

EFFECTS OF PLANT MATURITY AND BACTERIAL INOCULUM LEVEL ON THE
SURFACE CONTAMINATION AND INTERNALIZATION OF *ESCHERICHIA COLI*
O157:H7 IN GROWING SPINACH

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ABSTRACT

The incidence of foodborne outbreaks linked to fresh produce has increased in the United States. Particularly noteworthy was the 2006 *Escherichia coli* O157:H7 outbreak associated with pre-packaged baby spinach. Factors affecting the contamination of spinach leaves with *E. coli* O157:H7 are not yet well understood. This study aimed to determine whether *E. coli* O157:H7 would be present in the aerial leaf tissue of a growing spinach plant when introduced at various plant maturities and different inoculum levels in the growth media in a greenhouse setting. Spinach seeds of a standard commercial variety were sown individually in 8-inch pots, watered daily and fertilized weekly after germination. Two levels (10^3 and 10^7 CFU) of an *E. coli* O157:H7 green fluorescent protein (GFP)-expressing strain were introduced into the plant growth media on a weekly basis after germination. Inoculated spinach plants were examined weekly for the presence of *E. coli* O157:H7 on leaves and in surrounding growth media. Among 120 spinach plant samples examined for internal leaf contamination, only one yielded positive result. Surface leaf contamination occurred occasionally and clustered between 4 to 5 weeks of age, but not among leaves younger than 3 weeks of age. Additionally, when inoculated at 10^7 CFU level, the *E. coli* O157:H7 GFP strain survived the entire cultivation period although with gradually reduced levels. The experiments demonstrated that internalization of *E. coli* O157:H7 of growing spinach plant leaves under greenhouse conditions was a rare event, but surface contamination did occur, primarily when the plants reached 3 weeks of age. The study provided important data to further assess the association between spinach age and potential contamination of *E. coli* O157:H7.

CHAPTER 1

INTRODUCTION

In the past few years, outbreaks of human illness associated with the consumption of fresh and fresh-cut fruits and vegetables have rapidly increased in the United States (Doyle and Erickson, 2008). Numerous fresh produce items, including cantaloupe, herbs, lettuce, tomatoes, sprouts, and spinach, have been implicated in foodborne outbreaks. Lettuce/leafy greens and tomatoes are the top two produce categories, accounting for 30% and 17% of all produce-associated outbreaks, respectively (Buchanan, 2006). *Escherichia coli* O157:H7 and *Salmonella* are the two leading foodborne pathogens implicated in these produce outbreaks. Data from the U.S. Food and Drug Administration (FDA) showed that between 1995 and 2005, 19 fresh or fresh-cut lettuce and spinach-associated *E. coli* O157:H7 outbreaks occurred, resulting in 409 reported illnesses and two deaths (USFDA, 2005). Particularly noteworthy was a prebagged spinach outbreak caused by *E. coli* O157:H7 in August and September 2006 in multiple states (26 U.S. states and one Canadian province), causing 205 confirmed illness (including 31 cases of HUS, 103 hospitalizations, and 3 deaths), as well as an estimated \$37-74 million loss to the California produce industry (Kotewicz et al., 2008; USFDA, 2007).

Reduction of human illness risks associated with raw produce consumption can be better achieved through controlling points of potential contaminations in the field, during harvesting, during processing or distribution, in retail markets and food-service facilities, or at home. In the agricultural field, the growth soil, irrigation water, workers, and unclean utensils are the most likely source of contamination with foodborne pathogens (Natvig et al., 2002). A

previous study (Islam et al., 2004) has shown that *E. coli* O157:H7 could survive long period of time (150 to 200 days) in the soil, which is of special concern to produce safety. Once harvest, fresh produce undergoes minimum processing (no lethal kill step) which mainly cleaned and sanitized using chlorine or alternative sanitizers (Dong et al., 2003). One of the recurrent questions that emerge from high frequency of recent outbreaks is how *E. coli* O157:H7 could survive under harsh environmental conditions in the field or after commercial sanitizer treatments. Several studies indicated that post-harvest sanitizer wash was intended to reduce microorganisms on the produce surface but will not be effective if microbes are inside the tissues (Erickson and Ortega, 2006; Takeuchi and Frank, 2000). Moreover, some microbes could survive by biofilm formation or cellulose production (Solomon et al., 2005), which could not be effectively washed off by post-harvest sanitizers. Also, whether foodborne pathogens could internalize into the tissue of fresh produce has been a largely debated topic. It is therefore very important to investigate the interaction between these foodborne pathogens and produce products.

Multiple studies have shown that internalization of *E. coli* O157:H7 in growing lettuce or spinach occurred (Cooley et al., 2003; Franz et al., 2007; Hora et al., 2005; Jablasone et al., 2005; Solomon et al., 2002b; Warriner et al., 2003a) while others showed no internalization (Johannessen et al., 2005). Plant roots (rhizosphere) appeared to be the preferable site for attachment and entrance, and the contamination was reported to be dose-dependent (Wachtel et al., 2002a). Past research suggests that foodborne pathogens can enter lettuce plants through roots and end up in the edible leaves (Solomon et al., 2002b; Warriner et al., 2003a; Warriner et al., 2003b; Warriner et al., 2003c).

However, it remains controversial whether *E. coli* O157:H7 is capable of contaminating the edible part of a mature plant (phyllosphere) when introduced through soil or irrigation water. In spinach, internalization was observed in the root tissue or seedlings but not in mature leaves (Hora et al., 2005; Warriner et al., 2003a). Moreover, in most studies examining the attachment and internalization of enteric pathogens in plant tissues, inoculations were done at the seed or seedling stage only. Since contamination events may occur at any time throughout the cultivation period in the field, the effect of plants encountering *E. coli* O157:H7 at later growth stages on the contamination of mature plants needs to be established. Furthermore, most experiments were conducted under selected simplistic conditions instead of the complex natural systems found in the farms (Doyle and Erickson, 2008). Therefore, the mechanism of natural fresh leafy greens being internalized with *E. coli* O157:H7 is still uncertain and needs to be examined following conditions that closely mimic the agricultural field growth conditions.

Our objective in this study is to better understand whether *E. coli* O157:H7 would be present in the aerial leaf tissue of a spinach plant when introduced via soil inoculation at different growth stages under an experimental conditions that closely mimic the field conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology of Produce-Linked *E. coli* O157:H7 Outbreaks

In the past few years, outbreaks of human illness associated with the consumption of fresh produce have increased rapidly in the United States (Doyle and Erickson, 2008). According to the Centers for Disease Control and Prevention, the proportion of foodborne outbreak-associated illness due to the consumption of fresh produce jumped from 1% in the 1970s to 12% in the 1990s (Sivapalasingam et al., 2004). Between 1996 and 2006, seventy-two produce-related outbreaks were documented, resulting in over 8,500 reported illnesses and several deaths (Buchanan, 2006; USFDA, 2008).

This increase could be partly attributed to better outbreak surveillance systems implemented in the U.S. such as the national surveillance program termed the Foodborne Disease Active Surveillance Network (FoodNet) (Niemira, 2007). FoodNet conducts surveillance and monitors trends for major foodborne pathogens (e.g., *E. coli* O157:H7, *Salmonella*, *Campylobacter*, *Vibrio* and *Listeria monocytogene*) in more than ten states in the U.S. Some other reasons for the increase number are possibly due to the changes in demographic, harvesting, distribution, processing, and consumption patterns (Beuchat and Ryu, 1997).

Numerous fresh produce items, including cantaloupe, herbs, lettuce, potatoes, tomatoes, sprouts and spinach, have been linked to high-risk foodborne pathogens, primarily *Salmonella*, *Escherichia coli* O157:H7, *Shigella*, and *Listeria monocytogenes* (Doyle and Erickson, 2008; Sewell and Farber, 2001). Among all produce categories, lettuce/leafy greens

and tomatoes are the top two, accounting for 30% and 17% of all produce-associated outbreaks, respectively (Buchanan, 2006). *E. coli* O157:H7 and *Salmonella* are the two leading pathogen associated with such outbreaks (Sivapalasingam et al., 2004).

Recently, Centers for Disease Control and Prevention (CDC) reported that outbreaks associated with *E. coli* O157:H7 have increased dramatically, especially the produce-linked outbreaks (CDC, 2006). Data from Figure 1 were modified from CDC annual reports of *E. coli* O157:H7 illnesses, with both total cases and cases associated with produce. From 2001 to 2005, the *E. coli* O157:H7 illnesses related to produce remained less than 35% of all *E. coli* O157:H7 cases. However, in 2006, the percentage jumped to 78.5% (402/512).

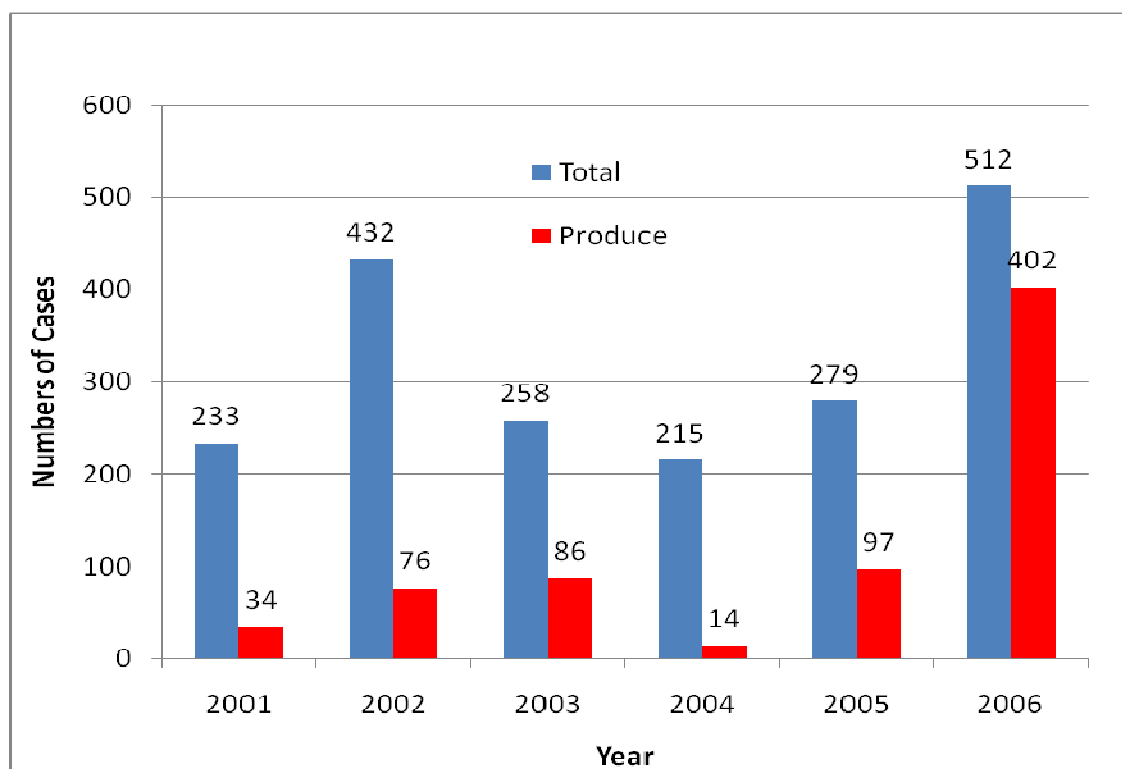


Figure 1. *E. coli* O157:H7 illnesses from 2001 to 2006 (CDC Annual Report)

Particularly, there were several outbreaks occurred during 2006 linked to fresh spinach or lettuce. From August to September, a prebagged spinach-associated outbreak of *E. coli* O157:H7 occurred through multistate area in the United States (Kotewicz et al., 2008),

causing 205 illnesses (including 31 cases of HUS; 103 hospitalizations; and 3 deaths) (CDC, 2006). Another two *E. coli* O157:H7 outbreaks were associated with lettuce during November and December, causing 152 illnesses, including 10 cases of HUS, and 79 hospitalizations (CDC, 2006). Therefore, produce safety presents an important public health concern, and more research is needed.

2.2 General Information on *E. coli* O157:H7

2.2.1 Microbiology

Escherichia coli is the most thoroughly studied species in the microbial world, which has gained much more information and benefited many areas of biological sciences (Fratamico et al., 2005). *E. coli* is a Gram-negative, non-spore-forming, rod-shaped bacterium, belonging to the family Enterobacteriaceae. It is facultatively anaerobic, with the optimal growth temperature of 37°C. *E. coli* was first considered as a low virulence potential microorganism in 1980's until many strains of *E. coli* acted as pathogens causing serious gastrointestinal diseases and even death in humans (Park et al., 1999). Pathogenic *E. coli* strains which have potential to cause diarrhea in human are termed diarrheagenic *E. coli*, which can be categorized into six major groups based on virulence properties, mechanisms of pathogenicity, and clinical syndromes. They are enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC) (Fratamico et al., 2005).

Among these diarrheagenic *E. coli* groups, EHEC is the most significant group based on the severity of illness (Doyle et al., 2001), particularly the commonly known *E. coli* O157:H7 (O for somatic antigen and H for flagella), which can cause serious bloody diarrhea and

hemolytic uremic syndrome (Boyce et al., 1995). In 1982, *E. coli* O157:H7 was first identified as a cause of two outbreaks of severe bloody diarrhea associated with the consumption of undercooked hamburgers in fast food restaurants (Riley et al., 1983).

2.2.2 Epidemiology

The first *E. coli* O157:H7 outbreak associated with eating hamburgers at restaurants in Oregon and Michigan was identified by the U.S. CDC in 1982 (CDC, 1982). The incidence began with sudden and severe abdominal cramps, followed by watery diarrhea within 24 hours. All four patients in this outbreak recovered within one week without special treatment (CDC, 1982).

After the first outbreak in 1982, cases of *E. coli* O157:H7 infections have been increasingly reported worldwide. In the United Kingdom, laboratory-confirmed *E. coli* O157:H7 infections increased from 1 in 1982 to 1039 in 1995 (Park et al., 1999). A report in North Ireland showed that the number of infections with *E. coli* O157:H7 rose from a few cases in the early 1990s to 54 reported in 1999 (Watabe et al., 2008). The largest *E. coli* O157:H7 outbreak occurred from November to December 1996 in central Scotland, resulting in 496 infections with 20 deaths (Cowden et al., 2001). After this outbreak, more efforts from the U.K. government were made to control and prevent such infections by increasing the funding for a wide variety of *E. coli* O157:H7 research on public health measures (Park et al., 1999).

In Sakai City, Japan, a massive outbreak of *E. coli* O157:H7 infection occurred among school children in 1996, which was associated with the consumption of uncooked white radish sprouts from a single farm (Michino et al., 1999). This is the largest outbreak ever in

Japan which affected about 6,000 people, mostly schoolchildren in 47 different elementary schools.

There is less information available on *E. coli* O157:H7 infection in most of the African countries, possibly due to the lack of surveillance systems for *E. coli* O157 cases in many countries. However, a study done in Egypt showed that *E. coli* O157:H7 was widely present in raw ground beef, chicken, lamb and unpasteurized milk and along the product processing chain, including slaughterhouses, supermarkets and farmer's homes (Abdul-Raouf et al., 1996).

In Australia, few outbreaks reported are related to O157:H7 strains, while non-O157 Shiga toxin-producing *Escherichia coli* (STEC) strains are more common and more frequently cause serious human disease (Goldwater and Bettelheim, 1995). Australian National Notifiable Diseases Surveillance System announced that only a small case number of 85 were reported as STEC infection in 2005, compared to 7,720 cases of salmonellosis and 15,313 cases of campylobacteriosis (Hall et al., 2008). The surveillance of STEC in all Australian States and Territories has low rates for STEC, except for South Australia where the rates are slightly higher (Combs et al., 2005).

In the U.S., after the first outbreak in 1982, more outbreaks were reported and accompanied with tighter regulations from the U.S. Department of Agriculture and better reporting systems. From CDC annual reports (Figure 1), we clearly observed that the numbers of *E. coli* O157:H7 outbreaks had increased and ranged from 215 to 512, between 2001 and 2006. Particularly, as mentioned above, there were three big *E. coli* O157:H7 outbreaks related to fresh lettuce and spinach in 2006 (USFDA, 2007).

In summary, *E. coli* O157:H7 is a common foodborne pathogen all over the world. With high outbreaks numbers, this pathogen is becoming a wide concern for food safety.

2.2.3 Clinical Symptoms

E. coli O157:H7 infection often leads to a mild non-bloody diarrhea or a severe, acute bloody diarrhea termed hemorrhagic colitis, and abdominal cramps. Also, it can be asymptomatic (Watabe et al., 2008). In some people, primarily infants, children, and the elderly, *E. coli* O157:H7 infections can cause hemolytic uremic syndrome (HUS), in which the red blood cells are destroyed and the kidney fails (Fratamico et al., 2005). In the U. S., infections among 2% to 7% of patients can lead to such a complication (Mead and Griffin, 1998), which is the principal cause of acute kidney failure in children. Most HUS cases occurred in the U.S. are caused by *E. coli* O157:H7 (Mead and Griffin, 1998).

Most people infected by *E. coli* O157:H7 can recover from hemorrhagic colitis without using antibiotics in 5-10 days. Less severe illness is seen in patients with non-bloody diarrhea, and they are less likely to develop systemic sequelae or die. However, HUS is always a severe, life-threatening condition, which needs intensive care. Blood transfusions and kidney dialysis are often required in roughly 50% of HUS patients (Ammon, 1997; Scheiring et al., 2008). With intensive care, approximately 3% to 5% die and about 5% develop chronic renal failure, stroke, and other major sequelae. Treatments with some antibiotics may precipitate kidney complications without improving the course of disease (Mead and Griffin, 1998; Skerka et al., 2009). Therefore, antibiotic treatment is generally not recommended for *E. coli* O157:H7 infections.

2.2.4 Shiga Toxins Produced by *E. coli* O157:H7

Usually, the most important factors in the pathogenesis of EHEC is considered as the ability to produce one or more types of Shiga toxins (Mead and Griffin, 1998). Shiga toxins (Stxs) are a family of bacterial cytotoxins produced by *Shigella dysenteriae* type 1 and Shiga toxin-producing *E. coli* (STEC) (Park et al., 1999). It also can be called vero-cytotoxins, and formerly known as Shiga-like toxins. The most common Shiga toxins produced by *E. coli* O157:H7 are Stx1 and Stx2 (Mead and Griffin, 1998).

Shiga toxins (Stx) produced by EHEC strains play a key role in the pathogenesis of hemorrhagic colitis and HUS (Robins-Browne et al., 2004). The binding of Shiga toxins to specific receptors on endothelial cells results in damage and death of the cells. There are clear evidences that platelets and fibrin can lead to abnormal white blood cell adhesion, reduced blood flow in small vessels of the affected organs, increased coagulation, and thrombosis formation (O'Loughlin and Robins-Browne, 2001). Therefore, Shiga toxins are probably critical to the development of bloody diarrhea due to its both local and systemic effects on the intestine (Mead and Griffin, 1998).

2.2.5 Transmission

It is known that *E. coli* O157:H7 can be present in animals as well as humans, but the most common reservoir of *E. coli* O157:H7 is cattle (Armstrong et al., 1996). EHEC existed in the bodies of cattle do not normally cause disease, but could transmit to humans by the consumption of undercooked beef products, because after cattle are slaughtered, the pathogens would remain in the cattle bodies and come into the food chain. In addition, it is also commonly agreed that the feces on the cattle farms are highly contaminated with *E. coli*

O157:H7. The fecal shedding of *E. coli* O157:H7 can stay even longer in calves than in adults (Brown et al., 1997). The Canadian group's report also confirmed that feces plays an important role in the cattle-to-human transmission, since *E. coli* O157:H7 were frequently isolated from the feces of healthy cattle (Rahn et al., 1997). Moreover, direct contact with cattle or their environment could be considered as another transmission vehicle. The report of the prevalence of *E. coli* O157:H7 isolates from Minnesota dairy farms and county fairs in 2006 demonstrated that the connection between the presence of *E. coli* O157:H7 at county fairs and the potential for transmission to the public (Cho et al., 2006).

Waterborne outbreaks of *E. coli* O157:H7 were reported as the source of transmission vehicle (Swerdlow et al., 1992). Drinking water (Akashi et al., 1994) and unchlorinated swimming water (Keene et al., 1994) contaminated with *E. coli* O157:H7 were reported to cause severe outbreaks of hemorrhagic colitis and HUS infections in 1994. In July 2001, twenty cases occurred in Minnesota among people who had visited a beach, and were finally confirmed as *E. coli* O157:H7 infections because of the high fecal coliform levels in the lake water (Yoder et al., 2004). It somehow suggests that *E. coli* O157:H7 has a high infectious potential for transmission through a water environment.

Another source of transmission vehicle is humans. Public facilities could become the place for person-to-person transmission, combining a high potential for transmission with a population at increased risk for severe outcomes (Belongia et al., 1993). The infected people in the Minnesota waterborne incident subsequently attended child care centers and caused secondary outbreaks (Yoder et al., 2004).

Additionally, produce including fruits and fresh vegetables were frequently involved in the *E. coli* O157:H7 outbreaks. Recently, the foodborne outbreaks associated with the

consumption of fresh produce have increased, linked to the food production chain “farm to fork” with the potential microbial pathogen contamination (Wachtel et al., 2002b). Sources of pre-harvest contamination include feces, soil, irrigation water, improperly composted manure, air, wild and domestic animals, and human handling. The source of *E. coli* O157:H7 outbreak associated with spinach in 2006 were demonstrated to link with feral pigs near spinach fields (Cooley, Carychao et al. 2007; Jay, Cooley et al. 2007).

2.2.6 Detection Methods for *E. coli* O157:H7

Generally speaking, the isolation and detection of *E. coli* O157:H7 methods can be divided into several categories: culture-based detection methods, immunochemical methods, and nucleic acid-based methods.

Due to not fermenting sorbitol by most *E. coli* O157:H7, Sorbitol-MacConkey agar (SMAC) is usually used as the selective media for O157:H7 serotypes. The nutritional requirements for most *E. coli* strains are similar, including O157:H7 serotype, but O157:H7 serotype, does not ferment sorbitol within 24 hours, resulting in the colorless colonies on SMAC. The use of SMAC offers a simple, inexpensive, and generally reliable method of screening stools for *E. coli* O157:H7 (Chapman et al., 1991). Regarding the deficiencies of SMAC, regular plating media SMAC agar has been modified with some supplements, to increase the selectivity and ability to differentiate *E. coli* O157:H7 from other microorganisms (Fratamico et al., 2005).

Immunochemical methods, which usually give high selectivity, speed, and simplicity, have been widely used for pathogen screening and detection. Many assays can use the antibodies to O-antigens of the O157 serotype as the antigen in immunoassays. However, these assays may produce false-positive results due to the antigen structures (Bennett et al.,

1996). The commercial enzyme-linked immunosorbent assay (ELISA) was developed to detect *E. coli* O157:H7 and decreased the false-positive rate (Chapman et al., 1997). Later, an immunomagnetic separation (IMS) was developed for *E. coli* O157:H7 detection.

The most widely used nucleic acid-based method for the detection of *E. coli* O157:H7 is polymerase chain reaction (PCR) assays, targeting on *stx* genes or the *eae* gene (Gannon et al., 1993; Meng et al., 1998; Oberst et al., 1998). Both two genes are used for detection and characterization of the Shiga Toxins produced by *E. coli* O157:H7. The *stx* gene based PCR was widely developed and different primers were used. The specificity of the *eaeA*-based 5' nuclease assay system developed by Oberst's group could be sufficient to correctly identify all *E. coli* O157:H7 strains evaluated, mirroring the previously described specificity of the PCR primers (Oberst et al., 1998). However, PCR-based methods targeting the two genes have their own limitations. The *stx* gene-specific PCR methods are for Shiga toxin-producing *E. coli* but not specific for *E. coli* O157:H7 serotype. On the other hand, the *eae* gene-specific methods are not specific for *E. coli* O157:H7 only; certain EPEC strains also contain *eae* gene. More recently, primers were developed, targeting some other specific genes for O157:H7 serotype.

Recently, as the risk of spreading outbreaks with the globalization of trade and increased cross-continental flow, more rapid, sensitive and reliable methods for screening of different foodborne pathogens, especially for these high-risk organisms are stringently needed (Mukhopadhyay and Mukhopadhyay, 2007).

2.2.7 Green Fluorescent Protein (GFP)-Expressing *E. coli* O157:H7 Strain

In the past 15 years, Green fluorescent protein (GFP) has been widely used as a novel marker system (Chalfie et al., 1994) in biochemistry and cell biology, particularly in food and

environmental studies (Simpson-Stroot et al., 2008). Fluorescent protein markers are both intrinsically fascinating and tremendously valuable because of the capacity to generate a highly visible, efficiently emitting internal fluorophore (Tsien, 1998). GFP is usually introduced into some microorganisms as a stable and easily identifiable tracer label (Errampalli et al., 1999).

GFP, the green fluorescent protein, was originally discovered from the jellyfish *Aequorea Victoria* (Tsien, 1998). In jellyfish, energy was transferred from a Ca⁺⁺-activated photoprotein aequorin, to the GFP, and green fluorescent light is emitted (Chalfie et al., 1994; Cody et al., 1993). The *gfp* gene could be cloned and expressed in both eukaryotic and prokaryotic system (Chalfie et al., 1994), encoding a 27-kDa green fluorescent protein which can absorb UV and blue light at 395 nm and emits green light at 509 nm (Cody et al., 1993). GFP is stable to heat (65°C), alkaline pH (6-12), and the presence of detergents and many proteases (Prasher et al., 1992), and it is independent of other protein, substrates, or cofactors (Chalfie et al., 1994; Kremer et al., 1995; Valdivia et al., 1996). Therefore, it appears to be a valuable reporter and marker system. Furthermore, some mutated GFP proteins have been produced to give much stronger fluorescence, so it gives more sensitivity when detecting microorganisms (Errampalli et al., 1999).

Studies have been validated that the use of GFP in *E. coli* O157:H7 have no effect on the organism behavior (Viallette et al., 2004). The specific GFP strain *E. coli* O157:H7 B6-914 was successfully constructed and characterized in 1997 (Fratamico et al., 1997). The green fluorescent protein was constructed by transforming the plasmid pGFP into the *E. coli* O157:H7 B6-914 strain. Because of the safety advantages, Shiga toxin Type 1 (Stx1) and 2

(Stx2) were no longer produced by this strain. Since the toxin genes Stx1 and Stx2 had little or no influence on the growth characteristics of *E. coli* O157:H7 cells (Kudva et al., 1998), this strain can be used for laboratory experiment. Another property of this strain is the resistance to antibiotic ampicillin, so it can be distinguished from regular *E. coli* O157:H7 strains by using selective medium supplemented with ampicillin (Franz et al., 2007). Moreover, as the plasmid was stable in this GFP strain (Fratamico et al., 1997), the loss of plasmid under greenhouse environment could be not a big concern.

2.3 Spinach Horticultural Information

Spinach (*Spinacia oleracea*), originally from central and southwestern Asia, is a flowering plant in the family of Amaranthaceae used as a vegetable. It is an annual herb, grown in fall to spring in temperate region as it is a cool-season plant (Bailey, 1917). It can grow up to 30 cm in height, and it runs to seed in warm weather (Robbins, 1917). The leaves may be large enough for eating within eight weeks after seedling (Bailey, 1917). During the developmental process, spinach throws out a number of large leaves crowded near the ground surface early in the season, and later a flower stalk is sent up to a distance of 2 or 3 feet. The mature leaves are large, alternate, petioled, and triangular-ovate or arrow-shaped in outline, variable in size from 2-30 cm long and 1-15 cm broad. The flowers occur in axillary clusters, appearing as inconspicuous, yellow-green, 3-4 mm diameter. During maturation, it will turn into a small hard dry lumpy fruit cluster 5-10 mm across containing several seeds (Robbins, 1917). Farmers annually sow the seeds from early spring to late summer every other week to provide a steady supply.

Spinach is commercially sold loose, bunched, in prepackaged bags, canned, or frozen. Fresh spinach other than processed spinach products, can keep more nutritional value such as

folate and carotenoid content, and subsequently pre-bagged fresh spinach is the most common product in most grocery stores. Although uncooked fresh spinach is recommended for its nutrition, canning, refrigeration or freezing are still commonly used in order to prolong the storage time, up to eight months (Robbins, 1917).

Driven by fresh-market use, the consumption of spinach has been on the rise in the United States. The fresh market now accounts for about three-fourths of all the U.S. spinach consumed (Charatan, 2006). Much of the growth over the past decade has been due to sales of triple-washed cello-packed spinach and, more recently, baby spinach.

2.4 Current Knowledge on the Interaction between *E. coli* O157:H7 and Fresh Produce

2.4.1 Contamination Sources in the Field

Fresh produce could be contaminated with human pathogens like *E. coli* O157:H7 at any point through the entire farm-to-table continuum. The most common contamination sources in the environment are the feces of healthy cattle and other farm or wild animals, as *E. coli* O157:H7 can survive in such condition over extended periods of time (Islam et al., 2004; Natvig et al., 2002).

For leafy greens like spinach grown in field, the primary sources of pre-harvest contamination of potential foodborne pathogens in the produce growth environment include soil amended with untreated or improperly composted manure, contaminated irrigation water, presence of wild and domestic animals, infected workers, and unclean containers and tools used in harvesting (Beuchat and Ryu, 1997; USFDA, 2008). Studies demonstrated that cattle feces are commonly considered as the major source of *E. coli* O157:H7 in dairy farms (Rahn et al., 1997; Wells et al., 1991). Also, the *E. coli* O157:H7 sources of spinach nationwide

outbreak in 2006 were traced to feces of feral swine (Jay et al., 2007). Through the irrigation and fertilization in contamination of agricultural fields and surface waterways, *E. coli* O157:H7 could be inoculated into produce growth environment (Jay et al., 2007). Solomon and colleagues (Solomon et al., 2002a) discovered that both spray and drip irrigation could possibly be responsible for the transmission of *E. coli* O157:H7 to lettuce. In the follow-up experiment on lettuce by the same group, the transmission of *E. coli* O157:H7 from manure-contaminated soil and irrigation water to lettuce plants were demonstrated (Solomon et al., 2002b).

2.4.2 Post-Harvest Treatment Methods and Their Effectiveness

After harvested, fresh produce undergoes minimum process (no lethal kill step), mainly cleaning and sanitizing using chlorine or alternative sanitizers (Doyle and Erickson, 2008; Sapers, 2005).

However, treatment of produce with chlorinated water reduces populations of pathogenic and other microorganisms on fresh produce but cannot eliminate them. At concentrations used in the produce industry (50-200 ppm), a typical commercial chlorine wash only results in 1-2 log CFU/g reduction of bacterial pathogens (Delaquis et al., 2002; Li et al., 2001; Takeuchi and Frank, 2000), although in the laboratory one study (Rodgers et al., 2004) reported approximately 5 log CFU/g reduction with inoculated produce. One report demonstrated that commercial washing processes for cantaloupe were limited in their ability to inactivate or remove the *E. coli* O157:H7, and it is suggested to refrigerate the products as soon as possible following harvest in case of any possible contamination (Annous et al., 2004). In 2003, Warriner and colleagues reported that the bioluminescent *E. coli* strains

inoculated on seeds can become internalized within the germinating mung bean sprouts, and cannot be removed by postharvest biocidal washing (Warriner et al., 2003c).

Niemira's group compared the method of sodium hypochlorite wash with irradiation to inactivate *E. coli* O157:H7 internalized in leaves of romaine lettuce and baby spinach (Niemira 2007). Their results showed radiation was more effective than chemical sanitizers against the *E. coli* O157:H7 cells internalized in leafy green vegetables, but with different responses on spinach and lettuce leaves. The follow-up experiment conducted by the same group (Nemecek et al., 2008) showed that the irradiation could effectively reduce viable *E. coli* O157:H7 cells internalized in lettuce, but with different responses due to the variety of lettuce. However, irradiation has not been commonly used for commercial post-harvest fresh produce yet.

Limitations in the effectiveness of chemical sanitizers are due partly to the formation of microbial biofilms and the physical structure of the plants that limit the accessibility of sanitizers to the sites where microorganisms are resided (Annous et al., 2005; Doyle and Erickson, 2008).

2.4.3 Internalization and Contamination Studies

Post-harvest sanitizer wash is intended to reduce microorganisms on the produce surface but will not be effective if microbes are inside the tissues (Doyle and Erickson, 2008). Multiple studies have shown that internalization of *E. coli* O157:H7 in growing lettuce or spinach occurred (Cooley et al., 2003; Franz et al., 2007; Hora et al., 2005; Jablasone et al., 2005; Solomon et al., 2002b; Warriner et al., 2003a) while others showed no internalization (Johannessen et al., 2005).

Plant roots (rhizosphere) appeared to be the preferable site for attachment and entrance, and the contamination was reported to be dose-dependent (Wachtel et al., 2002a). However, it remains controversial whether *E. coli* O157:H7 is capable of contaminating the edible part of a mature plant (phyllosphere) when introduced through soil or irrigation water. In spinach, internalization was observed in the root tissue or seedlings but not in mature leaves (Hora et al., 2005; Warriner et al., 2003a). Moreover, in most studies examining the attachment and internalization of enteric pathogens in plant tissues, inoculations were done at the seed or seedling stage only. However, since contamination events may occur at any stage throughout the cultivation period in the field, the effect of plants encountering *E. coli* O157:H7 at later growth stages on the contamination of mature plants needs to be established.

Some previous studies indicated that pathogens can be incorporated into fresh produce (Burnett et al., 2000; Seo and Frank, 1999; Zhuang et al., 1995). However, Multiple studies have shown that internalization of *E. coli* O157:H7 in growing lettuce or spinach occurred (Cooley et al., 2003; Franz et al., 2007; Hora et al., 2005; Jablasone et al., 2005; Solomon et al., 2002b; Warriner et al., 2003a) while others showed no internalization (Johannessen et al., 2005). In 2002, Guo and colleagues confirmed the possibility of uptake of *Salmonellae* by roots of hydroponically grown tomato plants (Guo et al., 2002). The Canadian group demonstrated that *E. coli* O157:H7 became established on the roots of growing plants while the risk associated with internalized bacteria in salad vegetables was low at harvest. A similar article published in 2005 reported that *E. coli* O157:H7 internalized in cress, lettuce, radish and spinach seedlings cannot be recovered within the tissues of mature plants (Jablasone et al., 2005).

However, it remains controversial whether *E. coli* O157:H7 is capable of contaminating the edible part of a mature plant (phyllosphere) when introduced through soil or irrigation water. In spinach, internalization was observed in the root tissue or seedlings but not in mature leaves (Hora et al., 2005; Warriner et al., 2003a). Moreover, in most studies examining the attachment and internalization of enteric pathogens in plant tissues, inoculations were done at the seed or seedling stage only. However, since contamination events may occur at any stage throughout the cultivation period in the field, the effect of plants encountering *E. coli* O157:H7 at later growth stages on the contamination of mature plants needs to be established.

Although these conclusions somehow imply that it is a low risk exists for internalized *E. coli* O157:H7 in leafy greens, these experiments were all under selected simplistic conditions instead of the complex natural systems found in the farms (Doyle and Erickson, 2008). Therefore, the mechanisms of natural fresh leafy greens being internalized with *E. coli* O157:H7 is still uncertain and needs to be examined following environment protocols mimicking the agricultural growth conditions.

To enhance produce safety, substantial research is needed to better understand factors that contribute to the contamination and internalization of pathogens in produce. The objective of this study was to determine the effects of plant maturity and bacterial inoculum level on the colonization and contamination of *E. coli* O157:H7 in the aerial leaf tissue of growing spinach plants in a greenhouse setting.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial Strain Characterization

In this study, the specific GFP strain *E. coli* O157:H7 B6-914 (Fratamico et al., 1997) was used for its characters of green fluorescence, lacking Shiga toxins 1 and 2, and carrying an ampicillin resistance marker. Once it is inoculated into the spinach growth media, the GFP-labeled strain could help to trace the contamination on the surface and internal leaves. For the safety reason in the greenhouse setting, Shiga toxins associated with regular *E. coli* O157:H7 cells could be avoided by this GFP strain. Moreover, with the ampicillin resistance marker, it was easier to differentiate inocula bacteria from other bacteria in the greenhouse environment by using a selective media with ampicilin supplement.

To better understand the characters of the specific GFP-labeled *E. coli* O157:H7 strain B6-914, the presence of Shiga toxins 1 and 2, and the ampicillin resistance marker needed to be confirmed before actual work on spinach plants. Also, overnight culture growth concentration was observed by the OD₆₀₀ so that the exact serial dilutions of bacteria concentrations could be made for the preparation of inoculum levels.

3.1.1 Bacterial Strains

Along with *E. coli* O157:H7 strain B6-914, two other enterohemorrhagic *E. coli* O157:H7 strains in our strain collection, CVM 97 (CDC strain G5244) and UMD 263 (CDC strain EDL 932 NaI^{R+}, ATCC 43894) were also tested as a comparison for producing Shiga toxins and ampicillin resistance marker and fluorescence under UV light. The strains were kindly provided by Dr. Jianghong Meng, at the Department of Nutrition and Food Science, University of Maryland.

3.1.2 Bacterial Growth Conditions and Morphology

All the three *E. coli* O157:H7 strains were streaked onto different growth media, including tryptic soy agar (TSA), TSA with 100 µg/ml of ampicillin, MacConkey (MAC) agar, and sorbitol MacConkey (SMAC) agar. After overnight incubation at 37°C, all the cultures on the agar plates were observed for growth, colony morphology (color, shape, and size), and green fluorescence activity under UV light. All media were obtained from Difco, Becton Dickinson, Sparks, MD. All chemicals obtained from Sigma-Aldrich, St. Louis, MO.

3.1.3 Detection of Shiga Toxin Genes

Three fresh pure bacterial strains were obtained from TSA agar plates. For each culture, one single colony was selected and suspended in a 1.5 ml centrifuge tube containing 500 µl of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The bacterial suspension was then heated for 10 min at 95°C in a dry heating block to make DNA template and stored at -20°C.

Two PCR assays were performed using the following primers: VT1-f: 5'-TGT AAC TGG AAA GGT GGA GTA TAC A-3' and VT1-r: 5'-GCT ATT CTG AGT CAA CGA AAA ATA AC-3' for *stx 1* and VT2-f: 5'-GTT TTT CTT CGG TAT CCT ATT CC-3' and VT2-r: 5'-GAT GCA TCT CTG GTC ATT GTA TTA C-3' for *stx 2* (Meng et al., 1998). The PCR product for *stx 1* was a 210 bp fragment whereas a 484 bp fragment for *stx 2*. The PCR mixture contained: 1.5 mM MgCl₂; 0.2 µM of each dNTP (dATP, dCTP, dGTP, dTTP); 5 µl of template; 0.5 µM of each primer, and 0.02 U of *Taq* DNA polymerase (Promega, San Luis Obispo, CA) in a total of 25 µl. The prepared master reagent mix was distributed to PCR microcentrifuge tube reaction vessels. DNA templates were finally added into each tube. The thermocycling program contained 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C in C100 Thermal Cycle PCR machine (Bio-Rad, Hercules, CA)

The PCR products were analyzed by running in 1.5 % agarose gel containing 1 µg/ml ethidium bromide submerged in $1 \times$ Tris-acetate-EDTA (TAE) buffer. Under a UV transilluminator, the two pairs of primers listed could give 210 bp and 484 bp bands relative to molecular weight marker migration.

3.1.4 Growth Concentration and OD₆₀₀ Observation

Aliquots of 1 ml of fresh *E. coli* O157:H7 B6-914 overnight culture from TSB broth were centrifuged at 9,000 *g* for 5 min, washed with 2 times of saline, and measured for OD₆₀₀ values in duplicate in a UV/Visible Spectrophotometer (Beckman Coulter DU 530, Fullerton, CA.). The same amount of sterile TSB broth was used as the zero bases. Serial dilutions of the overnight culture from 10^0 to 10^{-7} were made using the TSB broth (1 ml of culture into 9 ml of broth). Dilutions from 10^0 to 10^{-3} were measured similarly for OD₆₀₀ values. The exact cell concentration was quantified by using the standard spread-plating method with 0.1 ml of 10^{-5} , 10^{-6} , and 10^{-7} dilutions on TSA agar plates. After overnight incubation, a standard curve of OD₆₀₀ vs. Log CFU/ml was plotted out and used for the spinach inoculation experiment.

3.2 Spinach Plant Cultivation

3.2.1 Seeds Information

A standard commercial variety of spinach seeds, El Grinta (Rogers/Syngenta Seeds, Inc., Boise, ID) was used in this study. A simple germination rate was pretested in the laboratory. Around 50 seeds were rinsed by tap water and incubated at room temperature in a pertri-dish with 10 layers of moist paper towel.

3.2.2 Plant Growth Conditions

Spinach was cultivated in a greenhouse facility located on the Louisiana State University campus as approved by the Inter-Institutional Biological and Recombinant DNA Safety

Committee. The greenhouse had a 14-h photoperiod and air-conditioned control of day and night temperatures of 24°C and 18°C, respectively. The humidity range was kept between 50% and 85% throughout the cultivation period. Spinach seeds were chilled at 4°C for 3 days before randomly sowing in 8-inch pots filled with Sunshine Mix #1 (Sun Grow Horticulture, Bellevue, WA). The pots were randomly arranged on the greenhouse benches, with at least 10 cm distances in between to avoid cross-contamination (splashing) during watering. After sowing, the pots were watered daily for 2-5 days to allow for seed germination. The plants were continuously watered daily after germination and fertilized weekly using fish emulsion (5-2-5; Ferti-Lome, BWI Cos., Inc., Jackson, MS) throughout the cultivation period until the final harvest time on day 44. During watering, special attention was paid so that the water level did not exceed 5 cm above the growth media levels in the pots, to avoid vertical bacterial transfer from the growth media to spinach leaves.

3.3 Greenhouse Experiment

3.3.1 Inoculum Preparation

The GFP-labeled *E. coli* O157:H7 strain B6-914 was routinely cultured on TSA agar plate or in TSB broth containing 100 µg/ml of ampicillin at 37°C overnight with shaking. On the day of inoculation, fresh *E. coli* O157:H7 B6-914 culture was washed twice with sterile saline (0.85 % NaCl) and adjusted to the OD₆₀₀ of 0.05 (ca. 1×10^7 CFU/ml). Serial dilutions were made in saline for inoculation at two levels, 10^3 and 10^7 CFU/ml. The exact cell numbers were enumerated by standard plating methods.

3.3.2 Plant Inoculation

Inoculation regime and harvest scheme are detailed in Table 1. Starting on day 9 after sowing, the bacterial inoculums were introduced into the plant growth media on a weekly

basis for a total of five times, i.e., on days 9, 16, 23, 30, and 37. Each plant was inoculated only once. Inoculation was done through surface addition of 1 ml of bacteria at two concentrations (10^3 CFU/ml and 10^7 CFU/ml) into the plant growth media within 5 cm radius of the plant. The total number of plants maintained in the greenhouse consisted of five replicates for each inoculation level/harvest week combination and five control plants with no bacteria inoculated for each harvest week.

Table 1. Experimental variables for inoculation regime and harvest scheme

Variables	No.	Specific levels for each variable					
Inoculation time	5	Day 9	Day 16	Day 23	Day 30	Day 37	
Inoculation level	3	10^7 CFU/ml	10^3 CFU/ml	Blank control			
Harvest time	6	Day 11	Day 18	Day 25	Day 32	Day 39	Day 44
Microbial analysis	4	Total leaf	Surface leaf	Internal leaf	Growth media		

3.3.3 Plant Harvest

Following inoculation, spinach plant samples were collected on the third day and weekly thereafter until the final harvest on day 44 (Table 2). On each sample collection day, three healthiest plants out of the five replicates designated for the inoculation level/harvest week combination were randomly selected. Each plant was aseptically removed from the pot using scissors to cut the plant leaf tissues 5 cm above the ground to avoid growth media contamination. The leaf tissues from the same plant were divided equally in half and placed into two Ziploc bags. Additionally, approximately 20 g of growth media in the pot within 5 cm radius of the plant was collected with sterile gloves and placed into the third Ziploc bag. Collected spinach and growth media samples were sealed and transported to the laboratory on ice. The weight of each sample was recorded.

Table 2. Spinach plant inoculation with GFP-labeled *E. coli* O157:H7 strain B6-914 and sample harvest scheme

Harvest week (days after sowing)	No. of plants harvested										Control plants	Total
	Experimental plants inoculated once on different weeks at two bacterial inoculum levels (CFU)											
	1 st (day 9)		2 nd (day 16)		3 rd (day 23)		4 th (day 30)		5 th (day 37)			
	10 ³	10 ⁷	10 ³	10 ⁷	10 ³	10 ⁷	10 ³	10 ⁷	10 ³	10 ⁷		
1 st (day 11)	3	3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3	9
2 nd (day 18)	3	3	3	3	N/A	N/A	N/A	N/A	N/A	N/A	3	15
3 rd (day 25)	3	3	3	3	3	3	N/A	N/A	N/A	N/A	3	21
4 th (day 32)	3	3	3	3	3	3	3	3	N/A	N/A	3	27
5 th (day 39)	3	3	3	3	3	3	3	3	3	3	3	33
6 th (day 44)	3	3	3	3	3	3	3	3	3	3	3	33
Total	18	18	15	15	12	12	9	9	6	6	18	138

3.4 Microbiological Analysis

For microbiological analysis, the entire process was finished in a biosafety Level 2 laboratory. A flowchart (Figure 2) illustrates the detailed procedure. First, two leaf samples (Leaf 1 and 2) from the same plant and one growth media sample were obtained from each spinach pot. Leaf 1 sample was used for total *E. coli* O157:H7 enumeration, whereas Leaf 2 was used for examining the *E. coli* O157:H7 internalization. Additionally, rinse waters used for the internalized *E. coli* O157:H7 analyses, as described below, were enumerated for *E. coli* O157:H7.

The spinach leaf samples used for total *E. coli* O157:H7 counts (Leaf 1) and the growth media samples were processed similarly. After adding 100 ml of TSB, the samples were homogenized for 1 min in a food stomacher (model 400, Tekmar Company, Cincinnati, OH). Aliquots (100 μ l) of the homogenate and appropriate dilutions were spread-plated onto TSA supplemented with 100 μ g/ml ampicillin. After overnight incubation at 37°C, the numbers of colonies were counted and also observed under UV light (for green fluorescent colonies). For samples containing no visible colonies, enrichment was performed in TSB overnight which was followed by spread-plate counting.

Spinach leaf samples used for internalized *E. coli* O157:H7 counts (Leaf 2) were first rinsed with 100 ml of sterile distilled water, then submerged in 100 ml of 2% (w/v) calcium hypochlorite (Sigma-Aldrich) solution for 20 minutes to inactivate the residual surface microflora, which was followed by three rinses in 100 ml of sterile distilled water and one final rinse in 50 ml of sterile distilled water. After the final rinse, spinach leaf samples used for internal *E. coli* O157:H7 were enumerated similarly as described above for total *E. coli*

O157:H7 counts. Aliquots (100 μ l) of the first and final rinses were spread-plated on TSA with ampicillin to examine the counts or presence/absence of the *E. coli* O157:H7 GFP strain.

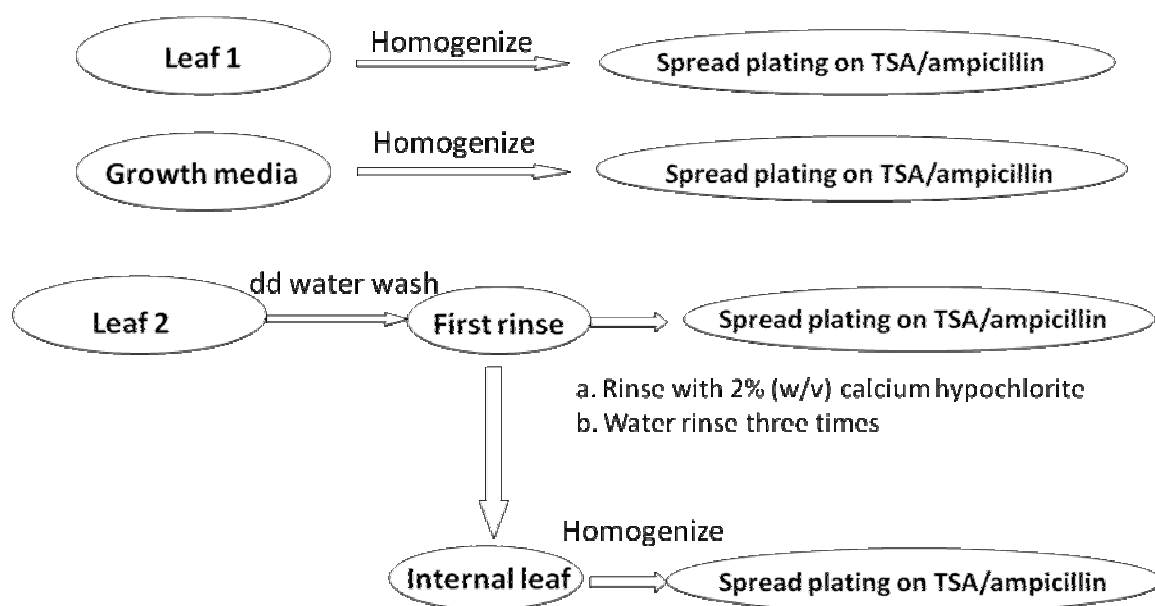


Figure 2. Microbiological analysis procedures for spinach leaves and growth media

*Leaf 1 & 2: evenly separated same leaf samples; Growth media: soil-like media for spinach growth in pot; TSA/ampicillin: tryptic soy agar with 100 μ g/ml of ampicillin supplement; dd water: double distillation water.

3.5 Statistical Analysis

The analysis of variance (ANOVA) followed by post-hoc multiple comparisons using the Least Significant Difference (LSD) test (SAS for Windows, v 9.1; SAS Institute Inc., Cary, NC) was performed to compare the contamination incidences of the *E. coli* O157:H7 GFP strain among spinach leaf samples harvested at different plant ages and at different time intervals between inoculation and harvest, as well as differences in the *E. coli* O157:H7 GFP strain counts (Log CFU/g) among growth media samples from plants inoculated at 10^7 CFU. Additionally, the independent Chi-square test was used to compare the contamination incidences of the spinach leaves for plants inoculated with two bacterial inoculums levels. Differences between the mean values were considered significant when $P < 0.05$.

CHAPTER 4

RESULTS

4.1 Bacterial Strain Characterization

4.1.1 Morphological Characteristics

The morphology of all the strains could be observed on either growth media or selective media. Differences in the morphology, as depicted by color, colony size and growth/ no growth on TSA, TSA with ampicillin, MacConkey agar, and Sorbitol MacConkey agar are illustrated in Table 3. On TSA with ampicillin, only B6-914 strain showing growth demonstrating that the B6-914 strain has an ampicillin resistant marker, which could be used to differentiate it from other *E. coli* O157:H7 strains by using a growth media with ampicillin supplement. Also, the results showed that only B6-914 strain gave the green fluorescence color under the UV light.

Table 3. Characteristics of *E. coli* O157:H7 strains used in the study

Strain	Growth, Color, and Morphology on Agar Plates				Fluorescence under UV light
	TSA	TSA with ampicillin	MacConkey agar	Sorbitol MacConkey agar	
B6-914	White, medium Size	Growth	Pink	Colorless	Green
UMD 263	White, medium Size	No Growth	Pink	Colorless	None
CVM 97	White, medium Size	No Growth	Pink	Colorless	None

4.1.2 The Absence of Shiga Toxins

B6-914 strain does not have the *stx* gene, including both *stx* 1 and *stx* 2. For the purpose of inoculation spinach with B6-914 for safety reason, the strain proposed in our project was tested by general PCR. Another two *E. coli* O157:H7 strains were also tested by PCR as the comparison. The results from Table 4 showed that the B6-914 strain in our stock did not have

either *stx* 1 or *stx* 2. As a result, this B6-914 without the *stx* genes, which can meet the requirement for greenhouse use, was utilized for *E. coli* O157:H7 inoculation on growing spinach plants.

Table 4. PCR results of *E. coli* O157:H7 strains used in the study

Strain	Shiga toxin types	
	Stx 1	Stx 2
B6914	Negative	Negative
UMD 66	Negative	Negative
UMD 263	Positive	Negative

4.1.3 OD₆₀₀ vs. Log CFU/ml Standard Curve

The result of a triplicate test for the OD₆₀₀ vs. Log CFU/ml standard curves is shown in Figure 3. Based on the figure, the relationship of *E. coli* O157:H7 B6-914 between OD₆₀₀ and Log CFU/ml in TSB broth could be converted into the formula: $\text{Log CFU/ml} = 0.45 \text{ Ln} (\text{OD}_{600}) + 9.3$. Therefore, the two inoculum levels (10^3 and 10^7 CFU/ml) broths could be adjusted to correlative concentrations by serial dilutions.

4.1.4 Summary of Strain Characteristics

From series of testing in the laboratorial experiment, the specific GFP-labeled *E. coli* O157:H7 strain B6-914 was confirmed for its characters of green fluorescence, lacking Shiga toxins 1 and 2, and carrying an ampicillin resistance marker. Therefore, once used as the inoculation strain into the spinach growing plants, the GFP-labeled strain could help to track the contamination on the surface, internal leaves, or growth media.

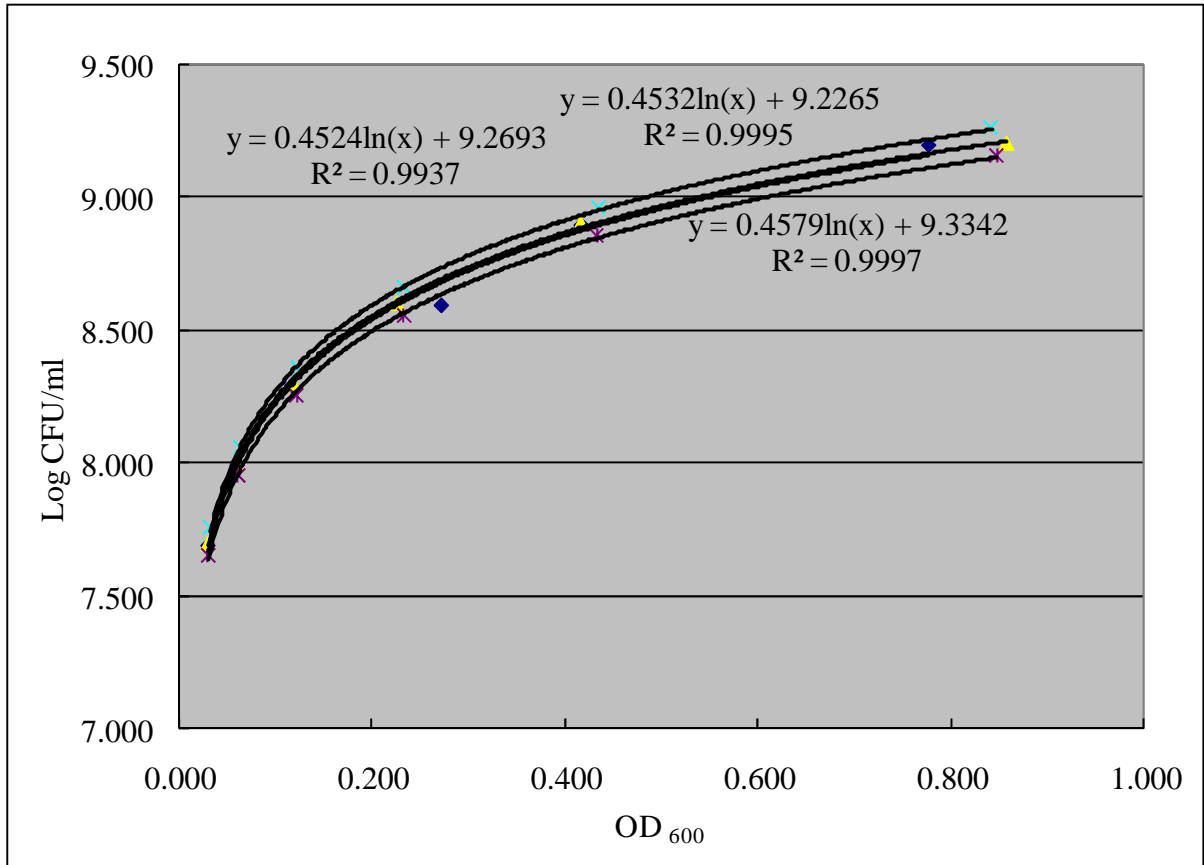


Figure 3. Standard curves of OD₆₀₀ vs. Log CFU/ml of *E. coli* O157:H7 B6-914 strain

*Three curves stand for a triplicate test for the standard curves. The final standard curve equation is $\text{Log CFU/ml} = 0.45 \text{ Ln} (\text{OD}_{600}) + 9.3$. The bacteria concentration 1×10^8 CFU/ml equals to 0.05 (OD₆₀₀).

4.2 Greenhouse Parameters during Cultivation Period

Although the greenhouse setting in LSU has an air-condition system, the temperature and humidity were monitored daily. The day and night (24-h period) temperature changes were recorded by the data logger and are showed in Figure 4. The daily temperatures ranged between 15°C and 30°C, with the highest average temperature 29.27°C at 3 pm, and lowest average temperature 17.44°C at 8 a.m. The average humidity was manually recorded and ranged from 50% to 85% during the entire experimental period.

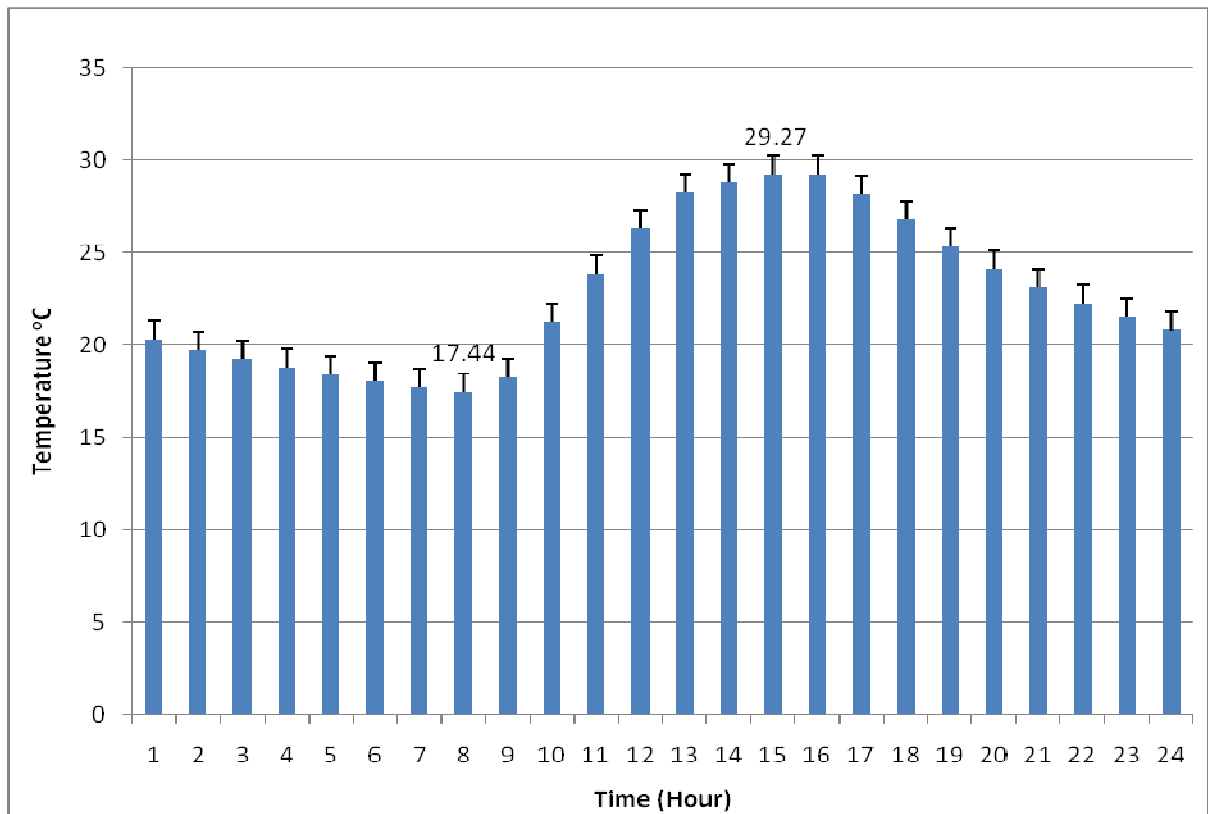


Figure 4. Temperature Monitoring during 24-h period in the Greenhouse

*The average temperatures were measured by the entire period (44 day) data.

4.3 Microbiological Data

4.3.1 Harvested Sample Weight

The leaf samples and growth media samples were first weighed before microbiological analysis. Figure 5 shows the spinach leaf weight by different harvest day from day 11 to day 44. The weight ranged from 0.11 g to 2.00 g with an increasing trend according to the growth period. The soil weight ranged from 10.5 g to 80.2 g with a mean of 32.3 g. All the weight data were used to convert the CFU colony count into CFU/g in the microbiological analysis.

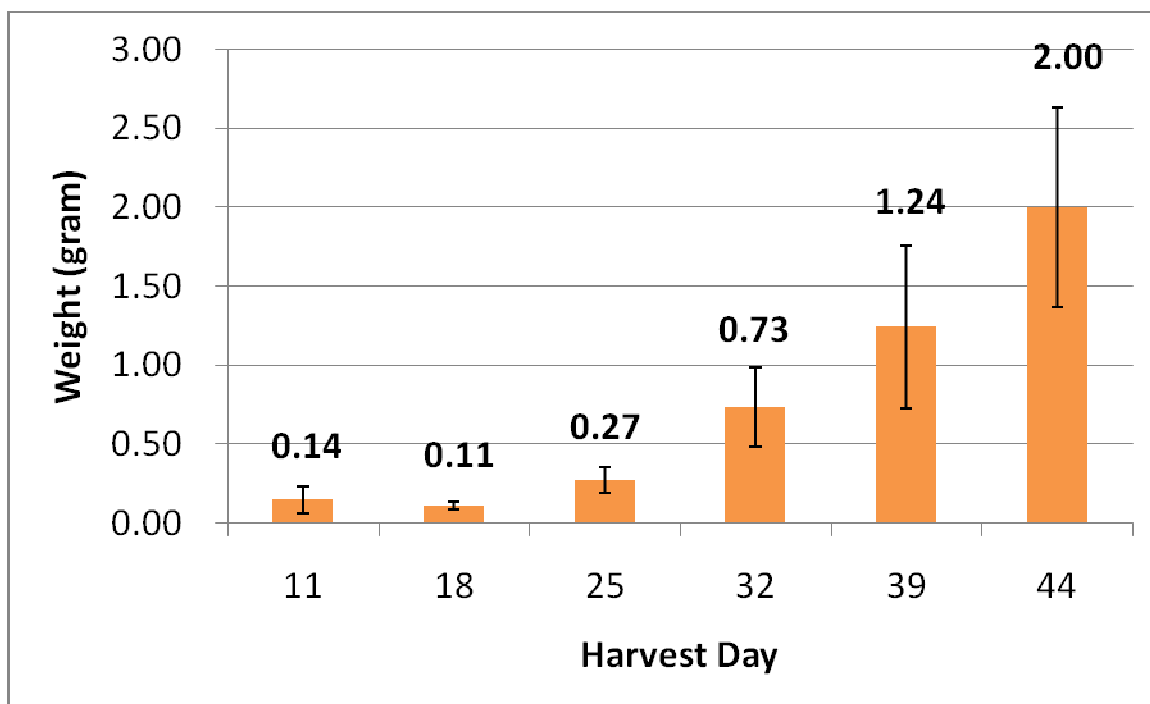


Figure 5. Spinach Leaf Weight by Harvest Day

*Average of all the leaf samples on each harvest day was included.

4.3.2 Internalization of *E. coli* O157:H7 in Spinach Leaves

Among 120 spinach samples examined for internal *E. coli* O157:H7 contamination, only one yielded positive result (bold and underlined in Table 5). This incidence occurred in one of the three plants which were inoculated at 10^7 CFU level in the third week (day 23) and harvested in the same week (day 25). The contamination level was below the detection limit (10 CFU/ml) for direct plating but was observed after enrichment. The actual internal contamination level was less than 3.3×10^3 CFU/g when taken into account of the sample weight. Internal *E. coli* O157:H7 was not detected in subsequent sampling of plants inoculated at the same time and level.

Table 5. Incidences of Spinach Leaf Contamination Following Experimental Inoculation of GFP-labeled *E. coli* O157:H7 Strain B6-914 at Various Plant Maturities and Different Bacterial Inoculum Levels

Inoculation day	Inoculum level (CFU)	Leaf sample type	Spinach age in days at the time of harvest							
			11	18	25	32	39	44	Total	
9	10^3	Total	0/3	0/3	0/3	1/3	2/3	0/3	3/18	
		Surface	0/3	0/3	0/3	0/3	0/3	0/3	0/18	
		Internal	0/3	0/3	0/3	0/3	0/3	0/3	0/18	
	10^7	Total	0/3	0/3	0/3	1/3	0/3	0/3	1/18	
		Surface	0/3	0/3	0/3	1/3	0/3	0/3	0/18	
		Internal	0/3	0/3	0/3	0/3	0/3	0/3	0/18	
	16	10^3	Total	N/A	0/3	0/3	1/3	1/3	0/3	2/15
			Surface	N/A	0/3	0/3	0/3	1/3	0/3	1/15
			Internal	N/A	0/3	0/3	0/3	0/3	0/3	0/15
10^7		Total	N/A	0/3	0/3	0/3	0/3	0/3	0/15	
		Surface	N/A	0/3	1/3	0/3	0/3	0/3	1/15	
		Internal	N/A	0/3	0/3	0/3	0/3	0/3	0/15	
23		10^3	Total	N/A	N/A	0/3	0/3	2/3	0/3	2/12
			Surface	N/A	N/A	0/3	1/3	1/3	0/3	2/12
			Internal	N/A	N/A	0/3	0/3	0/3	0/3	0/12
	10^7	Total	N/A	N/A	3/3	0/3	3/3	0/3	6/12	
		Surface	N/A	N/A	2/3	1/3	1/3	0/3	4/12	
		Internal	N/A	N/A	<u>1/3</u>	0/3	0/3	0/3	1/12	
	30	10^3	Total	N/A	N/A	N/A	2/3	1/3	0/3	3/9
			Surface	N/A	N/A	N/A	1/3	1/3	0/3	2/9
			Internal	N/A	N/A	N/A	0/3	0/3	0/3	0/9
10^7		Total	N/A	N/A	N/A	1/3	1/3	1/3	3/9	
		Surface	N/A	N/A	N/A	0/3	1/3	0/3	1/9	
		Internal	N/A	N/A	N/A	0/3	0/3	0/3	0/9	
37		10^3	Total	N/A	N/A	N/A	N/A	1/3	0/3	1/6
			Surface	N/A	N/A	N/A	N/A	0/3	0/3	0/6
			Internal	N/A	N/A	N/A	N/A	0/3	0/3	0/6
	10^7	Total	N/A	N/A	N/A	N/A	2/3	1/3	3/6	
		Surface	N/A	N/A	N/A	N/A	1/3	1/3	2/6	
		Internal	N/A	N/A	N/A	N/A	0/3	0/3	0/6	
	Total	10^3	Total	0/3	0/6	0/9	4/12	7/15	0/15	11/60
			Surface	0/3	0/6	0/9	2/12	3/15	0/15	5/60
			Internal	0/3	0/6	0/9	0/12	0/15	0/15	0/60
10^7		Total	0/3	0/6	3/9	2/12	6/15	2/15	13/60	
		Surface	0/3	0/6	3/9	2/12	3/15	1/15	9/60	
		Internal	0/3	0/6	1/9	0/12	0/15	0/15	1/60	

4.3.3 Surface Contamination of *E. coli* O157:H7 on Spinach Leaves

Among 120 spinach leaf samples examined for total leaf contamination, eleven out of 60 (18.3%) plants inoculated at 10^3 CFU level were contaminated, compared to 13 out of 60 (21.7%) for those inoculated at 10^7 CFU level (Table 5). Similarly, a higher incidence of contamination was observed for surface leaf samples, with 5/60 (8.3%) and 9/60 (15%) for inoculations at 10^3 and 10^7 CFU levels, respectively. Nonetheless, the differences in contamination incidences observed between the two inoculum levels for both total leaf and surface leaf samples were not statistically significant ($P > 0.05$) as analyzed by Chi-square test. Additionally, most of the contamination incidences were detected after enrichment, indicating levels lower than the detection limit (10 CFU/ml) for direct plating. The five spinach leaf samples with countable numbers by direct plating were the total leaf samples for all three plants which were inoculated at 10^7 CFU level in the third week (day 23) and harvested in the same week (day 25), one surface leaf sample among the same three plants, and one total leaf samples among the three plants which were inoculated at 10^7 CFU level in the fourth week (day 30) and harvested in the same week (day 32).

When grouping the incidences of leaf contamination by spinach age at the time of harvest, interestingly, none of the plants harvested during the first three weeks or the last week were contaminated for the 10^3 CFU inoculum level, regardless of the time of inoculation (Table 5). The contamination incidences clustered among leaf samples harvested when the plants were 4 or 5 weeks old. The association between contamination incidence and specific plant ages (4 and 5 weeks) was found to be statistically significant ($P < 0.05$). Similar findings were observed among plants inoculated at the 10^7 CFU level, although

contamination also occurred when the plants were at 3 or 6 weeks age (Table 5). Nonetheless, plants harvested at weeks 4 and 5 were found to have significantly higher incidence of contamination with the *E. coli* O157:H7 GFP strain ($P < 0.05$).

The effect of harvesting at different days after inoculation on the contamination incidences of the spinach leaf samples is shown in Table 6. For 30 experimentally inoculated spinach plants harvested on day 44, none of the 15 plants inoculated at 10^3 CFU level were contaminated whereas 3 out of the 15 plants inoculated at the 10^7 CFU level were contaminated. One of the three contaminated plants was inoculated on day 30 and the other two were inoculated on day 37, one to two weeks before the harvest. Similar analyses were performed for plants harvested on 39, 32, and 25 days and data are shown in Table 6. Across these four harvest dates, close to 40.5% (15 out of 38) spinach leaf contamination occurred within 1 week after inoculation, and 60.5% (23 out of 38) contamination occurred within 2 weeks after inoculation. Based on the statistical analysis, spinach samples harvested with 1 and 2 weeks inoculation had significantly higher incidences of contamination compared to those harvested at a later time ($P < 0.05$).

4.3.4 Survival of *E. coli* O157:H7 in the Growth Media

The *E. coli* O157:H7 GFP-expressing strain was found in all growth media samples collected throughout the cultivation period. For plants inoculated at 10^3 CFU ($n = 60$), although recovered after enrichment, the GFP-expressing *E. coli* O157:H7 could not be detected for the majority of samples by direct plating, indicating the contamination levels below the detective limit (10 CFU/ml). In contrast, from plants inoculated at 10^7 CFU ($n = 60$), all except one growth media sample had countable numbers via direct plating, indicating

higher levels of contamination. Figure 6 shows changes in the *E. coli* O157:H7 GFP strain concentrations (log CFU/g) in the growth media for plants inoculated at the 10^7 CFU level. A gradually reduced level of contamination was observed and this decreasing trend was found to be statistically significant ($P < 0.05$).

Table 6. The Effect of Harvesting at Different Days after Inoculation (Time Intervals between Inoculation and Harvest) on the Incidences of GFP-labeled *E. coli* O157:H7 Strain B6-914 Contamination of the Spinach Leaf Samples

Harvest day	Inoculum level (CFU)	Leaf sample type	Contamination incidence	No. of contaminated samples (days between inoculation and harvest)
44	10^3	Total	0/15	N/A
		Surface	0/15	N/A
	10^7	Total	2/15	1 (7); 1 (14)
		Surface	1/15	1 (7)
39	10^3	Total	7/15	1 (2); 1 (9); 2 (16); 1 (23); 2 (30)
		Surface	3/15	1 (9) ^a ; 1 (16); 1 (23) ^a
	10^7	Total	6/15	2 (2); 1 (9); 3 (16)
		Surface	3/15	1 (2); 1 (9); 1 (16) ^a
32	10^3	Total	4/12	2 (2); 1 (16); 1 (23)
		Surface	2/12	1 (2) ^a ; 1 (9)
	10^7	Total	2/12	1 (2); 1 (23)
		Surface	2/12	1 (9); 1 (23)
25	10^3	Total	0/9	N/A
		Surface	0/9	N/A
	10^7	Total	3/9	3 (2)
		Surface	3/9	2 (2) ^a ; 1 (9)

^a Both total leaf and surface leaf samples from the same plants were contaminated with GFP-labeled *E. coli* O157:H7 strain B6-914.

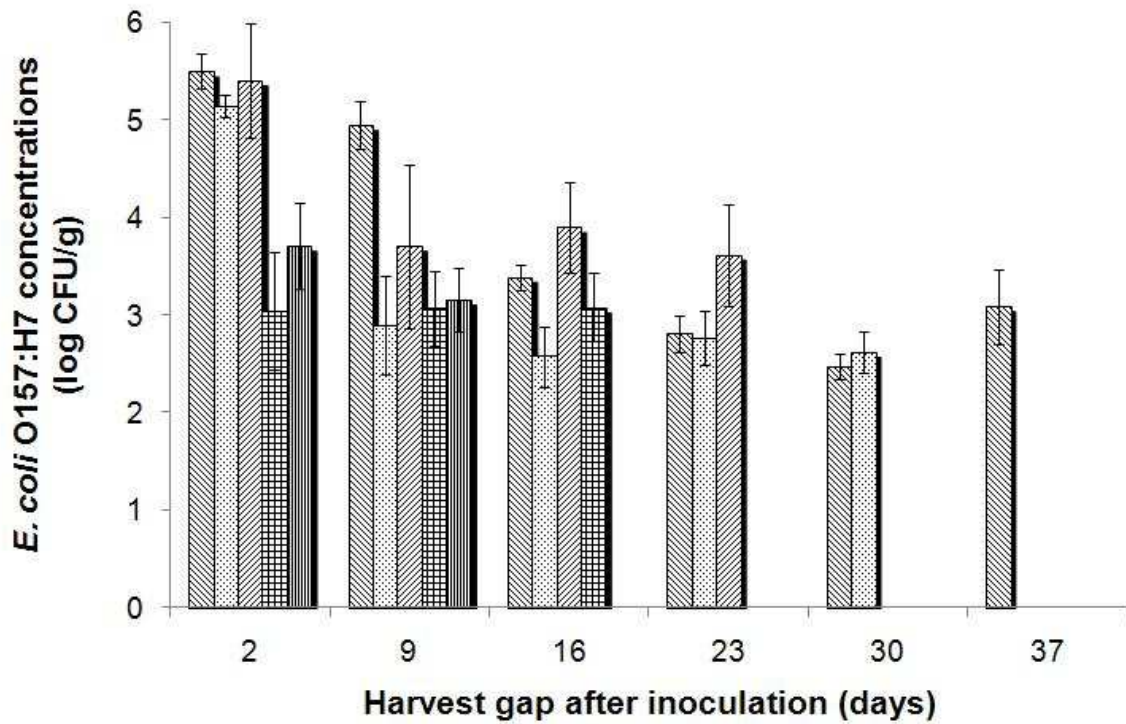


Figure 6. Fluctuation of *E. coli* O157:H7 GFP strain concentrations (in log CFU per gram) in the plant growth media when inoculated at 10^7 CFU/ml once on a weekly basis for five weeks, and analyzed 2 days after inoculation and weekly thereafter.

*The weeks of *E. coli* O157:H7 GFP strain inoculation are indicated as follows: first week (right-slashed bars), second week (light dotted bars), third week (left-slashed bars), fourth week (plaid bars), and fifth week (vertical strip bars). When analyzed by ANOVA and least-significant difference among the means of the *E. coli* O157:H7 concentrations in the growth media after different gaps between inoculation and harvest.

CHAPTER 5

DISCUSSION

From series of testing in the laboratorial experiment, the specific GFP-labeled *E. coli* O157:H7 strain B6-914 was confirmed for its expression of green fluorescent protein, lacking Shiga toxins 1 and 2, and carrying an ampicillin resistance marker. Our study result suggested that the markers were stable in our greenhouse experiment.

Leafy greens such as lettuce and spinach are a leading produce category among many fresh and fresh-cut produce items that have been increasingly involved in foodborne outbreaks (Buchanan, 2006; USFDA, 2008). A better understanding of the occurrence and factors affecting the internalization and contamination of foodborne enteric pathogens in leafy greens will have significant implications for prevention and control. It remains uncertain whether the adherence of enteric pathogens to produce tissues is due to passive physicochemical forces of the plants or active attachment processes involving specialized bacterial cellular mechanisms (Delaquis et al., 2007). Some studies reported that bacterial strains varied widely in their endophytic colonization abilities, which could be related to the plant defense mechanisms that targeted bacterial extracellular components (Dong et al., 2003; Iniguez et al., 2005). Interestingly, Solomon et al. (Solomon and Matthews, 2006) recently reported that the uptake of *E. coli* O157:H7 in lettuce is independent of any bacterial process, since dead cells and fluorescent microspheres were present in the aerial part of mature lettuce plants in a similar manner as live *E. coli* O157:H7 cells. The authors suggested that the uptake process may be governed by the plant instead of the organism, recommending commodity-specific investigations. Previous studies have demonstrated the internalization of

enteric pathogens in mature lettuce tissues via soil inoculation (Bernstein et al., 2007; Franz et al., 2007; Solomon et al., 2002b), but for spinach grown in soil inoculated with *E. coli* O157:H7, internalization was observed in roots but not in mature spinach leaves (Hora et al., 2005; Jablasone et al., 2005; Warriner et al., 2003a). In this study, we found one incidence of internalization (out of 120 spinach samples examined) in the spinach leaves harvested on day 25, suggesting that internalization was not readily occurring when the plants were grown in a greenhouse setting. Generally speaking, under greenhouse conditions, the possibility of external vectors (e.g. bird droppings or grazing wild animals) contaminating the spinach plants were largely minimized. In this study, we further reduced cross-contamination by randomly arranging the pots with sufficient separations and avoiding splashing during watering. Nonetheless, the risk of *E. coli* O157:H7 internalization needs to be further evaluated in field studies with conditions closely mimic the environmental conditions spinach encounters during the growing seasons.

A previous report (Franz et al., 2007) underscored the importance of using a proper surface sterilization method in studies examining internalized pathogens in plants. In this study, a common chlorine solution, 2% (w/v) calcium hypochlorite solution for 20 minutes (Wachtel and Charkowski, 2002) was used and found to be an effective mechanism to remove residual surface microflora. The method was also found to be logistically easy when handling a large number of plant samples compared to methods using multiple sanitizing steps.

In contrast to internalized contamination, surface contamination of the aerial leaf tissues was readily observed in this study, both introduced at 10^3 and 10^7 CFU level. Two mechanisms may help explain how surface contamination occurred. We did not cover the

plants with plastic at the growth media line which may result in cross-contamination of the *E. coli* O157:H7 GFP strain from the soil to the leaves during watering. Secondly, the ventilation system in the greenhouse may help bacterial cells to migrate from the soil onto the spinach leaves. An interesting observation was that surface spinach leaf contaminations clustered between 4 to 5 weeks of age at harvest, but not among leaves younger than 3 weeks of age. We hypothesize that plant defense systems may play a role here which are dependent on the developmental stage of the plant. Very few reports have examined the effect of plant age on the contamination of enteric pathogens. Bernstein et al. (Bernstein et al., 2007) assessed the contamination potential of lettuce of *Salmonella enterica* Serovar Newport added to the plant growing medium and reported that internalization of *Salmonella* via the root to the aerial tissues was identified in 33-day-old plants but not 17- or 20-day-old plants, and such contamination was stimulated by root decapitation. Another recent study reported that leaf age was a risk factor in contamination of lettuce with *E. coli* O157:H7 and *Salmonella enterica*, with the population size of the pathogens *ca.* 10-fold higher on the younger (inner) leaves than on the older (middle) leaves (Brandl and Amundson, 2008). The authors correlated the nitrogen content in the leaves with the likelihood of contamination as young-leaf exudates were 2.5 and 1.5 times richer in total nitrogen and carbon, suggesting nutritional content of the plant tissue played a role (Brandl and Amundson, 2008). Further studies assessing the association between spinach age and potential contamination of *E. coli* O157:H7 would provide practical means for developing strategies for control.

Besides plant age as a factor affecting the interaction between foodborne pathogens and produce, previous studies have shown the effects of phytopathogens and protozoa to enhance

the internalization and persistence of human pathogens on produce leaves (Barak and Liang, 2008; Brandl, 2008; Gourabathini et al., 2008). Other studies, on the other hand, showed no effect or even beneficial effect of phytopathogens on the survival of human pathogens on plants (Aruscavage et al., 2008; Cooley et al., 2006). Additionally, several studies have investigated genetic factors involved in the attachment and colonization of produce by human pathogens (Danhorn and Fuqua, 2007; Klerks et al., 2007; Palumbo et al., 2005). Future studies in these areas will lead to a better understanding of various factors that affect the contamination of produce by human enteric pathogens.

In most reports examining the contamination of fresh produce with enteric pathogens, the levels of bacteria used were far greater than what may be found in an agricultural field, therefore, may only indicate the worst case scenario. Our data showed that inoculation levels (10^3 CFU and 10^7 CFU) affected the contamination incidences, corroborating previous studies (Wachtel et al., 2002a).

Another interesting finding from this study was the effect of harvesting at different days after inoculation on the contamination incidences on the surface of spinach leaf samples. In the study by Bernstein et al. (Bernstein et al., 2007), the authors observed the presence of *Salmonella* in lettuce leaves 2 days post-inoculation but not 5 days later. Similar to their findings, we found that 60.5% of contamination incidences occurred within 2 weeks after inoculation, although some occurred as many as 30 days post-inoculation. As reviewed by Doyle and Erickson (Doyle and Erickson, 2008), the longer the interval between application of the contaminated vehicle and the harvest of the plant, the greater the likelihood that the produce would not be contaminated. It is therefore important to incorporate sufficient

intervals between potential contamination events and harvest, such as the minimum 120 days fertilization-to-harvest interval recommended by the National Organic Program and validated by some studies (Ingham et al., 2005).

Similar to many previous studies which reported that *E. coli* O157:H7 can survive extended period in soil and environment (Islam et al., 2004; Natvig et al., 2002), our data indicated the presence of *E. coli* O157:H7 recovered from the growth media throughout the cultivation period (44 days) at both inoculum levels.

However, only one strain of *E. coli* O157:H7 was used in the study and hence it had the limitation to represent other *E. coli* O157:H7 strains in the similar experimental procedure. It is possible that the pathogenic *E. coli* O157:H7 strains could have some other mechanisms to facilitate attachment of bacteria onto leaves. In addition, the effect of phytopathogens to enhance the ability of pathogenic *E. coli* O157:H7 internalization could not be known throughout this study.

CHAPTER 6

SUMMARY AND CONCLUSION

This study aimed to determine whether *E. coli* O157:H7 would be present in the aerial leaf tissue of a growing spinach plant when inoculated via growth media by different levels of inoculums.

The experiment used the specific GFP-labeled *E. coli* O157:H7 strain B6-914 as a trace marker in the inoculation during the entire cultivation period. By detecting the B6-914 strain in 120 harvested spinach leaf samples, it demonstrated that internalization of *E. coli* O157:H7 of growing spinach plant leaves under greenhouse conditions was a very rare event, but surface contamination did occur, primarily when the plants were harvested at age of 4 and 5 weeks. Interestingly, for the mature plants (after age of 5 weeks), the contamination rate was significantly lower than found in plants of younger age. Also, the effect of harvesting at different days after inoculation on the incidence of contamination of the spinach leaf samples was significant. The result showed spinach samples harvested with 1 and 2 weeks after inoculation had significantly higher incidences of contamination of *E. coli* O157:H7 compared with those harvested at a later time.

This study also demonstrated that *E. coli* O157:H7 strain could survive in the growth media throughout the entire cultivation period (35 days) at either lower inoculation level (10^3 CFU) or higher (10^7). However, a decreasing trend of the concentration of *E. coli* O157:H7 was observed with a gradually reduced level of contamination.

However, the findings in this study are subject to at least four limitations. First, as mentioned above, the study was conducted in a greenhouse rather than a field setting. The sample size included in this study was relatively small. Second, the bacterial inoculation levels used were greater than what may be found in an agricultural field. Third, stomaching was used in this study to assess the

uptake of *E. coli* O157:H7 into spinach leaves, which may not effectively release internalized populations from within leaves and hence underestimate the contamination levels. Additionally, the study used a single *E. coli* O157:H7 strain, hence, it is not clear how the results relate to other strains.

In conclusion, the present study demonstrated that internalization of *E. coli* O157:H7 of growing spinach plant leaves under greenhouse conditions was a rare event, but surface contamination did occur, primarily when the plants reached 3 weeks of age. The finding that greater contamination occurred on the surface than internalized tends to support that the pathogen would have been deposited on the leaf surface before being internalized and hence would not have entered via the roots. Furthermore, the study provided important data to further assess the association between spinach age and potential contamination of *E. coli* O157:H7.

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July 31, 2009

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