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EVIDENCE OF TRANSMISSION OF *ESCHERICHIA COLI* O157:H7 TO THE TISSUES OR PHYLLO-PLANE OF WHEAT, FROM CONTAMINATED SOIL, SEEDS OR WATER

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by

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Escherichia coli O157:H7 is a human pathogen that can cause a wide spectrum of disease symptoms, such as bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS). *Escherichia coli* O157:H7 illness are mainly associated with undercooked beef; however, in recent years outbreaks have been linked to fresh produce such as spinach, lettuce, and sprouts. In 2009, flour was implicated as a contamination source in the consumption of raw cookie dough resulting in 77 illness-cases. The objective of this research was to determine the possible route of transmission of *E. coli* O157:H7 into the phyllo-plane of wheat using contaminated seed, soil or irrigation water. Levels of contamination were 6.88 log CFU/g, 6.60 log CFU/g and 6.76 log CFU/ml of Kanamycin resistant *E. coli* O157:H7. One hundred plants per treatment were sown in pots trays with 50 g of sterile soil, watered every day with 5 ml of dilution water and harvested after 9 days post-inoculation. In a fourth experiment, flowering wheat heads were spray-inoculated with water containing 4.19 log CFU/ml of *E. coli* O157:H7 and analyzed for survival after 15 days, close to the harvest period. To detect low levels of internalization, BioTecon *q*-PCR detections assays were used to determine the presence of *E. coli*

O157:H7 in the wheat plants using a Roche Applied Science LightCycler 2.0. Results showed that internalization was possible using contaminated seed, soil, and irrigation water in wheat seedlings with an internalization rate of 2%, 5% and 10% respectively. Even though this rate is low, this is the first study to demonstrate the ability of this strain to reach the phyllo-plane in wheat. In the head contamination experiment, all samples tested positive, showing the ability of *E. coli* O157:H7 to survive on the wheat head phyllo-plane. Although possible this research does not provide evidence for efficient uptake of *E. coli* O157:H7 into the internal tissue of wheat plants from a contaminated environment. However, surface contamination and the ability of *E. coli* O157:H7 to survive long-term on the wheat plants is an issue to be considered when addressing food safety issues in products derived from wheat.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1. LITERATURE REVIEW

1. History and background

Escherichia coli were discovered by a German scientist named Theodor Escherich, who identified, characterized and isolated these bacteria from neonate feces in 1885 (Meng and Schroeder 2007). This bacterium had been named *Bacterium coli commune* by the discoverer; in 1911 the name was changed to *Escherichia coli* in honor of its discoverer (Meng and Schroeder 2007). Since then, investigations on this microorganism have been extensive, allowing a better understanding of the different features and implications that these microorganisms have in human life.

According to O'Brien and Kaper (1998) these bacteria were first described by Gasser et al. in 1955 as illness-inducing with symptoms such as: renal failure, thrombocytopenia and microangiopatic hemolytic anemia. Interestingly, the cause of human Hemolitic Uremic Syndrome (HUS) was unknown until 1968, when Kibel and Bernard conducted a survey in South-Africa to better understand this disease. They found that a possible cause was the presence of an entero-pathogenic *E. coli* strain. It was not until 1977, when Knowalchuck and colleagues showed that certain diarrheagenic strains of *E. coli* can produce toxin (Shigatoxin) with the capacity to kill verocells. That the possibility that this bacteria could be involved in this disease was shown.

In 1982, two outbreaks that occurred in Michigan and Oregon were linked to hemorrhagic colitis and caused abdominal cramps, mucosal edema, bloody stools, along with other symptoms (Mead and Griffin 1998; O'Brien and Kaper 1998). After these

outbreaks, *E. coli* O157:H7 was unmistakably linked to illness and was considered the first time a human pathogen capable of producing hemorrhagic colitis was discovered (Mead and Griffin 1998).

Another, confirmatory investigation in 1983 showed that sporadic Hemolytic Uremic Syndrome (HUS) cases were linked to the presence of shigatoxin (sometimes called verotoxin) produced by *E. coli* strains; this conclusion resulted from the study of HUS patient stools (Mead and Griffin 1998; O'Brien and Kaper 1998). Those strains that were involved in the entero-hemorragic disease including *E. coli* O157:H7 and were categorized as the "Enterohemorragic *Escherichia coli"* group (known as EHEC) due to their production of toxin (Mead and Griffin 1998). Since then, scientists have been focusing on understanding, preventing and monitoring food-borne illness from these strains.

In 1993, 700 foodborne illness cases were reported from a single outbreak and resulted in 4 deaths; this outbreak triggered a demand to increase the food safety of some products. In 1994, *E. coli* O157:H7 was declared as an adulterant in raw ground beef (Hollingsworth and Kaplan 1998; Rangel J.M. 2005). In the following year, The Council of State and Territorial Epidemiologists established *E. coli* O157:H7 as a nationally notifiable disease and since then increased monitoring data has shown an increase in occurrence caused by this agent from 0.8 to 1.1 per 100,000 person until 2003 (Rangel J.M. 2005; Meng and Schroeder 2007). The government agency went further by conducting a survey of about five thousand samples with the purpose of motivating the

industry to act during the first two years after this regulation was approved (Hollingsworth and Kaplan 1998).

Outbreaks associated with produce were first reported in 1991, and since then, scientists have determined that around 21% of all outbreak cases between 1982 - 2002 involved produce (Rangel J.M. 2005). The presence of *E. coli* O157:H7 in produce or other products (that are usually eaten raw) is seen as example of pathogen in a new food vehicle that has high risk of contamination (Matthews 2006) .

2. Characteristics and classification of *E. coli* O157:H7

Taxonomically, the genus *Escherichia* is part of Enterobacterieae family. This genus has six species described (*E. adecarboxilata, E. blattae, E. fergusonni, E. hermanni, E. vulneris* and *E. coli*) (Meng and Schroeder 2007). *E. coli* is a gram negative, non-sporeforming, straight rod with size ranging between 1.1 – 1.5 µm x 2.0 - 6.0, arranged in pairs or single, may be motile (peritrichous flagella) and may have a capsule or microcapsules (Flatamico and Smith 2006). A facultative anaerobe, *E. coli* grows optimally at 37°C with a generation time of approximately 20 min in rich media (Meng and Schroeder 2007). It is catalase positive and oxidase negative (Flatamico and Smith 2006; Meng and Schroeder 2007). *E. coli* strains do not grow under refrigeration conditions but can survive in temperatures of 4 or -20 °C for weeks, water activity (a_w) of at least 0.95, and in NaCl concentration of 8.5% (Flatamico and Smith 2006).

E. coli strains can be differentiated using serological techniques based on three major cell surface antigens: O (somatic), H (flagella) and K (capsular) (Meng and Schroeder 2007). At least 167 O antigens, 53 H antigens and 74 K antigens have been described (Meng and Schroeder 2007). This is useful due to the distinguishing features between serotypes in *E. coli* species. In the case of *E. coli* O157:H7, the name is derived from the expression of somatic antigen (O) 157 and flagella (H) antigen 7.

E. coli O157:H7 has some important characteristics that allow it to survive in the intestinal tract. The capability to survive in low pH is considered one of the most important characteristics due to high acid environment in the stomach. This also allows *E. coli* O157:H7 to cause infection with only a few cells in the human intestinal tract (Yuk and Marshall 2004). At least three different systems allow *E. coli* O157:H7 to survive the acid environment: 1) the oxidative system (required *Rpos*); 2) the arginine-depending system; and 3) the glutamate-dependent system (Flatamico and Smith 2006).

Another important characteristic is the pathogenicity of *E. coli* O157:H7. This is explained by two important features of this bacterial group. One of the key features is the presence of a gene island known as LEE (Locus of Enterocyte Effacement- with length of 43 Kb) that encodes for attachment (type III secretion system- permitting bacteria to bind to intestinal cells) and production of diarrhea (Karmali 2004; Chahed et al. 2006; Flatamico and Smith 2006). In the same gene island, *eae* (which encodes for a bacterial adhesion protein known as Intimin) has been associated with the development of Hemorrhagic Colitis (HC) and Hemolytic Uremic Syndrome (HUS) (Blanco et al. 2003)

The second important key feature is the production of shigatoxins which are responsible for the production of hemolytic uremic syndrome (HUS) (Karmali 2004; Chahed et al. 2006; Flatamico and Smith 2006). Moreover, *Escherichia coli* O157:H7 can produce at least one form of the two different types of shigatoxin. Shigatoxin 1 is practically indistinguishable from the *Shigella dysenteriae* type 1 with only one amino acid difference in one of the sub-parts; shigatoxin 2 is similar to shigatoxin 1, sharing 56% amino acid homology (Mead and Griffin 1998; Melton-Celsa and O'Brien 1998). It is important to note that any *stx*- producing strains will be able of producing HUS and this is exclusive to the EHEC group, which includes the serogroups O26, O103, O111, O118, 128, O145, O157 (Melton-Celsa and O'Brien 1998; Chahed et al. 2006). Shigatoxins are responsible for blocking the synthesis of proteins in the host cells; therefore eventually the cell will stop functioning due to this deficiency (Acheson et al. 1998). Moreover, shigatoxin 2 is 1000 fold more toxic than shigatoxin 1; this is an important feature because *E. coli* O157:H7, which has *stx2*, has been highly associated with the development of HUS (Melton-Celsa and O'Brien 1998).

3. Infectious dose and disease characteristics

The infectious dose necessary to cause illness is between 10 to 100 cells (Feng and Weagant 2011). This dose of this microorganism has been determined through analysis of the numerous outbreaks that have occurred. An outbreak of *E. coli* O157:H7 related to the consumption of salami was analyzed to measure the minimal dose necessary to produce a food-borne illness. Tilden et al. (1996) showed that, according to their

calculation, the infectious dose present in the salami was less than 50 cells of *E. coli* O157:H7 bacteria which was enough to produce 17 cases of illness. Other outbreaks related to the consumption of hamburgers patties showed that the infections dose could be even lower. Investigators reported that in some cases the dose could be as low as 1.5 microorganisms per gram or 67 microorganisms per patty were enough to cause illness (Tuttle et al. 1999). *E. coli* O157:H7 produces a higher hospitalization rate (47%) than any other pathogen (Griffi 1998). Due to this low infectious dose and the severity of the illness, *E. coli* O157:H7 is one of the most important human foodborne pathogens. Therefore, it is necessary to enforce zero tolerance for this microorganism in food (Tuttle et al. 1999).

E. coli O157:H7 can produce an asymptomatic infection, but may manifest into more severe symptoms such a mild diarrhea or acute bloody diarrhea termed "hemorrhagic colitis" (Flatamico and Smith 2006). Incubation periods can range between 3 to 8 days, but in some cases can be as short as 1 or 2 days (Karmali 2004; Flatamico and Smith 2006). Investigators found that after this incubation time, this strain can produce hemorrhagic colitis along with other characteristics, including the presence of necrotic cells in the stools.

The process of developing HUS is not well understood but it is known that shigatoxins are produced in intestine and ultimately transferred into the bloodstream, where it binds the polymorphonuclear leukocytes which can be transported to vital organs, especially the kidney (Karmali 2004). The toxin can also be translocated to the lungs, or brain in

extreme cases (Karmali 2004). This disease produced by this type of bacteria, can be very serious for patients as around 70% need red blood transfusions, 50% need dialysis and around 25% have neurologic involvement, such as stroke, seizure and coma (Noris and Remuzzi 2005). Finally, in the worse cases, patient can die as a consequence of the production of the toxins.

EHEC strains represented around 70% of the total development of diarrheic HUS; and 80% of these HUS cases are produced from *E. coli* O157:H7 in recent years (Boyer and Niaudet 2011). Some investigators considered that *E. coli* O157:H7 has the strongest association worldwide with HUS (Tarr et al. 2005). It is considered one of the primary causes of renal failure in the western world with death rate about 5% (Delaquis et al. 2007). The rate of renal failure in North America is around 15% of the cases (Wong et al. 2000).

Overall, HUS produced by Stx is estimated to be 2.1 cases per 100,000 persons per year. If this data is analyzed by age, the data shows a peak in younger children (1-5 years old) with a frequency of 6.1 per 100,000; and lower in adults (50-39 years old) at 0.5 per 100,000 persons in recent years (Ruggenenti et al. 2001; Noris and Remuzzi 2005). In the United States around 60 deaths are attributed every year to the HUS caused by Stx-producer *E. coli* (Noris and Remuzzi 2005).

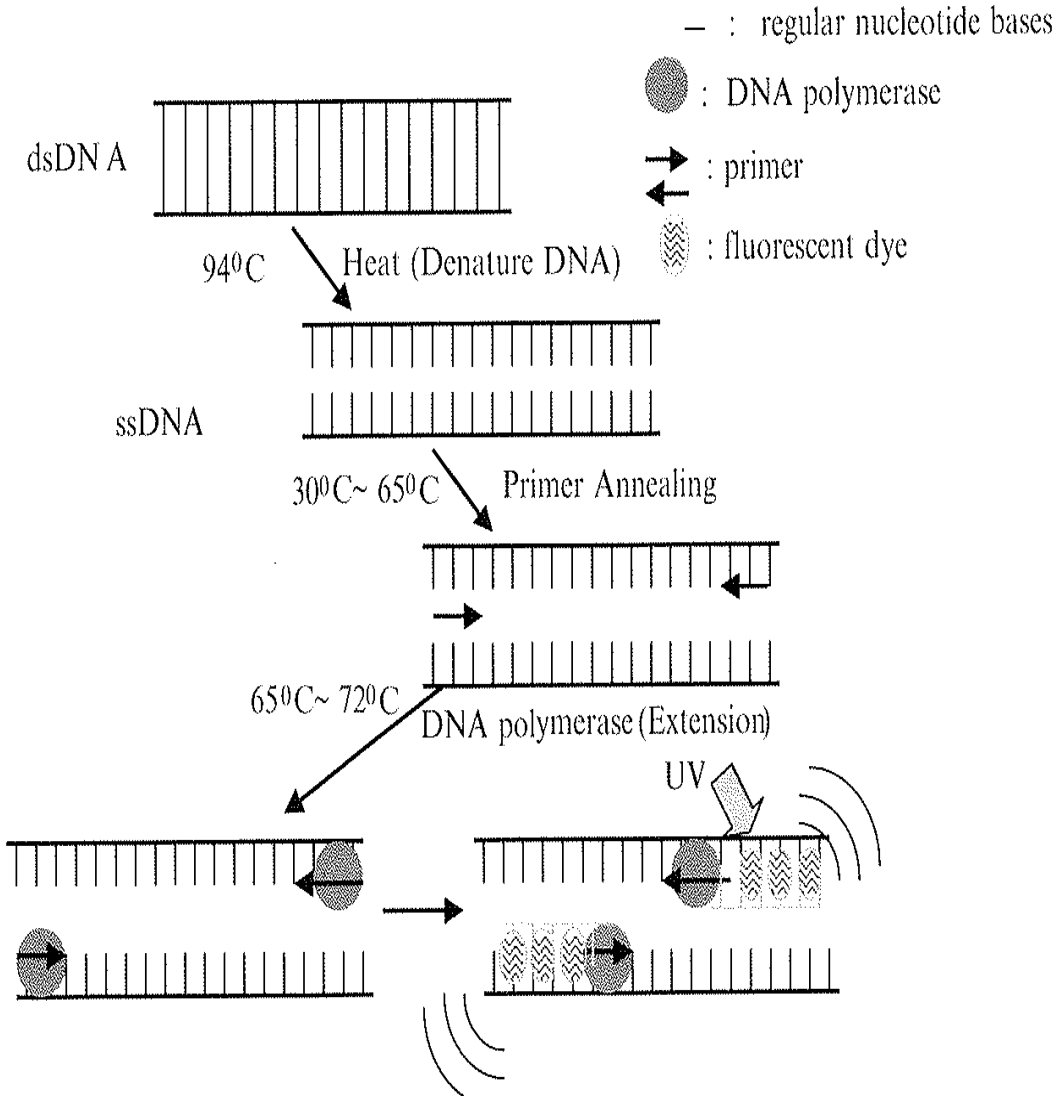
4. Detection of *E. coli* O157:H7 in food Matrix

Using traditional methods, *E. coli* O157:H7 can be isolated using selective media that is based on some distinctive characteristics compared with other bacteria. Because traditional plating methods are labor-intensive and time-consuming, scientists have developed accurate and rapid methods to identify this strain. In recent years, the development of Polymerase Chain Reaction (PCR) methods for detecting microbial pathogens have been used extensively due to their precision and speed when compared to traditional methods.

This method is based on the concept that all organisms (that includes bacteria) have sequences of DNA that are unique to their species. PCR is a DNA based method, which was developed in 1983 by Dr. Kary Mullis and colleagues (Fairchild et al. 2006). DNA is consisted of complementary oligonucleotide base pairs that are bound in a ladder configuration and twisted in a cork-shape. Based on the complementarity of the base pairs in the DNA, the PCR method uses just one oligonucleotide strand from DNA to find the complementary oligonucleotide. These short pieces of single strand of DNA are specific to a target section of DNA known as primers. These primers play a key role in the specificity and accuracy of this method due to complementary match with the target DNA. The PCR principle is based on making millions of copies of target DNA from a microorganism. According to Roche (2012) for PCR reaction, it is necessary to separate the two strands of the DNA helix and this is done by increasing the temperature to 95°C. The following step consists of decreasing the temperature of the mix to 55°C thereby allowing the primers to match with the DNA of the target microorganism (known as

annealing). Finally, the temperature is increased again to 72°C during which the polymerases attach the complementary nucleotides from the primer to the developing strand of DNA. After this cycle is done, the DNA copy is complete. This process is explained in figure 1. This cycle is repeated between 35 and 45 times (cycles) to ensure the DNA amplification process is adequate for detection of the target DNA.

Figure 1. Procedure to perform PCR (Fairchild et al. 2006)



Real-Time or quantitative PCR (*q*PCR) consists of monitoring and quantifying the PCR products (or amplicon) as the reaction cycle progress (Fairchild et al. 2006). Different detection methods have been developed to monitor the PCR product. For the experiments described in this thesis, the detection method utilized a "hybridization probe". This detection method uses fluorescence resonance energy, from one probe to another, after annealing of the primers to the template strand of DNA (Fairchild et al.

2006). One of the probes has a donor dye (located in the 3' end) that will accept it by the other probe located on the 5' end (Fairchild et al. 2006). When both probes anneal to the target sequence, the dyes are located adjacent one to one another, and the energy emitted by the donor probe dye excites the acceptor dye, which produces fluorescent light (Fairchild et al. 2006). The ratio between the two fluorescent emissions increases as the PCR progresses and is proportional to the amount of amplifications produced from the PCR (Fairchild et al. 2006). This fluorescence is plotted in a graph showing the increase in fluorescence by the number of cycles. If this fluorescence increase seem that DNA is amplifying.

5. *E. coli* O157:H7 in produce and actual issue

A large survey conducted by the USDA (2004) analyzed both imported and domestic fresh produce including cantaloupe, celery, leaf lettuce, romaine lettuce, and tomatoes. Investigators found that around 0.62 % of the produce samples were contaminated with *E. coli* that possessed virulence genes. Even though this percentage is low, there is a high risk due to these products being consumed raw. According to Tyler and Triplett (2008), 4.2% of outbreaks were from non-meat sources during the period of 1998-2002; these outbreaks caused 8.2 % of illness and 8% of deaths for all the outbreaks in the category of fresh fruits, nut and grains (Tyler and Triplett 2008).

Scientists have been investigating the economic cost of foodborne-illnesses, and the results show that the cost of illness due to *E. coli* O157:H7 in United States (including

medical cost, and quality of life losses) represented around $993 million dollars per year (Scharff 2010). This investigator also analyzed the data collected from the period 2003-2007 and categorized outbreaks according to different pathogens found in fresh, canned and processed produce. It was found that 39% of the outbreaks were attributed to *E. coli* O157:H7 establishing it as the primary pathogen causing illness in this period. The data further suggested that the cost of illnesses could reach about $ 15.21 billion dollars.

6. Contamination routes in plant and produce

Escherichia coli O157:H7 outbreaks have been reported in fresh produce since the 1990's (Delaquis et al. 2007); although the transmission route has often been unclear, the contamination source appears to have come from the farm in which produce is grown (Solomon et al. 2002a). According to Brandl (2006) and Berger et al. (2010), the contamination source in fresh produce comes from different sources such soil, air or irrigation water; or possibly from more complex interactions such as insects that act as vectors for human pathogenic bacteria.

To better understand the problem, it is necessary to examine the environment in which this pathogen is found. Cattle are considered the main reservoir for this pathogenic strain. The carriage rate has been reported to be around 8.3% with *E. coli* O157:H7 in an asymptomatic stage, which has been confirmed through analysis of cattle feces (Solomon et al. 2002b). Other surveys conducted on cattle indicate that this number is underestimated. It has been reported that animals have shed this strain with carrying rate

as high as 36.8% in summer months with an annual overall mean of 15.7% (Chapman et al. 1997). The organism survival time in these feces can be as long as 162 days (Avery et al. 2004; Looper et al. 2009). Usually cattle excrete around 20 to 50 kg of feces per day and when *E. coli* O157:H7 is present in the intestinal tract, they may shed a population between 10^1 to 10^7 CFU/g (Solomon and Sharma 2009). Therefore, a significant amount of bacteria could contaminate farmland. This leads to the conclusion that it is possible that the manure is a potential soil contaminant when used as a fertilizer.

Manure incorporation is a routine practice in the production of some crops as fertilizer or soil amendment (Islam et al. 2005; Delaquis et al. 2007). This incorporation increases water holding capacity, improves aeration and provides beneficial microorganisms (Islam et al. 2005). The use of this practice has been increasing (nearly 20% per year) from 2007-2012, mainly because of the trend toward organic produce (Laux 2012). Studies have shown when manure has been piled specific populations of bacteria can survive for long periods (Franz et al. 2008), but their survival is also dependent on which species the manure came from. For bovine manure, *E. coli* O157:H7 can survive for at least 47 days; in aerated ovine manure the survival can be as long as 4 months or 21 months when piling is specific for this species (Kudva et al. 1998) . This shows the possibility that manure or wild animal feces that has been improperly decomposed can contaminate crop fields and allow pathogenic bacteria to come in contact with produce (Brandl 2006).

It is possible that manure or slurry manure can contaminate surface water sources or crop production soils during the rainy seasons. Survey data taken in 2005 showed that 58% of

the irrigation water came from surface water (USGS 2005) demonstrating a high risk that *E. coli* O157:H7 could reach water sources during the rainy season by runoff. According to this data, there is a high probability that surface water could end up in irrigation water for crops, a potential food source.

Researchers report that the slurry manure survival is not shorter than soil survival, but variations due to soil type, bacterial strain, and experimental design cause slightly different results. In some cases the survival can range from weeks to more than 6 months (Franz et al. 2008). Even though composting is an ideal practice because increases in temperature may inhibit the presence of pathogenic bacteria, this method has been impractical for cattle manure due to the large amounts that this animal produces (Kudva et al. 1998). Slurry manure or solid manure has been used as a fertilizer without the proper decomposition procedure (Kudva et al. 1998). This could increase the risk of contamination on crops.

According to current regulations for organic production by the United States Department of Agriculture (USDA 2012), raw manure can be applied to crops providing there is at least 90 days from the application to the harvested period where the edible parts are not in contact with soil. This regulation changes to 120 days when the edible parts are in direct contact with soil. Previous studies have been described that show survival of *E. coli* O157:H7 beyond 90 days, meaning this time period may be not enough to decrease levels of these bacteria in the soil. Investigators have found *E. coli* and *Salmonella* in detectable levels when manure was applied during warm weather after 119 days (17 weeks) of

application in washed radish and arugula (Natvig et al. 2002). Crops with production cycles more than 90 days, such as wheat -when allowed to use raw manure - may possibly become contaminated with *E. coli* O157:H7 from the soil during production.

Other scientists have been found additional information about the survival of *E. coli* O157:H7 in soil. It can survive for at least two months in plain soil (Semenov et al. 2008; Zhang et al. 2009). Therefore, soil needs to be considered important source of contamination for plants (Mootian et al. 2009) along with irrigation water (Habteselassie et al. 2010). During winter, the survival of *E. coli* O157:H7 could increase due to the low temperature. One study demonstrated that *E. coli* O157:H7 can survive for at least 6 months at 4°C, starting at a level of 10^6 CFU/g (Vidovic et al. 2007) or longer in frozen samples (around 500 days) (Gagliardi and Karns 2002). These temperatures could enhance survival in some crops that could handle the winter climate, such as wheat. Production cycles are short for some crops (around 3 months), therefore *E. coli* O157:H7 could be present throughout the cycle and into the next one. This characteristic increases the possibility that plants could be infected by *E. coli* O157:H7. Islam et al. (2005) showed that *E. coli* O157:H7 can survive 168 days in a crop soil (manure soil amended or contaminated irrigation water) and therefore can reach the harvest period in carrots (126 days). Also, this strain was present throughout the production cycle and may compromise the safety of vegetables growing in this soil.

Some researchers believe that *E. coli* O157:H7 faces too many barriers to enter the edible parts using different ways of transmission. However, *E. coli* O157:H7 has the capacity to

manage different environments. According to Duffitt et al. (2011), *E. coli* O157:H7 has at least 18 different genes it can express as a response to environmental stress. These genes were expressed in higher proportion when *E. coli* O157:H7 was inoculated into sterile soil compared with enriched solution in just 14 days of incubation. This suggests that these genes increase this organism's survival in inhospitable environments such soil.

Many outbreaks of *E. coli* have been linked to the consumption of sprouts (Breuer et al. 2001; Mohle-Boetani et al. 2001; Berger et al. 2010). According to Berger et al. (2010), the majority of these cases involved contaminated seeds as the source of contamination, and through their distribution this may explain the wide geographic area of the outbreaks. This type of contamination (by seeds) is also important to consider because seeds may become contaminated during agricultural production and could be a vector to contaminate other farms' soil, thus perpetuating in the next generation of plants.

Wang and Doyle (1998) found that *E. coli* O157:H7 can survive long period of time in water (91 days in water at 8 °C and 49-84 days at 25 °C). This shows the possibility that this strain can be transmitted by the irrigation water due to the long survival period. Apparently, survival time in farm water could be less using real environment conditions. According to McGee et al. (2002), farm land water with 10^3 CFU/ml of *E. coli* O157H7 population was tested during time in a crop field. They found that *E. coli* O157:H7 can survive around 12 to 18 days post-inoculation in the field conditions and 20 - 28 in laboratory conditions.

6.1 Via root

Gagliardi and Karns (2002) found that *E. coli* O157:H7 can survive better when roots are present such as in rye and alfalfa. Moreover, *E. coli* O157:H7 has been shown to enter into the edible parts of the plant using the root as a way of transmission (Solomon et al. 2002a; Solomon et al. 2002b; Warriner et al. 2003a; Sharma et al. 2009). This introduction of bacteria in plant tissue is called "internalization" and usually the bacterium is located in the intercellular space or the inside of the plant cells (Bartz 2006).

Using the root as a way of internalization, lettuce could become contaminated with *E. coli* O157:H7 from soil or water fertilized with manure (Solomon et al. 2002a; Solomon et al. 2002b). It has been shown that after five days of inoculation with a population of 10^7 CFU, *E. coli* O157:H7 become internalized (Solomon et al. 2002b). Other studies have shown that crops such as salad spinach can also become contaminated with *E. coli* O157:H7 (Warriner et al. 2003a). The organism was able to internalize into the edible parts of radish sprouts (hypocotyls and cotyledons) (Itoh et al. 1998). In alfalfa sprouts, *E. coli* O157:H7 colonized the emerging radicule along the root cell junction and on the second day were observed in developing hair root (Charkowski et al. 2002). In maize, *E. coli* O157:H7 can enter the vascular system via the root and become internalized in the leaves when the root tip is cut, thereby exposing the vascular system of the plants (Bernstein et al. 2007). This study showed the likelihood of *E. coli* O157:H7 to be translocated from the roots to the leaves

No study has been done describing the internalization of *E. coli* O157:H7 in wheat plants, although other members of the cereal family (barley) have been studied. Barley roots are susceptible to colonization by *Salmonella enterica* at high cell densities, where the bacterium become internalized by crossing the epidermis of the root and transferring to the edible part of the plant (Kutter et al. 2006). This study used a PCR method to determine that *Salmonella* could translocate from the soil (quartz sand) to the root (Kutter et al. 2006). Moreover, this result was confirmed by other investigators who showed that the root appears to be one of the main routes of contamination in other crops (Solomon et al. 2002a; Solomon et al. 2002b).

To understand how this happens, it is necessary to learn about the physiology of these plants. Roots are usually coated by mucilage (hemicellulose and pectin) that acts as a barrier to avoid interaction with bacteria, but physical or chemical damage can expose the root as well as its exudates. The exudate composition has some nutrients that work as chemo-attractants for bacteria (Mandrell et al. 2006).

According to Jablasone et al. (2005), some root plant exudates are produced with the purpose of making symbiotic interactions with the endogenous microflora. The composition of plant exudates and external factors such as a poor nutrient environment which decreases the endogenous micro-flora, can affect the ability of the pathogen to colonize the root junction (Jablasone et al. 2005). Root exudates are species-specific metabolites of plants that affect the probability of internalization in each species (Bertin et al. 2003). Soil microorganisms or plants can be affected by the composition of the

exudates and their main components are sugars (mono, di and poly saccharides), organic compounds and phenolic compounds. These last compounds are the major cause of changes in the microflora and antagonistic characteristics (Bertin et al. 2003). Sugars present in this exudate can be used by microorganisms as a carbon source (Bertin et al. 2003). Some wheat species can have allelophaty (capability to inhibit growth of other plants) due to the phenolic compounds (p-hydroxybenzoic, valic, p-coumaric, syringyc and hydroxamic acid) present in the exudates (Bertin et al. 2003).

Onions, when sowed in manure-amended soil contaminated with *E. coli* O157:H7; However, this microorganism has less survival time than carrot when they were grown in the same soil matrix (Islam et al. 2005). These differences may be due to phenolic compounds in the exudates (present in edible and non-edible parts in onions) (Islam et al. 2005). According to these studies, the survival and internalization of *E. coli* O157:H7 could depend on plant composition and exudate produced during growth. There can be a variation between crop species, allowing differences in the survival of the bacteria. This difference also shows that even though internalization could occur in different crops, the specific factors involved are highly variable, thus making predictions about this phenomenon occurring in all crops difficult to determine.

Human pathogens can grow using the nutrient sources present in the root exudate, allowing them to compete with the endogenous micro-flora (Jablasone et al. 2005). According to this study (Jablasone et al. 2005), the root junction subpart is responsible for secreting these exudates, and is also the part that *E. coli* O157:H7 prefers to colonize

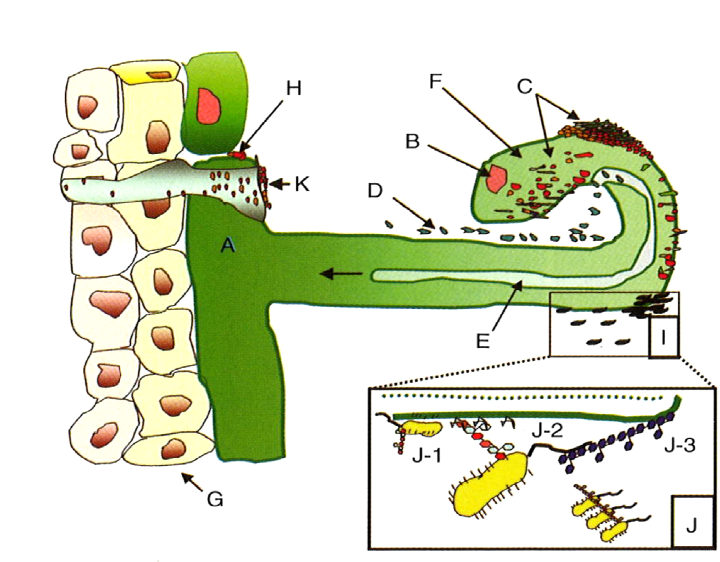
thus becoming internalized into the complete plant. *E. coli* O157:H7 was found to grow strictly in the root of some plants; and studies also suggest that emerging seedlings are able to better support the growth of bacteria due to rich nutrients available during this stage (Jablasone et al. 2005). Although some pathogens (*E. coli* O157:H7, *Salmonella* spp.) are internalized in early stages (day 9) of certain crops, they were not found after day 49, close to end of cultivation of lettuce and radish (Jablasone et al. 2005).

It is not completely understood exactly how internalization happens in the root, and it is not clear to which exact part bacteria is translocated. Other investigators are trying to explain this occurrence in other ways, and they suggest that them is a sequencial order of events that *E. coli* O157:H7 needs to accomplish to cross the root and mucilage barrier (Cooley et al. 2003). It is suggested that these bacteria will invade when the second (lateral) roots are forming, because during this stage the root layers that are surrounding the root are broken by expansion in order to allow the emerging of the second root (Cooley et al. 2003). This appears to be the window that *E. coli* O157:H7 can use to enter the root and finally reach the primary root.

Another possible route for internalization is though the hair of the root. Rhizobial bacteria commonly attach to these sub-parts of the root, and therefore may be the site in which human pathogens can do it as well (Mandrell et al. 2006). In Figure 2, these authors explain the different interactions on epidermal cells that are present on root surface. According to figure 2, root hairs are modifying cells that are exposed on the root surface (A). A second layer is formed by cortex cells below this hair cell (G). Rhizobial bacteria

can infect the root hair cell making hair cells to be curly (D), but other bacteria also can attach using different patterns. Bacteria can attach between the epidermal cells root and hair root cell in some cases (H). Others possible patterns could be the attachment of bacteria biofilms or aggregates (C) or more complex systems in which bacteria attach itself directly on the root hair (I). Moreover in Figure 2, bacterial attachments are magnified (J) to show that at least three different methods for attachment are used by bacteria: 1) pilli or fimbriae bind to root cell (J-1); 2) lectins (sugar attached to proteins) bind sites with bacterial carbohydrates (EPS, LPS, etc.); or 3) bacterial flagellin interacting with the plant receptor. These attachments are believed to be the first step to internalization into the root of the plants.

Figure 2. Graphical Explanation of root anatomy and microbial interaction (Mandrell et al. 2006)



(A) Hair root cell
(B) Nucleus
(C) Bacteria bound to epidermial cells (aggregates or biofilms)
(D) Rhizobial bacteria
(E) Root hair infection
(F) Curling root hair tip
(G) Cortex cell
(H) Junction between epidermal cells
(I) Bacteria binding as a singles cells
(J) Magnification of I
(J-1) Pili/Fimbriae binding
(J-2) Bacterial carbohydrate binding
(J-3) Bacterial flagellin bonding

E. coli O157:H7 attachment has been studied specifically within the root environment. Jeter and Matthysse (2005) found that this strain can attach to the epidermis of the root (root hairs and seed coat) in *Arabidopsis*, showing that this strain can attach according to the different mechanism explained by Mandrell et al. (2006). Also, this makes sense when considering results reported by Hora et al. (2005), which showed that nematode or mechanical damage do not enhance the internalization of *E. coli* O157:H7 in spinach. It

was also observed that internalization occurred in all the samples tested and that it occurs in undamaged parts of the roots showing that *E. coli* O157:H7 can internalize with the epidermal root surface intact.

Usually, antimicrobial compounds are inside of plant cells or in specialized cells (Bartz 2006). Nonpathogenic plant bacteria do not damage cells tissues of plant; therefore, their immune response does not attack these type of bacteria (Bartz 2006). This observation that *E. coli* O157:H7 do not penetrate the cells were observed by Auty et al. (2005). In this study, *E. coli* O157:H7 was found in the intracellular space using scanning micrograph, in depth of 60-80 μm. This could be the same pattern described by Figure 2 (H). Attachments could differ depending on the food matrix, making the internalization really specific for each produce (Auty et al. 2005).

According to the literature, *E. coli* O157:H7 can attach and become internalized in the root. However, it is still unknown if the bacteria can use the vascular tissue to migrate to the leaf tissue (Solomon and Sharma 2009). Even though this potential use of the vascular system as a transported system is not clear; some investigators suggest that this could happen primarily in seedlings rather than in mature plants (Jablasone et al. 2005).

Arabidopsis thaliana (Thale cress) has been used as a plant model to observe the colonization by *E. coli* O157:H7 and *Salmonella*, and it has been found that *E. coli* O157:H7 can colonize the entire plant, including seeds and flowers, in the absence of competition using the roots as a way of infection (Cooley et al. 2003). One explanation is

that *E. coli* O157:H7 reaches the vascular system and spreads throughout the whole plant. This is an important fact because seeds are a known contamination vector (Berger et al. 2010), extending the contamination cycle to the next generation. This also could explain the mechanism of contamination of alfalfa seeds previously described.

When using contaminated soil and subsequent surface sanitization of lettuce (Zhang et al. 2009), investigators have found that *E. coli* O157:H7 internalizes at a rate of 0.3%. In this study it is important to note that soil contains the original endogenous microflora which may have out-competed *E. coli* O157:H7 (Cooley et al. 2003; Zhang et al. 2009).Vidovic et al. (2007) found that *E. coli* O157:H7 survive better in autoclaved soils than in un-autoclaved soils, apparently due to the low endogenous microflora of the soil which may have an antagonistic effect on the survival of *E. coli* O157:H7. These results were also supported by Cooley et al. (2003) who found that *Enterobacter asburiaer* out-competes *E. coli* O157:H7, resulting in a decrease of their internalization in the plants. Other bacteria, such as *Pseudomonas* can also inhibit the growth of *E. coli* O157:H7 when the pathogen is in small quantities (Johannessen et al. 2005). Studies conducted by Zhang et al. (2009) and Johannessen et al. (2005) in lettuce using soil with their natural micro-flora did not find a high transmission rate of *E. coli* O157:H7, apparently due to competition. Johannessen et al. (2005) found that contamination by *E. coli* O157:H7 could occur in prevalences ranging from 0 to 2.6% of the cases using a 95% confidence limit; but this contamination rate only applies when lettuce has reached the seedling stage, when they may be less susceptible to uptake of *E. coli* O157:H7. Even though these results are more conservative, it is important to note that *E. coli* has the tools to become internalized in

produce and therefore the possibility of causing disease exists. Rhizobial microflora appears to play a key role in the internalization of human pathogens in produce.

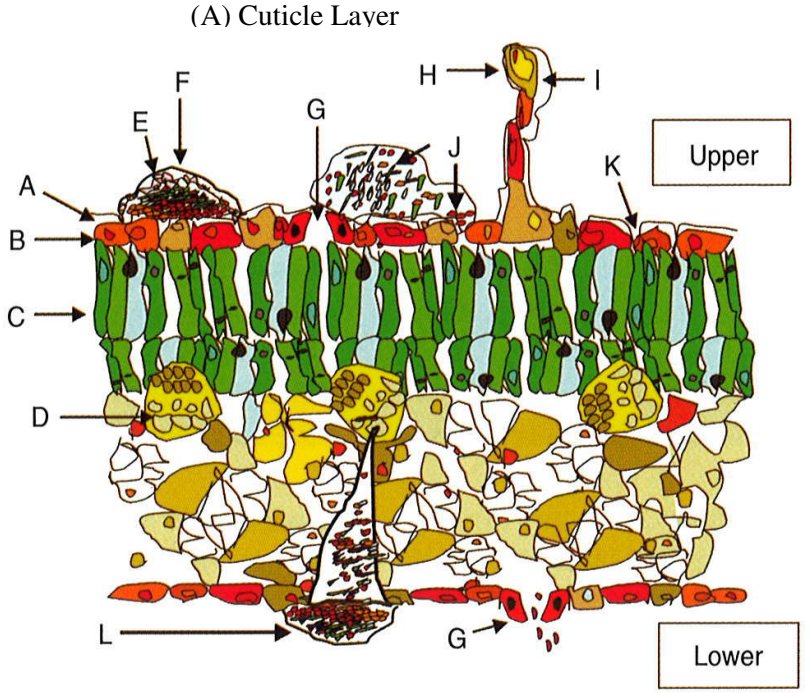
6.2 Via leaves

Other contamination sources include air movement during rainfall, in which floating bacteria can be transported to crop in the field (Bartz 2006). According to Mandrell et al. (2006) the phyllo-plane is the interface between the leaves and the environment. To understand how pathogenic bacteria interact with the leaves, it is necessary to understand the different parts the leaves that could influence the attachment or the survival of some bacteria in the phyllo-plane. Usually leaves are covered by a cuticle and other polymers or wax, the main purpose of which is to avoid the loss of water. Some bacteria such as ephiphytic strains can attach to this cuticle and obtain nutrients without damaging the surface (Mandrell et al. 2006). Also the microscopic topography of leaves or other aerial parts are irregular and epidermal cell junctions could produce hills and depressions that could help the bacteria survive in this environment (Solomon et al. 2006).

E. coli O157:H7 can internalize via the stomata of the leaf and on shoots of other plants such as alfalfa (Jeter and Matthysse 2005). Figure 3 represents the different subparts that leaves contain and the possible attachment of bacteria on surface of the leaves. As shown, it is believed that bacteria can form aggregates on the surface of leaves in different concentrations and commonly are reported at the base of the trichome structure, or near the stomata and epidermal cell grooves along the veins (Monier and Lindow 2004). This

could enhance the internalization of these bacteria due to their close distance and high concentrations near these natural openings.

Figure 3. Graphical explanation of anatomy of leaves and microbial interactions (Mandrell et al. 2006).



During irrigation or pesticide application, water can be a source of contamination in the field. For some produce such as lettuce, spray irrigation is used in nearly 50% of these crops in United States (Matthews 2006), making this a potentially significance source of contamination. Research has shown that *E. coli* O157:H7 can be internalized via natural openings such as stomata of the leaves (pores for gas exchange) as they lack the ability to penetrate the plant epidermis (Bartz 2006; Melotto et al. 2006). Although this route of contamination is more complex for the bacteria, it has been shown to be possible (Monier and Lindow 2004; Bartz 2006).

Other investigators have a slightly different theory. They believed that human pathogens and some epiphytic bacteria who are members of the family *Enterobacteriaceae* have certain attachment factors that are multifunctional depending on the host (Delaquis et al. 2007) similar to the previous described in the root attachments. To colonize and be transmitted to plants, bacteria first need to be attached in the root section (Berger et al. 2010). The mechanism as to how these factors work in the attachment process are not well understood, but is believed that some of these mechanisms used to attach to animal cells have some role in attachment to plant cells.

According to Boyer (2006) and Berger et al. (2010), Shiga toxin-producing *E. coli* has at least 3 different ways to be attached to plant leaves:

1. Curli mediated attachment
2. Filamentous type III secretion system
3. Flagellin
4. Other (lipopolisacharides)

Curli mediated attachment is described as an extracellular structure, very thin and oily, present on the surface of most *E. coli* (Patel et al. 2011). This method of attachment could be used for some crops such as Alfalfa roots, spinach leaves, lettuce, cabbage, and tomato skin (Jeter and Matthysse 2005; Patel et al. 2011; Macarisin et al. 2012). *E. coli* O157:H7 can attach in a small period of time, such as five minutes, with increasing attachment occurring with more time (Patel et al. 2011). As it was described, other attachments factors such as: Fimbriae (Pili) could play a role in the process. Fimbriae is a proteinaceous hair-like appendage that is common in gam-negative bacteria (Solomon et al. 2006). Flagellin is conserved in many entero-pathogenic bacteria such as *E. coli* O157:H7 and *Salmonella* and could also have an effect in the attachment plants (Cooley et al. 2003). It is important to note that these methods of attachment are not only useful in leaves but also in other regions such as the root or fruit of the plants (Berger et al. 2010).

Studies have shown that internalization in lettuce can occur when the surface of leaves are exposed to high concentrations of *E. coli* O157:H7 (greater than 4.4 log CFU) (Solomon et al. 2002b; Erickson et al. 2010a). Although the percentage is low (<1%),

when *E. coli* O157:H7 colonized alfalfa leaves by crossing the stomata, the bacteria could not be removed by washing (Jeter and Matthysse 2005).

Related studies show that in lettuce leaves, *Salmonella enterica* can use the stomata as an effective portal to enter deep into the tissue; an observation that was detected by confocal microscopy (Kroupitski et al. 2009). Internalization of *Salmonella* will occur in higher rates when the stomata are open, an event that occurs when light is present due to the plant needing to undergo gas exchange, thereby allowing *Salmonella* to spread to nearby tissue (Kroupitski et al. 2009). It is interesting to observe that in some cases *Salmonella* spp. will make micro-colonies near the vein and base of leaves, and this could be explained by bacterial chemo-taxis because the glandular trichomes can exudate carbohydrates. Itoh et al. (1998) also found that radish was colonized by large numbers of *E. coli* O157:H7 apparently via the stomata of leaves.

Moreover, it has been found that some bacteria (*Pseudomona* spp.) can move toward selected, opened stomata and the plant (*Arabidopsis*) reacts to this action by closing the stomata (Melotto et al. 2006). To test this hypothesis, *E. coli* O157:H7 was introduced to determine if this reaction can occur under controlled environment (Melotto et al. 2006). It was found that the closing of the stomata was indeed induced by *E. coli* O157:H7, showing the defensive reaction of the plant as soon as two hours of incubation (Melotto et al. 2006). This is supported by other early studies that found *E. coli* O157:H7 to induce the stomata on lettuce leaves to close (Seo and Frank 1999).

This mechanism of internalization for *E. coli* O157:H7 has also been studied in spinach. In simulated irrigation experiments, although investigators could not detect internalization of bacteria immediately (day 0), they did find it was present internally in the leaves at day 7 post-inoculation with a rate of 20% (Mitra et al. 2009). This level then declines to 0.5% at 14 days post irrigation with contaminated water (Mitra et al. 2009).

E. coli O157:H7 has the capacity to survive in harsh conditions such as UV irradiation, and low water availability (Barker-Reid et al. 2009). This allows it to compete with the epiphytic and phytobacteria present in the phyllospheres and rhizospheres (Fink et al. 2012). Certain transcriptional genes are affected due to environmental stress, and this gene expression enhances the ability of *E. coli* O157:H7 to survive the stress condition in the leaves of lettuce (Fink et al. 2012). Heaton and Jones (2008) report that *E. coli* O157:H7 may have ability to survive some UV irradiation due to the presence of a gene homologue to *rul*AB; other studies have shown this gene to improve tolerance of UV irradiation in *Pseudomonas syringe.*

In some studies, the survival of *E. coli* O157:H7 has been shown to be greater than 20 days in lettuce after inoculation (Solomon et al. 2002a). *E. coli* O157:H7 has a greater chance of surviving until harvest if contamination occurs in mature plants rather than in young, even in small concentrations (< 4 log CFU/ ml of soil or water) (Mootian et al. 2009) (Solomon *et al.* 2003). *E. coli* O157:H7 and *Salmonella* Typhimurium persisted for

long periods (3-6 month) in phyllospheres after the seedling of parsley and lettuce were exposed to these pathogenic bacteria using manure and irrigation water (Bartz 2006).

6.3 Via fruit and flowers

Similarly, other studies show that fruit can be contaminated by *E. coli* O157:H7 both internally and externally. According to Burnett et al. (2000), contamination in fruit may occur as a consequence of rain, dew or irrigation systems. One particular study suggests that *E. coli* O157:H7 can be internalized in oranges using the stem scar as the route to the interior of the pulp at a rate around 2.5% (Eblen et al. 2004).

E. coli O157:H7 has been detected inside parts of apples as a consequence of surface contamination (Burnett et al. 2000). This strain could become internalized into core of the apple using the floral tube of the apple. As a result, the bacteria were found near the trichomes, crevices, cavities and seed locules of intact apples (Burnett et al. 2000).

Doyle and Erickson (2008) suggest that these ways of internalization are not the only ones that have a key role on the possible infection. They suggest that bacterial contamination can persist depending on the time period from inoculation to harvest explaining that the longer times decrease the likelihood of survival and risk of contamination.

In summary, the literature indicates that the possibility exists that *E. coli* O157:H7 can survive in different types of produce and become internalized via 3 different routes: by

the root, leaves or the fruit of some plants. It is critical to analyze the interaction between the pathogen and the plant environment in order to better understand the routes of plant contamination resulting in food borne illnesses.

7. Interventions strategies to control pathogens in produces

Intervention strategies have been used to reduce bacteria in fresh produce and they may involve washing with 100-200 ppm of hypochlorite (Seo and Frank 1999) . This and other antimicrobial treatments need to be in contact with the bacteria in order to be effective. Therefore, if the bacteria are inside the plant structure the chemical agent cannot reach them and bacteria will survive. Also, when the contamination is present, the reduction at best is only around 2 log (Warriner et al. 2003a). Moreover, some products such as seeds will be difficult to decontaminate because the seed will increase in moisture and they will start to germinate.

8. Wheat contamination

Wheat is usually considered a low risk product for pathogens because the attributes of the grain are not suitable for the growth of *E. coli* O157:H7. The water activity is low in the kernels (0.40-0.65) and in flour (0.68-0.70), but that does not mean that *E. coli* O157:H7 cannot survive in it (Berghofer et al. 2003). Wheat can be contaminated with *E. coli, Salmonella spp.* and *Bacillus cereus* (Kim et al. 2006). Furthermore, wheat flour can be contaminated if it is produced from contaminated grains. In the US, it was determined that 12.8% of flour and 22% of durum flour were contaminated with pathogenic bacteria (Aydın et al. 2009). In other countries as Turkey, 50.7% of the surveyed samples tested

positive for *E. coli* (Aydın et al. 2009), indicating a high probability of the presence of pathogenic strains as well. The Department of agriculture of the United States (USDA 2010) reported that the United States is a major wheat-producing country, with output typically exceeded only by China, the European Union, and India. United States is also a major consumer of wheat.

Microorganisms present on the wheat plant survive the milling process and ultimately could end up in the wheat flour. Surveys indicate that the normal load of microorganisms (aerobic plate counts) in white flour produced in North America has been almost the same from 4.17 log CFU/g in 1989 to an average of 3.79 log CFU/g in 2003-2005 (Sperber 2007). However in whole wheat flour the microbial load is higher, with an average of 4.42 log CFU/g (Sperber 2007).

Evidence from Australian, European and US studies indicates that some outbreaks can be linked to wheat as the source of contamination (Aydın et al. 2009). According to CDC (2009) and Neil et al. (2012), *E. coli* O157:H7 was linked to a multistate outbreak for consumption of raw cookie dough producing 77 cases of illness and although 10 people developed HUS, none of them died. Historically, this is the first time that raw dough was reported as the vehicle of transmission of *E. coli* O157:H7. It is important to note that the people involved in the outbreak consumed the dough uncooked. Raw product consumption is not an unexpected behavior by the customers, in this outbreak around 11% of patients declared they ate the cookie dough and some of them explained that they have the intention to eat it unbaked (Neil et al. 2012). According to this investigation,

around 3.5 million packages of cookie dough and reformulated products were subjected to a recall (Neil et al. 2012). At least 157 unopened samples tested negative for *E. coli* O157:H7. Although an extra two additional samples were positive according to company's testing program, none of these products were shipped to the stores showing the possibility that flour could be a vector of transmission (Neil et al. 2012).

Other outbreaks have also been associated with the consumption of raw flour. *Salmonella* spp. has been found in raw flour in New Zealand and was the cause of an outbreak resulting in 66 cases of illness (Eglezos 2010). *Salmonella* Typhimurium in cake batter ice cream caused a food borne outbreak in 2005 in the U.S. (FDA, 2005). Again, it was suspected that the cake batter being added to the ice cream was contaminated (Sperber 2007). Gilbert et al. (2010) reported that *Salmonella* can be present in the range of 0.0% to 1.3% in flour based on surveys from Australia and North America. Although pathogens tend to have low counts in this kind of product*;* there is enough to cause illness when the flour is eaten raw.

Contamination of flour by microorganisms is not historically significant, because traditionally flour or basic product are been baked. This heat treatment would normally eliminate any pathogenic bacteria. These outbreaks demonstrate that the food preparation behavior of the general public is changing; particularly in the way of flour is consumed. The consequence of this behavior is the increase of foodborne illnesses. New legislation and guidelines were issued by the government after these outbreaks to try to reduce the

risk of illnesses by requiring the label of flour products to read, “Need to be baked for safety”.

Peak water requirement in wheat is during the formation of the heads (Yonts et al. 2009); hence irrigation water seems necessary to achieve this requirement during drought. Yonts et al. (2009) suggest that this peak is equivalent to 2 inches of water per week in this stage, or in other words 254 m^3 of water per hectare. According to the Environmental Protection Agency of the United States, western states use more water for irrigation (>50%) especially areas such as Nebraska, California, Great Plains and western Kansas (data from 1997). As it was discussed before, irrigation water can be contaminated with *E. coli* O157:H7 by runoff. Survey data taken in 2005 showed that 58% of the irrigation water came from surface water (USGS 2005) demonstrating a high risk that *E. coli* O157:H7 could reach water sources and may contaminate crops in the field.

Summary

The microbiological quality of water and soil is an important attribute that needs to be addressed when growing crops. Based on the literature, it is possible that *E. coli O157:H7* can be internalized in some crops. Currently, no studies have been done demonstrating that wheat can be contaminated with *E. coli* O157:H7 by water or soil contamination. It is important to determine if this possibility exists to improve the safety of flour by understanding where the potential sources of contamination may occur.

Objectives

This study was designed to determine if *E. coli* O157:H7 can be transmitted to the tissue or phylloplane of wheat, using contaminated soil or seed or irrigation water in four possible ways of contamination:

1. Determination of *E. coli* O157:H7 transmission into wheat plants by contaminated seeds.
2. Determination of *E. coli* O157:H7 transmission in wheat sowed in a contaminated soil.
3. Determination of *E. coli* O157:H7 transmission using contaminated irrigation water
4. Determination of *E. coli* O157:H7 survival in wheat heads contaminated by irrigation water

Chapter 2. MATERIALS AND METHODS

2.1 Bacterial strains and preparation of inoculum

Five different strains of *Escherichia coli* O157:H7 with resistance to Kanamycin (516 T2, 1181 lee3, 726 Lee2, 1809 Lee2, and Am 1882.3) were used for the experiments. A pure culture of each strain was stored in 10% glycerol at -80°C until needed. To generate the inoculum, 100 µl of each stock culture was transferred to test tubes containing 9 ml of Tryptic Soy Broth (Acumedia-Neogen corp.) with 50 µg/ml of kanamycin (Fisher BioReagents, N.J.) and incubated for 24 h at 37ºC. Three transfers were performed to allow recovery of the bacteria after frozen storage. *E. coli* O157:H7 were pelleted by centrifugation (Eppendorf 5810R, Eppendorf) at 5500 g for 10 min at 4 °C in 50 ml conical tubes and resuspended in Phosphate Buffer Solution (PBS) to a final cell density of $\geq 10^8$ CFU/ml. Equal volumes of 5 strains (10 ml) of *E. coli* O157:H7 suspensions were then combined in a 50-ml conical tube to produce a single cocktail.

2.2 Sanitization procedures

2.2.1 Seed and its sanitization

Seeds of two different wheat varieties, COOKER9835 and VAO3W-433, were obtained from the Plant Pathology Laboratory at the University of Nebraska - Lincoln. The COOKER 9835 variety is classified as a soft red winter wheat with susceptibility to *Fusarium* head blight. The VAO3W-433 variety is classified as a soft red winter wheat and also is resistant to *Fusarium* head blight. These two varieties were used in the seed, soil, and irrigation contamination experiments.

Seed sanitization was performed using the protocol described by Mitra et al. (2009). This protocol was used to eliminate all endogenous micro-flora present in the seeds that may out compete *E. coli* O157:H7. Seeds were submerged in 70% ethanol (1 min), followed by submerging them in 6% sodium hypochlorite (1 min), and finally three rinses of sterile distilled water were done to remove any remaining sanitizer. Seeds were allowed to dry for at least two hours inside of the biosafety cabinet and stored aseptically in sterile bags for subsequent use. The storage time was no longer than one day after the sanitization procedure was done.

For the head contamination experiment, the Wheaton variety was used. This variety is a hard red spring wheat that is also susceptible to *Fusarium* head blight (Zhang et al. 2008). For these experiments seeds were not sanitized and were cultivated with their natural microflora in the greenhouse.

2.2.2 Soil preparation and sanitization

Soil mix (33% of clay loam soil, 33% peat soil, 16.5% sand, and 16.5% vermiculite) was passed through a 4 mm pore size sieve. This mixture was pasteurized using steam as a heating media similar to the method used on the farm or green houses for horticulture applications (82 °C for 30 min). In addition to this procedure, this soil was autoclaved twice during a period of 1 hour at 121 °C to assure lower bacterial counts. Soil was distributed in a pot tray with 50 g of soil per pot.

2.3 Inoculation experiments

2.3.1 Seed inoculation

Sanitized wheat seeds were contaminated using two sterile aluminum pans containing pinhole perforations in the bottom and placed inside 200 ml beakers for containment. Two grams of seeds (around 30-35 seeds) were added to each pan inside the beakers. Seeds were submerged in the beaker containing 40 ml of phosphate buffered cell suspension (> 8 log CFU/ml) of the five-strain cocktail of *E. coli* O157:H7 for 40 minutes in a biosafety cabinet. Using a forceps, the pan was lifted and the inoculum was drainage into the bottom of the beaker without losing the seeds. The seeds were air dried on a sterile filter paper in a petri dish inside the biosafety cabinet for 2 hours with air flow of 0.3 inches/W.C. One gram of the seeds was retained for plating on TSA + kanamycin (50µl/g) to determine the initial counts on inoculated seeds. The remaining inoculated seeds were cultivated and grown according to standard protocols described for wheat, following biosafety precautions. The pots with contaminated seeds were housed in a water-bath equipped with a fluorescent lamp and the whole unit placed in Biosafety level

II laboratory (BSL-2). For this experiment, seedlings were grown for 9 days after planting and subsequently analyzed using *q*PCR detection kits.

2.3.2 Soil inoculation

Previously sanitized soil mix was inoculated with at least 6 log CFU/g *E. coli* O157:H7. To spread the culture homogeneously, the soil was placed in a tray at a depth of 1 inch and sprayed evenly with the bacterial inoculum using an atomizer. This procedure was performed inside of a Type II Biosafety cabinet. To control the inoculum used in the atomizer, a known volume of inoculum was placed into a sterile tube used to feed the atomizer. The volume sprayed was calculated according to soil present in the tray to achieve the desired population level (6 log CFU/g). For this experiment, seedlings were grown in this soil until they reached 9 days after inoculation and subsequently analyzed using *q*PCR detection kits.

2.3.3 Irrigation water inoculation

The effect of contaminated irrigation water was also analyzed during the germination period. For this experiment, sterile seeds were planted in sterile soil. The contamination procedure took place once, when contaminated water was added in a circular area around the seeds during sowing. Using a pipet tip, water was added carefully into an area of 1 cm in diameter from the center of the seed. This water was slowly infiltrated through the soil until it reached the seed. After 9 days, seedlings were collected, enriched, and analyzed by *q*PCR.

2.3.4 Head Inoculation

Plants were grown until they reached maturity for formation of heads, which is the flowering stage of wheat also known as 10.1. Using a laboratory atomizer similar to the soil contamination experiment, head were spray with 5 ml of culture (close to 4 log CFU/ml). Three different heads per plant were contaminated per pot assuring at least 3 log CFU per head. Some heads were removed after 24 hours and analyzed to determine the inoculation level achieved. At least three heads were contaminated and analyzed per plant to determine the survival rate of *E. coli* O157:H7 in the heads after 15 days. The usual time period between flowering and harvesting is of 20 days.

2.4 Growth conditions

Seeds were planted at a depth of 15 mm in the soil and watered daily with 5 ml of Butterfield's stock solution for germination of the seeds. The temperature ranged from 23.5 - 25.5 °C for all the experiments. Soil was placed in black-cell trays with a size of 5x4x5.7 cm. For the seedlings experiments (seed, soil, and irrigation experiments), 50 g of soil per cell was used to assure homogeneous conditions from one cell to another. The plants were grown by placing the cell-tray in a serological water bath (Bockel industries, Model 148007) retrofitted with a light supply (10-12 light hours per day).

For the head experiments, four seeds were planted in each 6 inch-pot. Plants were allowed to grow in the plant pathology greenhouse at University of Nebraska-Lincoln for around 70 days until they reached stage 10.5. The temperature in the green house ranged from 21°C (night-time) to 27° C (maximum day-time). Plants were watered twice a day (morning and evening) and were fertilized with water soluble 20-10-20, N-P-K fertilizer

(Peter's Professional Peat-Lite Special) at a concentration of 250 ppm, 5 days a week. Plants were transported to a BSL-2 laboratory for the inoculation experiments. After inoculation, plants were placed in a plant-growth incubator (Thermo-Scientific Precision) with a light that supplied 12 hours of light and a constant 23.7 °C temperature. These plants were watered every two days with 60 ml of potable water.

2.5 Plants preparation *E. coli* O157:H7 before detection step

2.5.1 Seed, Soil and irrigation experiments

After seeds germinated under the growth conditions described, seedlings were collected after 9 day post-inoculation. Wheat seedlings (from both varieties) were cleaved from the pots with a sterile scalpel and forceps. Seedlings were sanitized following the protocol used by Mitra *et al*. (2009). This protocol was used to eliminate the presence of *E. coli* O157:H7 outside of the plants as well as other bacteria on the surface that could interfere with detecting any *E. coli* O157:H7 on the present in the phyllo-plane. This consisted on submerging seedlings in 70% ethanol (1 min), following by submersion in 10% sodium hypochlorite (1 min). Finally three rinses with sterile distilled water were done to remove any remaining sanitizer. The cleaved and sanitized seedlings from seed and soil experiment were cut into small pieces (1cm) inside of a sterile bag and diluted (approximately 1:10) using mTSB + Novobiocin as enrichment. The bag was placed in a stomacher for 2 min and finally incubated for 48 hours at 37 ºC.

For the irrigation water experiment, sanitization was performed as described for the seed and soil experiments. However, after sanitization the seedlings were macerated using a

tissue homogenizer. The macerated seedling was then diluted 1:10 with mTSB + Novobiocin as enrichment in a sterile bag and incubated at 37 °C for 48 hours as described in soil and seed experiment.

2.5.2 Head contamination experiment

For the experiment with wheat heads, the sanitization procedure previously described was not applied due to the interest in evaluating the survival of *E. coli* O157:H7 in the heads. After 15 days, heads were cleaved and broken into small pieces inside of a sterile bag and diluted (1:100) with mTSB + Novobiocin as enrichment media. The bag was then placed in a stomacher for 2 min. From this dilution, enumeration analysis was performed to determine the bacterial population on each wheat head. Finally, the enrichments were incubated for 48 hours at 37ºC to confirm the presence of *E. coli* O157:H7 by *q*PCR.

2.6 *E. coli* O157:H7 detection

Following enrichment, samples were tested for *E. coli* O157 using the AOAC validated method known as Foodproof® *E. coli* O157 detection kits (Hybridization probes-LC 2.0) manufactured by Biotecon Diagnostic. All analyses were conducted using a Roche LightCycler® 2.0 carousel-based system. The reagents in the kit were stored at -20 °C and contained the polymerase enzyme, primers, templates, internal controls, master mix and purified water that was used as a negative control. To extract the DNA of the bacteria present in the enrichments, Foodproof® StartPrep kits containing a lysis buffer were used.

To avoid false positives, Ethidium Monoazide-Loop mediated isothermal amplification method (EMA-LAMP) was used to eliminate amplification of DNA from dead cells. This Ethidium Monoazide-Loop method is known by its commercial name "Reagent D" (Biotecon Diagnostic, Potsdam, Germany). This reagent has the ability to penetrate into *Enterobacteria* dead cells presented in 100 µl bacterial enrichment culture. In the presence of light, the substances present in this solution form a covalent bond with DNA of dead cell. This action prevents the DNA of dead cell from being amplified via PCR methods (Biotecon 2012). Moreover, the presence of light also inactivates the remaining compounds, thus eliminating the possibility that these substances could interfere with the DNA that is going to be extracted. After this step, DNA extraction of the live cells was performed for *Escherichia coli* O157:H7.

The DNA procedure to avoid false positives and extraction of the DNA from live cells is described in the following steps:

1. Enrichments were shaken gently and let settle for 5-10 min.
2. 100 µl of enrichment cultures (supernatant) were transferred to 1.5 ml reaction tubes.
3. 300 µl of "Reagent D" were pipet into 1.5 ml (transparent) reaction tube previously added with enrichment.
4. This tube was incubated in the dark for 5 min at room temperature.
5. A high power halogen lamp was used to expose the reaction tube to light. The tube needs to be placed 20 cm from the light (on ice) to minimize any increase of

temperature in the sample. The light needs to pass through the cap and reach the sample.

6. Reaction tube was centrifuged for 5 min at 8,000 g.
7. Supernatant was removed immediately after centrifugation and discarded.
8. 200 µl of Lysis buffer were added.
9. The pellet was resuspended by pipetting the contents of the reaction tube gently up and down.
10. Tubes with suspension were incubated on heating units for 10 min at 95-100 °C using a heating block (Thermo mixer).
11. Reaction tubes were carefully removed from the heating units and allow to sit.
12. Tube was mixed by vortex for 2 seconds.
13. Tube was centrifuged for 2 min at 13,000 g. The supernatant now containing the extracted DNA used directly for the *q*PCR reaction. The sediments may inhibit the PCR and must not be used for the reaction.
14. Supernatant was transferred to a clean centrifuge tube, and cooled down to 4°C. This supernatant containing the DNA was stored for no longer than two hours at this temperature to assure better results.

2.6.1 PCR preparation

After the DNA extraction was done, samples were ready for *q*PCR analysis. The Foodproof ® *E. coli* O157 detection kits – hybridization probes (LC1.x, 2.0) - contains 5 different centrifuge tubes:

- Master Mix: This solution is prepared by the kit manufacturer and contains ready-to-use primers and hybridization probe mixes specific for *E. coli* O157 DNA and *E. coli* O157 Internal control (IC).
- Enzyme Solution: FastStart Taq polymerase and Uracil-DNA Glycosylase for prevention of carry-over contamination.
- Internal control: contains a stabilized solution of plasmid of DNA, for use as an internal amplification control only.
- PCR-Grade H_2O
- Control Template: This solution contains the DNA of *E. coli* O157 for the positive control to assure the accuracy of the *q*PCR.

To perform the *q*PCR, it was necessary to follow these instructions:

1. Micro-centrifuge tubes were allowed to warm up room temperature. After the solutions were thawed, the five tubes were briefly spun in a micro-centrifuge before opening. Finally, the centrifuge tubes were opened and mixed carefully but thoroughly by pipetting the contents up and down.
2. In a 1.5 ml reaction tube, PCR Mix was prepared by adding the following components described in the Table1. and then mixed gently by pipetting up and down. The volumes shown in Table 1. are based on a single 20 µl standard reaction.

Table 1. Preparation of PCR mix

Component	**Volume**
Foodproof® *E. coli* Master mix	13 µl

Foodproof® *E. coli* Enzyme solution	1 µl
Foodproof® *E. coli* O157 internal control	1 µl
Total volume	**15 µl**

3. Using a pipet, 15 µl PCR mix was transferred into each LightCycler capillary. For the samples of interest, 5 µl sample DNA were added to a capillary, which was sealed with a stopper. For the negative control, 5 µl of PCR-Grade H_2O were added to capillary, which was sealed with stopper. For the positive control, 5 µl Foodproof® *E. coli* O157 control template were added to a capillary, which was sealed with stopper.
4. Capillary tubes were placed into the adapters from the carousel Lightcycler®. This adapter was placed in a LC Carousel centrifuge for spinning the capillaries for 15 seconds.
5. The adapter was placed into the LightCycler® and the lid was closed.

Lightcycler® equipment was operated by the software Lightcycler® software 4.x. Food-proof *E. coli* O157:H7 detection kit recommended the following parameters described in Table 2 to perform this test.

Table 2. Parameters required for analysis by the Food-proof® *E. coli* O157 detection kit

Parameter	**Setting**
Seek temperature	30 °C
Default channel • During run • Analysis	Fluorescence channel 640 nm or 705 nm 640/back 530 nm
Florescence gains	Not required

"Max. Seek pos"	Enter the number of samples including controls
"Instrument Type"	6 Ch.: LightCycler ® 2.0
Capillary size	20 µl

The PCR programs used for the amplification of DNA as well as the cooling program are shown in Table 3.

Table 3. Program cycles for Food-proof® *E. coli* O157 detection kit

Pre-incubation			
Programs/ cycle program data	Value		
Cycles	1		
Temperatures targets	**Segment 1**	**Segment 2**	
Target/ Target temperature (°C)	40	95	
Hold/ Incubation time [h:min:s]	00:02:00	00:10:00	
Ramp rate/ temperature transition rate [°C/s]	20	20	
Sec target/ secondary target temperature [°C/s]	0	0	
Step size [°C]	0.0	0.0	
Step delay [cycles]	0	0	
Acquisition mode	None	None	
Amplification (of the target DNA)			
Programs/ Cycle program data	Value		
Cycles	45		
Analysis mode	Quantification		
Temperature targets	**Segment 1**	**Segment 2**	**Segment 3**
Target/ target temperature (°C)	95	59	72
Hold incubation time [h:min:s]	00:00:00	00:00:30	00:00:05
Ramp rate [°C/s]	20	20	20
Sec target [°C/s]	0	0	0
Step size [°C]	0.0	0.0	0.0
Step delay [cycles]	0	0	0
Acquisition mode	None	Single	None
Cooling (the rotor and thermal chamber)			
Programs/cycle program data	Value		
Cycles	1		
Analysis mode	None		
Temperature targets	Segment 1		
Target temperature (°C)	40		

Hold/incubation time	00:00:30
Ramp rate [°C/s]	20
Sec target temperature [°C/s]	0
Step size [°C]	0.0
Step delay [cycles]	0
Acquisition mode	None

After the cycles are completed, a positive or negative result is displayed based on the amplification signals given by the amplification curves. Moreover, all positive enrichments were streaked in TSA + kanamycin to assure viable *E. coli* O157:H7 was recovered. If the sample was not recognized as a positive by the the *q*PCR method, also traditional isolated techniques were used to confirm the results as negative.

2.7 Experimental and statistical design

E. coli O157:H7 internalization has never been studied before in wheat. Therefore, the first step in this process was to determine the probability of internalization in wheat in the different experiments (soil, seed or irrigation water). Each pot was an experimental unit (with one seed, soil and water during the growing period); which was assumed to be completely independent from each other. The target number of samples per experiment was 100 seedlings divided in two varieties; every variety with 50 samples tested. In each variety, seeds were planted in at least two different batches of 35 plants or more because of space limitations. From the seedlings that germinated, 25 were analyzed after 9 days of plating as previously described. A total of 50 negative control samples were included with these seedlings experiments.

For the wheat head inoculation experiment, 100 contaminated heads divided into three different batches were done using a single wheat variety. For this experiment, three heads were contaminated per plant, and at least 12 plants were used per batch. Thirty-three heads per batch were analyzed, along with 10 negative controls heads (without contamination).

After all of the experiments were performed, statistical evaluation of the data was done using Chi-squared analysis to determine of the samples from the different experiments were any different from the control samples. .

Chapter 3. RESULTS

3.1 Seed Contamination Experiment

Sterile seeds were inoculated with *E. coli* O157:H7 using the protocol described under material and methods. Analysis of bacterial contamination was performed using TSA + Kanamycin medium. Table 4 shows the contamination levels achieved for each batch. The results are expressed as the average in CFU log per gram of seeds.

Table 4. Levels of E. coli O157H7 in contaminated seed in Log CFU/g

Sample ID	**Log CFU/g**
VAO3W-433- Batch 1	6.16
VAO3W-433-Batch 2	6.60
COOKER9835-Batch 1	6.75
COOKER9835- Batch 2	7.22
Average ± standard deviation	6.68 ± 0.44

After seeds were contaminated, they were planted and analyzed after 9 days by *q*PCR to detect internalization in the seedlings. During the experiments, seedlings were cleaved 1 cm above the soil level to avoid contamination from the soil. After sanitizing these samples, they were enriched with mTSB as previously described (selective media for Enterohemorragic *E. coli*). Using these enrichments, amplification curves using *q*PCR

were generated as result (Figure 4). It is important to note that only when *E coli* O157 DNA is present, the fluorescence will be detected by the *q*PCR method.

The *q*PCR results are explained in Figure 4.Graphs A and B show amplification curves of *q*PCR for seedlings of the variety COOKER9835. Graph (A) presents 24 plant samples that were analyzed during this batch. Graph (B) shows the amplification curves for 26 samples tested for the second batch. Four negative controls (none inoculated seeds) are presented in these amplification curves. Moreover, positive controls (template control) and negative controls were performed to assure the accuracy of the *q*PCR reaction. A positive result (peak line) is present in each graph to indicate the correct performance of the method. From both graphs, it was observed only one positive result (graph B) out of 50 plants analyzed. Graphs C and D show amplification curves by *q*PCR for seedlings of the variety VAO3W-433. Graph (C) shows the analysis of 21 samples of this variety; while graph (D) shows the amplification curves of 25 samples. Four negative controls (non-inoculated) were tested during these experiments. Control positive (template control) and negative for the PCR reaction were also analyzed to determine the accuracy of the PCR reaction kit, as previously described. A positive result is present in Graph (D) showing that *E. coli* O157:H7 was present in the enrichments analyzed.

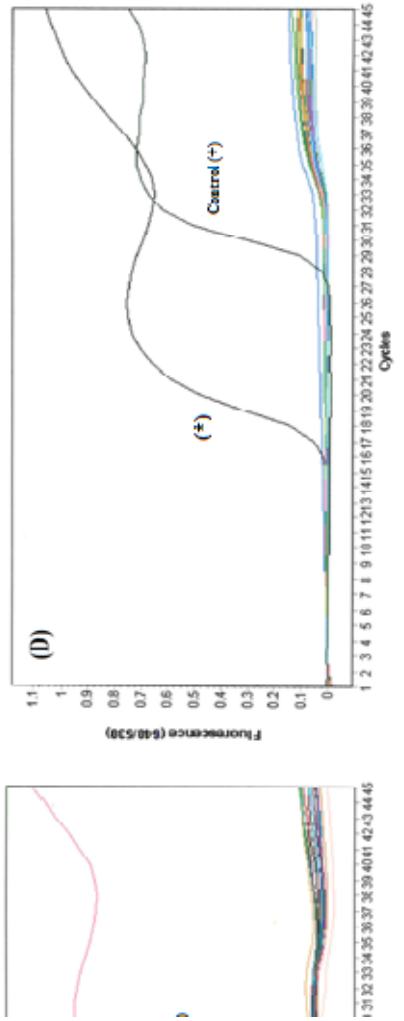


Figure 4. Amplification curves for contaminated seed experiment with *E. coli* O157:H7

(*) Samples tested positive in the *q*-PCR reaction

A total 96 plants were analyzed, including both varieties. COOKER9835 and VAO3V 3 showed one positive result for *E. coli* O157:H7 transmission. Negative control s (un-inoculated) were analyzed during this experiment and *E. coli* O157:H7 was not d in any of them (Figure 4).

E. coli ':H7 strains used in these experiments are kanamycin resistant that enabled the isolation of only the test strains from the enrichment broth. To perform this method, enrichments were plated out on Tryptic Soy Agar (TSA) supplemented with 50 µg/ml of

Kanamycin used as a selective medium allowing the growth of only *E. coli* O157:H7. This method was used to confirm the PCR results in all enrichments.

Enrichments that were positive for *E. coli* O157:H7 by *q*PCR matched the results on TSA + Kanamycin resistant, showing presence and survival of viable cells of *E. coli* O157:H7. The presence of viable cells indicated the possibility and risk for product contamination with pathogenic bacteria.

According to the data collected, the probability that *E. coli* O157:H7 to be present or may become internalized in the phylloplane, is around 2% (2 positive samples found in both varieties tested). To confirm that *E. coli* O157:H7 was still present in the soil pot, a small samples of the complete pot contents (containing individual pot, soil and root) were analyzed. For this soil analysis, all of the samples were positive (3 samples), showing the ability of *E. coli* O157 to survive after 9 days post inoculation in the soil.

3.2 Soil Contaminated Experiment

Sterile seeds were sampled to confirm that the population of endogenous bacteria was eliminated after the sanitization process. During this procedure, seeds were submerged in alcohol and sodium hypochlorite, which allowed the removal of most of the initial microbial contamination. The final population on the seeds were below the detection limit (<10 CFU/g) (Data do not shown).

Sterile soil was contaminated to achieve at least 6 log of *E. coli* O157:H7 per gram. Enumeration of the organisms in the soil was done to assure that the target population of *E. coli* O157:H7 was reached. The results of this analysis are showed by replication and variety on table 5.

Table 5. Levels of *E. coli* O157:H7 in contaminated soil in Log CFU /g

Sample ID	**Log CFU/ g**
VAO3W-433-Batch 1	6.08
VAO3W-433-Batch 2	7.08
COOKER9835-Batch 1	6.11
COOKER9835-Batch 2	7.13
Average ± standard deviation	6.60 ± 0.58

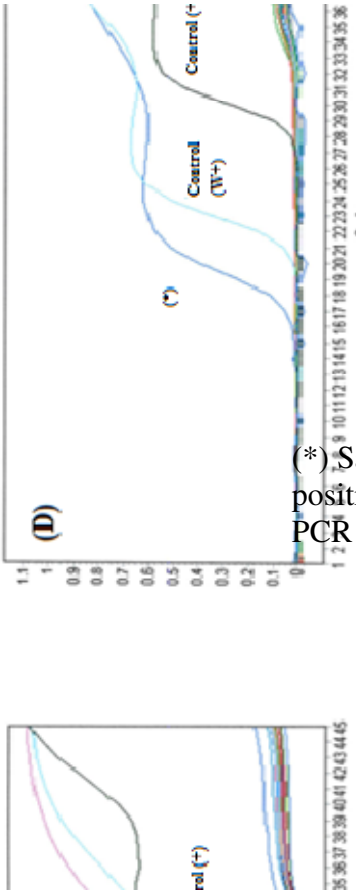
Seeds were planted and after 9 days and they were cleaved (as described before) and sanitized. Seedlings were then enriched for 48 hours and analyzed by *q*PCR. Results of the soil contamination experiments are shown in Figure 5.

In Figure 5, Graphs A and B show amplification curves of *q*PCR for seedlings of the variety COOKER9835 using contaminated soil. Graph (A) represents 25 plant samples that were analyzed during this batch. Graph (B) shows the amplification curves for 25 samples tested for the second batch. In both graphs, positive and negative controls for PCR were run to assure the accuracy of the *q*PCR reaction. In total 6 negative controls from wheat samples were tested. One extra positive control was run to ensure the accuracy of the DNA extraction used (control W+). According to the result of the

experiment, one positive result was observed in the first 25 samples (Graph A) and 2 positive results were found in the second replication. For the control samples, all of them were negative.

In the same figure, Graphs C and D show amplification curves by *q*PCR for VAO3W-433 Variety. Section (C) shows the analysis of 25 samples of this variety. Graph (D) represents the second replication of samples for this variety and in this case 25 samples were analyzed for the first batch. In both amplification curves, both positive (template) and negative controls were analyzed to determine the accuracy of the PCR reaction kit. To test the DNA extraction procedure, one extra control positive was run in each replication to demonstrate the extraction procedure was performing correctly. Graph C showed one positive result from 25 samples analyzed as well as one in Graph D. Therefore, two positive results were observed from 50 plants analyzed by the variety VAO3W-433. Six negative controls were tested, all of them were negative.

Figure 5. Amplification curves for contaminated soil experiment with *E. coli* O157:H7



(*) Samples tested positive in the *q*-PCR reaction.

Amplification curves showed that three samples from the variety COOKER9835 were positive for *E. coli* O157:H7 in a total of fifty samples analyzed. For the VAO3W-433 variety, two samples were positive from fifty samples analyzed. Therefore, the total number of positives was 5 out of 100 plants analyzed. A total of 12 negative controls (non-inoculated) were evaluated, all of them were negative as expected.

Moreover, according to the plating results using TSA with 50 µg/ml of Kanamycin, all of the positive results indicated by *q*PCR were positive as well on the TSA+ Kanamycin agar. This confirms that the bacteria were viable in the samples collected after the sanitization treatments.

In this experiment, *E. coli* O157:H7 appears to internalize at a higher rate than in the seed experiments. The rate of internalization in this experiment was close to 5% (3 positive samples found in the variety COOKER9835 and 2 positive samples found in the variety VAO3W-433 during this experiment) in comparison with the 2% observed in the experiment with contaminated seeds.

3.3 Irrigation Water Experiment

The irrigation experiment consisted of water application in the surrounding soil area where the seeds were located. Usually infiltration through the soil is necessary when the seeds are irrigated. Therefore, the importance of this irrigation experiment is critical to understand contamination routes through normal crop production. Using contaminated

water, *E. coli* O157:H7 were introduced into 50 g soil pots in which seeds were planted. For this experiment, three batches were performed due to low germination of wheat in one of the batches. Table 6 shows the contamination levels of *E. coli* O157:H7 in irrigation water used during this experiment in log CFU/g.

Table 6. Levels of E. coli O157:H7 in water used for irrigation in Log CFU/ml

Sample ID	**Log CFU/ml**
VAO3W-433-Batch 1	6.82
VAO3W-433- Batch 2	6.83
VAO3W-433- Batch 3	6.77
COOKER9835- Batch 1	6.59
COOKER9835-Batch 2	6.92
COOKER9835- Batch 3	6.65
Average ± standard deviation	6.76 ± 0.11

During this procedure, the sterile seeds were planted into sterile soil and the contamination occurred when 5 ml of contaminated water was pipetted onto the soil surface of each pot into a circular area. It is important to note that the addition of this contaminated water performed an extra 10-fold dilution, resulting in an actual contamination level about 6 log of *E. coli* O157:H7 in the soil.

With the same principle as the previous experiment, samples were analyzed after 9 days using the sanitization procedure previously described. The amplification curves are shown in Figure 6, indicating that five positive results were found in each variety from 100 plants analyzed.

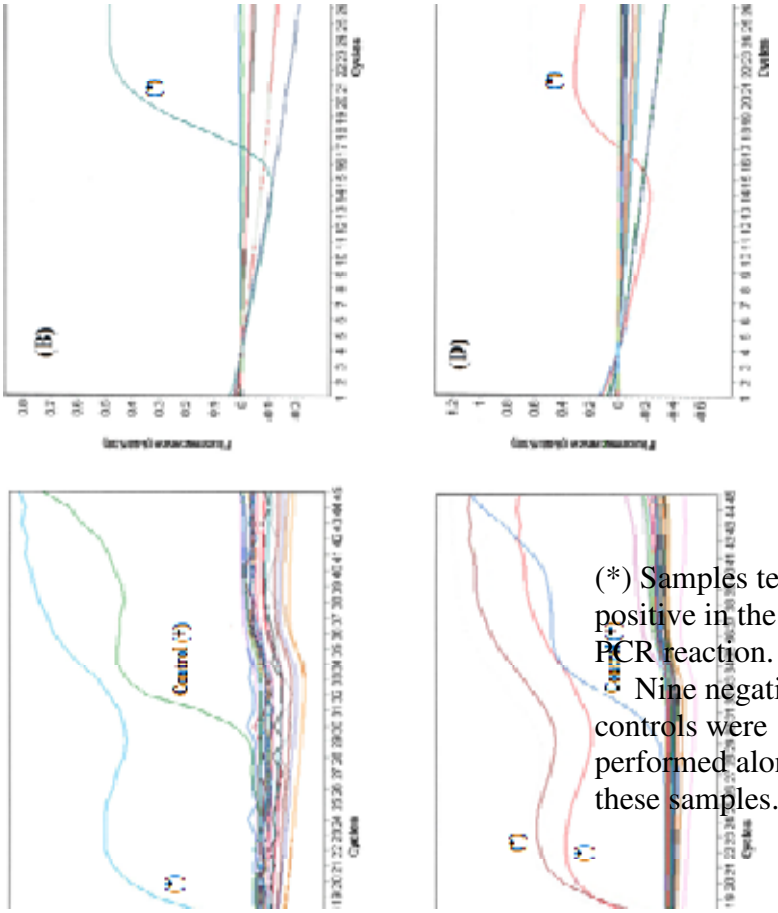
Moreover, TSA + Kanamycin analysis showed that all of the positive results determined by *q*PCR were confirmed by the plating method. The ratio of internalization of this experiment was 10% (10 positive samples from 100 samples analyzed during this experiment).

Figure 6 shows the amplification curves for irrigation water experiment. Graphs A, B, C and D shows the results for COOKER9835 variety using irrigation water as a source of contamination. Graph (A) represents 18 plant samples that were analyzed during the first batch. Graph (B) shows the amplification curves for 7 samples tested as a continuation of this batch. Graph (C) shows 15 samples tested in the second batch. Graph (D) shows 10 samples tested in the third batch. In all of the graphs, positive controls and negative controls for PCR were performed to assure the accuracy of the *q*PCR reaction. Nine negative controls were analyzed. According to these amplification curves, five positive results were found in 50 samples for the variety COOKER9835 analyzed. All of the negative controls were negative.

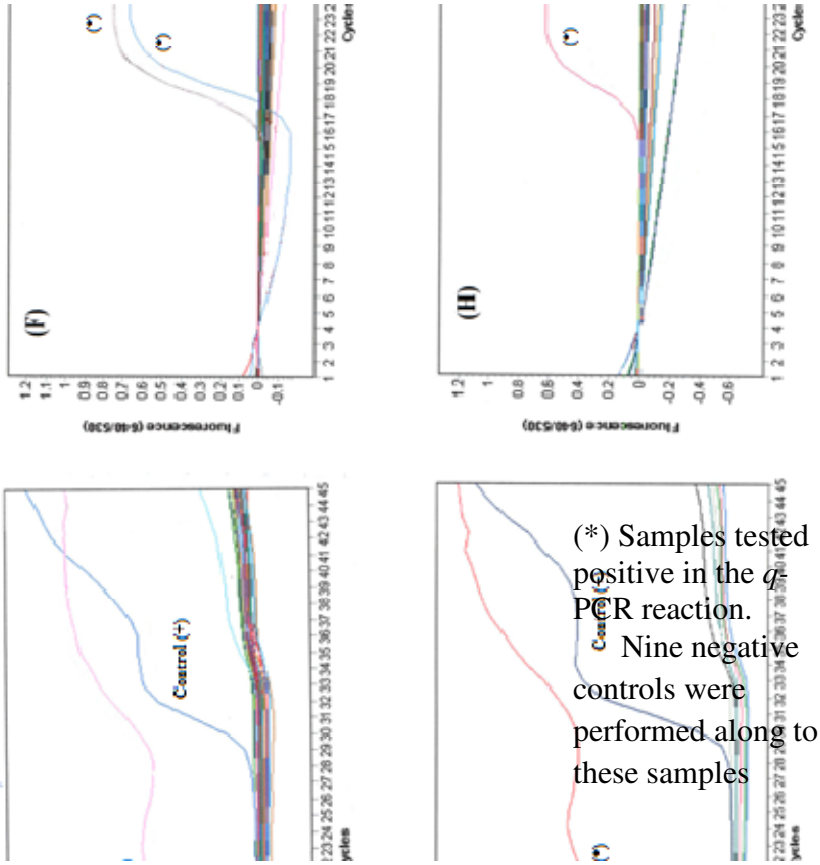
In the same figure, Graphs E, F, G and H shows the *q*PCR amplification curves for the VAO3W-433 variety using irrigation water as a contamination source. Graph (E) represents 25 plant samples that were analyzed during this replication. Graph (F) shows the amplification curves for 12 samples tested. Graph (G) shows results from 3 samples tested. Graph (H) contains 10 samples tested. In all of the graphs, positive and negative controls for PCR were performed to assure the accuracy of the *q*PCR reaction. A total of nine negative controls were tested. As a summary of these amplification curves, 5 samples tested positive (three for the variety COOKER9835 and two for the Variety

VAO3W-43) out of 50 samples analyzed and all of the nine negative controls were negative.

Figure 6. Amplification curves for irrigation water experiment



(*) Samples tested positive in the *q*-PCR reaction. Nine negative controls were performed along to these samples.



(*) Samples tested positive in the *q*-PCR reaction.

Nine negative controls were performed along to these samples

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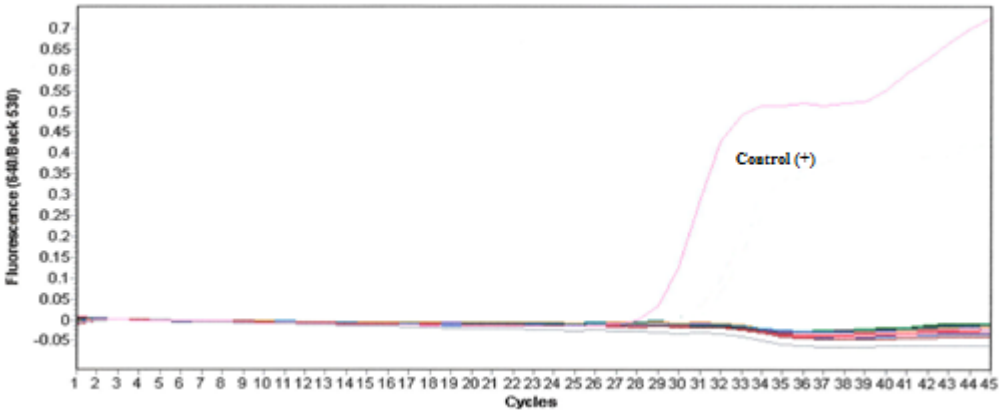
3.4 Control samples

Negative control samples were distributed in all of the experiments (seed, soil of irrigation water) and all of them where negative as expected. The numbers of controls presented in the amplification curves are: 8 control samples tested in seed experiment, 12 in the soil experiment and 18 during the irrigation water experiment. As previously described in the experimental design, 50 negative controls were tested; the remaining 12 samples are presented in the following graph. All of the negative controls were negative as expected. Positive controls were performed to assure the accuracy of the *q*PCR and it is shows as a single peak in the chart.

Figure 7. Amplification curves for remain control samples



3.5 Head contamination experiment

The Wheat variety used was Wheaton. This variety does not require vernalization (process in which the seed is kept in cold temperature, usually 4 °C, in the early stages of germination) to produce heads since it is a spring wheat. During the contamination procedure, heads were sprayed with a known population of *E. coli* O157:H7 and the source was contaminated water. Analysis was performed using TSA+ Kanamycin. The results obtained from contaminated water are shown in Table 7 in Log/g.

Table 7. Levels of *E. coli* O157:H7 in the contaminated water in Log CFU/ml used in the head experiment

Sample ID	Log CFU/ml
Water spray irrigation- Batch 1	4.9
Water spray irrigation- Batch 2	3.81
Water spray irrigation-Batch 3	3.85
Average ± standard deviation	4.19 ± 0.61

To determine the initial contamination level in the heads, analysis was conducted after 24 hours. It is important to note that the levels of contamination were at least 100 times higher than the levels in the irrigation water experiments (Table 8). This contamination difference showed that after 24 hour *E. coli* O157:H7 may grow on the surface of the heads of wheat plant. During the head contamination procedure, 5 ml were sprayed to each head, but not all were absorbed by the head. It was determined that was close to 120-200 µl collected by the heads. It is important to remark with this inoculum concentration used is 4.19 as showed in the Table 7. With this amount of inoculum is no way that *E. coli* O157:H7 could reach 6 log CFU/g in the head after 24 hours without

growth of *E. coli* O157H7 in the head during this time (Table 8). Therefore, this showed that *E. coli* O157:H7 could multiply during this first 24 hours.

Table 8. *E. coli* O157:H7 counts in log CFU/ml after 24 hours of inoculation

Sample ID	**Log CFU/g**
Head irrigated Batch 1	6.33
Head irrigated Batch 2	6.25
Head irrigated Batch 3	6.95
Average ± standard deviation	6.51± 0.38

After 15 days post-inoculation, *E. coli* O157:H7 still had high counts showing that this strain has the ability to survive in ambient conditions provided by in this experiment. Enumeration of *E. coli* O157:H7 on the heads using TSA + Kanamycin after 15 days of inoculation are shown in Table 9.

Table 9. Counts of *E. coli* O157:H7 in Log CFU/g in wheat heads after 15 days of inoculation

Sample ID	**Log/g (*)**
Head- Batch 1	5.89 ± 0.61
Head- Batch 2	5.98 ± 0.38
Head- Batch 3	5.69 ± 1.13

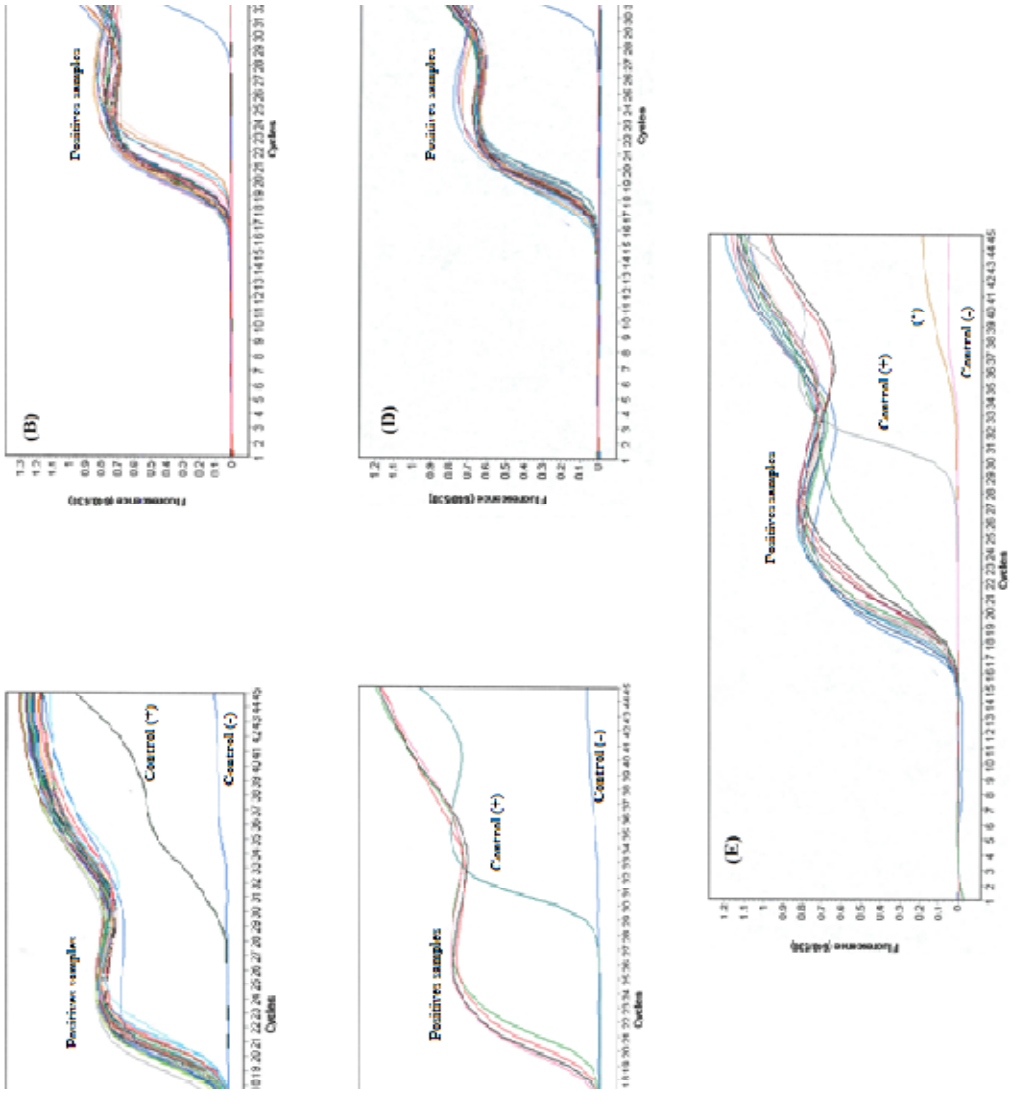
(*) Average and standard deviation were obtained from counts in samples in every batch.

These results indicate that *E. coli* O157:H7 can survive for long time (at least for 15 days) on the wheat heads in optimum conditions. The number of bacteria is surprisingly high, showing the possibility that these bacteria can survive until harvesting. Wheat heads samples were also analyzed as described before using *q*PCR. Amplification curves from the enrichments samples are showed in the Figure 8.

Graphs A, B, C shows *q*PCR amplification curves for the Wheaton variety in the head contamination experiment. Graph (A) represents 29 plant samples that were analyzed during this replication. Graph (B) shows the amplification curves for 25 samples tested as a continuation of this replication. Five positive controls were tested. Graph (C) shows four samples tested. All samples tested positive. Negative controls were negative as is showed in the graph B, showing there was no cross contamination between the samples.

Section (D) presented 25 plant samples were analyzed during this batch as well as 5 controls. Section (E) shows the amplification curves for samples fifteen samples tested. For both graphs showed that a positive control and a negative control. Results showed that *E. coli* O157H7 was presented in all of the case. Just one of the samples was tested negative for the *q*PCR. This sample was tested again using TSA+ Kanamycin and SMAC to assure that *E. coli* O157:H7 was presented and this media result showed positive results. Moreover, these samples were tested positive previously in the enumeration after 15 days.

Figure 8. Amplification curves for irrigation head contamination experiment



3.5 Statistical analysis

Data from all of the experiments were summarized in the Tables 10, 11 and 12, along with the results of the statistical analysis. Three different experiments with seedlings were analyzed using Chi square to evaluate if there were any statistical differences between the two varieties tested. Results showed that there was not a statistical internalization difference between varieties for the internalization of *E. coli* O157:H7.

Table 10. Results of internalization of *E. coli* O157:H7 in the experiments with seedlings along with statistical analysis by varieties

Experiment	Variety	Positive samples/Total of samples	% infection
Seed contamination	COOKER9835	1/50 [(a)]	2.0
	VAO3W-433	1/46 [(a)]	2.1
Soil contamination	COOKER9835	3/50 [(a)]	6.0
	VAO3W-433	2/50 [(a)]	4.0
Irrigation water contamination	COOKER9835	5/50 [(a)]	10.0
	VAO3W-433	5/50 [(a)]	10.0

(a, b, c) Varieties with the same letter did not show statistical differences using Chi square.

Since there were no statistical differences found between varieties, results from different varieties were combined to detect the differences between contamination methods and the control samples. The results of this analysis showed that irrigation water showed an internalization rate of *E. coli* O157:H7 that was higher than the control experiment (Table 11).

Table 11. Results of internalization of *E. coli* O157:H7 in the experiments with seedlings along with statistical analysis by contamination treatment and comparison with control samples

Experiment	Variety	Positive samples/Total of samples	% infection
Seed contamination	Both	2/96 (2)	2.1
Soil contamination	Both	5/100 (2)	5.0
Irrigation water	Both	10/100 (1)	10.0
Total Controls	Both	0/50 (2)	0.0

(1, 2) Treatment with the same numbers as the control, did not showed statistical differences using Chi square

Statistical analysis of the data from the experiment with heads, the results indicated that the survival of *E. coli* O157H7 was statistically different than the control (Table 12).

Table 12. Results of internalization of *E. coli* O157:H7 in the experiments with seedlings along with statistical analysis by head experiment

Experiment	Variety	Positive samples/Total of samples	% infection
Head contamination	Wheaton	98/98 (1)	100.0
Control	Heads	0/10 (2)	0.0

(1, 2) Treatment with the same numbers as the control, do not showed statistical difference using Chi square

Chapter 4. DISCUSSION

E. coli O157:H7 has been linked to the consumption of wheat flour in recent years. To date, no study has been conducted to determine the probability of *E. coli* O157:H7 internalizing into the phyllo-plane or vegetative tissue from possible contamination sources (seed, soil or irrigation water) in wheat plants. This research is the first attempt to answer this question.

According to the data collected, results showed that *E. coli* O157:H7 could be internalized into wheat seedlings at different rates, depending upon the contamination source. When seeds were contaminated, *E. coli* O157:H7 was internalized and translocate after 9 days post-inoculation into the wheat seedlings at a rate of 2%.

This is not an isolated observation; this behavior has been observed in different plant varieties and produce with *E. coli* O157:H7 and related microorganisms. In previous studies, *E. coli* O157:H7 was able to internalize into *Arabidopsis Thaliana* (Cooley et al. 2003), lettuce sprouts (Habteselassie et al. 2010) and bean sprouts contaminated with *E. coli* P36 (Warriner et al. 2003b).

Salmonella enterica and *E. coli* O157:H7 were shown to internalize in the root tip of *Arabidopsis thaliana* and move through veins of leaves and stems, using contaminated seed (Cooley et al. 2003). This seems possible for wheat; although the root was not tested during this experiment, *E. coli* O157:H7 was found in the tissue above the surface soil, meaning it had to arrive there using the root system. Unfortunately, the exact location where *E. coli* O157:H7 entered the plant was not determined during this experiment. Investigators have also suggested that *E. coli* may enter the root due to active motility or

simple diffusion. Once inside, bacteria will move up through the stem of the plant and finally into the leaves and flowers (Cooley et al. 2003). Seeds from contaminated *Arabidopsis* plants were tested and the recovery of *E. coli* O157:H7 after the complete cycle of production was around 26.7%, much higher than the rate reported in this study.

It is not well understood; however, *E. coli* O157:H7 becomes internalized and it is believed that these bacteria could reach the vascular system of the plant and produce a systemic infection without any plant response (Itoh et al. 1998; Bartz 2006). Researchers suggested that young plants could be internalized more efficiently by *E. coli* due to low plant defenses that are not well developed during germination when seed breakage occurs (Habteselassie et al. 2010). This may explain the occurrence of this internalization in wheat seedlings from contaminated seeds, soil or water.

E. coli O157:H7 also reached the internal parts of the plant through contaminated soil, with a slightly higher internalization rate (5%) observed in wheat seedlings after 9 days. A similar study showed that internalization can occur in produce as well using a similar method. Mootian et al. (2009) analyzed the internalization of *E. coli* O157:H7 in lettuce using low contamination levels (<4 log CFU/g) in soil after 12 and 30 days-old seedlings. For 12 day-old lettuce seedling, results showed no internalization from any of the samples tested after 10, 20, 30 days post-inoculation using contaminated manure or soil. For 30 day-old lettuces were contaminated at day 15th and results showed 4 out of 36 samples tested positive for the different levels of contamination. This represents an internalization level that could vary between 0% for 12 day-old lettuce to 11% for 30 day-old lettuce using contaminated manure or soil. This range seems in agreement with

the observations of internalization of *E. coli* O157H7 in wheat seedlings in this research when the soil was contaminated.

Solomon et al. (2002b) found that *E. coli* O157:H7 could be transmitted to the plant tissue and internalized (1 cm above the soil surface). Their results showed that *E. coli* O157:H7 was presented in 3 samples out of 16, when population of 6 log CFU/g was applied to the soil after 6 days post-inoculation. This result shows an internalization rate of around 18.75%. Similar results were found when the samples were tested at day 9 pos-inoculation. These experiments established that *E. coli* O157:H7 could become internalized (sub-surface tissue) using contaminated soil as a vector.

Looper et al. (2009) used slurry manure to contaminate tall-fescue (175 days old) using a population of 5 log CFU/g of *E. coli* O157:H7 in soil and similar results were found. For this study, inoculation of *E. coli* O157:H7 was done at the soil surface level. Samples were collected from the top (10 cm) of the plant tissue and disinfected with alcohol prior to analysis. The results showed *E. coli* O157:H7 translocated on 18% of the cases (9 out of 50 plants analyzed) after 14 days post-inoculation.

Other studies have showed the internalization of *E. coli* O157:H7 using contaminated soil (6 log CFU/ml) in spinach (Mitra et al. 2009). Leaves were analyzed 7 days post-inoculation using three different varieties of spinach and 1 positive sample out of 20 was detected in one of the varieties. This showed a relatively low rate of infection when soil or water were the source of contamination in spinach after 7 days. *E. coli* O157:H7 was found after 7 days at a rate of 5% using the BAX PCR method (without root inclusion)

and at 10% after 14 days using enrichments of the plants with root inclusion (Mitra et al. 2009).

The data from the experiments described in this research indicate that *E. coli* O157:H7 may have the ability to internalize and translocate from a contaminated soil matrix into the wheat plants. The internalization rate is much lower in the wheat experiment varying from the 5% reported by contaminated soil experiment in compared to 18% found in the literature. It is important to note that the studies with higher internalization rates tested plants 15 days post-inoculation, and this maybe a source of the difference in the results because *E. coli* O157:H7 may have had more time to translocate from the root to the stem. Even though the internalization rate showed by this research seems low, it is important to note that the seedlings had only 9 days and if more time was allowed the rate may have been increased.

Other factor that could make a difference in the internalization rate is the composition of the exudates in the rhizospheres microbiota (Jablasone et al. 2005). It has been showed that wheat exudates has some phenolic compounds (Bertin et al. 2003) that may inhibit the attachment and internalization of *E. coli* O157:H7, which could explain the lower internalization rate found in wheat in compared to produce.

For the last seedling experiments, irrigation with contaminated water was used as a source of *E. coli*. In these experiments, *E. coli* O157:H7 had an internalization rate of 10% in wheat seedlings at 9 days post-inoculation. This experiment showed the highest

internalization rate of among the different methods of contamination under evaluation. This was the only contamination method that was statistical different from the control.

The literature reports that *E. coli* O157:H7 can be internalized in plant tissue via contaminated water. Habteselassie et al. (2010) found that contaminated irrigation water (6 log CFU/ml population of *E. coli* O157:H7) resulted in contaminated lettuce after 10 days post-inoculation. *E. coli* O157:H7 was translocated to the leaves without direct contact with the irrigation water.

E. coli O157:H7 became internalized in lettuce at low levels (2 positive samples out of 24 at the day 10 post-inoculation) when this produce were contaminated at the time of 12 days-old (Mootian et al. 2009). This represents an internalization rate of 8.33%. This study also evaluated internalization of *E. coli* O157:H7 when contamination occurred after 30 days from germination. These plants were then tested after 15 days post inoculation, and in this case *E. coli* O157:H7 was internalized at a rate around 12.5 % (3 plants out of 24).

The internalization rates described in the literature when water was the source of contamination agrees with the observations made during the wheat irrigation experiment described here (10% internalization rate). The slight variations between the results described here and the values previously described in the literature could be attributed to different inoculation levels, different plant species or even different days of inoculation or protocols used of each experiment (size of samples).

For the last experiment, wheat heads were contaminated with irrigation water and the results showed that this strain could grow and survive in the wheat heads even after 15 days post-contamination in all tested samples. It is important to note that this experiment was done with the presence of the natural microflora of the wheat heads. The results of this experiment are interesting, because even though slightly less inoculum was added (around 3 log CFU per head), it was observed that *E. coli* O157:H7 can permit in the wheat heads at the levels of 6.51 log CFU/g after 24 hours post-inoculation. Considering that each wheat head weighted around 1 gram (data not shown), the levels of *E. coli* O157:H7 in the head expected to be around 3 log CFU/g after inoculation. This growth observed may increase the survival time of *E. coli* O157:H7 in wheat heads and also the probability of kernel contamination. After 15 days *E. coli* O157:H7 survival on wheat heads was 100% at this time, wheat plants are close to their harvest period, demonstrating the possibility that *E. coli* O157:H7 could contaminate the wheat kernels.

Field experiments have found survival of *E. coli* O157:H7 in the phyllo-plane on lettuce for more than 5 days when contaminated water was used for irrigation just before harvest (Barker-Reid et al. 2009). Erickson et al. (2010b) has also found that irrigation water could be a source of contamination. In their study, they found that inoculation levels play a key role in the internalization of *E. coli* O157:H7 (shigatoxin negative strains) in spinach. Three levels of contamination: low-dose (2 log CFU/ml), medium-dose (4 log CFU/ml) and high dose (6 log CFU/ml) were applied using a spray irrigation method. The results showed that immediately after contamination low inoculation levels showed no internalization of bacteria, but for high-doses (6 log CFU/ml) the internalization rate

was 25% of the samples immediately after contamination. This study showed that attachment to the phyllo-plane (surface of the leaves) can occur mostly immediately when high doses are applied (Erickson et al. 2010b). Moreover, they tested the survival of the bacteria on the leaves using high inoculum concentrations (10^8 CFU/ml) and it was observed that *E. coli* O157:H7 can survive until day 14 post-inoculation.

Other studies showed longer survival times for *E. coli* O157:H7 (non-shigatoxin producer strain); as long as 77 days on lettuce and 177 days on parsley (Islam et al. 2004). Similar results have been found in related studies using other produce. Solomon et al. (2003) found that sprayed populations (2 log CFU/ml) of *E. coli* O157:H7 could increase the population with each watering on the phyllo-plane of lettuce. Moreover, this study showed that even with low dose (2 log CFU/ml), *E. coli* O157:H7 can survive for at least 10 days on the leaves of lettuce after inoculation.

Produce leaves were believed to be a harsh environment for *E. coli* O157:H7; but as the evidence shows, *E. coli* O157:H7 can survive quite well. Comparing this environment to wheat heads would be appropriate since they are both under similar environmental conditions. Usually heads at the flowering stage (as was used in this experiment), are starting to open their hulls in order to produce the kernels. Therefore more cavities are exposed, in which *E. coli* O157:H7 could hide from light or dehydration, allowing these bacteria to survive well for 15 days after inoculation as showed in these experiments.

Important observations were gathered during these experiments, such as the ability of *E. coli* O157:H7 to increase their numbers during the first 24 hours post-inoculation in

wheat heads. This ability to grow on the plant surface has been seen previously by Solomon et al. (2003). This observation is remarkable because it may lead to internalize in wheat kernel as was previously discussed by Cooley et al. (2003).

Even though during this study the survival of *E. coli* O157:H7 after 15 days was determined in all of the heads, one of the experimental limitations was that kernels themselves were not analyzed. The quality of irrigation water plays a key role in the transmission *E. coli* O157:H7 into wheat kernels during the agricultural cycle production.

Future studies need to be done to determine the possibility that this strain could reach the kernel and potentially contaminated products such as flour. During wheat harvesting, combines harvester are used to de-hull the grains form the spikes by friction. Hence during this process, *E. coli* O157:H7 that may be contaminating the hulls could be transferred to the surface of the kernels, thereby increasing the risk that *E. coli* O157:H7 may reach the milling process.

Chapter 5. CONCLUSION AND FUTURE RESEARCH

Wheat is a cereal grain that is commercialized as a raw agricultural product and consumed by large populations as wheat flour. Recently *E. coli* O157:H7 has been linked to the consumption of wheat based products. However, no studies have been done to determine the probability of *E. coli* O157H7 to be internalized into wheat plants. This research is the first report analyzing the possible route of transmission of E. coli O157H7 into wheat phyllo-plane or vegetative tissue form possible contamination sources (seed, soil or irrigation water).

According to the data collected, *E. coli* O157:H7seems to have a the ability to be internalized into the tissues of wheat seedlings. Irrigation water experiments showed the highest frequency with 10% of internalization rate, followed by soil contamination experiment with internalization rate of 5% and seed contaminated with 2% of internalization. The experiment with irrigation water were significantly different from the control, while experiment with seed and soil were not significantly different. Literature has been found a slightly higher internalization rate in some cases using different crops; but since these experiment are really complex, they are really hard to make a judgment between them.

Researchers have reported internalization rates that vary from 0% to 18% in certain type of produce. However, comparisons are difficult due to their complexity. Small difference in strains (hydrophobicity, flagella, etc.), amount of inoculum, root exudate, sanitization and protocol procedure and inoculation time could make a difference in the internalization rate. Even though these differences can influence the outcome, most of

these studies assure that internalization of *E. coli* O157:H7 can occur even in low levels; in some cases similar or close to wheat experiments.

More investigation is necessary to determine the survival of *E. coli* O157:H7 internally in the wheat plant environment. No information is available showing the ability of *E. coli* O157:H7 could the vascular system yet in close related crops; therefore, the capacity of this strain internalized in the kernel of the wheat producing a contaminated seed is uncertain. Even though this experiment is not conclusive about if this route can reach the kernels in the maturity plants, it was demonstrated that *E. coli* O157:H7 could internalize in seedlings showing a possible window to investigate.

Surface head contamination was more relevant than internalization in seedling at day 9, since the survival rate and contamination were higher. Moreover, this experiment was conducted using lower levels of *E. coli* O157:H7 (4 log CFU/g) as well as the natural microflora, being most significant findings due to the similarities to the real environment. *E. coli* O157:H7 showed excellent survival on wheat heads, with a high probability of reaching the kernel. All samples were positive after 15 days, meaning 100% survival and recovery after this period. Head contamination seems to be the most likely route to reach the kernel, thus giving a possible opportunity to reach the milling process and ultimately reach the consumers. Crop management or good agricultural practices are important to control the microbial quality of the irrigation water due to the risk of contamination of *E. coli* O157:H7 based on the results found in this research.

In conclusion, the present study demonstrates that internalization of *E. coli* O157:H7 in wheat plant can occur using the conditions applied (sterile soil, absence natural microflora, ambient lighting, temperature, etc.) but it was rare. These conditions may not simulate the environmental conditions present in real-world crop production, hence this information may not be directly transfer to the field. The most important finding was that irrigation water on wheat heads (surface contamination), in which *E. coli* O157:H7 showed a high survival rate, is the most likely route of contamination in wheat plant in real environmental conditions. Furthermore, this study provided important information on understanding the possible routes of contamination of *E. coli* O157:H7 in wheat.

While these initial findings suggest that *E. coli* O157:H7 can be internalized into wheat at low levels, future research is necessary to understand if this could happen in the natural environment. A number of new approaches to address this issue are outlined below:

- Analysis and determination of internalization rate of *E. coli* O157:H7 in different stages of the wheat plant
- Determining the ability of *E. coli* O157:H7 to reach the vascular system in wheat and the possibility of reaching the kernel using the vascular system
- Determining the internalization during environmental conditions on-farm by applying a surrogate organism
- Using non-sterile soil as a possible variable to understand the effect the rhizobial microflora in the internalization rate in these plants

To analyze further the surface contamination on heads, more research is necessary to address to determine if this *E. coli* O157:H7 can reach the flour chain. New research addressing this possible contamination route of the flour could be include such as:

- Determination of the level of contamination on the kernel alone using real environmental conditions
- Determination of the attachment location of *E. coli* O157:H7 in the heads to determine if the kernel is contaminated.
- Determination of the ability of *E. coli* O157:H7 to survive during storage in wheat and whether it can survive the milling process and flour production
- Finally, if these contamination routes seems possible, intervention strategies and solutions to increase food safety in this products should be developed.

APPENDIX

1. Statistical analysis

1.1 Statistical Analysis by Variety using the same contamination method

Seed contamination experiment

```
Data contaminationSeed;
input Variety$ result$ count;
datalines;
Cooker positive 1
Cooker negative 49
Vao negative 45
Vao positive 1
;
PROC FREQ DATA=contaminationSoil;
WEIGHT count;
TABLES Variety*result/NOPERCENT NOROW NOCOL
EXPECTED CHISQ;
RUN;
```

The SAS System 18:48

The FREQ Procedure

Table of Variety by result

Variety Frequency Expected	negative	positive	Total
Cooker	49 48.958	1 1.0417	50
Vao	45 45.042	1 0.9583	46
Total	94	2	96

Statistics for Table of Variety by result

Statistic	DF	Value	Prob
Chi-Square	1	0.0036	0.9525
Likelihood Ratio Chi-Square	1	0.0035	0.9525
Continuity Adj. Chi-Square	1	0.0000	1.0000
Mantel-Haenszel Chi-Square	1	0.0035	0.9527
Phi Coefficient		0.0061	
Contingency Coefficient		0.0061	
Cramer's V		0.0061	

WARNING: 50% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Fisher's Exact Test

Cell (1,1) Frequency (F)	49
Left-sided Pr <= F	0.7730
Right-sided Pr >= F	0.7314
Table Probability (P)	0.5044
Two-sided Pr <= P	1.0000

Sample Size = 96

Soil contamination Experiment

```
Data contaminationSoil;
input Variety$ result$ count;
datalines;
Cooker positive 3
Cooker negative 47
Vao negative 48
Vao positive 2
;
PROC FREQ DATA=contaminationSoil;
WEIGHT count;
TABLES Variety*result/NOPERCENT NOROW NOCOL
EXPECTED CHISQ;
RUN;
```

The SAS System

The FREQ Procedure

Table of Variety by result

Variety / Frequency / Expected	negative	positive	Total
Cooker	47 47.5	3 2.5	50
Vao	48 47.5	2 2.5	50
Total	95	5	100

Statistics for Table of Variety by result

Statistic	DF	Value	Prob
Chi-Square	1	0.2105	0.6464
Likelihood Ratio Chi-Square	1	0.2119	0.6453
Continuity Adj. Chi-Square	1	0.0000	1.0000
Mantel-Haenszel Chi-Square	1	0.2084	0.6480
Phi Coefficient		-0.0459	
Contingency Coefficient		0.0458	
Cramer's V		-0.0459	

WARNING: 50% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Fisher's Exact Test

Cell (1,1) Frequency (F)	47
Left-sided Pr <= F	0.5000
Right-sided Pr >= F	0.8189
Table Probability (P)	0.3189
Two-sided Pr <= P	1.0000

Sample Size = 100

Irrigation contaminated experiment

```
Data contaminationIrrigation;
input Variety$ result$ count;
datalines;
Cooker positive 5
Cooker negative 45
Vao negative 45
Vao positive 5
;
PROC FREQ DATA=contaminationIrrigation;
WEIGHT count;
TABLES Variety*result/NOPERCENT NOROW NOCOL
EXPECTED CHISQ;
RUN;
```

The SAS System 1

The FREQ Procedure

Table of Variety by result

Variety	result		
Frequency Expected	negative	positive	Total
Cooker	45 45	5 5	50
Vao	45 45	5 5	50
Total	90	10	100

Statistics for Table of Variety by result

Statistic	DF	Value	Prob
Chi-Square	1	0.0000	1.0000
Likelihood Ratio Chi-Square	1	0.0000	1.0000
Continuity Adj. Chi-Square	1	0.0000	1.0000
Mantel-Haenszel Chi-Square	1	0.0000	1.0000
Phi Coefficient		0.0000	
Contingency Coefficient		0.0000	
Cramer's V		0.0000	

Fisher's Exact Test

Cell (1,1) Frequency (F)	45
Left-sided Pr <= F	0.6297
Right-sided Pr >= F	0.6297
Table Probability (P)	0.2593
Two-sided Pr <= P	1.0000

Sample Size = 100

1.2 Statistical Analysis respect to the control

Statistical analysis using Head experiment and Control

```
Data contamination;
input method$ result$ count;
datalines;
head positive 98
head negative 0
control positive 0
control negative 50
;
PROC FREQ DATA=contamination;
WEIGHT count;
TABLES method*result/NOPERCENT NOROW NOCOL
EXPECTED CHISQ;
RUN;
```

The SAS System 1

The FREQ Procedure

Table of method by result

method result

Frequency / Expected	negative	positive	Total
control	50 16.892	0 33.108	50
head	0 33.108	98 64.892	98
Total	50	98	148

Statistics for Table of method by result

Statistic	DF	Value	Prob
Chi-Square	1	148.0000	<.0001
Likelihood Ratio Chi-Square	1	189.3189	<.0001
Continuity Adj. Chi-Square	1	143.5636	<.0001
Mantel-Haenszel Chi-Square	1	147.0000	<.0001
Phi Coefficient		1.0000	
Contingency Coefficient		0.7071	
Cramer's V		1.0000	

Fisher's Exact Test

Cell (1,1) Frequency (F)	50
Left-sided Pr <= F	1.0000
Right-sided Pr >= F	1.122E-40
Table Probability (P)	5.496E-37
Two-sided Pr <= P	1.122E-40

Sample Size = 148

Statistical analysis using Soil and Control

```
Data contamination;
input method$ result$ count;
datalines;
soil positive 5
soil negative 95
Control negative 50
Control positive 0
;
PROC FREQ DATA=contamination;
WEIGHT count;
TABLES method*result/NOPERCENT NOROW NOCOL
EXPECTED CHISQ;
Run;
```

The SAS System

The FREQ Procedure

Table of method by result

method result

Frequency Expected	negative	positive	Total
Control	50 48.333	0 1.6667	50
soil	95 96.667	5 3.3333	100
Total	145	5	150

Statistics for Table of method by result

Statistic	DF	Value	Prob
Chi-Square	1	2.5862	0.1078
Likelihood Ratio Chi-Square	1	4.1404	0.0419
Continuity Adj. Chi-Square	1	1.2672	0.2603
Mantel-Haenszel Chi-Square	1	2.5690	0.1090
Phi Coefficient		0.1313	
Contingency Coefficient		0.1302	
Cramer's V		0.1313	

WARNING: 50% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Fisher's Exact Test

Cell (1,1) Frequency (F)	50
Left-sided Pr <= F	1.0000
Right-sided Pr >= F	0.1273
Table Probability (P)	0.1273
Two-sided Pr <= P	0.1698

Sample Size = 150

Statistical analysis using seed and Control

```
Data contamination;
input method$ result$ count;
datalines;
seed positive 2
seed negative 94
Control negative 50
Control positive 0
;

PROC FREQ DATA=contamination;
WEIGHT count;
TABLES method*result/NOPERCENT NOROW NOCOL
EXPECTED CHISQ;
RUN;
```

```
                          The SAS System                          1

                        The FREQ Procedure

                Table of method by result

method      result

Frequency
Expected  |negative |positive |  Total
----------+---------+---------+
Control   |      50 |       0 |     50
          |  49.315 |  0.6849 |
----------+---------+---------+
seed      |      94 |       2 |     96
          |  94.685 |  1.3151 |
----------+---------+---------+
Total     |     144 |       2 |    146


                Statistics for Table of method by result

Statistic                       DF       Value      Prob
------------------------------------------------------
Chi-Square                       1      1.0561    0.3041
Likelihood Ratio Chi-Square      1      1.6915    0.1934
Continuity Adj. Chi-Square       1      0.0770    0.7814
Mantel-Haenszel Chi-Square       1      1.0489    0.3058
Phi Coefficient                         0.0851
Contingency Coefficient                 0.0847
Cramer's V                              0.0851

 WARNING: 50% of the cells have expected counts less
          than 5. Chi-Square may not be a valid test.

              Fisher's Exact Test
----------------------------------
Cell (1,1) Frequency (F)      50
Left-sided Pr <= F        1.0000
Right-sided Pr >= F       0.4308

Table Probability (P)     0.4308
Two-sided Pr <= P         0.5465

             Sample Size = 146
```

Statistical analysis using seed and Control

```
Data contamination;
input method$ result$ count;
datalines;

control positive 0
control negative 50
Irrigation positive 10
Irrigation negative 90
;
PROC FREQ DATA=contamination;
WEIGHT count;
TABLES method*result/NOPERCENT NOROW NOCOL
EXPECTED CHISQ;
RUN;
```

The SAS System

The FREQ Procedure

Table of method by result

method	result		
Frequency Expected	negative	positive	Total
Irrigati	90 93.333	10 6.6667	100
control	50 46.667	0 3.3333	50
Total	140	10	150

Statistics for Table of method by result

Statistic	DF	Value	Prob
Chi-Square	1	5.3571	0.0206
Likelihood Ratio Chi-Square	1	8.4624	0.0036
Continuity Adj. Chi-Square	1	3.8705	0.0491
Mantel-Haenszel Chi-Square	1	5.3214	0.0211
Phi Coefficient		-0.1890	
Contingency Coefficient		0.1857	
Cramer's V		-0.1890	

WARNING: 25% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

Fisher's Exact Test

Cell (1,1) Frequency (F)	90
Left-sided Pr <= F	0.0148
Right-sided Pr >= F	1.0000
Table Probability (P)	0.0148
Two-sided Pr <= P	0.0311

Sample Size = 150

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