AGRICULTURE, FOOD & ANALYTICAL BACTERIOLOGY

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BRIEF COMMUNICATION

Attachment of *Escherichia coli* O157:H7 and *Salmonella* on Spinach (*Spinacia oleracea*) Using Confocal Microscopy

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ABSTRACT

Foodborne illness outbreaks associated with fresh produce have significantly increased. Researchers must investigate sources of these pathogens as well as new modes of transmission including internalization within plant vascular systems. Confocal scanning laser microscopy (CSLM) was used to observe the location of *Escherichia coli* O157:H7 and *Salmonella* on and within fresh spinach leaves. Sections of leaves measuring 1 cm^2 and stems measuring 0.5 cm^2 were inoculated in a suspension of green fluorescent protein (GFP) *E. coli* O157:H7 and red fluorescent protein (RFP) *Salmonella* transformed by electroporation to express and at initial levels of 10^6 to 10^7 CFU/cm^2. Samples were washed before preparing for CSLM, therefore, all microorganisms visualized were assumed to be strongly attached. Both pathogens were found attached to the surface, cut edges and within tissue layers. Internalization was determined on leaves and stems by taking multiple images of the same sample at different layers. Fluorescent cells not seen on the surface layer of the sample appeared in the interior of spinach sample. These images demonstrate the ability of pathogens to congregate in areas on the leaf surface as well as internalization within the plant possibly escaping chemical decontamination treatments.

Keywords: Confocal microscopy, spinach, *E. coli* O157:H7, *Salmonella*

Agric. Food Anal. Bacteriol. 2: 275-279 2012

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INTRODUCTION

The number of reported foodborne illness outbreaks associated with fresh produce has increased in the past thirty years (Alkertruse *et al.*, 1996; Hedberg and Osterholm, 1994; Sivapalasingam *et al.*, 2004). This increase can be attributed not only to changes in consumption patterns but also changes in production and processing technologies, new sources of produce as well as the manifestation of pathogens such as *Salmonella* and *Escherichia coli* O157:H7 that have not been previously associated with raw produce (Burnett and Beuchat, 2001; Sivapalasingam *et al.*, 2004; Hanning *et a*l., 2009).

Bacteria can be introduced to leafy greens at any step from planting to consumption and once they are introduced, their colonization can have a tremendous effect on both the quality and safety of the product. The attachment and colonization of microorganisms on fresh produce have significant public health implications due to the fact that these processes may be related to the inability of sanitizers and decontamination treatments to remove or inactivate human pathogens (Beuchat, 2002; Frank, 2001). Bacteria attach to fruits and vegetables in pores, indentations and natural irregularities on the produce surface where there are protective binding sites as well as cut surfaces, puncture wounds, and cracks in the surface (Sapers, 2001; Seo and Frank, 1999).

Although several studies have demonstrated that human bacterial pathogens have the ability to penetrate the interior of cut leaf edges or become internalized within lettuce tissue (Seo and Frank, 1999; Solomon *et al.*, 2002; Takeuchi and Frank, 2001; Takeuchi *et al.*, 2000; Wachtel *et al.*, 2002), studies on spinach, particularly those aimed at simulating postharvest operations, are less obtainable. A recent review of literature on the internalization of produce by pathogens (Erickson, 2012) shows how most studies involving spinach have focused on the internalization of pathogens during growth. The scenario where spinach is subjected to a postharvest wash where pathogens may be transferred to the leaves has not been profusely studied. The purpose of this study was to determine how pathogens associate with spinach leaves after washing cut spinach leaves, simulating incorrect washing practices during postharvest processing of spinach.

MATERIALS AND METHODS

Source of spinach leaves

Fresh spinach leaves typical of leafy greens entering the U.S. food supply were kindly provided by the Winter Garden Spinach Producers Board (Crystal City, TX). The spinach was harvested at approximately 45 days and placed in coolers with an internal temperature of 4°C for 6 h and transported 250 miles to the Texas A&M Food Microbiology Laboratory, College Station, TX, where it was stored at 4°C for up to 24 h. In the laboratory, spinach leaves were manually sorted to remove leaves that were bruised, cut or had decay. Spinach leaves were not washed or decontaminated in any manner before the spinach was obtained for this study.

Sources of bacteria and plasmids

Isolates from the Texas A&M Food Microbiology Laboratory culture collection were previously transformed by electroporation using the plasmid vectors pEGFP and pDsRed-Express (Clontech Laboratories, Inc., Mountain View, CA) to express GFP or RFP and resistance to ampicilin. The GFP plasmid was inserted in the strain of *E. coli* O157:H7, which had been isolated from cattle fecal samples, whereas the RFP plasmid was inserted into *S.* Typhimurium ATCC 13311. Three days prior to the experiment the microorganisms were resuscitated by two consecutive transfers to tryptic soy broth (TSB; Becton Dickinson, Sparks, MD) and incubated at 37°C for 24 h. A 24 h TSB culture of GFP-producing *E. coli* O157:H7 (GFP-EC) and RFP-producing *S.* Typhimurium (RFP-ST) was harvested, washed in sterile phosphate buffer saline (PBS; EMD Biosciences, Inc. La Jolla, CA) and resuspended in 0.1% peptone water (Becton Dickinson).

Preparation of inoculum and sample preparation for confocal scanning laser microscopy

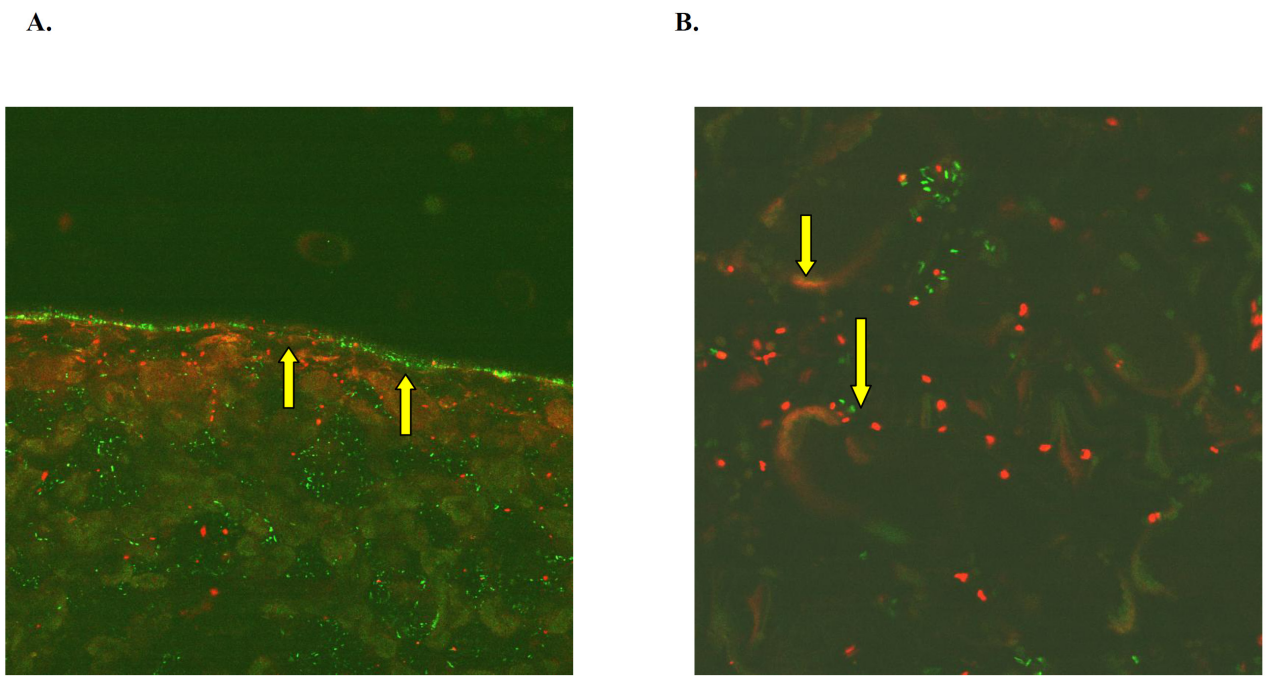
A bacterial cocktail was prepared consisting of 1 mL each of GFP-EC .and RFP-ST. The cocktail then was added to 8 mL 0.1% peptone water to produce a suspension containing 7.0 to 8.0 log CFU/mL. Samples consisting of 4 spinach leaf pieces measuring 1 cm^2 and 4 stem samples measuring 0.5 cm^2 were placed in the cocktail and stored in an incubator at 37°C for 24 h to promote growth. The spinach leaf and stem samples then were washed twice in 0.1% peptone water. This wash had been validated to remove loosely attached cells. Strongly attached bacteria were those not removed by washing, and were verified by plate counting on tryptic soy agar (Becton Dickinson) supplemented with 100 µg/mL ampicillin (TSA + Amp). The washed spinach samples were observed using a BioRad Radiance 2000MP confocal microscope (Zeiss, Heltfordshire, UK) using an excitation wavelength of 488 nm. The confocal microscopy was conducted at the Image Analysis Laboratory at Texas A&M University (College Station, TX).

RESULTS AND DISCUSSION

For the confocal microscopy study, the spinach leaf provided a thin, relatively flat sample, which produced meaningful images. Internalization of *E. coli* O157:H7 and *Salmonella* was seen on leaves and stems by taking multiple images of the same sample at different layers. Fluorescent cells not seen on the surface layer of the sample appeared in the interior images of the same sample. Microorganisms near the cut surface of a spinach leaf can be seen in Figure 1A. The preferential gathering of pathogens to the stomata and cracks in the cuticle are seen in Figure 1B. Fluorescent bacteria allocated in the interior of the spinach stem are shown in Figures 2A and 2B.

These images demonstrate the ability of pathogens to congregate in areas on the leaf surface as well as internalization within the plant possibly escaping chemical decontamination treatments. One possible reason for the congregation of pathogens in specific areas on the leaf surface may be due in part to high hydrophobic leaf surfaces allowing surface water to accumulate in depressions of leaf veins suggesting that more free water is available

Figure 1. Confocal scanning laser microscopy (CSLM) photomicrographs of spinach leaves inoculated with GFP-expressing *E. coli* O157:H7 and RFP- expressing *Salmonella*. (A) Pathogens lined along the cut edge of the spinach leaf (arrows). (B) Pathogens at the stomata and cracks (arrows).



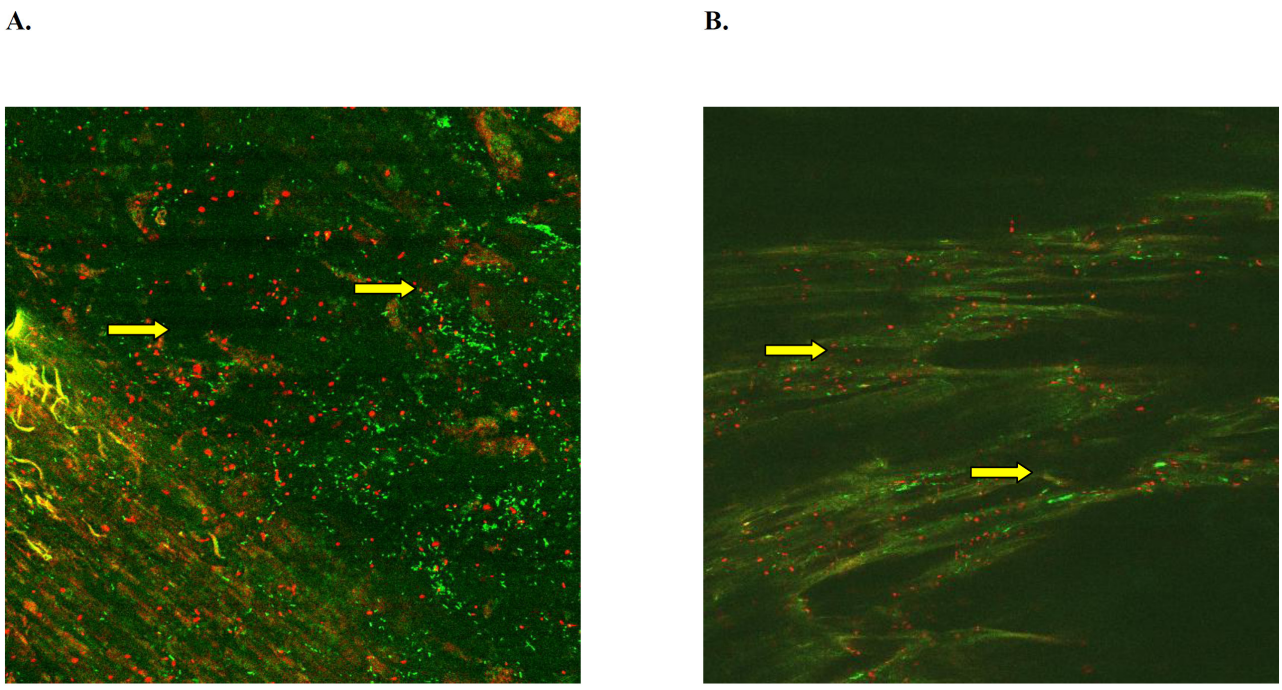
to pathogens at these locations. The accumulation of bacteria in the stomata or intercellular spaces of lettuce and spinach has been reported in different studies and seems to be induced by light and colonization mechanisms (Brandl and Mandrell, 2002; Gomes *et al.*, 2009; Kroupitski *et al.*, 2009; Solomon *et al.*, 2002; Xicohtencatl-Cortes *et al.*, 2009). This internalization seems to result in the microorganisms being out of reach of antimicrobial compounds used for washing and disinfecting produce (Xicohtencatl-Cortes *et al.*, 2009).

In addition, lesions on lettuce and spinach leaves provide sites for internalization of microorganisms where they may be protected from adverse conditions and provide a higher availability of substrates (Brandl, 2008). Seo and Frank (1999) described the preferential attachment of *E. coli* O157:H7 to cut edges rather than intact surfaces and the penetration of the pathogen into the interior of lettuce leaf. Takeuchi and Frank (2000) reported similar findings and suggested that *E. coli* O157:H7 may attach to less favorable attachment sites once all of the preferred initial attachment sites were occupied.

CONCLUSIONS

From our findings, it is apparent that pathogens such as *E. coli* O157:H7 and *Salmonella* can not only lodge themselves onto exterior locations inaccessible to chemical sanitizers but can also be internalized within the plant structure. Therefore, both farmers and processors must realize that chemical sanitizers may not reach all microorganisms when washing leafy greens, such as spinach. Efforts must be taken to reduce the overall microbial load of the produce and begins with preventing contamination by implementing Good Agricultural Practices.

Figure 2. Confocal scanning laser microscopy (CSLM) photomicrographs showing GFP-expressing *E. coli* O157:H7 and RFP-expressing *Salmonella* in the interior of spinach stems. (A) Pathogens throughout stem fissures (arrows). (B) Pathogens lodged within crevices in the stem interior (arrows).



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