had never been investigated. Wells and Butterfield (1997) conducted research studying the interactions between bacterial soft rot (*Erwinia* spp.) and *Se* in fresh fruits and vegetables. They discovered that *S. enterica* populations increased by up to 10 fold when co-inoculated with *Erwinia* spp. as compared to inoculating the fruits and vegetables with *S. enterica* alone. A more recent study conducted by Barak and colleagues (2008) found that *S. enterica* populations on tomato plants were increased in the presence of *Xanthomonas campestris* (the causal agent of bacterial spot). Since previous research suggests that plant pathogens promote increases in *S. enterica* populations on fruits and vegetables, the effect of *R. solanacearum* on tomato plant and fruit susceptibility to *S. enterica* is of interest.

Objectives:

The primary goal of this research was to design and conduct applied scientific experiments that investigated the relationship between the human pathogen, *S.* Newport, and the plant pathogen, *R. solanacearum*, in tomato plants. This information will aid in identifying factors that influence *S. enterica* internalization in tomato as well as identifying the role bacterial plant pathogens play in human pathogen contamination of fresh produce. This research also involved establishing a Biological Safety Lab 2 laboratory and greenhouse at Virginia Tech's Eastern Shore Agricultural Research and Extension Center which will create innumerable opportunities for future studies involving human pathogens in an agricultural setting.

The research objectives were:

1. Identify potential interactions of the human pathogen, *S.* Newport, and the plant pathogen, *R. solanacearum*, in tomato plants under greenhouse conditions.
2. Compare *S. enterica* persistence in tomato fruit collected from plants expressing symptoms of bacterial wilt (symptomatic) and plants not expressing those symptoms (asymptomatic) from commercial tomato fields on the ESV.

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Table 1.1. Tomato Salmonellosis outbreaks since 1999. [a]

Year	Serovar	Source	Illnesses	Hospitalizations
1999	Baildon	FL	86	15
2000	Thompson	FL or GA	29	14
2002	**Newport**	**VA**	**512**	**31**
2002	Newport	FL or MX	12	2
2002	Javiana	FL or MX	90	3
2004	Javiana	FL or GA	471	129
2004	Braenderup	FL	123	29
2005	**Newport**	**VA**	**71**	**8**
2005	Enteritidis	CA	77	1
2005	Braenderup	FL	76	18
2006	Typhimurium	OH	186	22
2006	**Newport**	**VA**	**107**	**8**
2007	**Newport**	**VA**	**NA**	**NA**
Total	**-**	**-**	**1,840**	**280**

[a] Red text entries originated from one strain of *S.* Newport from the Eastern Shore of Virginia. Information retrieved from the Centers for Disease Control and Prevention (CDC).

Chapter 2

Potential Interactions between *Salmonella enterica* and *Ralstonia solanacearum* in Tomato Plants

Abstract:

Over the past decade, the Eastern Shore of Virginia (ESV) has been implicated in at least four outbreaks of Salmonellosis associated with tomato all originating from the same strain, *Salmonella enterica* serovar Newport. In addition to *S.* Newport contamination, the devastating plant disease, bacterial wilt, caused by the phytopathogen, *Ralstonia solanacearum,* threatens the sustainability of ESV tomato production. Bacterial wilt is present in most ESV tomato fields and causes devastating yield losses each year. Due to the ESV's connection with bacterial wilt and tomato-related salmonellosis outbreaks, the relationship between the two pathogens is of interest and has never been investigated. The objective of this study is to identify potential interactions between the two bacteria. Tomato plants were root-dip inoculated with one of four treatments: 1) *S.* Newport, 2) *R. solanacearum*, 3) a co-inoculation of *S.* Newport + *R. solanacearum*, and 4) a control group with no inoculation. Leaf, stem, and fruit samples were collected from the plants at the early green fruit stage and *S. enterica* presence from the internal tissues was observed. *S. enterica* was recovered from a low percentage of fruit and leaf samples. There were significantly more stem samples from plants co-inoculated with *S.* Newport + *R. solanacearum* positive for *S. enterica* (17.46 %) than from other treatments. Results suggested that *R. solanaearum* can influence *S. enterica* survival and transportation throughout the internal tissues of tomato plants.

Keywords: Bacterial wilt, food safety, *Ralstonia solanacearum*, *Salmonella enterica* Newport, tomato

Introduction:

Foodborne illnesses are estimated to cause over 183,000 hospitalizations, 47 million sicknesses, and 3,000 deaths annually in the United States (Scharff, 2012). Non-typhoidal *Salmonella enterica* is the second leading cause of foodborne illnesses each year in the United States, with norovirus being the leading cause, resulting in 35% of total hospitalizations and 28% of total deaths due to all foodborne pathogens (Scharff, 2012). Fresh produce has gained notoriety in recent years as a vehicle for human salmonellosis. Between 1990 and 2005, contaminated produce yielded the greatest number of foodborne cases annually and the second highest number of outbreaks (seafood being the highest) (CSPI, 2007). In the United States, diseases caused by the major human pathogens alone are estimated to cost up to $51 billion annually in medical costs and lost productivity (Scharff, 2012).

The Eastern Shore of Virginia (ESV) is responsible for approximately 80% of Virginia's tomato production. Since 2002, the ESV has been implicated in at least four outbreaks of Salmonellosis associated with tomatoes, and threatens the sustainability of this industry. Not only does *S.* Newport contamination threaten the fresh market tomato industry, but so does the devastating plant disease, bacterial wilt, caused by the phytopathogen *Ralstonia solanacearum. R. solanacearum* is a soilborne pathogen that infects the roots of plants through natural openings or wounds. Bacterial wilt is the number one disease problem on tomato on the ESV causing devastating yield and economic losses each year. In some instances, growers have had to forfeit harvesting a field because the bacterial wilt infestation was so severe that it was not possible to make a profit from the harvestable fruit. Good Agricultural Practices (GAPs) guidelines urges growers not to harvest fruits from diseased plants in fear that the plant's compromised immune system would make it more susceptible to human pathogens such as *S. enterica* (Anonymous,

2012; Rangarajan *et al.*, 2000). These recommendations are made without the support of scientific evidence. These recommendations are not enforced by law and fruit from health-compromised plants do inevitably end up in our food system. Studies have been conducted investigating the effects of plant pathogens on *S. enterica* populations on plants and produce. One such study was conducted by Wells and Butterfield (1997) who investigated interactions between the causal agent of bacterial soft rot, *Erwinia* spp., and *S. enterica* in fresh fruits and vegetables. They discovered that *S. enterica* populations increased by up to 10 fold when co-inoculated with *Erwinia* spp. as compared to inoculating the fruits and vegetables with *S. enterica* alone. A study conducted by Barak and colleagues (2008) found that *S. enterica* populations on tomato plants were increased in the presence of *Xanthomonas campestris,* the causal agent of bacterial spot. Since previous research suggests that plant pathogens can cause an increase in *S. enterica* populations on plants and fruits and vegetables, the effect of *R. solanacearum* on tomato plant and fruit susceptibility to *S. enterica* is of interest.

Due to the ESV's endemic population of *R. solanacearum* and *S. enterica*, the relationship between these two pathogens needs to be investigated. The objective of this research was to investigate the interactions between *S.* Newport and *R. solanacearum* in tomato plants. A series of greenhouse trials were conducted to assess the effect of *R. solanacearum* infection on *S.* Newport uptake via tomato plant root system. Understanding the interactions among these two pathogens can provide insight into *S. enterica* contamination on tomato fruit.

Materials and Methods:

Tomato Seedling Propagation

Tomato seed of the cultivar BHN602 were planted in a 128 cell Styrofoam plug tray (Speedling Inc., Sun City, FL) using ProMix BX Mycorise Pro (Premier Horticulture Inc., Rivière-du-Loup, Quebec) growing medium. The plants were grown in a biological safety level 2 (BSL-2) greenhouse facility located at Virginia Tech's Eastern Shore Agricultural Research & Extension Center (ESAREC) in Painter, VA. Approximately two weeks after seeding, seedlings were transplanted into 24.6 L plastic pots (Wetsel, Harrisonburg, VA) containing the same growing medium. The plants were irrigated with well water and fertilized with Miracle Gro (The Scotts Company LLC, Marysville, OH) as needed for optimal growth (Figure 2.1). A total of three greenhouse trials and multiple initial trial-and-error pilot studies were completed from June 2011 through July 2012.

Cultivar Susceptibility to R. solanacearum

Initial studies were conducted to determine specific experimental parameters optimal for *S.* Newport-*R. solanacearum* interaction experiments using BHN602 tomato plant cultivar including: 1) a sub-lethal bacterial inoculation concentration of *R. solanacearum* that would cause bacterial wilt symptoms (wilting) but not death of the plant before fruit production to simulate field bacterial wilt occurrence, 2) optimal plant growth stage for inoculations as the plants needed to be vigorous enough to survive the *R. solanacearum* infection but young enough for the pathogen to cause symptoms, 3) and root dip inoculation duration. Three different *R. solanacearum* inoculum concentrations were examined (5, 6, and 7 log CFU/ml), two different root dip inoculation durations (2.5 and 5 min), and three different plant growth stages for inoculation timings (at the time of initial transplanting 24.6 L pots – two week old; pre-flowering – 4-6 week old; and at flowering – 7-8 week old. Statistics were not performed on these

experiments as these were trial-and-error tests with the ultimate goal of setting optimal parameters for the remaining interaction trials.

Interaction Trials

Interaction trial 1 was conducted Fall 2011 in Blacksburg, VA whereas trials 2 and 3 were conducted at Virginia Tech's off-campus Eastern Shore Agricultural Research and Extension Center (ESAREC) in Painter, VA. All trials were conducted in BSL-2 greenhouses. Trial 1 consisted of eight plants per treatment for a total of thirty-two plants, trial 2 consisted of twelve plants per treatment for a total of forty-eight plants, and trial 3 consisted of fifteen plants per treatment for a total of sixty plants. There were a total of 35 plants per treatment including all trials.

Statistical Analyses

JMP 9 statistical software was used to compute a oneway analysis of leaf, stem, and fruit samples positive for *S. enterica* from each trial by treatment. A Tukey-Kramer Honest Significant Difference (HSD) means comparison test was performed for all pairs ($\alpha = 0.05$). A combined analysis was also performed with mean *S. enterica* positive samples from all trials.

Plant Inoculation

When plants reached flowering, each plant was inoculated with one of four treatments: 1) 8 log CFU/ml *S.* Newport, 2) 5 log CFU/ml *R. solanacearum*, 3) a co-inoculation of 8 log CFU/ml *S.* Newport and 5 log CFU/ml *R. solanacearum*, and 4) water control via root dip in a sterile hood (Figure 2.3). Plants were inoculated by root-dip method to mimic natural infection of *R. solanacearum*, which infects the plants through natural openings or through wounds in the

roots (Agrios, 2005). Plants were uprooted and the roots were submerged in water to remove potting debris, then the roots were submerged in the designated bacterial suspension for 2.5 min followed by re-planting. During the uprooting process, the roots were minimally damaged, which created minor wounds which aid in bacterial infection (Tans-Kersten *et al.*, 2001). Bacterial inoculation concentrations, root-dip duration, and plant growth stage at inoculation were chosen based on results from the initial pilot studies.

Bacterial Cultures

S. Newport strain J1892 was isolated from a tomato-related salmonellosis outbreak and originally obtained from the Center for Disease Control and Prevention (CDC) in Atlanta, GA. The frozen isolate was plated onto Xylose-Lysine-Desoxycholate (XLD) media (Becton, Dickinson and Company Franklin Lakes, NJ) and incubated for 24 h at 37˚C for preliminary confirmation of *S. enterica* species. Colonies presumptively positive for *S. enterica* were red with black centers, indicating hydrogen sulfide production. Serotype identification was confirmed by the Boyer lab using the slide agglutination test (Becton, Dickinson and Company, Sparks, MD).

The *R. solanacearum* isolate used for the studies was obtained from a local commercial tomato field on the ESV infected with bacterial wilt. The isolate was plated onto Tripheny Tetrazolium Chloride (TTC) (Becton, Dickinson and Company Franklin Lakes, NJ) media and incubated overnight at 27 ˚C and bacterial growth was confirmed to be *R. solanacearum* via AgDia (Elkhart, IN) *Rs* Rapid Test Kit.

Measuring Inoculum Concentration

S. Newport was incubated overnight on Xylose-Lysine-Tergitol 4 (XLT-4) media (Dot Scientific Inc., Burton, MI) and suspended in sterile water to an $OD_{600\ nm}$ of 0.2 (~ 8 log CFU/ml) (Barak and Liang, 2008). The bacterial suspension was serially diluted to a concentration of 2 log CFU/ml and plated for manual colony counting to confirm concentration.

R. solanacearum was grown on TTC media for 24 h at 27 ˚C and suspended in sterile water. The bacterial concentration of the suspension was measured using a hemacytometer (Hausser Scientific, Horsham, PA) and an Olympus BX41 microscope (Olympus Imaging America Inc., Center Valley, PA).

S. enterica Presence

After inoculation, plants were staked and strung to ensure upright growth and irrigated as needed. Early green fruit were sampled from each plant when two or more green fruit were produced. 235, 199, 202, and 284 fruit were harvested from *S.* Newport, *R. solanacearum*, *S.* Newport + *R. solanacearum*, and water control treated plants, respectively, and were analyzed for *S.* Newport presence inside the fruit pulp. 68 leaf samples were collected from *S.* Newport + *R. solanacearum* treated plants, and 70 leaf samples were collected from each of the remaining treatments. Leaf samples were collected from each plant from the first and fifth node. Similarly, 70 stem samples were collected from *S.* Newport and water control treated plants, and 63 stem samples were collected from each of the *R. solanacearum* and *S.* Newport + *R. solanacearum* treated plants at approximately 2.5 cm below the first and fifth nodes.

Plants were sampled aseptically using scissors sterilized by submerging in 100% ethanol for 30 sec between each sample. All collected samples were placed into individual labeled whirl-pak sterile sampling bags (eNasco, Fort Atkinson, WI). Plant samples (stem and leaf)

were surface sterilized with 70% ethanol until runoff. Samples were placed into individual labeled whirl-pak sterile sampling bags. Fruit samples were surface sterilized by submerging in a solution of 70% ethanol for ≥ 2 min and placed into individual labeled whirl-pak sterile sampling bags (Klerks *et al.*, 2006; Miles *et al.*, 2009; Hintz *et al.*, 2010). All samples were homogenized by hand and the liquid exudates were collected in labeled, sterile 2 ml eppendorf tubes (Sigma-Aldrich, St. Louis, MO) for storage at -80˚C until further analysis. The liquid exudate samples were spiral plated onto semi-selective XLT-4 media using the Eddy Jet 2 spiral plater (IUL Instruments, Barcelona, Spain) mode c100. Each sample was plated in triplicate, 100 µl per plate and incubated at 42˚C for 24 h. After incubation, observations were made for presumptive (black) colonies (Figure 2.4) with a single colony streaked onto XLT-4 for isolation. Polymerase chain reaction (PCR) was performed on these isolates with primers targeting the *inv*A gene of *S. enterica*. The *inv*A gene is unique to *S. enterica* and assists in the invasion of epithelial cells (Gorski *et al.*, 2011). Bacteria isolated from the leaf, stem, and fruit samples were confirmed to be *S. enterica* via semi-selective plating onto XLT-4 media which allows for differentiation of *Salmonella* from other organisms based on the fermentation of carbohydrates (Lactose, Xylose, Sucrose) with the resulting production of hydrogen sulfide which causes the colonies to appear black or black-centered (Miller and Tate, 1990). The bacteria was further confirmed to be *S. enterica* via PCR utilizing *S. enterica* specific primers, followed by gel electrophoresis to observe the size PCR product.

Molecular Methods

The PCR protocol selected included 95˚C for 10 min followed by 35 cycles of 95˚C for 30 s, 60˚C for 30 s, and 72˚C for 1 min, followed by one cycle of 72˚C for 7 min and then holding at 4˚C until analysis (Gorski *et al.*, 2011). The primers used were INVA-1 (5’ –ACA

GTG CTC GTT TAC GAC CTG AAT -3') and INVA-2 (5' –AGA CGA CTG GTA CTG ATC GAT AAT -3') (Gorski *et al.*, 2011). PCR mixtures contained 300 nM of each primer, SsoFast EvaGreen Supermix (Bio Rad, Hercules, CA), and molecular grade water. The PCR products were analyzed via gel electrophoresis on a 1.5% agarose gel.

Results:

Cultivar Susceptibility to R. solanacearum

It was found that the *R. solanacearum* inoculum concentration of 7 log CFU/ml killed all of the plants at all growth stages. The *R. solanacearum* concentration of 6 log CFU/ml killed the plants at the youngest growth stages tested and killed 50% of the plants inoculated at flowering. The *R. solanacearum* concentration of 5 log CFU/ml killed the plants at the transplant growth stage, 50% plants at the 4-6 week old stage, and no plants at flowering (Figure 2.2). There was no difference observed in bacterial wilt severity or *S. enterica* recovery between the two inoculation duration times of 2.5 and 5 min. Based upon these initial results, all subsequent interaction trials used an inoculum concentration of 5 log CFU/ml *R. solanacearum* with a root dip duration of 2.5 min, with inoculations occurring at flowering.

Interaction Trial 1

S. enterica was isolated from one leaf sample (6.25%) from plants inoculated with *S.* Newport only from the fifth node and from two leaf samples (12.5%) from plants co-inoculated with *S.* Newport + *R. solanacearum*, one from the first node of a plant and one from the fifth node of a different plant. *S. enterica* recovery was low and there were no significant differences

in recovery between the different treatments (Table 2.1). For all trials, bacterial isolates were confirmed to be *S. enterica* via PCR and gel electrophoresis (Figure 2.5).

S. enterica was also isolated from three stem samples (18.75%) from plants co-inoculated with *S.* Newport + *R. solanacearum* all from the first node of different plants. *S. enterica* was not recovered from any other stem samples in this trial. There was significantly more stem samples positive for *S. enterica* from plants co-inoculated with *S.* Newport + *R. solanacearum* than other treatments.

S. enterica was isolated from 4.84% of fruit sampled from plants inoculated with *S. enterica* only (n = 81), 0.76% of fruit sampled from plants inoculated with *R. solanacearum* only (n = 132), 6.09% of fruit sampled from plants co-inoculated with *S.* Newport + *R. solanacearum* (n = 115), and 1.71% of fruit from control plants (n = 117). No significant difference among treatments for *S. enterica* recovery from fruit pulp were observed.

Interaction Trial 2

S. enterica was isolated from 16.67% of stem samples from plants inoculated with *S. enterica* only (n = 24) and 4.17% of stem samples from plants co-inoculated with *S.* Newport + *R. solanacearum* (n = 24) (Table 2.2). *S. enterica* was not recovered from any leaf or fruit samples for this trial. There was significantly greater *S. enterica* positive stem samples from plants inoculated with *Se* only than plants inoculated with *R. solanacearum* only and control plants.

Interaction Trial 3

There were significantly more *S. enterica* positive stem samples from plants co-inoculated with *S.* Newport + *R. solanacearum* (30.43 %) than other treatments (Table 2.3). *S. enterica* was not recovered from any leaf or fruit samples from this trial.

Combined Analysis

A combined analysis was performed using *S. enterica*-positive samples to report pathogen presence from all trials revealing significantly more *S. enterica* positive stem samples from plants co-inoculated with *S.* Newport + *R. solanacearum* compared to other treatments (Table 2.4). There were no other significant differences in *S. enterica* presence among the treatments.

Discussion:

S. enterica positive samples from inoculated tomato plants were low, indicating that plant tissues may not present an optimal environment for *S. enterica* persistence. Although *S. enterica* positive samples were low, the bacterium was isolated from the internal tissues of leaf, stem, and fruit samples from a percentage of inoculated plants. Because plants were inoculated via root dip, these findings indicate that *S. enterica* can enter the plant through the roots and move throughout the plant's vascular system to contaminate internal leaf, stem, and fruit tissues. These results support previous research reporting *S. enterica* movement throughout and colonization of internal plant tissues. Guo and colleagues (2001) report *S. enterica* isolation from internal tissues of tomato fruit collected from plants inoculated via stem injection and by flower brush inoculation (Guo *et al.*, 2001). Klerks and colleagues (2007) reported *S. enterica* internalization of lettuce plants in a study investigating the physiological and molecular responses of lettuce to colonization of *S.* Enteritidis (Klerks *et al.*, 2007). Furthermore, studies

conducted by Kroupitski and colleagues (2009) reported *S. enterica* invasion into the inner leaf tissue of lettuce via the stomata (Kroupitski *et al.*, 2009).

The pilot studies identified factors that influence experimental success including environment temperature, *R. solanacearum* inoculum concentration, and optimal plant growth stage at the time of inoculation. It was found that high temperatures along with higher *R. solanacearum* inoculum concentrations amplified bacterial wilt disease symptoms in tomato plants and thus killed the plants before fruit set. Previous studies investigating the affect of soil and air temperatures on bacterial wilt found an increase in bacterial wilt in tomato plants subjected to warmer soil temperatures from 22 ˚C to 36 ˚C and warmer air temperatures from 16 ˚C to 28 ˚C (Gallegly and Walker, 1949). It was also found that inoculation with *R. solanacearum* at early plant growth stages (before flowering) killed the plants before fruit set. Through a series of trial-and-error experiments, a sub-lethal inoculation concentration of *R. solanacearum* was identified (10^5 CFU/ml), along with optimal plant growth stage for inoculations (flowering). The greenhouse facility did not allow for exact temperature regulation, but the average temperatures for trial 1, 2, and 3 were approximately 20, 30, and 35 ˚C, respectively.

Interaction trial 1 was conducted in Blacksburg, Virginia in the Fall of 2011. The highest recovery rate of *S. enterica* was from this trial. The temperature conditions were much cooler for this study as compared to all other studies that were conducted late spring/summer. Previous research has shown that *S. enterica* survival rates are higher at lower temperatures, but multiplies more quickly at higher temperatures (Semenov *et al.*, 2007; Natvig *et al.*, 2002). Previous research reported that *S. enterica* persists in tomato plants but does not grow (Gu *et al.*, 2011; Gu *et al.*, 2012). These findings suggest that the lower temperature conditions for trial 1 could have

enhanced *S. enterica* survival in the tomato plants, thus producing a greater *S. enterica* recovery rate.

In the first and third trials, as well as the combined analysis, there was significantly more *S. enterica* recovery from stem samples that were co-inoculated with *S.* Newport + *R. solanacearum* than from stem samples from other treatments. *R. solanacearum* clogs the vascular system of the plant it infects, which could also congest the *S.* Newport bacteria, causing a higher recovery rate from stem vascular tissue.

It is also important to note the recovery of *S. enterica* from two plants not originally inoculated with *S.* Newport as the bacteria was isolated from one plant inoculated with *R. solanacearum*, and from one control plant. It is possible that *S. enterica* was dispersed onto non-inoculated plants during irrigation. Previous research has shown that *S. enterica* may be dispersed by rain to contaminate tomato plants (Cevallos *et al*., 2012) and the plants were irrigated with an automatic overhead sprinkler system. The overhead irrigation along with the close proximity of plants could have permitted splash dispersal of *S. enterica* onto non-inoculated plants (Figure 2.6). Once the bacteria reached the surface of the plant, there were many options for internal colonization including natural openings such as the plant stomata along with plant wounds (Hallman *et al*., 1997; Kroupitski *et al.*, 2009).

The second and third trials were conducted in the late Spring and Summer of 2012 in Painter, Virginia at Virginia Tech's ESAREC facility. *S. enterica* was not recovered from any of the fruit or leaf samples from either trial 2 or 3; only from stem samples. As mentioned, temperature effects *S. enterica* growth and survival and could have been the reason for the low recovery rate of *S. enterica* from these two trials. During the two trials the greenhouse reached

temperatures as high as 43 ˚C. These high temperatures enhanced bacterial wilt disease onset and deterred *S. enterica* survival. The elevated bacterial wilt infection caused some plants to die before fruit set. Although these temperature conditions were not ideal for plant survival, the conditions most closely reflected field conditions on the ESV.

Improvements were made to the second and third trials including an improved drip irrigation system (as opposed to the overhead irrigation used in trial 1), and more space for separation of the plant treatments (Figure 2.7 and 2.8). These changes could contribute to the absence of *Se* recovery from non-inoculated plants.

In conclusion, it is possible that *R. solanacearum* influences *S. enterica* survival and transportation throughout the internal tissues of tomato plants. Exploring factors that have an impact on *S. enterica* contamination on fresh produce is essential in determining the cause of contamination events. More research is needed to better pinpoint how various factors contribute to *S. enterica* contamination of fresh produce.

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Figure 2.1. Percent disease incidence of tomato plants inoculated with three different concentrations of *R. solanacearum* at three different plant growth stages during inoculation.

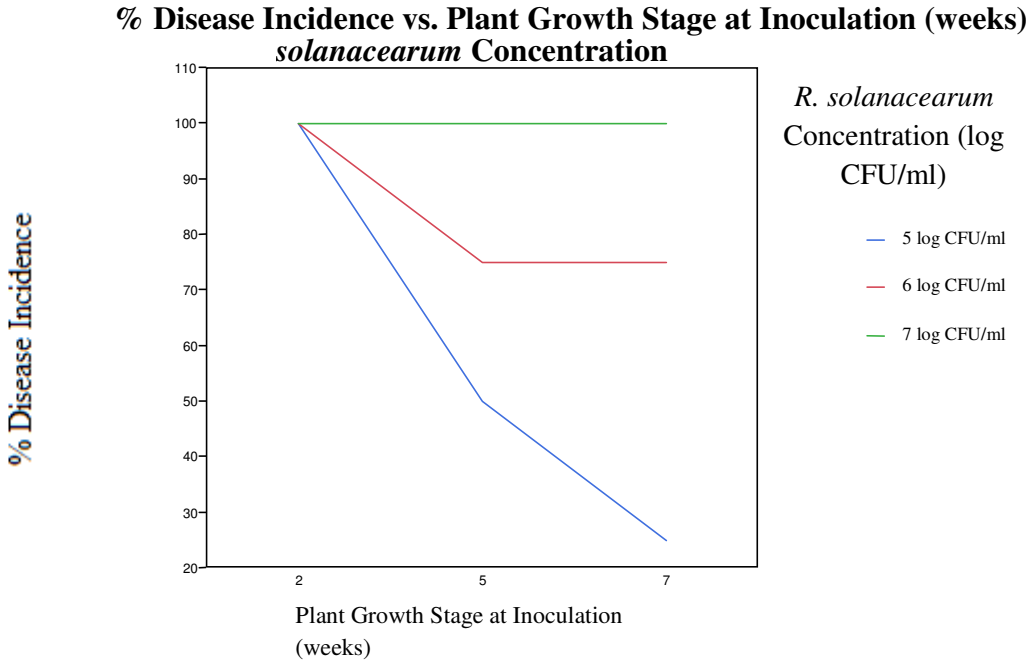
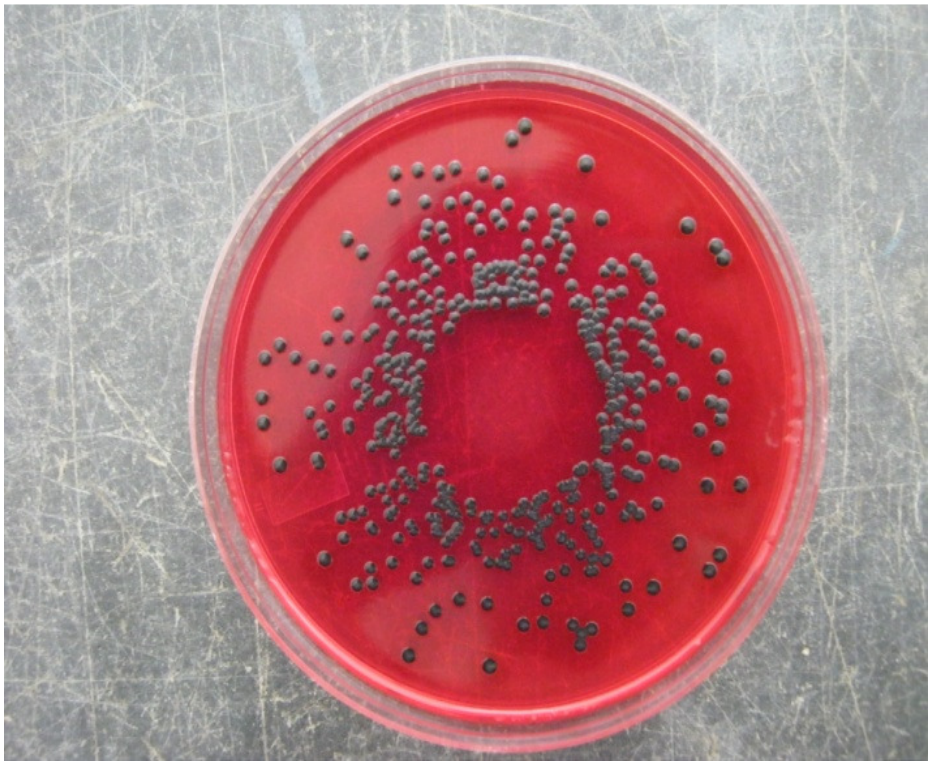


Figure 2.2. Tomato plants in BSL-2 greenhouse in Painter, VA after root dip inoculations with *S.* Newport and *R. solanacearum*.

Figure 2.3. Root dip inoculation method with *S.* Newport, *R. solanacearum*, and *S.* Newport + *R. solanacearum* co-inoculations in sterile hood in BSL-2 greenhouse.

Figure 2.4. Presumptive *S. enterica* positive black colonies on XLT-4 media plated using the Eddy Jet 2 spiral plater mode c40.



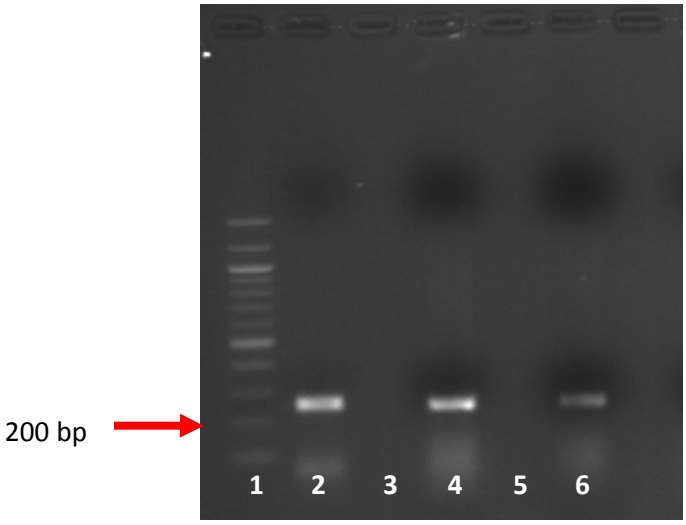


Figure 2.5. Agarose gel. Presumptive (black) colonies isolated from the interactions trials were further confirmed via PCR using *S. enterica* specific PCR primers *invA*-1 and *invA*-2 (Gorski *et al.*, 2011). This gel picture is from a typical gel electrophoresis of PCR products from presumptive colonies for all interaction trials. Lane 1) 100 bp DNA ladder, 2) *S.* Newport strain J1892, 3) Negative control, 4) 301/1 – 1N stem, 6) 301/4 – 1N stem. Those isolates that displayed an amplicon of the correct size with the *invA* primers (244 bp) on a 1.5 % agarose gel were considered to be *S. enterica*.

Figure 2.6. Interaction Trial 1. Plants were inoculated with *S.* Newport, *R. solanacearum*, or *S.* Newport + *R. solanacearum* and grown in limited space in a BSL-2 greenhouse and irrigated with automatic overhead sprinklers.

Figure 2.7. Interaction Trial 2 and 3. Plants were inoculated with *S.* Newport, *R.* solanacearum, or *S.* Newport + *R. solanacearum* and irrigated with a drip tubing irrigation system which reduced *S.* Newport dispersal via splash.

Figure 2.8. Interaction Trials 2 and 3. Plants were inoculated with *S.* Newport, *R.* solanacearum, or *S.* Newport + *R. solanacearum* and grown in a BSL-2 greenhouse utilizing more space to better separate plants of different treatments.

Table 2.1. Trial 1: *S.* Newport recovery from the internal tissues of leaf, stem, and fruit samples from inoculated tomato plants grown in a BSL-2 greenhouse [a]

Treatment	Concentration (CFU/ml) [b]	*S. enterica* Recovery Leaf(%) [c]	*S. enterica* Recovery Stem (%) [c]	*S. enterica* Recovery/Total Sampled Fruit (%) [d]
S. Newport	10^8	6.25a	0b	04.94a/81
R. solanacearum	10^5	0a	0b	0.76a/132
S. Newport + *R. solanacearum*	10^8+ 10^5	12.5a	18.75a	6.09a/115
Control	0	0a	0b	1.71a/117
P value	-	0.2293	0.0107*	0.0408*

[a] Oneway means comparison analysis of *Se* recovery using Tukey-Kramer HSD was performed utilizing JMP 9 statistical software. Letters followed by the same letter are not significantly different ($\alpha = 0.05$).
[b] Bacterial concentration in 2.5 minute root dip inoculation procedures.
[c] A total of 16 leaf and stem samples were analyzed for *S. enterica* recovery from each treatment.
[d] All fruit samples from each plant were collected and analyzed for *S. enterica* recovery.

Table 2.2. Trial 2: *S.* Newport recovery from the internal tissues of leaf, stem, and fruit samples from inoculated tomato plants grown in a BSL-2 greenhouse [a]

Treatment	Concentration (CFU/ml) [b]	*S.* Newport Recovery Leaf(%) [c]	*S.* Newport Recovery Stem (%) [c]	*S.* Newport Recovery/Total Sampled Fruit (%) [d]
S. Newport	10^8	0a	16.67a	0a/104
R. solanacearum	10^5	0a	0b	0a/48
S. Newport + *R. solanacearum*	$10^8 + 10^5$	0a	4.17ab	0a/69
Control	0	0a	0b	0a/92
P value	-	-	0.0270*	-

[a] Oneway means comparison analysis of *S. enterica* recovery using Tukey-Kramer HSD was performed utilizing JMP 9 statistical software. Letters followed by the same letter are not significantly different ($\alpha = 0.05$).
[b] Bacterial concentration in 2.5 minute root dip inoculation procedures.
[c] A total of 24 leaf and stem samples were analyzed for *S. enterica* recovery from each treatment.
[d] All fruit samples from each plant were collected and analyzed for *S. enterica* recovery.

Table 2.3. Trial 3: *S.* Newport recovery from the internal tissues of leaf, stem, and fruit samples from inoculated tomato plants grown in a BSL-2 greenhouse [a]

Treatment	Concentration (CFU/ml) [b]	*S.* Newport Recovery Leaf(%) [c]	*S.* Newport Recovery Stem (%) [d]	*S.* Newport Recovery/Total Sampled fruit (%) [e]
S. Newport	10^8	0a	0b	0a/50
R. solanacearum	10^5	0a	0b	0a/19
S. Newport + *R. solanacearum*	$10^8 + 10^5$	0a	30.43a	0a/18
Control	0	0a	0b	0a/75
P value	-	-	<0.0001*	-

[a] Oneway means comparison analysis of *S. enterica* recovery using Tukey-Kramer HSD was performed utilizing JMP 9 statistical software. Letters followed by the same letter are not significantly different ($\alpha = 0.05$)
[b] Bacterial concentration in 2.5 minute root dip inoculation procedures.
[c] A total of 28 leaf samples were collected from treatment *S.* Newport + *R. solanacearum* and analyzed for *S. enterica* recovery. A total of 30 leaf samples were collected from each of the remaining treatments and analyzed for *S. enterica* recovery.
[d] A total of 30 stem samples were collected from each of the *S.* Newport and control treatments and a total of 23 stem samples were collected from each of the *R. solanacearum* and *S.* Newport + *R. solanacearum* treatments.
[e] All fruit samples from each plant were collected and analyzed for *S. enterica* recovery.

Table 2.4. Mean *S.* Newport recovery from the internal tissues of leaf, stem, and fruit samples from inoculated tomato plants grown in a BSL-2 greenhouse [a]

Treatment	Concentration (CFU/ml) [b]	*S. enterica* Recovery Leaf(%) [c]	*S. enterica* Recovery Stem (%) [d]	*S. enterica* Recovery/Total Sampled Fruit (%) [e]
S. Newport	10^8	1.43a	5.71b	2.13a/235
R. solanacearum	10^5	0a	0b	0.50a/199
S. Newport + *R. solanacearum*	10^8, 10^5	2.94a	17.46a	3.47a/202
Control	0	0a	0b	0.70a/284
P value	-	0.2305	<0.0001*	0.0739

[a] Oneway means comparison analysis of *S. enterica* recovery using Tukey-Kramer HSD was performed utilizing JMP 9 statistical software. Letters followed by the same letter are not significantly different ($\alpha = 0.05$)

[b] Bacterial concentration in 2.5 minute root dip inoculation procedures.

[c] A total of 68 leaf samples were collected from treatment *S.* Newport + *R. solanacearum* and analyzed for *S. enterica* recovery. A total of 70 leaf samples were collected from each of the remaining treatments and analyzed for *S. enterica* recovery.

[d] A total of 70 stem samples were collected from each of the *S.* Newport and control treatments and a total of 63 stem samples were collected from each of the *R. solanacearum* and *S.* Newport + *R. solanacearum* treatments.

[e] All fruit samples from each plant were collected and analyzed for *S. enterica* recovery.

Chapter 3: Effects of Bacterial Wilt on Tomato Fruit Susceptibility to *S. enterica* Newport

Abstract:

Over the past decade, the Eastern Shore of Virginia (ESV) has been implicated in at least four outbreaks of Salmonellosis associated with tomato all originating from the same strain, *Salmonella enterica* serovar Newport. In addition to *S.* Newport contamination, the devastating plant disease, bacterial wilt, caused by the phytopathogen *Ralstonia solanacearum* threatens the sustainability of ESV tomato industry. *R. solanacearum* is a soilborne pathogen that causes severe wilting of the leaves and stem along with inevitable plant death. BW is the most severe market disease of tomato on the ESV and causes devastating yield losses each year. Due to the ESV's endemic population of *R. solanacearum* and *S.* Newport, the relationship between the two pathogens is of interest and has never been investigated. This study examined the relationship between the two bacteria via vacuum infiltration inoculations of tomato fruit collected from commercial production fields on the ESV with *S.* Newport. Tomato fruit were collected from plants expressing symptoms of bacterial wilt (symptomatic) and plants not expressing bacterial wilt symptoms (asymptomatic). After fruit infiltration with *S.* Newport, recovery of *S. enterica* from internal fruit tissues was measured. *S. enterica* recovery was greater in fruit originating from asymptomatic (5.15 log CFU/g) versus symptomatic (4.91 log CFU/g) plants across five studies. Fruit collected from asymptomatic plants had a significantly higher internal pH (4.60) than fruit collected from symptomatic plants (4.37). These results suggest that *R. solanacearum* plays a role in internal tomato fruit pH, which could potentially impact *S.* Newport survival in the fruit.

Keywords: Bacterial wilt, food safety, *Ralstonia solanacearum*, *Salmonella enterica* Newport, tomato, tomato fruit pH

Introduction:

Ralstonia solanacearum is the causal agent of bacterial wilt of tomato (*Solanum lycopersicum*). Bacterial wilt is a devastating disease worldwide of solanaceous and other crops grown in temperate regions. Bacterial wilt is a vascular disease that causes wilting of the leaves and stem of the infected plant leading to eventual plant death (Agrios, 2005). It is the most damaging disease of tomato on the Eastern Shore of Virginia (ESV), where land limitations prohibit crop rotation. Limited rotation has led to a monoculture production system that is optimal for persistent soilborne pathogens such as *R. solanacearum*. Fresh market tomatoes are the most valuable and most widely cultivated crop on the ESV. Approximately 80% of Virginia's tomato production comes from two counties that comprise the ESV, (Accomack and Northampton) (NASS, 2009). Just as threatening to ESV tomato industry as bacterial wilt infestation, there have been four recent implications to Salmonellosis outbreaks associated with ESV tomato fruit (Table 3.1).

There is limited research exploring the relationship between plant and human pathogens. However, a few notable studies have shown that the presence of a plant pathogen can increase the contamination likelihood of *Salmonella enterica* (*Se*). Wells and Butterfield (1997) studied the association of *S. enterica* contamination with bacterial soft rot of fresh fruits and vegetables, and it was found that *S. enterica* contamination was increased up to ten fold when co-inoculated with the soft rot bacterium, *Erwinia carotovora*, than when inoculated with *S. enterica* alone. It has also been demonstrated that the plant pathogen, *Xanthomonas campestris* pv. *vesicatoria*, causal agent of bacterial spot, permit increased multiplication of *S. enterica* in tomato plants (Barak and Liang, 2008). Research has also proven infection by the proteolytic, alkalinizing yeast, *Geotrichum candidum,* which causes a postharvest disease known as sour rot, in raw

tomato fruit enhances conditions for growth of *S. enterica* (Wade *et al.*, 2003). No studies have examined the impact of a vascular wilt, such as *R. solanacearum*, on tomato fruit susceptibility to *S. enterica*.

There are many methods of tomato fruit contamination with *S. enterica* including pre and post harvest contamination routes. This study imitated the potential of tomato fruit contamination with *S.* Newport during the post harvest process of tomato fruit in packinghouse dump tanks. Packinghouses subject fruit to water flumes or dump tanks containing 70 to 100 or 100 to 150 mg/liter free chlorine, respectively, to reduce pathogen populations (Bartz *et al.*, 2001). The minimum free chlorine concentration required to prevent the inoculation of fruits and vegetables in packinghouse water systems is not clear (Bartz *et al.*, 2001). This research mimicked the process of *S. enterica*-contaminated flume or dump tank water on the potential of pathogen internalization in fruit collected from bacterial wilt symptomatic plants verses fruit collected from asymptomatic plants by applying comparable pressure on fruit via vacuum infiltration that would exist in these water flume or dump tank systems (Hadjok *et al.*, 2008; Young, 1974).

The objective of this research was to determine the effect of *R. solanacearum* infection on *S.* Newport tomato fruit contamination. Good Agricultural Practices (GAPs) urges growers not to harvest fruits from diseased plants in fear that the plant's compromised immune system would make it more susceptible to human pathogens such as *S. enterica* (Anonymous, 2012; Rangarajan *et al.*, 2000). Although the growers are advised not to harvest fruit from diseased plants, these recommendations are not enforced by law. This research was conducted in effort to evaluate those recommendations as well as determine the effect of *R. solanacearum* infected plants on *S. enterica* tomato fruit contamination.

Materials and Methods:

Tomato Fruit Collection

Tomato fruit were collected from ESV commercial tomato fields that were infested with bacterial wilt infection. Fruit were collected from plants expressing symptoms of bacterial wilt (symptomatic) along with fruit from plants not expressing those symptoms (asymptomatic). Plants expressing symptoms of bacterial wilt were confirmed to have the disease based on visual wilting symptoms. Within each trial, the symptomatic and asymptomatic fruit were collected from the same field (from plants ≥ ten rows apart) (Figures 3.1 and 3.2). Tomato fruit were hand harvested and transported to the laboratory in polyethylene buckets, imitating the methods used by ESV tomato producers. This study was repeated five times from June 2011 to September 2012.

Tomato Surface Sterilization

Tomato fruit were surface sterilized by submerging the fruit in a solution of 70% ethanol for ≥ 2 min (Hintz *et al.*, 2010; Klerks *et al.*, 2007; Miles *et al.*, 2009). Fruit were then placed on the bench top for 30 min to dry and placed in individual labeled whirl-pak sterile sample bags (eNasco, Fort Atkinson, WI). Fruit weight was measured with an average weight of 154.8 and 134.4 g for fruit collected from asymptomatic and symptomatic plants, respectively. Fruit were stored in a 13 ˚C walk-in storage room overnight.

S. Newport Inoculation Preparation

S. Newport strain J1892 was isolated from a tomato-related salmonellosis outbreak and obtained from the Center for Disease Control and Prevention (CDC) in Atlanta, GA. The strain

was incubated overnight at 37 ˚C on Xylose-Lysine-Tergitol 4 (XLT-4) media and suspended in sterile water to an $OD_{600\ nm}$ of 0.2 (~10^8 CFU/ml) (Barak and Liang, 2008). The bacterial suspension was serially diluted to a concentration of 8 log CFU/ml and plated for manual colony counting to confirm concentration.

Vacuum Infiltration

Fruit were inoculated using a Rainin Classic 1 ml pipette (Mettler-Toledo International Inc., Columbus, OH) to dispense 100 µl of a bacterial suspension of 8 log CFU/ml *S.* Newport, with the aliquot of bacterial suspension transferred to the fruit stem scar for vacuum infiltration (Figure 3.4). Fruit were placed in a vacuum chamber (Fisher Scientific, Hampton, New Hampshire) where a negative pressure gradient of -0.01MPa was utilized to induce infiltration of *S.* Newport into the fruit through the stem scar (Figure 3.5). Three vacuum and release cycles (2 min per cycle) were applied to facilitate internalization at a vacuum pressure of -0.01 MPa (Figure 3.6) (Hadjok *et al.*, 2008; Young, 1974). Ten fruit from the symptomatic samples and ten from the asymptomatic samples were inoculated in the same manner with sterile water for use as a control. After inoculation, fruit were placed in individual labeled whirl-pak sterile sampling bags and placed back into the cooler for two days to imitate commercial practices of fresh fruit storage in packinghouse prior to distribution.

S. enterica recovery

After the two day storage period, fruit were surface sterilized using 70% ethanol as previously described. Fruit were stored in new individual labeled whirl-pak sterile sampling bags and homogenized manually. The last three trials included an added step of measuring the pH of the tomato fruit pulp using an Orion Thermo Scientific probe (Thermo Scientific,

Barrington, IL). pH measurement occurred after liquid exudate collection from homogenized fruit. Liquid exudates were kept in sterile 50 ml conicals (Thermo Scientific, Barrington, IL) and stored at -80 ˚C until further analysis. Samples were spiral plated on XLT-4 media using the Eddy Jet 2 spiral plater (IUL Instruments, Barcelona, Spain) mode c40. XLT-4 media allows for differentiation of *Salmonella* from other organisms based on the fermentation of carbohydrates (Lactose, Xylose, Sucrose) with the resulting production of hydrogen sulfide which causes the colonies to appear black or black-centered (Miller and Tate, 1990) Each sample was plated in triplicate, 40 µl per plate. Plates were then incubated at 37 ˚C for 24 h, at which time observations were made for presumptive (black) colonies and *S. enterica* was quantified.

Bacterial Quantification

Colony counting was performed manually for the first two trials following the Food and Drug Administration's (FDA) biological analytical method (BAM) for aerobic plate count. (FDA, 2001). The last three trials were quantified using the Neutec Flash & Go automated colony counter (Neutec Group Inc., Farmingdale, NY).

Experiment Design and Statistical Analyses

The first trial consisted of thirty-two and twenty-seven inoculated fruit from symptomatic and asymptomatic plants, respectively. Five fruit from each symptomatic and asymptomatic plant sampling was kept as a control comparison by inoculating with sterile water instead of the bacterial suspension. The remaining trials consisted of fifty inoculated fruit and ten control fruit for each of the symptomatic and asymptomatic plant samples. JMP 9 statistical software was used to compute a oneway analysis of *S. enterica* recovery from the internal tissues of the fruit samples for each trial as well as fruit pulp pH measurements. A Tukey-Kramer Honest

Significant Difference (HSD) means comparison test was performed for all pairs ($\alpha = 0.05$). A combined analysis was also performed with mean *S. enterica* recovery from the fruit samples for all five trials. A Tukey-Kramer HSD means comparison test was also used to compare fruit weight from asymptomatic and symptomatic plants.

Results:

There were no differences in the percentage of fruit from which *S. enterica* was recovered between fruit collected from asymptomatic verses symptomatic plants from any of the five trials (Table 3.2). There was no significant difference in *S. enterica* concentration in fruit pulp between fruit collected from symptomatic and asymptomatic plants in the first trial (mean concentrations of 5.46 and 5.50 log CFU/g for fruit samples from symptomatic and asymptomatic plants, respectively). In the second trial, a significantly greater concentration of *S. enterica* was recovered from fruit collected from symptomatic plants with a mean concentration of 3.70 log CFU/g versus 3.00 log CFU/g in fruit collected from asymptomatic plants (Table 3.2). For the third trial, the mean concentration of *S. enterica* in the fruit pulp was calculated to be 4.00 and 4.51 log CFU/g for fruit collected from symptomatic and asymptomatic plants, respectively, demonstrating significantly greater *S. enterica* internalization from fruit collected from asymptomatic plants (Table 3.2). In trial four, 4.98 log CFU/g of *S. enterica* was recovered from fruit collected from symptomatic plants and of 5.12 log CFU/g in fruit collected from asymptomatic plants. Trial five consisted of 5.01 and 5.41 CFU/g *S. enterica* recovered from fruit collected from symptomatic and asymptomatic plants, respectively. For both trials, this resulted in significantly more *S. enterica* recovery from fruit collected from asymptomatic plants versus symptomatic plants. A combined analysis was performed using the mean *S. enterica* recovery from all trials revealing significantly more *S. enterica* recovered from fruit collected

from asymptomatic plants (5.15 log CFU/g) compared to fruit collected from symptomatic plants (4.91 log CFU/g) (Table 3.2). There was no *S. enterica* recovered from control fruit for any trials. A combined analysis was also performed using the mean fruit weight (g) of fruit collected from asymptomatic and symptomatic samples which determined that there was no significant difference in fruit weight between the samples.

Tomato Fruit Pulp pH

There was a significant difference in fruit pulp pH between the fruit samples collected from asymptomatic and symptomatic plants for the third and fourth trial, with fruit from asymptomatic plants having a higher internal pH (Table 3.3). There was not a significant difference in pH from two of the fruit sample sets in trial 5 (Table 3.3). A combined analysis was performed using the mean fruit pulp pH from all trials revealing a significantly higher internal fruit pH in fruit collected from asymptomatic plants (4.60) compared to fruit collected from symptomatic plants (4.37).

Discussion:

Though previous research has proved that plant pathogens can enhance persistence of *S. enterica* populations on fresh fruits and vegetables (Wells and Butterfield, 1997; Barak and Liang, 2008), *R. solanacearum* infection does not appear to have that effect on the potential for *S.* Newport internalization in tomato fruit. In fact, in three of the five trials conducted (and in combined analysis) there were significantly greater concentrations of *S. enterica* recovered from fruit produced on asymptomatic versus symptomatic plants. The first two trials were quantified via manual colony counting, which introduced a segment of human error into *S. enterica* quantification. Also, when collecting fruit for trials one and two, fruit collected from

asymptomatic plants were at the mature green stage whereas fruit from symptomatic plants were mostly red and smaller. Fruit from bacterial wilt-diseased plants ripen much quicker than fruit from healthy plants (Agrios, 2005). A greater effort was made to collect more comparable fruit from the asymptomatic and symptomatic plants for the latter three trials by altering the time of fruit collection to earlier in the growing season before fruit on the symptomatic plants ripened. *S. enterica* quantification in the latter three trials was standardized with the use of an automated colony counter, utilizing computer software to count the *S. enterica* colonies. The software was able to distinguish colony color and morphology which enhanced quantification accuracy.

The addition of internal tomato fruit pH measurements were recorded for the last three trials. Trials three, four, and combined analysis revealed fruit collected from asymptomatic plants possessed a significantly higher internal fruit pH as compared to fruit collected from symptomatic plants. It is possible that *R. solanacearum* clogs the xylem of the plant, preventing nutrient transport that contribute to internal fruit pH, which directly effects the survival of *S. enterica*. The xylem is responsible for transportation of water and minerals such as K^+ and Ca^{2+}, which effect fruit acidity (Anthon *et al.*, 2010). Because *R. solanacearum* clogs the xylem of plants, this could prevent mineral transport that contribute to the significantly lower internal fruit pH found in fruit collected from symptomatic plants as compared to asymptomatic plants. It has also been reported that *S. enterica* growth and survival is effected by pH and can grow over a range of pH values from 3.7-9.5, with an optimum of 6.5-7.5 (Blackburn *et al.*, 1997). Fruit collected from asymptomatic plants had a significantly higher pH, which could have contributed to the significantly greater *S. enterica* survival observed in those fruits.

In conclusion, although tomato fruit susceptibility to *S. enterica* contamination does not appear to be enhanced by *R. solanacearum*, *R. solanacearum* does appear to have an effect on

the internal pH of tomato fruit. To our knowledge, this is the first time the effect of *R. solanacearum* on the internal pH of tomato fruit has been reported. *R. solanacearum* and *S.* Newport may not interact directly, but this research suggests that *R. solanacearum* lowers the internal pH of tomato fruit, indirectly effecting the survival of *S.* Newport.

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Figure 3.1. Plants expressing symptoms of bacterial wilt caused by *R. solanacearum* (symptomatic) on the ESV. Fruit were collected from these plants and inoculated with *S.* Newport via vacuum infiltration.

Figure 3.2. Plants not expressing symptoms of bacterial wilt (asymptomatic) caused by *R. solanacearum* on the ESV. Fruit were collected from these plants and inoculated with *S.* Newport via vacuum infiltration.

Figure 3.3. Surface sterilization of tomato fruit in 70% ethanol solution for 2.5 min at Virginia Tech's Eastern Shore Agricultural Research and Extension Center (ESAREC) prior to inoculation with *S.* Newport.

Figure 3.4. Dispensing 100 µl of *S.* Newport bacterial suspension onto the stem scar of the tomato fruit prior to vacuum infiltration.

Figure 3.5. Tomato fruit inoculation with 8 log CFU/ml *S.* Newport via vacuum infiltration with a negative pressure gradient of -0.01 MPa at Virginia Tech's Eastern Shore Agricultural Research and Extension Center (ESAREC).

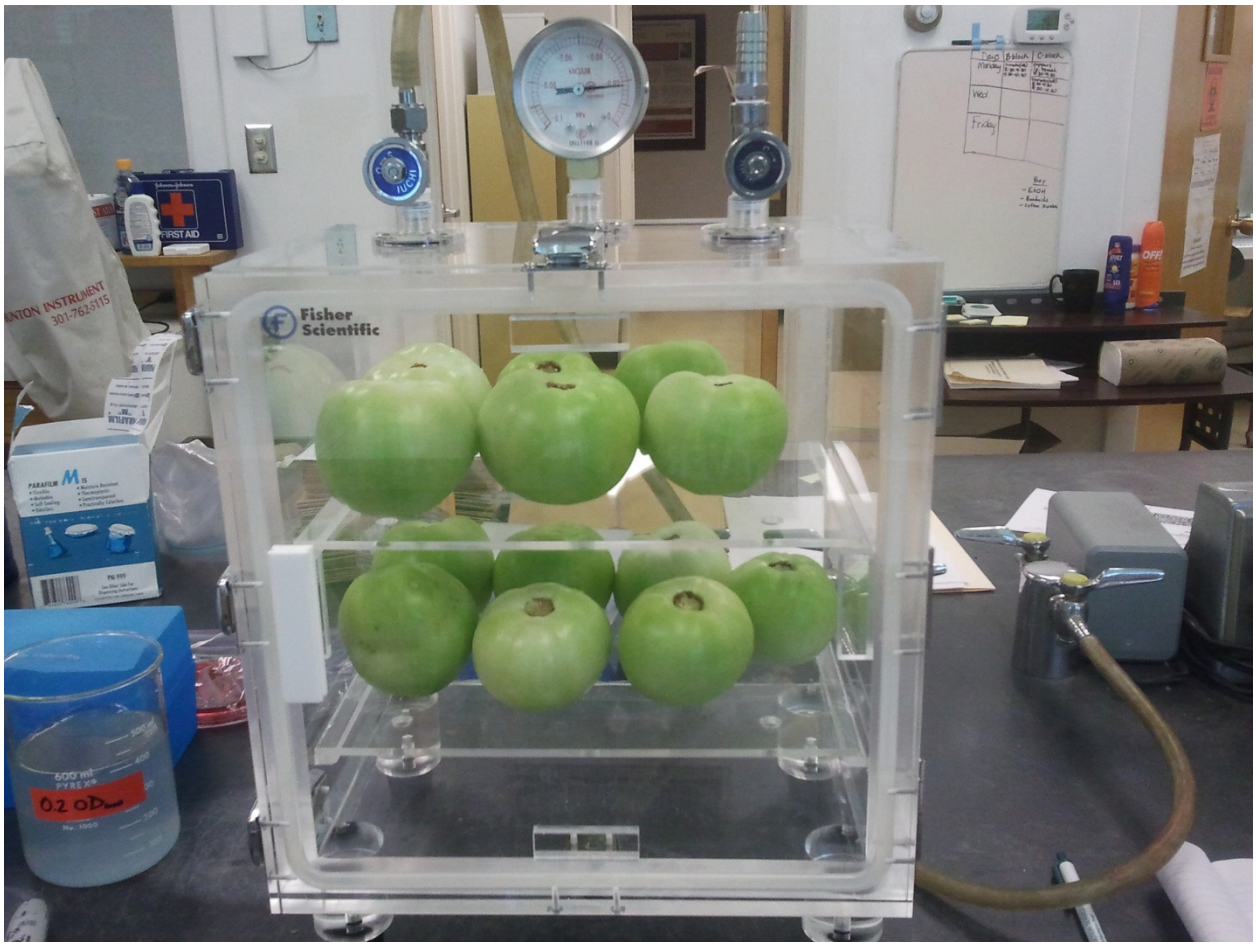

Figure 3.6. Vacuum infiltration utilizing a negative pressure gradient of -0.01 MPa to induce 100 µl bacterial suspensions of 8 log CFU/ml *S.* Newport internalization into tomato fruit through the stem scar.

Table 3.1. Tomato Salmonellosis outbreaks since 1999. [a]

Year	Serovar	Source	Illnesses	Hospitalizations
1999	Baildon	FL	86	15
2000	Thompson	FL or GA	29	14
2002	**Newport**	**VA**	**512**	**31**
2002	Newport	FL or MX	12	2
2002	Javiana	FL or MX	90	3
2004	Javiana	FL or GA	471	129
2004	Braenderup	FL	123	29
2005	**Newport**	**VA**	**71**	**8**
2005	Enteritidis	CA	77	1
2005	Braenderup	FL	76	18
2006	Typhimurium	OH	186	22
2006	**Newport**	**VA**	**107**	**8**
2007	**Newport**	**VA**	**NA**	**NA**
Total	**-**	**-**	**1,840**	**280**

[a] Red text entries originated from one strain of *S.* Newport from the ESV. Information retrieved from the Centers for Disease Control and Prevention (CDC).

Table 3.2. *S. enterica* quantification of inoculated fruit from bacterial wilt symptomatic and asymptomatic plants [a]

	% Symptomatic Samples Containing *S. enterica* [b]	% Asymptomatic Samples Containing *S. enterica* [b]	Mean ± SE *S. enterica* Concentration (log CFU/g) in Symptomatic Samples [c]	Mean ± SE *S. enterica* concentration (log CFU/g) in Asymptomatic Samples [c]	P Value [d]
Trial 1	37.5a	56a	5.46 ± 5.07 A	5.50 ± 5.10 A	0.6584
Trial 2	96a	80a	3.70 ± 2.58 A	3.00 ± 2.38 B	<0.0001*
Trial 3	96a	100a	4.00 ± 3.44 B	4.51 ± 3.70 A	0.0001*
Trial 4	84a	98a	4.98 ± 4.20 B	5.12 ± 4.18 A	0.0122*
Trial 5	98a	98a	5.01 ± 4.19 B	5.41 ± 4.37 A	<0.0001*
Combined	82.3a	86.4a	4.91 ± 4.19 B	5.15 ± 4.26 A	0.0127*

[a] Fruit samples from ESV commercial tomato fields collected from plants expressing symptoms of bacterial wilt (symptomatic) and from plants not expressing symptoms (asymptomatic) were inoculated with *S.* Newport via vacuum infiltration.

[b] Trial one consisted of thirty-two *S.* Newport-inoculated fruit from symptomatic plants and twenty-seven *S.* Newport-inoculated fruit from asymptomatic plants and five control fruit from each of the symptomatic and asymptomatic plant samples. All remaining trials consisted of fifty *S.* Newport-inoculated fruit and ten control fruit from each of the symptomatic and asymptomatic plant samples. A Tukey-Kramer HSD ($\alpha = 0.05$) test was used to compare the mean fruit positive for *S. enterica* internalization in fruit collected from asymptomatic and symptomatic plants. Lowercase letters signify comparison made across rows in columns two and three with Tukey-Kramer HSD test. Letters followed by the same letter are not significantly different.

[c] Oneway means comparison analysis of *S. enterica* quantification in inoculated fruit from asymptomatic and symptomatic plants using Tukey-Kramer HSD ($\alpha = 0.05$) was performed utilizing JMP 9 statistical software. Capital letters signify comparison made across rows in columns four and five with Tukey-Kramer HSD test. Letters followed by the same letter are not significantly different.

[d] P values presented are representative of *S. enterica* quantification in fruit collected from asymptomatic and symptomatic plants comparison across rows in columns four and five.

Table 3.3. Internal fruit pulp pH measurements from fruit collected from ESV tomato plants expressing BW disease symptoms and from plants not expressing disease symptoms [a]

	Fruit Pulp pH [b]			
	Trial			
Fruit Sample	3	4	5	Mean
Asymptomatic	4.63a	4.65a	4.53a	4.60a
Symptomatic	4.13b	4.49b	4.49a	4.37b
P value	<0.0001*	<0.0001*	0.6013	<0.0001*

[a] Internal pH measurements of tomato fruit samples from plants expressing BW symptoms (symptomatic) and from plants not expressing disease symptoms (asymptomatic).

[b] Oneway means comparison analysis of internal fruit pulp pH using Tukey-Kramer HSD ($\alpha = 0.05$) was performed utilizing JMP 9 statistical software. Letters not connected within a column by same letter are significantly different.

Appendix A

Biological Safety Level 2 Approval Process

For this research study we were able to obtain BSL-2 approval for two laboratories and one greenhouse facility. An overview of the process required to obtain BSL-2 approval is described below.

When working with human pathogens, it is necessary to take extra precautionary steps to ensure safety. There are four biological safety levels which range from working with low risk pathogens, such as non-pathogenic *E. coli* in level one, to working with dangerous and exotic agents that pose a high risk of infection for which vaccines or treatments are not available, such as the Ebola virus in level four. A biological safety level is the level of biocontainment precautions required to isolate dangerous biological agents in an enclosed facility. For this specific research study, the human pathogen, *Salmonella enterica* serovar Newport, was utilized and required a biological safety level 2 facility.

To obtain biological safety level 2 approval, many requirements had to be met and approved by appropriate university and health and safety committees including the Virginia Tech Biosafety Committee (VTBC). Research-specific safety and facility maintenance standard operating procedures (SOPs) and a biosafety manual was constructed and then reviewed and approved by a University Biosafety Officer. Appropriate engineering controls such as class II biosafety cabinet was in place along with personal protective equipment made available. All personnel involved in this research study was required to be trained on hazards, equipment use, protective measures, and incident response procedures prior to start of work. Personnel were specifically required to pass a two-part biological safety training course to ensure researchers were informed of appropriate procedures when working with biohazardous materials as well as participating in an autoclave use and verification program offered by the university. Medical surveillance requirements, including fit-testing for an N-95 respirator, were completed prior to start of work. Once all materials such as SOPs and biosafety manuals and training sessions were complete, a University Biosafety Officer evaluated the laboratory and greenhouse facilities to ensure compliance with applicable regulations and standards. Specific facility requirements include appropriate signage posted at all entrances to the facility warning of biohazard materials, an emergency contact information sheet posted at each entrance, and all entrances to the facility must be secure and kept locked when not in use. Bench top surfaces, tables, etc. must be made of a non-porous material that can be easily and effectively cleaned. Proper emergency structures must be in place, including an emergency shower and an eye wash station. All personnel were required to wear proper personal protective equipment (PPE) when handling *S.* Newport including a lab coat and gloves. In addition to lab coat and gloves, an N-95 respirator must be worn during the root-dip inoculation procedure described in chapter two. Proper waste management practices must be in order for proper disposal of biohardous materials including orange biohazard autoclave bags for waste materials which, after autoclaved, are placed in red

biohazard bags and are collected by a waste company (Sci Med Waste Systems Inc.) contracted through Virginia Tech who specialize in handling biohazardous materials.

The biological safety level 2 approval process is thorough and requires a great deal of planning and preparation. There must be a plan in place for specific methods for handling, transporting, and storing the pathogen, which must be addressed in the SOPs and biosafety manual.

References:

Virginia Tech Environmental Health and Safety. Biological safety. Available at: http://www.ehss.vt.edu/programs/biological_safety.php. Accessed 17 December 2012.

Appendix B

Plant Pathology, Physiology, and Weed Science Dept.

Virginia Polytechnic Institute and State University

LAB-SPECIFIC

Biosafety Level 2 Manual

for the

ESAREC Research Facility

Rm. #(s): Plant Growth Room C and Plant Pathology laboratory

Date: May 17, 2011

Authorized by:

Signature: _____

Steve Rideout, Principal Investigator
Date

Signature: _____

Stephanie Pollard, Laboratory Facility Manager
Date

Signature:

Charlotte Waggoner, University Biosafety Officer Date

TABLE OF CONTENTS

13. Risk Assessment Forms (and MSDS's if available)

List all Risk Assessments included in this manual:
1. Risk assessment for Glade Road and ESAREC locations
2.
3.
etc.

14. BSL-2 Agent-Specific Hazards/Precautions

List all Hazards/Precautions forms included in this manual:
1.
2.
3.
etc.

15. Lab-Specific Standard Operating Procedures

List all Lab-Specific SOPs included in this manual:
1. SOP for ESAREC Plant Growth Room C
2. SOP for ESAREC laboratory
3.
etc.

16. General Standard Operating Procedures

List all Lab-Specific SOPs included in this manual:
1.
2.
3.
etc.

17. Appendices (add as needed)

1. HOW TO USE THIS MANUAL

- **Enter text in the areas with yellow highlighting to render the manual specific to your laboratory. Add additional specific information in the appendices.**
- **All information in this manual is understood to be specific to the laboratory named below.**
- ***ADDING ESSENTIAL INFORMATION TO THE MANUAL:***

I. Attach in Section 13 a completed Risk Assessment (and MSDS sheet, if available) for BSL-2 agents currently used in the laboratory.

[The following website provides a source of MSDS information for many BSL-2 agents: <http://www.phac-aspc.gc.ca./msds-ftss/>]

II. In Section 14 document all BSL-2 agent-specific hazards/precautions requiring strict compliance by laboratory personnel IF THEY ARE NOT DESCRIBED IN THE RISK ASSESSMENTS.

- **Fill out the easy-to-use blank form (found in Section 14 for each BSL-2 agent for which the stipulation applies.**

III. In Section 15 document any other lab specific BSL-2 procedures used in the laboratory that are NOT otherwise addressed in this manual.

- **Such lab-specific Standard Operating Procedures (SOPs) must be developed, documented and reviewed by EHSS to secure approval for operation as a BSL-2 facility.**

IV. In Section 16 add any general building, department or area SOP's that are common for all labs.

V. In Section 17 add any other information that you feel is necessary for the safe operation of the lab.

2. PURPOSE

This laboratory-specific Manual is intended to:

- Supply biosafety information, guidelines, procedures and practices for using BSL-2 materials in the laboratory of Dr. Steven Rideout, ESAREC Plant Growth Room**.**

- Provide a complete reference list of all BSL-2 agents/materials/samples currently used in this laboratory.

  Enter list of agents here:
  1. *Salmonella enterica* serovar Newport
  2.
  3.
  etc.

  NOTE: *For on-site reference purposes, specific information for these agents should be included at the end of this document by attaching a risk assessment, or MSDS sheet if available, for each.*

  The following website provides a source of MSDS information for many BSL-2 agents: http://www.phac-aspc.gc.ca./msds-ftss/

- Provide a complete reference list of all personnel who work directly with or in close proximity to BSL-2 agents/materials/samples in this laboratory.

  Enter list of personnel here:
  1. Dr. Steven Rideout
  2.Stephanie Pollard
  3.
  etc.

  Add names of personnel below who come to work after BSL-2 project(s) has begun:
  (Fill out training sheet for each.)

  _____ _____
  _____ _____
  _____ _____
  _____ _____

- Provide a site for this laboratory to document its current approval number from the Virginia Tech Environmental Health and Safety Office (EHSS). ***EHSS approval must be obtained before BSL-2 work begins.***

  Enter EHSS approval number here:

  NOTE: *For all BSL-2 procedures used in the laboratory that are NOT addressed in this manual, lab-specific SOPs (Standard Operating Procedures) must be developed, documented and reviewed by EHSS to secure approval for operation as a BSL-2 facility. Lab-specific SOPs can be attached to the end of this document for a comprehensive EHSS review.*

3. EMERGENCY CONTACTS

- The door of this laboratory will be posted with current emergency contact information and biohazard information specific to the BSL-2 agents used therein.

- Posted information must include:
    a. BSL safety level, biohazardous agents(depending on location, agent list maybe posted on inside of lab door), & specific precautions (in brief) associated with those agents
    b. Principal Investigator, Lab Manager, other responsible personnel
    c. Telephone numbers for all responsible personnel

- Emergency contact sheets must be kept current.

NAME	WORK TELEPHONE	HOME TELEPHONE	CELL TELEPHONE
Dr. Steve Rideout Principal Investigator	757 414-0724; ext. 17	(757) 787-7470	(757) 694-7128
Stephanie Pollard Lab Manager		enter text	(434) 594-5159
enter text Second Lab Contact	enter text	enter text	enter text
enter text Lab Facilities & Res. Manager	enter text	enter text	enter text
Charlotte Waggoner University Biosafety Officer	540-231-1584	540-961-1301	enter text

4. SAFETY EQUIPMENT AND PRACTICES

4.1 PERSONAL PROTECTIVE EQUIPMENT (PPE)

MINIMUM REQUIREMENTS:

- While working with BSL-2 agent/material/samples in this laboratory, personnel must wear:
  a) disposable or cloth laboratory coat
  b) disposable gloves
  c) eye and face protection (goggles, mask, face shield or other splatter guard) will be used for anticipated splashes or sprays of infectious or other hazardous materials when the microorganisms must be handled outside the BSC or containment device, as determined by the risk assessment. Persons who wear contact lenses in laboratories should also wear eye protection.

PPE WILL BE LOCATED IN THESE AREAS: ESAREC Plant Growth Room C and in Plant Pathology laboratory

PPE RESTRICTIONS:

- **PPE worn while working with BSL-2 agents/samples in the laboratory or work area will not be worn *outside of the laboratory or work area.***

- BSL-2 material will be secured in closed, durable containers before being taken outside of laboratory or work areas. These secondary containers can be sprayed with disinfectant as an added precaution, and will be transported on carts to minimize spill hazards. Thus wearing PPE to transport BSL-2 material out of the laboratory in this manner *is not necessary.*

- BSL-2 material being transported to **Glassware/Autoclave Room** ESAREC Plant Pathology lab may need to be taken through hallways connecting to common-use and/or public area so compliance with these PPE policies is therefore of heightened importance.

PPE DISPOSAL, DECONTAMINATION AND CLEANING

- **Gloves:**
  - Will be changed when contaminated, integrity has been compromised, or when otherwise necessary.
  - Disposable gloves will not be washed or reused.
  - Gloves will be disposed of in Biohazard waste containers, NOT household trash.
  - Personnel will remove gloves and wash their hands after working with hazardous materials and before leaving the laboratory.

- **Lab Coats:**
  - Disposable lab coats may be reused for a period of 1 week if not contaminated during work processes. If they are contaminated they must be placed in a Biohazard waste container located within the laboratory BSL-2 work area.

    a. Cloth lab coats contaminated from work with BSL-2 materials should be autoclaved before they are sent to the laundry. ***Procedure:*** Put soiled coats in

Biohazard bags (preferably clear bags with red biohazard symbol, so contents can be readily identified and not mistaken for BSL-2 WASTE).

- . Autoclave in secondary containers (Nalgene pans). Retrieve bags after autoclaving, and place decontaminated coats in laundry pick-up area.

- Lab personnel must not take lab coats home to launder them.

- **All PPE will be removed in a manner that minimizes transfer of BSL-2 materials outside of the BSL-2 work area. These practices include: Not taking the PPE out of the BSL-2 area**

4.2. EYEWASH STATION

- Located by sink in Room # (s) ESAREC Plant Pathology lab. Eyewash will be flushed for a minimum of 5 minutes weekly.
- A log sheet is kept up to date by the laboratory manager.

4.3. SAFETY SHOWER

Located in Room #s basement hallway, near grad student office . EHSS checks showers annually.

4.4 FIRE EXTINGUISHERS AND FIRE ALARMS

- Extinguishers are located in: first floor hallway and basement hallway **.**
- Extinguishers which are located in laboratories *are checked monthly for proper charge by lab personnel*.
- Those located in hallways are checked monthly by custodial staff.

4.5 DISINFECTION AGENTS

- Sufficient quantities of bleach and ethanol are kept in the laboratory for disinfection purposes.

- Working dilutions of each disinfectant, 1:5 dilution of household bleach and 70% ethanol, are kept in sufficient quantities in the lab for disinfection purposes.

- *Bleach dilutions must be prepared fresh weekly, are inactivated by organic matter and are corrosive to metal surfaces and the skin.*

- For general disinfection of surfaces/ objects use a1:50 dilution (1 part undiluted bleach to 49 parts water). This provides 1000 ppm available chlorine.

- The minimum contact time or times (in minutes) which should be allowed for disinfectant (s) to be effective is as follows : 20 minutes for 1:5 bleach dilution, 2 minutes for 70% ethanol. enter contact time for each disinfectant used

- **Expiration dates of undiluted bleach and all other disinfectants must always be checked; up-to-date stocks must be maintained in the laboratory.**

4.6 CONDUCT OF LABORATORY PERSONNEL

To insure everyone's safety, all users of the BSL-2 facilities in this laboratory must agree to maintain the space in a clean and orderly state. Each individual will clean up safely after him/her self.

4.7 ROLES AND RESPONSIBILITIES OF LABORATORY PERSONNEL

- The Principal Investigator (PI) bears ultimate responsibility for laboratory BSL-2 practices, supplies, equipment, safety and procedural training, and documentation/record keeping.

- The PI is responsible for ordering BSL-2 agents.

- Under the supervision of the PI, a Laboratory Manager/Supervisor may be responsible for oversight of lab BSL-2 practices, supplies, equipment, safety and procedural training, and documentation/record keeping.

- PIs and/or Lab Managers are responsible for training laboratory personnel in lab-specific procedures and maintaining documentation of training. (Refer to Section *9. BSL-2 Training Record,* in this document.)
- Laboratory personnel are responsible for:
    a. Participating in all required training
    b. Following all departmental and lab-specific procedures as learned in training
    c. Informing supervisors of deficiencies in facilities, equipment or procedures
    d. Reporting accidents to supervisors

- Specific cleaning practices will be determined by PI and Lab Manager, and will be followed by all lab personnel.

- General cleaning practices for which lab personnel are responsible will include but are not limited to:
    a. Decontamination of work surfaces
    b. Decontamination and clean up of spills
    c. Decontamination and/or disposal of contaminated PPE
    d. Decontamination of lab equipment scheduled for repair or surplus
    e. Appropriate containment, removal and decontamination of biohazard waste
    f. Appropriate containment of chemical waste; making arrangements for disposal with EHSS

- Laboratory personnel are responsible for advising custodians and service providers on any safety awareness issues and procedures *prior to their entry into the laboratory*.

- Lab personnel are responsible for decontaminating refrigerated equipment before having interior surfaces exposed for defrosting. They will document those actions on a *Safety Notice for Defrosting Equipment* form and attach it to the equipment while defrosting is in process. [See Appendices for Form.]

  Lab personnel are responsible for decontaminating equipment scheduled for service or surplus, and they will document those actions on an *Equipment Decontamination Form*, to be taped onto the equipment upon completion of the tasks. For liability purposes, Decontamination Forms must be kept in laboratory records following service on equipment.

4.8 FOOD AND DRINK POLICY

- Eating, drinking, smoking, handling contact lenses, applying cosmetics, and

storing food for human consumption is not permitted in the BSL-2 lab area. Food will be stored outside the laboratory area in cabinets or refrigerators designated and used for this purpose.

4.9 VISITORS

- Visitors will be escorted into the BSL-2 laboratory area by authorized laboratory personnel, and only by prior arrangement with the Principal Investigator.

- Prior to entry into the laboratory, all visitors will be briefed on pertinent safety awareness, procedures and responses, including but not limited to:

    a) Instructions on avoiding physical contact with all research equipment, material and working surfaces, unless invited and/or approved to do otherwise by authorized personnel who will provide appropriate supervision.

    b) Information on any health hazards specific to the work being observed, as well as specific safety practices for avoiding those hazards.

    c) If visitors' time spent in the lab will exceed a short stay, they should be:
        - Shown the emergency exit route from laboratory
        - Given a review of the Emergency Procedures list posted in the lab
        - Shown the locations and proper use of emergency eyewash & shower
        - Shown the locations of the nearest fire alarm & extinguisher

4.10 SERVICE AND CUSTODIAL PERSONNEL

- Presence of custodians in the laboratory should be kept to a minimum. This will necessitate a greater level of housekeeping, cleanliness and equipment maintenance on the part of laboratory personnel.

- Custodians are responsible for:
    a) Emptying regular trash
    b) Regular mopping of floors <u>when requested</u>

- Laboratory personnel are responsible for advising custodians and service providers on any safety awareness issues and procedures *prior to their entry into the laboratory* and providing them with any necessary PPE.

5. BSL-2 WORK AREAS

5.1 LOCATION

- Use of BSL-2 materials will be limited to Room # (s) ESAREC Plant Growth Room C and Plant Pathology laboratory.
- No biosafety cabinets will be used.
- Appropriate Biohazard signage will be posted on all equipment (biosafety cabinets, centrifuges, refrigerators, water baths, etc.) used with BSL-2 agents.

5.2. SIGNAGE

- Appropriate "BSL-2—Biohazard" signs or labels will be posted in/on:
  - Entrance doors to work areas
  - biosafety cabinets
  - equipment such as centrifuges, refrigerators, etc.
  - transport containers
  - secondary containers for biohazardous waste

5.3 RESTRICTED OR LIMITED ENTRY

- Access to BSL-2 work areas or laboratories will be determined by the PI and will be restricted or limited in the following ways: Locked entry.
- The access doors to BSL-2 work areas will be closed when any work is being performed with BSL-2 materials.
- Appropriate Biohazard signage will be posted on doors when BSL-2 work is in progress.

5.4 PROTOCOL FOR MIXED BIOSAFETY LEVEL ACTIVITIES

- Sometimes BSL-2 work areas need to be established in a BLS-1 laboratory facility. The PI or lab manager must provide clear documentation of what procedures to follow to avoid confusion with multiple protocols, to protect personnel safety, and to preserve the integrity of biological agents or samples used in the lab.

5.5 STORAGE AND HANDLING

- There will be no storage in ESAREC Plant Growth Room C. Storage will be in ESAREC Plant Pathology lab.
- All other BSL-2 samples and agents (liquid cultures in tubes, cultures on solid media, tissue samples in formalin, etc.) will be secured on/within in taped Petri dishes in a BSL-2 refrigerator located in ESAREC Plant Pathology lab.
- All BSL-2 agent/material/sample containers will be opened inside a biological safety cabinet and subsequent procedures will be performed therein. This will minimize aerosol and contamination exposure for the user and/or reverse contamination potential for the agent/material/sample.
- The only procedures that will be performed with BSL-2 agents/materials/samples OUTSIDE OF THE BIOLOGICAL SAFETY CABINET are: Transportation from ESAREC Plant Growth

Room C to ESAREC Plant Pathology laboratory. All material will be transported in a double barrier container. (Refer to section 5.9)

- Procedures to be performed in the ESAREC Plant Growth Room C are as follows:

  1. Tomato plant root dip in *Salmonella enterica* liquid culture
  2. Tomato plant root dip in *Ralstonia solanacearum* liquid culture, causal agent of bacterial wilt
  3. Injection of *Salmonella enterica* (liquid culture) into plant stems via syringe needle

- Refer to section 15 for specific procedures

- Also, once tomatoes have matured, they will be transported to ESAREC Plant Pathology lab for *Salmonella* detection/quantification. All material will be transported in a double barrier container.

- Procedures to be performed in ESAREC Plant Pathology laboratory are as follows:

  1. Growth and maintenance of *Salmonella* culture on selective media
  2. *Salmonella* detection and quantification via PCR methods

- Refer to section 15 for specific procedures

5.6 WORK SURFACE DECONTAMINATION

- Work surfaces such as bench tops will be decontaminated/disinfected with 70% ethanol using paper towels to wipe the surface.

5.7 EQUIPMENT

- All equipment must be located in a BSL-2 approved area and posted with appropriate Biohazard signage.

- In the event of equipment failure during use of a biosafety cabinet with BSL-2 material, the cabinet's fan, filters and airflow plenums should be decontaminated by formaldehyde gas procedures or another approved method. Contact the Virginia Tech Biosafety Officer to schedule this procedure.

- Equipment failure experienced with refrigerators, freezers, water baths, centrifuges, etc. which are used with BSL-2 materials also require decontamination before being serviced and put back into use.

5.8 INCUBATION – NOTE: INCUBATION WILL BE DONE IN ESAREC PLANT PATHOLOGY LAB AND THEN TRANSPORTED TO ESAREC PLANT GROWTH ROOM C

- Culturing will be done in ESAREC Plant Pathology lab; no culturing in Plant Growth Room C. Refer to ESAREC Plant Pathology lab Biosafety manual.

- Liquid bacterial cultures will be incubated in 500 ml flasks with sterile gauze closures. Cultures will be incubated in ESAREC Plant Pathology lab in the PRECISION MODEL 4 incubator.

- There will be no tissue cultures

5.9 TRANSPORTATION

BSL-2 Materials/Agents/Samples/Extracts From One Work Area to Another

- Transportation of BSL-2 materials outside of ESAREC Plant Growth Room C will take place in closed, durable transport containers that are labeled with a Biohazard warning.

- Lab personnel will use Fisher biotransport carrier for containment of BSL-2 material for transport.

- For added safety, containers can be sprayed with disinfectant prior to removal from the lab or work area, and will be transported on carts to further minimize spill hazards.

BLS-2 Waste to Autoclave/Glassware Rooms

- Will be collected in orange autoclave bags displaying Biohazard symbol.

- Will be securely closed (at the end of the work session or when bag is 2/3 full), sprayed thoroughly with 70% ethanol, and allowed to dry.

- Will be placed within a secondary container (Nalgene or stainless steel pan) which is dedicated for this function with Biohazard label.

- Will be decontaminated by autoclaving ALONG WITH ITS SECONDARY CONTAINER.

- The user will be responsible for seeing that biohazardous waste is not left untreated over a weekend.

5.10 PEST MANAGEMENT

- Food and drink are not allowed in any BSL-2 area.

- Routine cleaning and mopping of floors will occur as will inspection for vermin.

- If insect or rodent pests are found in a laboratory work area/storage area, they present a possible contamination risk and containment breech. PI or Lab Manager should contact Virginia Tech Facilities to arrange pest control/removal by appropriate means.

6. VIRGINIA TECH BIOSAFETY LEVEL 2

STANDARD PRACTICES	✓
Restricted access; authorized entry only	
Doors to lab kept closed and labeled with Biohazard sign	
Decontaminate work surfaces by following specific protocol	
Remove waste frequently using leakproof secondary containers	
Decontaminate solid Biohazard waste by autoclaving	
Decontaminate liquid waste with bleach or by agent-specific disinfectant method	
Dispose of Sharps in hard-sided Biohazard containers only	
Minimize aerosols and splashes	
No direct handling of broken glass	
No mouth pipetting or label-licking	
No eating, drinking, etc. in laboratory or storing food/drink in laboratory	
Wash hands before exiting laboratory	
Wash hands after handling hazardous agents	
Wear clean or disposable coats over street clothes in work areas	
Do not wear lab coats outside of lab	
Do not wear open-toed shoes	
Personnel must know all lab-specific SOPs,	
Personnel are responsible for lab cleaning & waste removal	
Personnel must read & understand Biosafety Manual	
MSDS, risk assessments & safety manuals must be kept at known locations	
All labeling must be clear & complete	
SPECIAL PRACTICES	
Specified lab entry policies; limit entry into work area when it is in use	
Visitors—by permission only; PPE required	
Service personnel—PPE required; enter only upon arrangement by PI	
Wearing of gloves is mandatory	
Eye protection worn (including contact lens wearers) in areas of likely splash/spray	
Biohazard warning signs must be on all pertinent equipment	
Animals not involved in work not permitted in laboratories	
Remove BSL-2 agents from lab in closed, secure transport containers	
Dispose of decontaminated solid waste as Regulated Medical Waste	
Limit use of hypodermic needles/syringes	
Vacuum lines have in-line filters & traps with disinfectant	
Equipment is decontaminated before removal from area	
Spills/exposures reported to PI & EHSS immediately	
Medical surveillance provided as necessary	
Specific training is required in all risks & hazards	
All training is documented, & updated annually	
Biohazardous Agents Inventory and associate Risk Assessments are kept up-to-date	
All pertinent regulations are followed when packing and transporting BSL-2 material	
CONTAINMENT EQUIPMENT	
BSC function certified annually	
LABORATORY FACILITIES	
Impervious benchtops	
Sturdy furniture	
Good illumination	
Safe, adequate storage areas	
Work areas able to be easily cleaned	
Handwashing sink must be located in lab	
Autoclave available in building	

7. EMERGENCY PROCEDURES

7.1 MEDICAL EMERGENCIES

PHYSICAL INJURY:

- Provide immediate first-aid: stop bleeding of wounds and, if appropriate, wash the affected area with disinfectant/soap.
- If the incident is a medical emergency, DIAL 911 immediately.
- **Access the specific risk assessment sheets and/or MSDS sheets if chemical or biohazardous agents are involved. Give this documentation to emergency personnel caring for the victim; *emergency crews and hospital staff require these to provide appropriate medical treatment.***
- **For any incident that requires the non-emergency services of primary health care providers, BSL-2 risk assessments and/or MSDS sheets also must be provided so that appropriate treatment can be safely administered.**

EXPOSURE INCIDENT:

- **Immediately report any incident, accident or potential exposure to the PI, Lab Manager or other emergency contact for the laboratory. That person will advise and direct the appropriate course of action.**
- Elapsed time following exposure can be critical, so act quickly. In some instances, prophylactic medications can be given within the first few hours of exposure which will significantly lessen infection risk.
- If deemed appropriate, treatment must be obtained in the Emergency Room of the nearest hospital. The appropriate response: DIAL 911.

IN CASE OF EYE EXPOSURE:

- Immediately flush eyes for 5-10 minutes using an eyewash station.

IN CASE OF NEEDLE STICK:

- Clean and wash area thoroughly, using antimicrobial soap or mild disinfectant, for a minimum of 5 minutes. Gently massage the area to make it bleed during this time.

IN CASE OF MUCOUS MEMBRANE EXPOSURE:

- Immediately try to flush membranes if possible. Go to an appropriate healthcare provider for treatment (Emergency Room, primary care physician, Student Health Services, etc.)

ACCIDENT REPORTING:

- Accident reporting is critical to protect the employee. It is better to report a potential injury or exposure that may or may not be of consequence than to fail to report it and have complications.
- If you are injured:
  - Tell your PI, Lab Manager/Supervisor or coworker immediately
  - Contact the Virginia Tech Human Resources office to complete appropriate forms for worker compensation, should it be needed

- PIs or Lab Managers must complete an Employer's Accident Report, found at http://www.hr.vt.edu/downloads/forms/EmployersAccidentReport.doc. This should be emailed to Teresa Lyons (tlyons@vt.edu) and Kathy Gibson (gibsonk@vt.edu) within 24 hours of the incident.

7.2 BUILDING EMERGENCIES

FIRE:

- Immediately suspend work in the biosafety cabinet. Seal or close all open containers of infectious material, remove PPE and close the sash if you can safely do so. . If there is time, attach a sign to the cabinet, "SASH MUST STAY CLOSED," until you can return to it.

- Pull the nearest Fire Alarm in the hallway and/or DIAL 911.

- Evacuate the building following the red EXIT signs.

FIRE DRILL OR OTHER EVACUATION NOTICE:

- Suspend work in the Biosafety Cabinet as soon as possible, seal or close all open containers of infectious material, remove PPE and lower the sash.

- Attach a sign to the cabinet to keep the sash closed, and evacuate the building, as above.

7.3 LOSS OF ELECTRICAL POWER WHEN BSL-2 WORK IS IN PROGRESS

- Immediately stop work in the biosafety cabinet and seal any open containers of infectious material. Remain at the cabinet for a minute or two to see if power is restored.

- If power is not restored within a few minutes remove PPE, close the cabinet sash and post a sign that identifies the BSL-2 materials being used therein and explicitly states that the sash should stay closed until power is restored.

- When power is restored, don appropriate clean protective clothing when approaching the biosafety cabinet.

- Check to see if biosafety cabinet function has been restored.

- Open sash and discard any items exposed to unfiltered atmosphere while power was off. Place items in biohazard bag and autoclave immediately.

- Any BSL-2 materials (samples, agents, etc.) in lab work areas other than the biosafety cabinet should be secured in the most effective way possible to prevent an accidental exposure in the event of emergency personnel or service personnel requiring access to the laboratory.

7.4 SPILLS

A. CRITICAL THINGS TO KNOW

- **Major spill** --- a spill which, in your judgement, represents a significant health risk to people who may be exposed to a biohazardous agent as a result of the spill.

- Consider pathogenicity, concentration, volume, aerosol potential, etc. of the BSL-2 material when making this judgement. Consult the risk assessment for the BSL-2 agent(s) to find this information if necessary. Make this judgement as quickly as possible.

- Immediately notify everyone in the lab /area in the event of a major spill.

- Inform your PI or Lab Manager/Supervisor as soon as possible in the event of a major spill.

- In the event of a major spill, call 911 or 231-6411 for VT Campus Police; ask that EHSS be informed immediately.

IMPORTANT:

- Chemical disinfectants require contact time with the spill to effectively decontaminate it. Be aware of the specific contact time of the disinfectant you use, and allow that time to elapse before clean-up.
- If you use a chemical disinfectant (10% bleach, 70% ethanol, etc.) in a spill area and clean up with paper products, absorbent socks or pillows, etc., DO NOT AUTOCLAVE THIS MATERIAL TO INSURE DECONTAMINATION. THE CHEMICAL CONTENT OF THE DISINFECTANT CANNOT BE SAFELY EXPOSED TO THE HIGH HEAT IN THE AUTOCLAVE. INSTEAD, COLLECT THE MATERIAL IN A BLUE CHEMICAL WASTE BAG AND CONTACT EHSS FOR DISPOSAL.
- For metal surfaces, follow all bleach disinfectant treatments with a water rinse.
- In the event that a chemical disinfectant is not used (or cannot be used) with contaminated items, decontaminate by autoclaving or other method approved by EHSS if items can withstand the process (example: contaminated lab coats).

B. BIOHAZARD SPILL KITS

- BSL-2 work areas must contain a Spill Kit which meets the critical needs of a Biohazard spill. These kits will be supplied and maintained by Lab Facilities.

- Contents should be contained within a handled bucket and include a disposable lab coat, disposable gloves, face shield/mask, protective footwear, spray disinfectant and clean-up supplies (forceps, dustpan, autoclave bags, spill pillows & socks).

- If respiratory hazard is indicated on Risk Assessment Forms for the BSL-2 agents in question, respiratory protection in the form of respirators or PAPR units should be provided separately by the laboratory; it will not be included in the Spill Kit. These units must be fit-tested by EHSS. Contact EHSS to arrange.

- **LOCATION OF KIT:** Spill Kit will be located in ESAREC Plant Pathology laboratory to service all labs or work areas listed. Spill Kit directions must be displayed on the kit container, and a wall sign for quickly locating the Kit must be in place.

- **TRAINING WITH KIT:** All personnel working with BSL-2 materials should receive training for Biohazard Spill Kit use.

C. SPILL OCCURING INSIDE THE BIOLOGICAL SAFETY CABINET:

1. Immediately notify everyone in the lab/area of the spill. Remove any contaminated PPE/clothing and place in Biohazard bag to be autoclaved.
2. Put on clean disposable Personal Protective Equipment prior to initiating clean up.
3. Continue operating blower to help control any aerosols.
4. Lesser spills, even the smallest amount, should be **immediately** treated with 70% ethanol . After sufficient contact time, wipe up with paper towels.
5. Surfaces treated with 10% bleach should be rinsed immediately with sterile water to avoid damage to the surface metal of the cabinet.
6. Spills of greater volume require more extensive decontamination of cabinet surfaces with greater volumes of 70% ethanol. Allow the appropriate contact time, and clean up with absorbent materials followed by a sterile water rinse. Use Spill Kit if necessary.
7. Inform all users of the biosafety cabinet, as well as the laboratory supervisor and/or Principal Investigator, about the spill and status of clean-up as soon as possible.
8. For a major spill of BSL-2 material within a cabinet, the cabinet's fan, filters and airflow plenums should be decontaminated by formaldehyde gas procedures. Contact the Virginia Tech Biosafety Officer to schedule this procedure.
9. ***DISPOSAL OF ABSORBENT AND CLEANING MATERIALS SATURATED WITH DISINFECTANT:***
   - Collect in a blue Chemical Waste bag; contact EHSS for disposal.
   - Bags should not be overfilled, and should be closed securely.
   - Place bags in secondary, autoclavable containers (a Nalgene or stainless steel pan) until pickup..
   - Spray bag surface liberally with 70% ethanol and allow to dry.

D. SPILL OCCURING INSIDE LAB AND OUTSIDE BIOLOGICAL SAFETY CABINET:

1. Immediately notify everyone in the lab/area of the spill. Remove contaminated PPE/clothing and place in biohazard bag to be autoclaved.
2 ***Lesser Spills:***
Put on clean disposable Personal Protective Equipment prior to initiating clean up.
Clean up immediately with paper towels soaked in disinfectant.
3. ***Major Spills:***
   - Clear area of all personnel, close door and mark it with NO ENTRY (sign included in Spill Kit).
   - Notify the principal investigator or lab manager of the spill.
   - Wait 30 minutes for aerosol to settle before entering spill area. Assemble clean up materials and personal protective equipment during this time, using Spill Kit if needed.
   - Initiate clean-up as soon as possible following the 30 minute wait by flooding spill area with 70% ethanol . Make sure disinfectant is placed around edges of spill to prevent further spread.
   - Allow appropriate contact time (at least 20 minutes).
   - Absorb spill with paper towels or other absorbent material from Spill Kit.
   - Repeat flooding and absorbing process, and finish with a water rinse.
   - Place contaminated **reusable** items in biohazard bags, or lidded, heat-resistant pans/containers with lids before autoclaving. Place large equipment in separate bags and place bags on a lab cart for transport to autoclave. Initiate further clean-up, if needed, after autoclaving.
   - Expose non-autoclavable materials to disinfectant for 20 minutes.
   - Inform all lab personnel as well as the laboratory supervisor/principal investigator about the spill and status of clean-up as soon as possible.
4 ***DISPOSAL OF ABSORBENT AND CLEANING MATERIALS SATURATED WITH DISINFECTANT:***

- Collect in a blue Chemical Waste bag; contact EHSS for disposal.
- Bags should not be overfilled, and should be closed securely.
- Place bags in secondary, autoclavable containers (a Nalgene or stainless steel pan) until pickup..
- Spray bag surface liberally with 70% ethanol and allow to dry.

5 ***AUTOCLAVING CONTAMINATED, HEAT-RESISTENT ITEMS NOT TREATED WITh DISINFECTANT:***

- Collect in a Biohazard autoclave bag.
- Bags should not be filled more than 2/3 full. When full, close the bag securely.
- Place bags in secondary, autoclavable containers (a Nalgene or stainless steel pan).
- Spray bag surface liberally with 70% ethanol and allow to dry.
- Immediately transport to autoclave/glassware room and decontaminate by autoclaving as soon as possible.
- Immediately prior to autoclaving, loosen bag closure to allow steam penetration within bag.
- Dispose of all decontaminated waste in Regulated Medical Waste boxes.

E. SPILL OCCURING IN HALL OUTSIDE OF LABORATORY:

1. Warn personnel in the immediate area of the spill. Block off spill area as best you can
2. ***Minor spills:***
   - Cover spill with paper towel and saturate with 10% bleach or appropriate disinfectant.
   - If spill is on lab coat, remove and place lab coat in Biohazard bag; autoclave as soon as possible.
   - Notify lab supervisor or Principal Investigator, and EHSS.
3. ***Major spills:***
   - Remove lab coat and gloves and evacuate area. Allow 15 minutes for any aerosol to settle.
   - Notify lab supervisor and Principal Investigator.
   - Put on clean disposable protective clothing/PPE.
   - Re-enter area after 15 minutes and cover spill with either paper towels or spill control pillows found in Spill Kit. Saturate spill area with 70% ethanol. Make sure disinfectant is placed around edges of spill to prevent further spread.
   - Allow 20 minutes contact time. Clean up spill with absorbent materials and place in blue Chemical Waste bag for EHSS disposal.
   - For heat-resistant items not treated with disinfectant, collect in Biohazard autoclave bag.
   - Bags should not be filled more than 2/3 full. Close the bag securely and place in a secondary, autoclavable container (a Nalgene or stainless steel pan). Spray bag surface liberally with 70% ethanol and allow to dry. Immediately transport to autoclave/glassware room and decontaminate by autoclaving as soon as possible. Immediately prior to autoclaving, loosen bag closure to allow steam penetration within bag.Dispose of all decontaminated waste in Regulated Medical Waste boxes.
   - Disinfection process can be repeated in spill area if deemed necessary.

F. SPILL OCCURING INSIDE A SHAKING INCUBATOR

IMPORTANT:

- **Proceed quickly! Immediately turn off power to unit and unplug power cord from wall socket**.
- **Immediately notify everyone in the lab/area of the spill.**
- **If spill volume is large (>2 L), then close lid of incubator and call PI, lab manager or EHSS for assistance.**

- **If spill can be safely contained and removed by lab personnel, proceed as follows:**

1. Remove any clothing contaminated with spill and place in Biohazard bag to be autoclaved. If skin is contaminated, treat with non-bleach disinfectant and follow with a soap & water rinse.
2. Quickly place paper towels on spill inside incubator to absorb liquid before it leaks onto motorized parts below, then close lid.
3. Ask someone to contact the principal investigator or lab manager to advise them of the spill while you retrieve: a) the Biohazard Spill Kit from its storage location in the lab, and b) a sufficient quantity of 70% ethanol.
4. DO NOT LEAVE THE ROOM WITHOUT PUTTING A SIGN ON THE INCUABATOR THAT SAYS "HAZARDOUS SPILL INSIDE—DO NOT OPEN OR USE!"
5. DO NOT MIX DISINFECTANT TREATMENTS IN YOUR CLEAN-UP, ESPECIALLY BLEACH AND ETHANOL.
6. If you are wearing no Person Protective Equipment, put on the PPE in the Spill Kit.
7. Check to see if spill liquid is leaking out from the unit onto bench or floor. If so, apply disinfectant to the spill liquid as well as in a perimeter around the spill; wait for disinfectant to take effect, then clean up with paper towels or other absorbent material from Spill Kit. Discard soaked material into Biohazard waste.
8. Now direct your attention to the interior of the incubator once again. Spilled liquid cannot be absorbed all at once because of the way shaking incubators are constructed, therefore you must work from top to bottom. Spray disinfectant over the soaked paper towels you applied earlier, then position a Biohazard autoclave bag as close as possible for discarding the towels. Place wet towels in bag carefully, minimizing aerosols and drips however possible.
9. Immediately apply more absorbent material to the spill if needed. Use pads, socks or pillows from the Spill Kit according to the volume of the spill and the size of the area to cover.
10. Spray interior surface areas of unit with disinfectant, especially any broken vessels associated with the spill. Wait for disinfectant to be effective.
11. With forceps from Spill Kit, remove the pieces of broken vessels from the incubator interior. Place broken glass in Sharps container; decontaminate by autoclaving as soon as possible.
12. At this point you may need to remove the incubator's platform to get to lower regions for further spill clean up. Removal is often accomplished by using a hexagonal T wrench on 4 platform screws; a Phillips screwdriver may be needed to move flask clamps if they are covering the platform's hex screws. These tools should be located on or near the shaking incubator. (Call Lab Facilities staff for help if needed.)
13. Thoroughly spray platform with disinfectant before removal, and give disinfectant time to work.
14. Before taking platform out of incubator, spray paper towels with disinfectant and use them to cover an area on lab floor upon which to place the platform. Choose an area of the floor that is out of your way. Place removed platform onto paper towels and perform a more thorough clean up later. Spray tools with disinfectant.
15. Apply absorbent material to any spill liquid you see in lower regions of the incubator. TRY TO ABSORB AS MUCH OF THE SPILL FROM AS MANY SURFACES AS YOU CAN.
16. Flood the absorbent material in the incubator with enough liquid disinfectant to decontaminate, but not so much as will create a gross excess in the spill area. Wait for disinfectant to be effective.
17. Place absorbent material saturated with disinfectant into blue Chemical Waste bags provided in Spill Kit and securely close the bags. EHSS must be contacted for disposal. Bags must be kept in secondary containers while awaiting pickup.
18. For materials to be preferentially decontaminated by autoclaving (no alcohol or bleach), place directly into Biohazard autoclave bags and close bags securely. Place bags in secondary containers when transporting to autoclave room. Decontaminate by autoclaving.
19. After all spilled material has been removed, disinfect every surface of the incubator that is accessible, then repeat if necessary. Use cotton-tipped swabs for hard-to-reach areas. Do not use bleach on metal parts. If decontamination of enameled surfaces is performed with a bleach solution, apply a water rinse.

20. Put cleaned, dried platform back into position.
21. Leave lid incubator open for additional drying out.
22. Mop lab floor with disinfecting agent.
23. Autoclave any contaminated PPE.
24. Contact Lab Facilities before using the incubator again after spill clean-up. Lab Facilities will test the incubator for electrical safety and proper function before returning it to service.

G. SPILL OCCURING INSIDE CENTIFUGE:

1. Leave centrifuge closed for at least 30 minutes for aerosol to settle. During this time, get clean up supplies ready, including Spill Kit if needed.
2. Flood spill area with 70% ethanol. Allow contact time of 20 minutes. All exposed surfaces should be disinfected, including heads, cups, cushions, etc.
3. Absorb spill with paper towels.
4. Flood area again with 70% ethanol. Allow 20 minutes contact time, then repeat clean up and finish with a water rinse.
5. All disposable materials used in clean up must be collected in a blue Chemical Waste bag. Place bag in secondary container and contact EHSS for disposal.

H. SPILL OCCURRING IN WATER BATH OR SHAKER BATH

1. Turn power off.
2. Pour 70% ethanol directly into water bath in sufficient quantity to effect decontamination. (CAVICIDE is recommended over bleach to reduce likelihood of damage to metal parts from chloride exposure.)
3. Replace cover and wait for 20 minutes.
4. Discard the water/disinfectant solution by pouring down sink drain, and flush sink drain with water.
5. Disinfect the surfaces of the water/shaker bath, and allow to dry before returning unit to regular use.

I. SPILL OCCURRING IN INCUBATORS OR REFRIGERATORS

1. Minor spills which have not generated significant aerosols may be cleaned up with a paper towel soaked in disinfectant.
2. In the event of a major spill, the door should be left shut for 30 minutes to allow any aerosol to settle.
3. Cleanup should be initiated with 70% ethanol. Allowing for a contact time of 20 minutes, all exposed surfaces should be disinfected, including equipment, racks, tubes, bottles, etc.
4. Absorb the spill with paper towels, and flood the area again with the disinfectant.
5. After another 20 minute contact time, absorb with paper towels and finish clean up with a water rinse.
6. All disposables used in the clean up procedure should be collected in a blue Chemical Waste bag. Place bag in secondary container and contact EHSS for disposal.

J. SPILL INVOLVING BROKEN GLASS

1. DO NOT handle broken glass with your hands.
2. Using a dustpan or forceps, place the glass pieces in an approved sharps container and autoclave.

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8. ROUTINE PROCEDURES

8.1 BIOSAFETY CABINET WORK PRACTICES

- Cabinet blowers should be operated at least 5 minutes before beginning work to allow cabinet to “purge”. This purge will remove any particulates in the cabinet.
- The work surface, the interior walls (not including the supply filter diffuser), and the interior surface of the window should be wiped with a solution of 70% of ethanol.
- The surfaces of all materials and containers placed into the cabinet should be wiped with 70% ethanol to reduce the introduction of contaminants to the cabinet environment.
- Place all necessary materials in the biosafety cabinet before beginning work. This will serve to minimize disruptions across the fragile air barrier of the cabinet.
- Disruption of the air curtain occurs with rapid movement of a worker’s arms into and out of the cabinet, compromising containment provided by the BSC. Moving arms in and out slowly, perpendicular to the face opening of the cabinet, to reduce this risk.
- Other personnel activities in the room (e.g., rapid movement, opening/closing room doors, etc.) may also disrupt the cabinet air barrier. For this reason, access to the work area is restricted when work is in progress.
- Before beginning work, adjust stool height so that your face is above the front opening.
- Manipulation of materials should be delayed for approximately 1 minute after placing the hands/arms inside the cabinet. This allows the cabinet to stabilize and to “air sweep” the hands and arms to remove surface microbial contaminants.
- When the user’s arms rest flatly across the front grille, room air may flow directly into the work area, rather than being drawn through the front grille. Raising the arms slightly will alleviate this problem. The front grille must not be blocked with research notes, discarded plastic wrappers, pipetting devices, etc.
- All operations should be performed on the work surface at least 4 inches from the inside edge of the front grille.
- Equipment that causes turbulence (centrifuge, vortex, etc.) should be placed in the back 1/3 of the work surface. All other work in the cabinet should stop while the apparatus is running.
- The use of open flames such as Bunsen burners inside the biological safety cabinet is not recommended as the open flame creates turbulence that disrupts the laminar HEPA filtered airflow to the work surface.
- Separate clean and contaminated items. Minimize movement of contaminated items over clean items (work from clean to dirty).
- Only the materials and equipment required for immediate work should be placed in the BSC. Do not use as a storage area.
- All vacuum lines must have in-line filters and traps containing disinfectant; all vacuum filtering takes place in the biosafety cabinet. (See In-Line Filters SOP for details).
- At the end of the work session, all materials are surface decontaminated and removed from the cabinet. The work surface, the interior walls, and the interior surface of the window are again

wiped with 70% ethanol. UV light may be turned ON for 30 minutes as an additional precaution.

8.2 POTENTIALLY INFECTIOUS WASTE

Contaminated items generated in a biosafety cabinet can be removed after:

- Being decontaminated with disinfectant (rinsed in bleach solution or sprayed with 70% ethanol)
- Placement in a small SHARPS container, disposable pipette box or small Biohazard autoclave bag located inside cabinet, which can then be closed before removal

Liquid BSL-2 wastes:

- Liquid waste can be discarded into a container containing a sufficient quantity of pure bleach to yield a 1:5 bleach dilution.
- Alternatively, liquid waste can be poured into a well-labeled autoclavable container (containing no bleach), decontaminated by autoclaving on an appropriate LIQUIDS cycle (cycle time determined by liquid volume) and then flushed down the drain.
- Waste containers must be appropriately labeled as such, with biohazard signage.
- After 30 minutes exposure time in a biosafety cabinet, liquid waste + disinfectant may be disposed of down the lab sink drain, followed by a water flush.

Solid BSL-2 wastes:

- Are collected in an ORANGE autoclave bag with a Biohazard symbol.
- Autoclave bags for filling should be kept inside appropriately labeled biowaste container, equipped with a closeable lid.
- Container should remain closed except when in use.
- At end of session or when 2/3 full, the autoclave bag is securely closed, placed in a secondary container labeled for this purpose, and sprayed with 70% ethanol prior to transport on a cart to the autoclave room.
- Bags will be autoclaved daily when possible, or as soon as an autoclave is available. No full bags should be left in the laboratory or the autoclave/glassware room OVER THE WEEKEND awaiting decontamination.
- Bags should remain securely closed *until going into the autoclave, at which time their closures should be loosened to allow steam penetration.*
- All BSL-2 solid waste is to be decontaminated by autoclaving and disposed of as Regulated Medical Waste.

8.3 USE OF SHARPS

- Use of sharp objects such as Pasteur pipettes, syringe needles, etc. will be utilized only when an adequate, less hazardous substitute cannot be found. Plastic ware will be substituted for glassware whenever possible.

- Personnel will be trained for safe use and disposal of sharps in BSL-2 work areas.

- Needles must not be bent, sheared, broken, recapped, removed from disposable syringes, or otherwise manipulated by hand before disposal.

- Contaminated sharps will be disposed of in designated hard-sided Sharps containers; containers will be securely closed before transport to autoclave room for decontamination. All sharps containers are to be disposed of in Regulated Medical Waste.

- Broken glassware will not be handled directly. It must be removed using a brush and dustpan, tongs or forceps.

NOTE: In situations when collection of Sharps is intermittent or of smaller volume, avoid collecting them in large sharps containers. Use smaller Sharps containers whenever possible, as these will fill more quickly and thus can be decontaminated more expeditiously.

8.4 PIPETTES

- Disposable pipettes used in the biosafety cabinet will be discarded directly into lidded Sharps containers, submerged in a horizontal container with appropriate disinfectan, or discarded into a disposable pipette collection box.

- It is important for disposable pipettes to be discarded into appropriate receptacles inside biosafety cabinets to avoid repeatedly breaking the air barrier of the cabinet by discarding in a receptacle outside the cabinet.

- Aspirating 5% bleach solution in a pipette before discarding is recommended.

- Pipette receptacles will be closed when full and/or at the end of a work session. Autoclave in a timely way to decontaminate, then discard in Regulated Medical Waste.

- In situations when Sharps use is intermittent or of lesser volume, they will be discarded in smaller Sharps containers. These will fill and be removed more quickly than a larger Sharps container which may harbor contaminated materials for weeks before being filled removed from the lab.

8.5 PIPETTING

- *Never mouth pipette*. Automatic pipettors are to be used for all material.

- Automatic pipetters utilized in the BSL-2 Biosafety Cabinet will be properly identified and reserved for BLS-2 work. They will be sprayed within the hood with 70% ethanol disinfectant before work begins, and after work is finished.

- Larger volume pipettes to be utilized with the pipette aid device are recommended to be the disposable plastic type, with cotton plugged ends; this prevents aspiration of fluid or aerosols into the pipetting device.

- Avoid the following practices when pipetting:
  - Do not mix biohazardous fluids by repeated suction and expulsion from pipettes, which generates aerosols.
  - Do not bubble air through biohazardous fluids, which also generates aerosols.
  - Do not forcibly expel liquids from pipettes. Discharge as close as possible to the fluid or down the side of the container.
  - Avoid accidentally dripping infectious liquids from pipettes.

8.6 CENTRIFUGATION

Be completely familiar with your project's risk assessment and lab-specific SOPs, and follow their guidelines when undertaking a centrifugation step with BSL-2 materials. (Example: loading rotors with BSL-2 materials inside a biosafety cabinet when indicated.)

Do not run BSL-2 materials in the centrifuge without taking appropriate safety precautions and using required PPE.

Make sure a Biohazard Spill Kit is available when using the centrifuge.

All centrifuges used with BSL-2 materials must be labeled with the Biohazard symbol.

If BSL-2 agents must be centrifuged in buckets that do not have safety caps, the centrifuge will be left unopened for a minimum of 5 minutes after the spin has finished to allow initial aerosols to settle (in case a tube has broken during spin).

If, upon opening the centrifuge for spinning BSL-2 materials, it is discovered that there has been a release of liquid (outside of the sealed rotor bucket), close the centrifuge immediately and wait 30 minutes before initiating clean up to allow. Then follow "**Spill Procedure Inside Centrifuge**," found in this manual.

Some BSL-2 agents require that aerosol protection, such as respirators, be available in case of a spill when centrifuging. Consult with EHSS to fulfill this safety requirement, if applicable.

- After using a centrifuge for a BSL-2 agent, it is recommended to always wipe down the instrument with disinfectant.

9. PERSONNEL

9.1 TRAINING REQUIREMENTS

- All personnel working in a BSL-2 laboratory will receive general awareness training which relates to all infectious organisms/material being used within the lab.
- At a minimum the following training will be completed by each person directly performing work with the infectious material:
  - Specific BSL-2 training provided by the PI or Laboratory Manager/ Supervisor (see BSL-2 Training Record)
  - Introduction to Biological Safety Cabinets (Scholar: on-line)
  - Selected additional training provided by EHSS, such as Bloodborne Pathogens, Respiratory Protections, Autoclave Use, IACUC training (for working with lab animals)
- All personnel will abide by the BSL-2 protocols learned in training.
- The PI or Laboratory Manager will ensure and document that laboratory personnel demonstrate proficiency in standard and special microbiological practices at BSL-2 before working with any potentially infectious material.
- Personnel will receive annual updates or additional training when procedural or policy changes occur.
- The BSL-2 Training Record is found on the next page. This table is the required document for use an individual training record for every person working in a BSL-2 laboratory.

9.2 TRAINING RECORDS FOR LAB PERSONNEL

- Signed training records will be retained for the duration of each person's employment and for at least 3 years after.
- All BSL-2 training will be documented and kept in Biosafety Manual for the laboratory.
- Training completed:
  - Introduction to Biosafety cabinets, Virginia Tech Scholar site, completed 4/11/2011
  - Safe Autoclave Use and Verification, Virginia Tech Scholar site, completed 4/11/2011
  - Biosafety Training online Part I and II, Yale University http://www.yale.edu/ehs/trainingbio.htm, completed 4/27/2011

9.3 MEDICAL SURVEILLANCE

- Laboratory personnel will be provided medical surveillance and offered appropriate immunizations for agents handled or potentially present in the laboratory as determined by the risk assessment.

- Personal health status may impact an individual's susceptibility to infection, ability to receive immunizations or prophylactic interventions. Therefore, all laboratory personnel and particularly women of child-bearing age will be provided with information regarding immune competence and conditions that may predispose them to infection. Individuals having these conditions will be encouraged to self-identify to the institution's healthcare provider for appropriate counseling and guidance.

- Records of medical surveillance testing, including any testing deemed necessary, are maintained by EHSS.

- Records of medical surveillance, if any, will be kept for duration of each person's employment and for at least 3 years after.

10. BSL-2 TRAINING RECORD FOR _____
P.I. LABORATORY _____

TRAINING TOPIC	LOCATION OF INFORMATION ON TOPIC:	REVIEWED ON: (DATE/TRAINEE INITIALS)	REVIEWED BY: (TRAINER INITIALS)
BSL-2 Agent or Agents Used in the Lab:			
1. Specific hazards of BSL-2 agents (e.g., modes of transmission, signs and symptoms of disease, etc.)	Risk Assessment/MSDS		
2. Methods of disinfection & decontamination for specific BSL-2 agents	Risk Assessment/MSDS		
3. Use of hazard warnings & rationale for specific BSL-2 agents	Risk Assessment/BSL-2 Manual		
4. Ordering, shipping & receiving procedures for specific BSL-2 agents			
5. Handling and storage for specific BSL-2 agents			
Personal Protective Equipment, Lab Equipment & Lab Safety:			
1. PPE needed; location where PPE supplied in lab; proper use of PPE	BSL-2 Manual		
2. Operation procedures for biosafety cabinets	BSL-2 Manual/SOP		
3. Use & maintenance of pipetting aids	Lab-specific SOP		
4. Safe use & maintenance of centrifuges, incubators, etc.	Lab-specific SOP		
Emergency Response Training			
1. Procedure for spills & leaks; use of spill kit	BSL-2 Manual		
2. Decontamination techniques	BSL-2 Manual		
3. Fire/disaster response	BSL-2 Manual		
4. Power failure procedure	BSL-2 Manual		
Waste Disposal Procedure			
1. Collection,storage & disposal procedures for BSL-2 waste	BSL-2 Manual		
2. Segregation & destination of BSL-2 waste	BSL-2 Manual		
3. Decontamination/disinfection methods & efficacy	BSL-2 Manual		

This individual under my supervision has demonstrated proficiency in standard and special BSL-2 procedures used in this laboratory.

_____*(PI or Lab Manager signature)* **_____*Date***

SIGNATURE PAGE

This manual MUST be read before beginning work in this BSL-2 Laboratory. Please acknowledge that you have read this manual by printing and signing your name below.

PRINT NAME	SIGN NAME	DATE

12 SOURCES

Material in this manual has been obtained from the following sources:

1. *Biosafety for Laboratory Workers Manual*, Virginia Tech, November 2000.
2. *BSL-2 Laboratory Operations Manual,* VA-MD Regional College of Veterinary Medicine, October 2006.
3. "Primary Containment for Biohazards," Office of Health and Safety Information System, CDC.
4. "Biosafety Cabinet Work Practices," University of Maryland Biosafety Office.
5. *Biosafety in Microbiological and Biomedical Laboratories,* U.S. Department of Health and Human Services Public Health Services, Centers for Disease Control and Prevention and National Institutes of Health,5th edition, 2007
6. *Laboratory Biosafety Manual*, World Health Organization, 3rd edition, 2004.

13. RISK ASSESSMENT FORMS

INSTRUCTIONS: Insert completed and signed Risk Assessments and any MSDS'S that are available for the material you are using.

*See attached risk assessment form

14. BSL-2 AGENT-SPECIFIC HAZARDS/PRECAUTIONS

INSTRUCTIONS: **Please use this form to describe agent-specific procedural precautions, additional information, concerns, etc. which are NOT covered in this manual.** ***Fill out a separate form for each BSL-2 agent to which this stipulation applies.***

FORM COMPLETED BY: Click here to enter text.

DATE: Click here to enter text.

BSL-2 AGENT: Click here to enter text.

HAZARDS/PRECAUTIONS: Click here to enter text.

15. LAB-SPECIFIC STANDARD OPERATING PROCEDURES (S0P)

INSTRUCTIONS: Please add any lab specific SOP's that relate to any procedures not already covered in this manual. Especially any procedures taking place outside the biological safety cabinet.

Specific Procedures:

1. Tomato root dip in *Salmonella enterica*
    a. A culture of *S. enterica* will be grown in **ESAREC Plant Pathology lab** via selective media
    b. The culture will be suspended in water to make a 10^7 CFU/ml liquid culture of 500 ml. *The concentration may vary but will not exceed 10^7.
    c. The liquid culture of 500 ml will be transported to the **ESAREC Plant Growth Room C** in a sealed flask carried in a double barrier container
    d. Tomato plants will be grown in the **ESAREC Plant Growth Room C** until roots reach 3-5cm in length. At this time the plants will be inoculated.
    e. The plant roots will be dipped in the *S. enterica* liquid culture that was brought from ESAREC Plant Pathology lab. The roots will remain suspended in the bacteria culture for 5 minutes. This procedure will be performed under a static containment hood which will limit the area of potential splatter/droplet production. During this procedure lab coat, gloves, goggles, and a N-100 respirator will be worn. This PPE will also be worn in ESAREC Plant Pathology lab when working with *Salmonella*.
    f. Once plants have been laced with *Salmonella*, lab coat, gloves, and a N-100 respirator will be worn when handling the plants. Goggles are not needed since they were used for splash protection.
    g. The exact same procedure will be followed when inoculating the tomato plants with the plant pathogen *Ralstonia solanacearum*, the causal agent of bacterial wilt. Because *R. solanacearum* is not a human pathogen, only gloves are needed for PPE during this procedure.

2. *Salmonella* Injection into plant stem
    a. Tomato plants will also be inoculated with *S. enterica* via liquid culture injection. This procedure will be done in the **ESAREC Plant Growth Room C**. Proper PPE, lab coat, N-100 respirator, goggles, and gloves, will be worn during this procedure.
    b. The liquid culture of *S. enterica* will be grown in **ESAREC Plant Pathology lab** as done for the root dip procedure. A concentration no higher than 10^7 CFU/ml will be used.
    c. The plants will be injected in the stem with the bacterial culture at a pre-determined growth stage. The plants will be held with forceps in one hand and injected with the culture with a syringe in the other hand. This will reduce the chance of sticking your hand with the needle containing the Salmonella culture. This procedure will be performed under a static containment hood which will limit the area of potential splatter/droplet production.

3. Plant growth, *Salmonella* detection:
    a. All tomato plants (*Salmonella*-infected, *Ralstonia*-infected, and healthy controls) will be grown in the **ESAREC Plant Growth Room C**
    b. Plants will be transported to **ESAREC Plant Pathology lab** for *Salmonella* detection/quantification at pre-determined stages in plant growth development

c. The plants will be divided into sections: roots, stems, leaves, fruit. Each section will be tested for the presence of *Salmonella* via PCR methods. The plants will be divided in the **ESAREC Plant Growth Room C** and transported to **ESAREC Plant Pathology lab** for *Salmonella* detection. The sections will be stored in biohazard autoclave bags then put into a double barrier container for transportation. All necessary PPE will be worn during these procedures (lab coat, gloves, and a N-100 respirator).

16. GENERAL STANDARD OPERATING PROCEDURES (S0P)

INSTRUCTIONS: Please add any building, area or department operating procedures that you will be following. (e.g. biowaste decontamination and disposal, Biological safety cabinet use, using the biohazard spill kit, etc.)

17. APPENDICES

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