International Association for Food Protection WEBINAR

Pre-harvest Internalization: Water-mediated Biological Internalization of Pathogens into Produce

Moderators: Enrique Garcia, FirstFruits Farms

Organized by: IAFP's Fruit and Vegetable Quality and Safety PDG

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International Association for Food Protection

Today's Moderator

Enrique Garcia, FirstFruits Farms

Enrique is currently the Food Safety Manager at FirstFruits Farms in Prescott, WA. Enrique is also the current Vice-Chair for the IAFP Fruit and Vegetable Safety and Quality PDG.

Today's Panelists

International Association for Food Protection WEBINAR

Dr. Kellie Burris

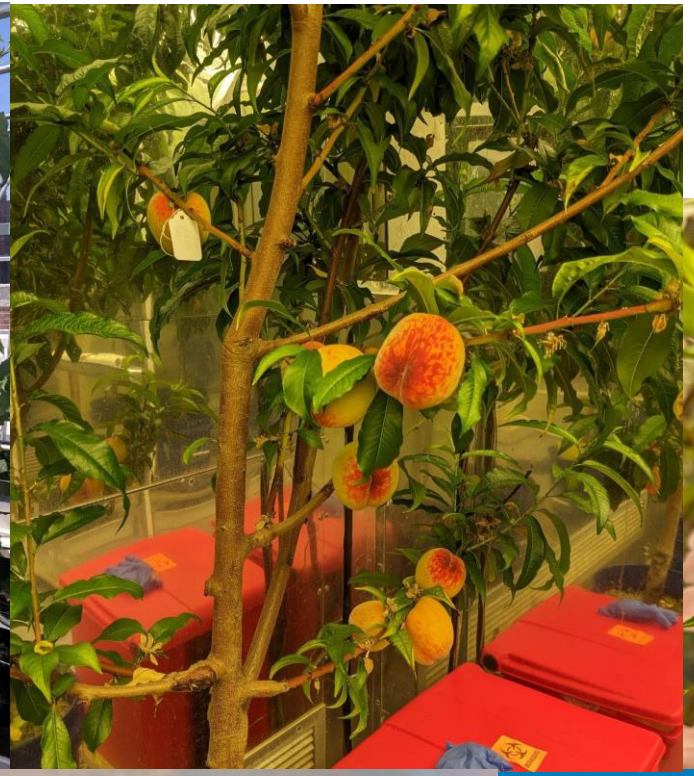
Dr. Burris is currently a Research Biologist at the US FDA/CFSAN. Her research focused on microbial food safety of fruits and vegetables, specifically examining how human pathogens associate with commodity crops as well as how these organisms interact with their environment.

Dr. Kalmia Kniel

Dr. Kniel is a Professor of Animal and Food Sciences at the University of Delaware. Her research specialized in microbial food safety, including the safety of fruits and vegetables, food science and food processing. Dr. Kniel works on understanding transmission, survival and risk associated with norovirus, hepatitis A, emerging enteric viruses, Salmonella, pathogenic E. coli and protozoa.

Dr. Shirley Micallef

Dr. Micallef is a Professor at the Center for Food Safety and Security Systems at the University of Maryland. Her research focused on microbial safety of fresh produce, assessing the impact of cropping practices on the persistence of foodborne enteric pathogens in the agricultural environment.


U.S. FOOD & DRUG ADMINISTRATION

Understanding Pre-Harvest Factors Contributing to Colonization and Internalization of Fruits with Foodborne Pathogens

Kellie P. Burris, Ph.D.

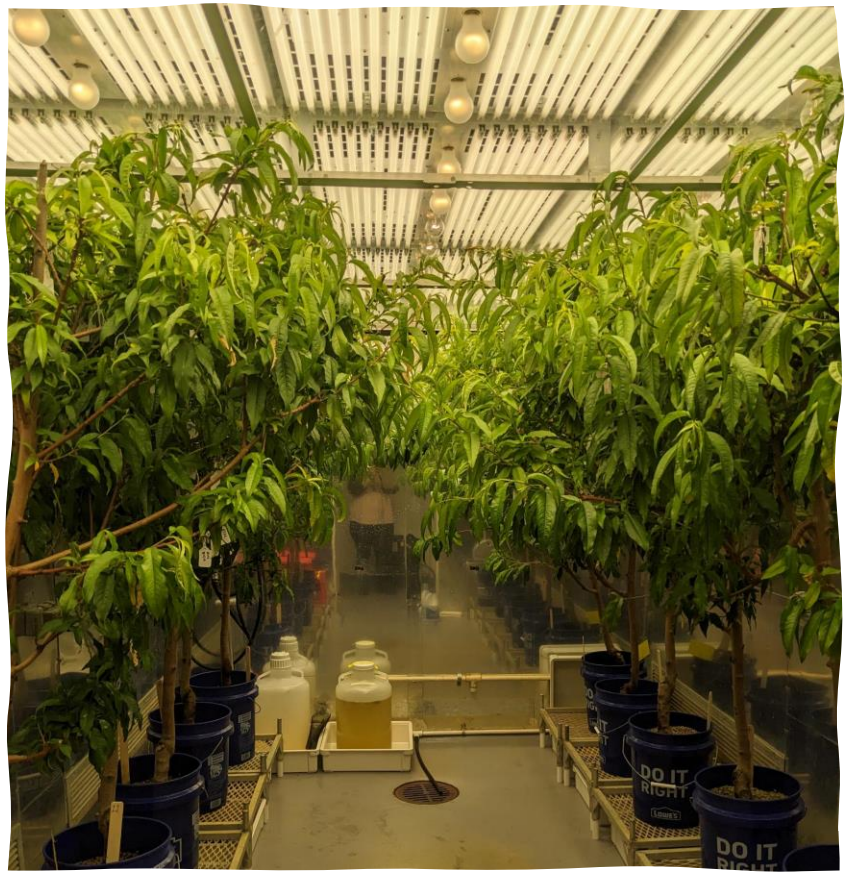
October 23, 2023

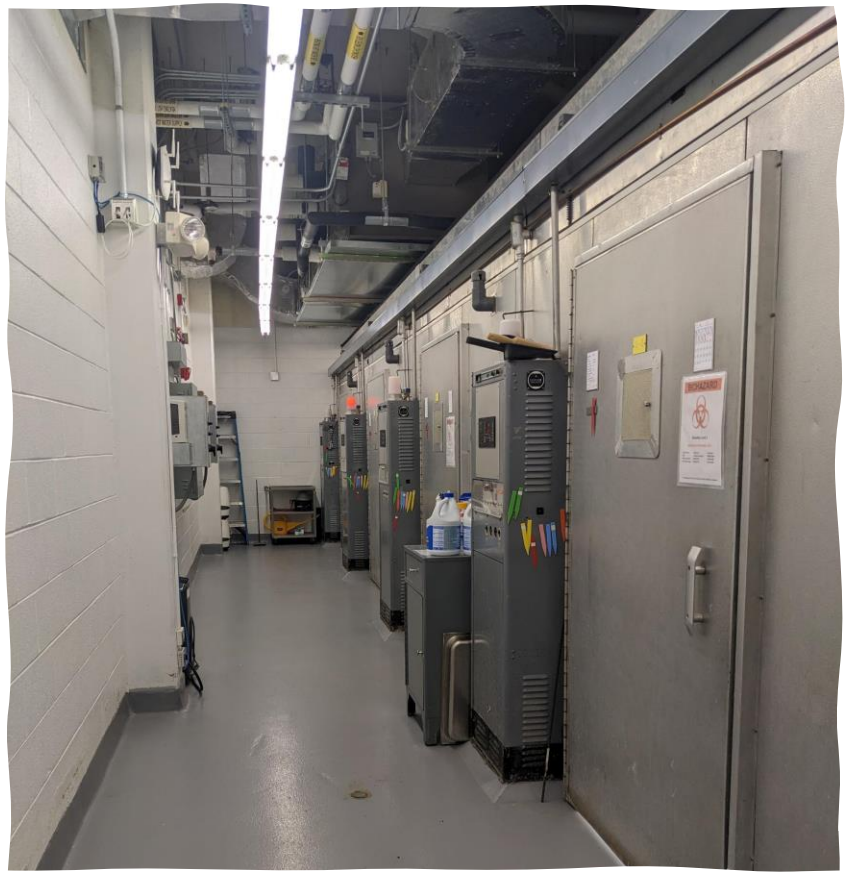
Importance of Phytotron Program

- Perform biological experiments using specific pathogen-plant commodity pairs
- To complement ongoing environmental surveillance studies, which look to answer overarching research questions related to outbreak prevention

Limitations to experiments within each type of growing environment

Field Environment	Controlled Growing Environment (Phytotron/Greenhouse)
Use of surrogates in place of outbreak pathogens-surrogates behave differently than pathogens	Use of outbreak pathogen strains
Environmental factors (such as UV exposure, temperature, rainfall) may affect pathogen survival	Environmental conditions are not identical to field and pathogens may have advantage
Presence of competing bacteria or microbes may influence pathogen survival	Plants are their healthiest and could bias against pathogen colonization
Natural pollination (wind, insects, etc.)	Artificial pollination is required
Soils with limited drainage	Soils with better drainage





A chambers

B chambers-used for vernalization

Benefits of these Phytotron/Greenhouse studies

- Produce-associated outbreak and environmental strains
- Commercial cultivars
- Single fruit inoculation and collection
- BSL-3P Phytotron greenhouse

Why blossoms?

- Previous research has demonstrated relatively high levels of inoculum necessary to observe contamination within plant tissues from exposure to roots
- Plant pathogens are well known to evade plant innate immune response when entering through blossoms
- Hospitable habitat with source of nutrients and protection from the environment

frontiers in Microbiology

ORIGINAL RESEARCH
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doi: 10.3389/fmicb.2020.01135

Colonization and Internalization of *Salmonella enterica* and Its Prevalence in Cucumber Plants

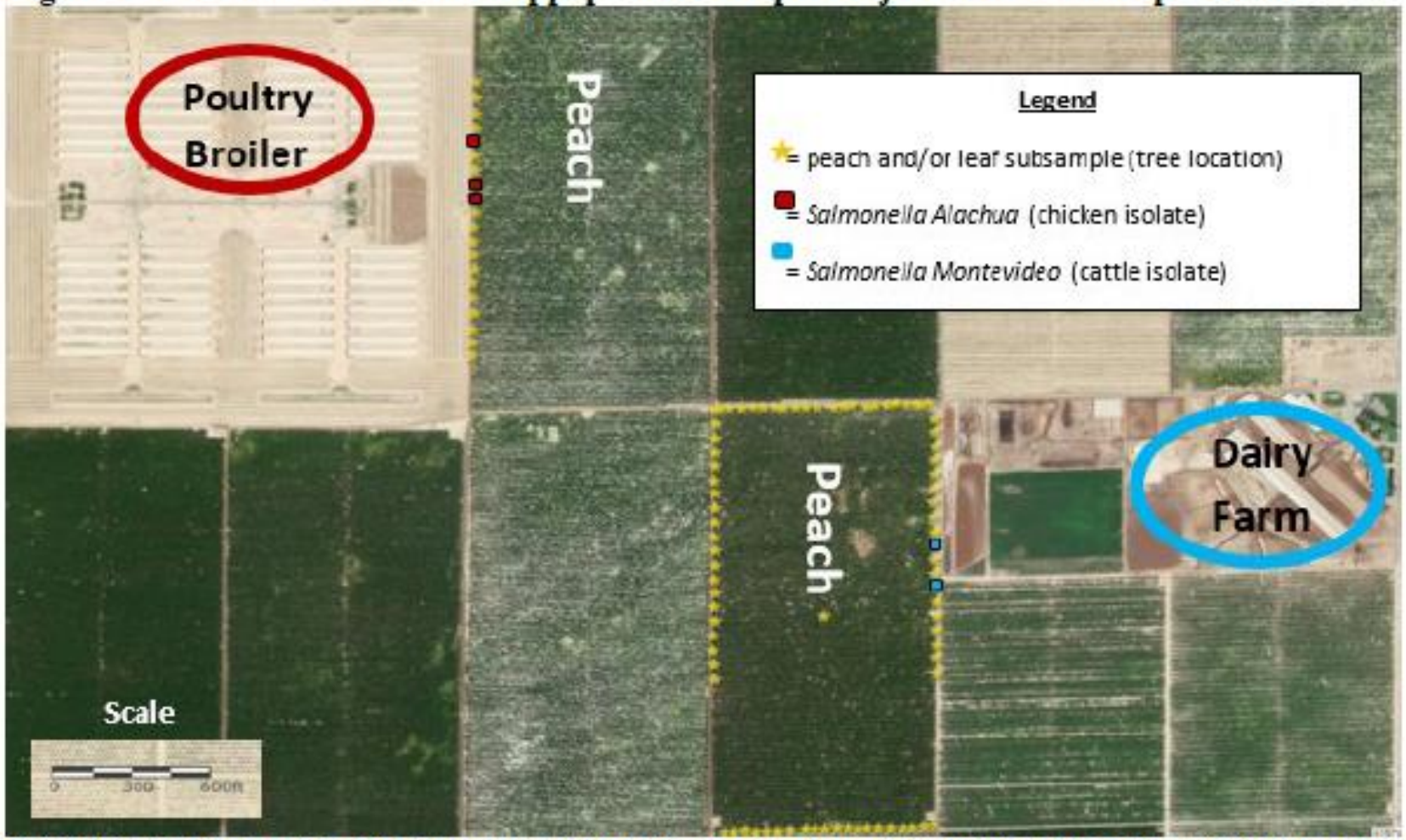
Kellie P. Burris[1], Otto D. Simmons III[2], Hannah M. Webb[1], Lauren M. Deese[1], Robin Grant Moore[1], Lee-Ann Jaykus[1], Jie Zheng[3], Elizabeth Reed[3], Christina M. Ferreira[3], Eric W. Brown[3] and Rebecca L. Bell[3]*

[1] Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, Raleigh, NC, United States, [2] Department of Horticultural Science, North Carolina State University, Raleigh, NC, United States, [3] Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, MD, United States

Cucumber Variety and Inoculation Status	Total # of cucumbers colonized/total # of cucumbers challenged (%)*	Proportion of *S. enterica*-positive cucumbers by colonization location: **	
		# Cucumbers colonized on surface only/total # *S. enterica*- positive cucumbers (%)	# Cucumbers colonized on surface and inside***/total # *S. enterica*-positive cucumbers (%)
HIGH INOCULUM (6.4 log_{10} CFU/BLOSSOM)			
Thunder			
Inoculated blossoms[a]	47/56 (83.9) a	9/47 (19.1) a	38/47 (80.9) a
Adjacent blossoms[b]	8/24 (33.3)	7/8 (87.5)	1/8 (12.5)
MEDIUM INOCULUM (4.5 log_{10} CFU/BLOSSOM)			
Thunder			
Inoculated blossoms	48/59 (81.4) a	27/48 (56.2) b	21/48 (43.8) b
Adjacent blossoms	3/32 (9.4)	3/3 (100.0)	0/3 (0.0)
LOW INOCULUM (2.5 LOG_{10} CFU/BLOSSOM)			
Thunder			
Inoculated blossoms	48/67 (71.6) a	35/48 (72.9) b	13/48 (27.1) c
Adjacent blossoms	1/54 (1.9)	1/1 (100.0)	0/1 (0.0)
Marketmore 76			
Inoculated blossoms	36/51 (70.6) a	23/36 (63.9) b	13/36 (36.1) c
Adjacent blossoms	0/50 (0.0)	0/0 (0.0)	0/0 (0.0)

[a]*Cucumbers collected from blossoms directly challenged with the S. enterica cocktail.* [b]*Cucumbers collected from blossoms that were in close proximity to S. enterica-inoculated blossoms.* **The Pearson Chi-Square Fisher's Exact test was used to determine significant differences in sample positivity (i.e., fruit colonization) obtained after inoculating blossoms for all cultivars over all inocula concentrations (within each column). Different letters indicate statistically significant differences ($P<0.05$).* ***No cucumber fruit were found contaminated inside only.* ****If a cucumber fruit was found positive for Salmonella inside the fruit, it also had surface contamination in every instance. External colonization with S. enterica was observed in a small number of the negative control cucumbers (5/167, 3.0%), i.e., those derived from uninoculated control plants.*

Figure 1: Location of *Salmonella* spp. positive samples adjacent to animal operations



Note: This map does not include all positive *Salmonella spp.* locations. *Salmonella* enterica subsp. Enterica serovar Montevideo isolates (in blue) are median of 16 SNPs from beef and cattle isolates from 2018, 2019 and 2020. *Salmonella* enterica subsp. Enterica serovar Alachua isolates (in red) are identical (0-2 SNP) to a surveillance sample of chicken from 2019.

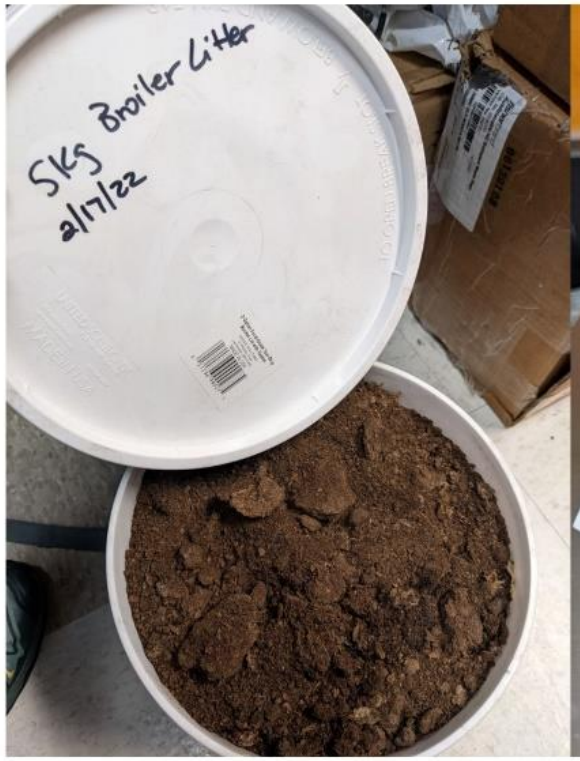
Current research focus

- Understanding pre-harvest factors contributing to colonization and internalization of fruits with foodborne pathogens
  - Simulate conditions of pathogen contamination through blossom route during pre-harvest

Purpose of this study

- To investigate the ability of *Salmonella* Poona to colonize and internalize cucumber fruit when applied to blossoms via contaminated poultry litter

A. Preparation of broiler litter



Broiler litter collected from poultry house

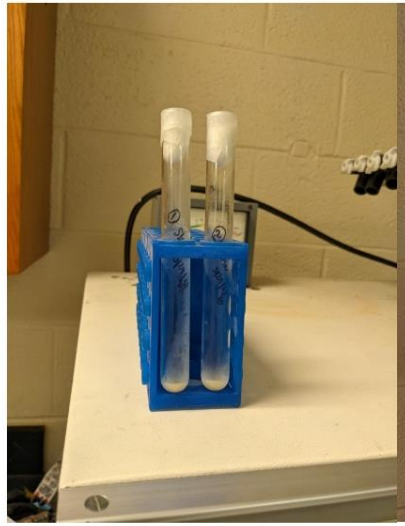
Wet weight (100 g)

Autoclaved and dried in biosafety cabinet overnight

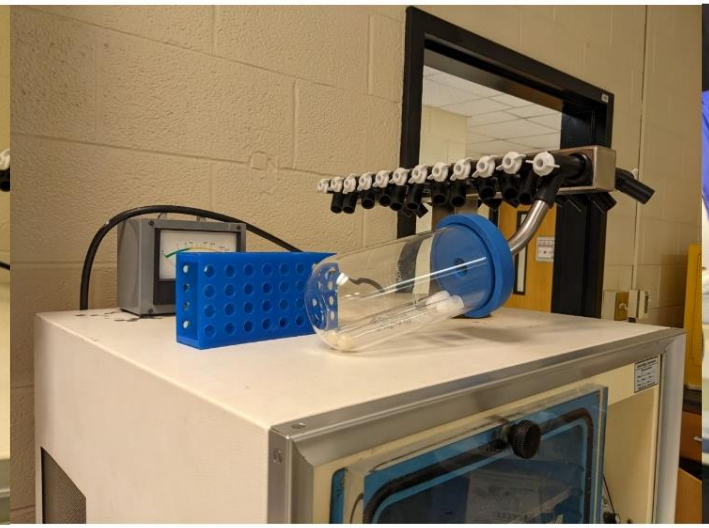
B. Hood-dried method

Add 1 ml overnight culture (washed 3 x PBS, pH 7.2 and resuspended in 5 ml PBS, pH 7.2) to 9 grams autoclaved and dried broiler litter, mix well and dry in biosafety cabinet overnight

C. Freeze-dried method



25 ml overnight culture, washed 3 x PBS, pH 7.2 and pellet resuspended in 250 µl skim milk and frozen for 1 h



Freeze dry 5.25 h

Mix pellet (ca. 40 mg) with 1.8 g poultry litter

D. Blossom inoculation

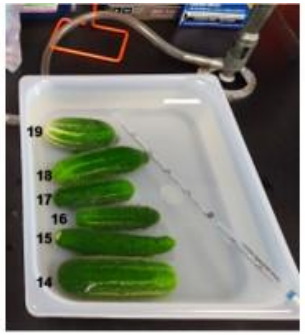
ca. 10 mg inoculated litter applied to cucumber blossom with forceps

Inoculum concentrations

Hood-dried method: 3.6 $\log_{10}$ CFU/blossom

Freeze-dried method: 6.2 $\log_{10}$ CFU/blossom

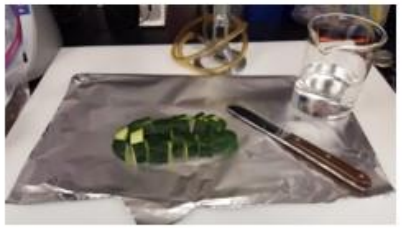
Schematic of mature fruit processing



Ethanol bath
(20 min)

Sterile water bath
(5 min)

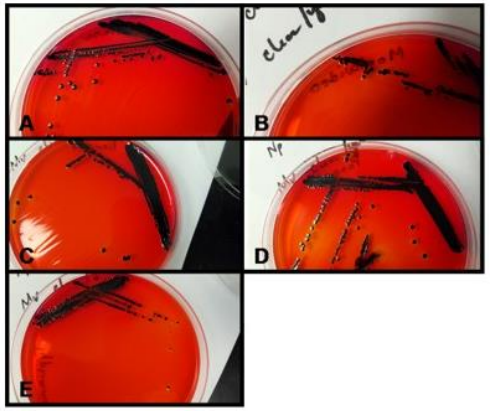
Dry in hood



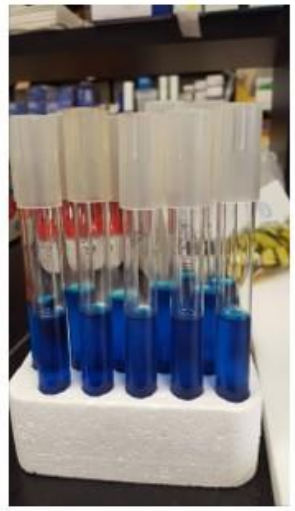
Cut

Pre-enrichment

Incubate at 37 C
for 20 h
(Inside)



Selection-XLD



Enrichment-RV medium

Incubate at 37 C
for 20 h
(Surface)

Pre-enrichment

Table 1. *Salmonella enterica* contamination of mature cucumber fruit when introduced through blossoms via contaminated poultry litter.

Method (ca. $\log_{10}$ CFU/blossom)	Total # of cucumbers colonized/total # challenged (%)	# of cucumbers colonized on surface only/total # *S. enterica*-positive cucumbers (%)	# of cucumbers colonized on surface and inside/total # *S. enterica*-positive cucumbers (%)
Hood-dried (3.6)	10/37 (27.0)	7/10 (70.0)	3/37 (8.1)
Freeze-dried (6.2)	15/24 (62.5)	13/15 (86.7)	2/24 (8.3)

Prevalence of internal contamination was equivalent when comparing inoculum methods (X^2=0.001, P=0.9750). Surface contamination was significantly lower in hood-dried versus freeze-dried method (X^2=7.573, P=0.0059).

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Modification of inoculum application

Weight of commercial poultry litter dust from one puff is approximately 10 mg

Table 1. *Salmonella enterica* contamination of mature cucumber fruit when introduced through blossoms via contaminated poultry litter.

Method (ca. $\log_{10}$ CFU/blossom)	# colonized/total # of fruit (%)	# surface only/total # of fruit (%)	# surface and inside/total # of fruit (%)
Hood-dried (3.6)	10/37 (27.0)	7/37 (18.9)	3/37 (8.1)
Freeze-dried (4.9)	17/47 (36.2)	15/47 (31.9)	2/47 (4.3)
Freeze-dried (6.2)	15/24 (62.5)	13/24 (54.2)	2/24 (8.3)

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Conclusions and Significance

- These results identified contaminated poultry litter as a means for *Salmonella* to colonize and internalize mature fruit when introduced to blossoms during pre-harvest

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Questions

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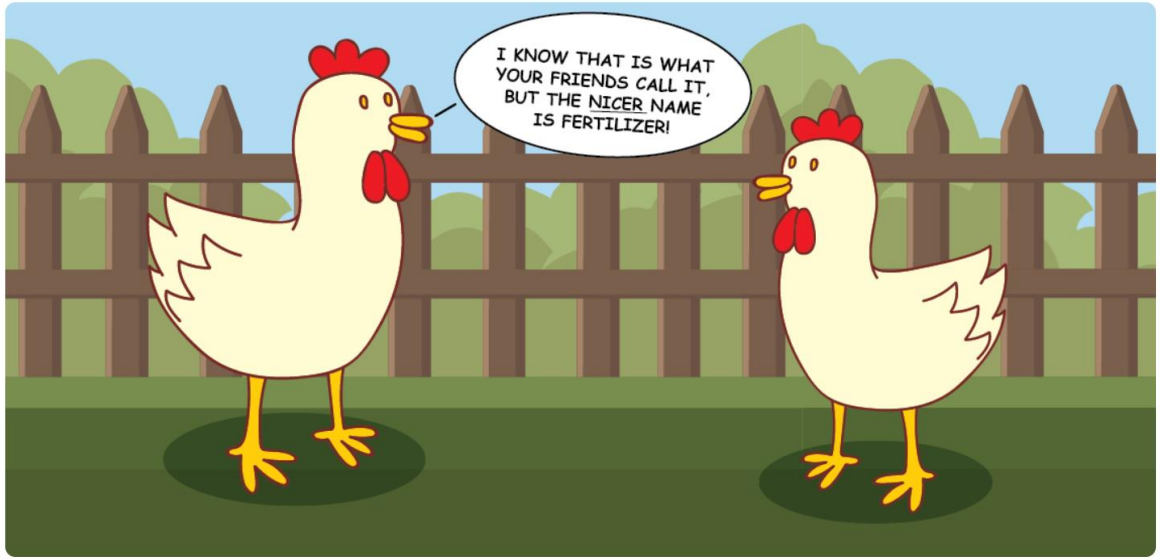
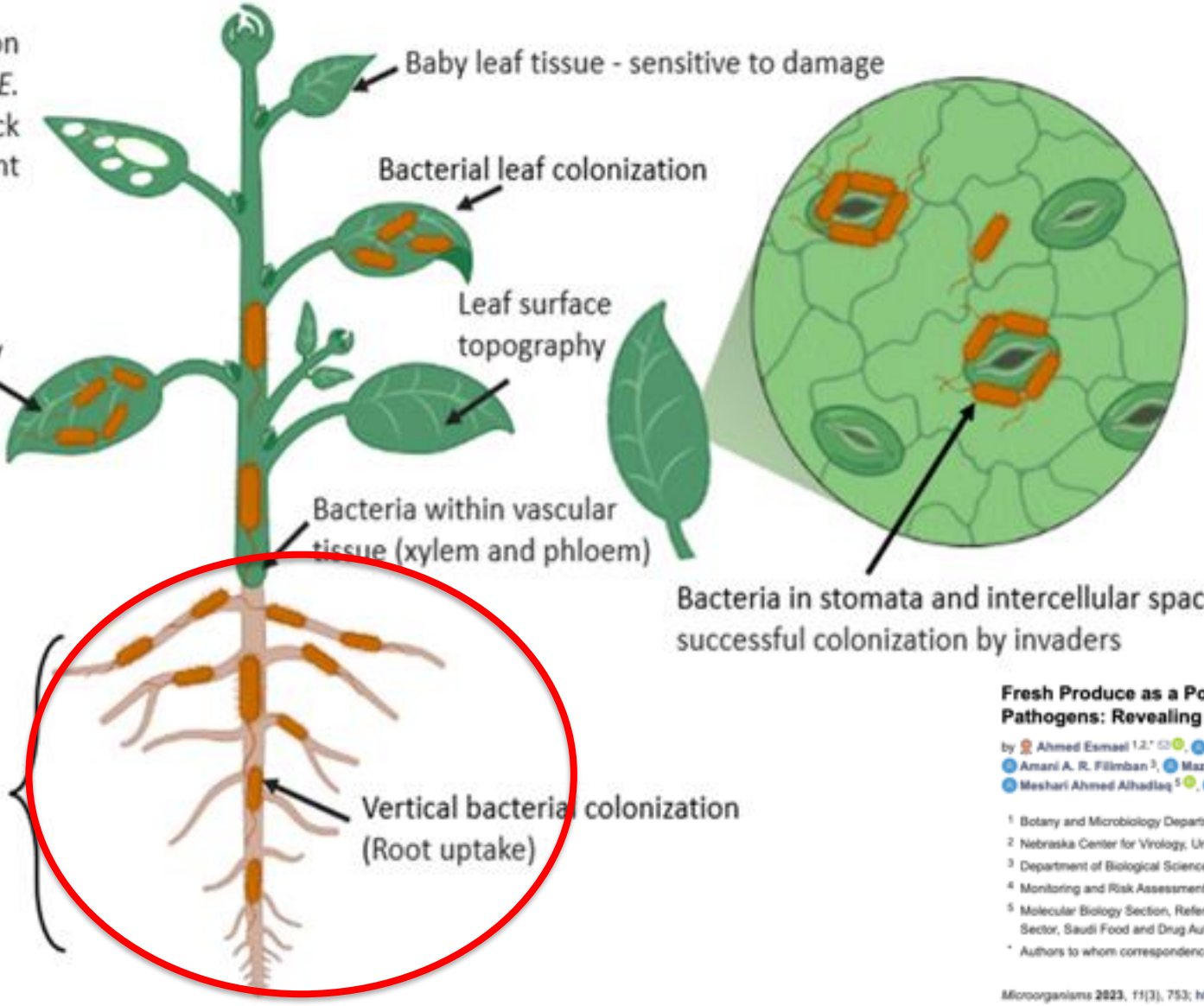


https://lawnsolutionsaustralia.com.au/lawn-care/lawns-and-manure/

Root uptake: Mechanism for Contamination of Produce Commodities

Kali Kniel, Ph.D.
Department of Animal and Food Sciences
College of Agriculture and Natural Resources
University of Delaware

Damaged and dead cells on plant surfaces (entry site for *E. coli* O157:H7 that lack enzymes for degrading plant cell wall components)



Determinantal factors - plant developmental stage, pathogen genus and/or strain, inoculum level, and plant species and cultivar

Fresh Produce as a Potential Vector and Reservoir for Human Bacterial Pathogens: Revealing the Ambiguity of Interaction and Transmission

by Ahmed Esmael [1,2,*], Rashad R. Al-Hindi [3,*], Raed S. Albiheyri [3], Mona G. Alharbi [3], Amani A. R. Filimban [3], Mazen S. Alseghayer [3,4], Abdulaziz M. Almaneea [3,4], Meshari Ahmed Alhadlaq [5], Jumaa Ayubu [3] and Addisu D. Teklemariam [3]

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Microorganisms **2023**, *11*(3), 753; https://doi.org/10.3390/microorganisms11030753

Overview

- **Water-mediated Biological Internalization of Pathogens into Produce**
- **Uptake via roots in hydroponic operations**

- Understanding pathogen interactions with different produce commodities in the growing environment is useful for our ability to reduce risk of contamination.

How do zoonotic bacteria and viruses become internalized?

Bacterial internalization has been studied rigorously since 2007 and continues today

M. Sharma, S. Ferguson, D. Ingram, USDA-ARS

http://blogs.usda.gov

FOODBORNE PATHOGENS AND DISEASE
Volume 9, Number 5, 2012

DOI: 10.1089/fpd.2011.1044

Human Enteric Pathogen Internalization by Root Uptake into Food Crops

Kirsten A. Hirneisen,[1] Manan Sharma,[2] and Kalmia E. Kniel[1]

Effect of growth substrate

- Internalization through roots is more likely to occur in hydroponic systems than in soil or bark matrices.
- Is this about attachment and interaction with soil or motility in hydroponic solutions?

Stressed bacterial cells

Wounding in root tissue

Motile bacterial cells

Bacterial populations

Effect of environmental stress

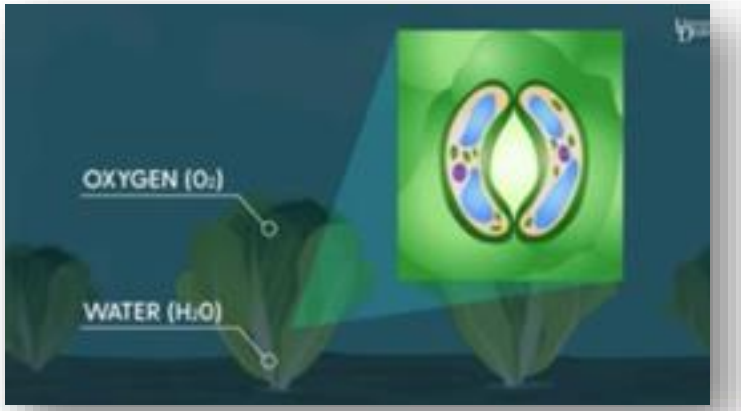
- Many internalization studies focus on extreme environmental conditions
  - High temperatures, flooding, drought

- Another potential issue is the root damage sustained from growth in soil?
  - Wounding has potential to expose vascular tissue. After root decapitation, internalization may occur
    - But varying internalization via roots in plants grown in soil
    - Often more internalization in hydroponically-grown plants
      - But in hydroponic systems bacteria are more motile
  - What about the state of the bacteria in the soil?
    - Stressed low initial populations, so less likely to occur in fields?

Effect of bacteria strain and serovar

- *Salmonella* and *E. coli* O157:H7 are most studied

- Some studied have shown that *Listeria* does not internalize as well, shown under same experimental conditions where *Salmonella* did internalize into plant tissues
  - *Listeria* remained detected on exterior root surfaces



frontiers in Microbiology

Front Microbiol. 2020; 11: 500.
Published online 2020 Apr 3. doi: 10.3389/fmicb.2020.00500

Evasion of Plant Innate Defense Response by *Salmonella* on Lettuce

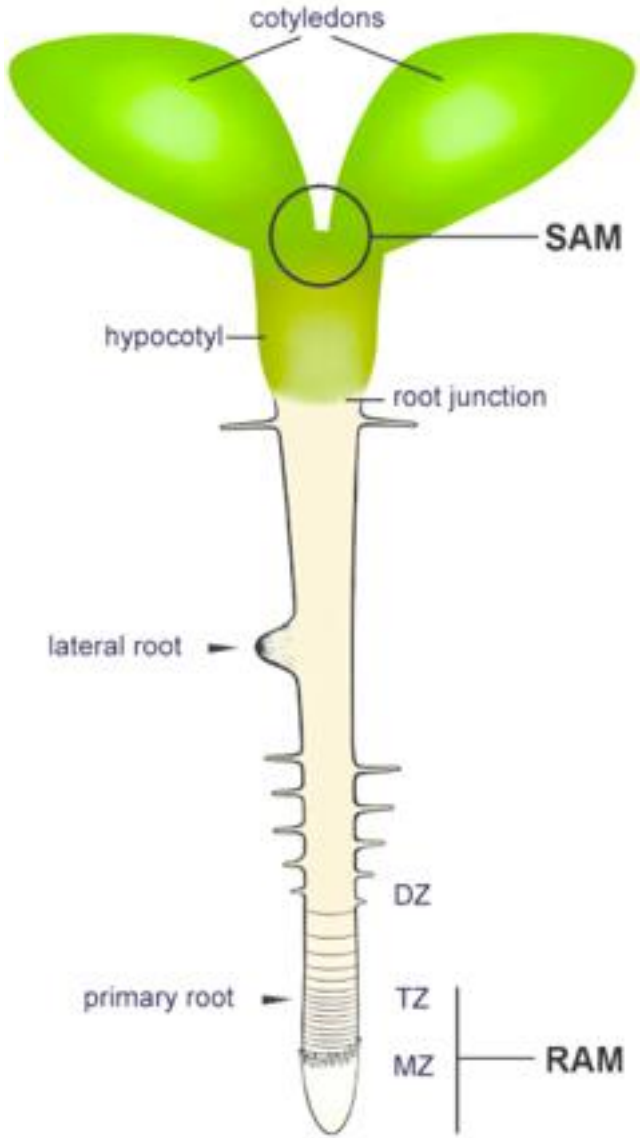
Nicholas Johnson,[1,2] Pushpinder K. Litt,[3] Kalmia E. Kniel,[3,*] and Harsh Bais[1,2,*]

Effect of Inoculum Level

- Internalization appears to be a factor of initial bacterial load
  - But higher inoculum levels do not always equate to higher internalization levels
  - This relates to the matrix of inoculum delivery (soil vs hydroponic media)
  - Still limited internalization in soil
  - Greater internalization via hydroponic solutions

- Higher bacterial inoculum often results in more uptake and internalization and lower inoculum results in little to no internalization.

As the inoculum level of the bacteria increases, the probability increases that the bacteria are translocated to more sites of the root tissue and more likely to internalize.



HANNA HELP-RINTA-RAHKO

The Interaction of Auxin and Cytokinin Signalling Regulates Primary Root Procambial Patterning, Xylem Cell Fate and Differentiation in *Arabidopsis thaliana*

State of the Inoculum

- This appears to be a gap in our knowledge
- In some studies, flagellin synthesis by *Salmonella* and motility functions important for invading lateral root junctions
  - Including ability to colonize the root

Effect of plant type, age, and exposure time

- Do antimicrobial properties of root vegetables limit pathogen internalization?
- Plant age at time of exposure? (similar to adult humans)

Photo:://www.moleaer.com/blog/high-plant-yields-oxygen-uptake-by-roots-is-critical-for-plant-respiration

Localization within the plant

- Variable results across the literature
- Bacteria may colonize roots well, perhaps due to availability of nutrients at the roots
- Systemic movement within a plant?
  - Less likely in many studies
  - Detected in intracellular space in the roots
  - This may be different if seeds are colonized?

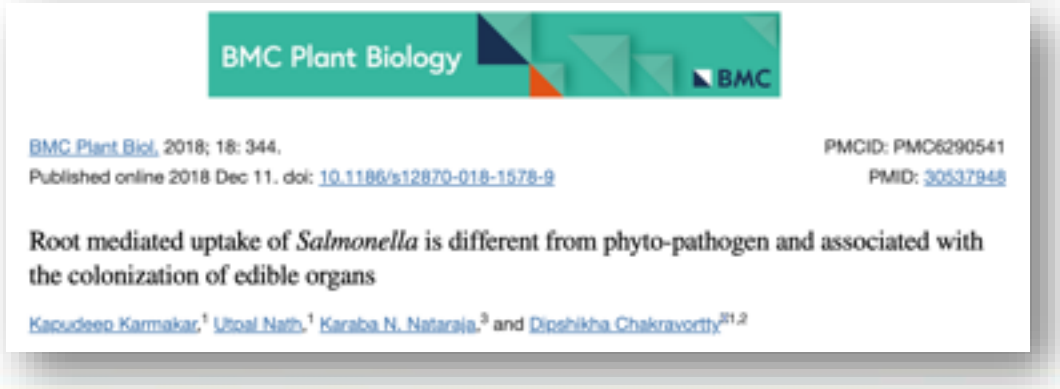
Mechanism of internalization

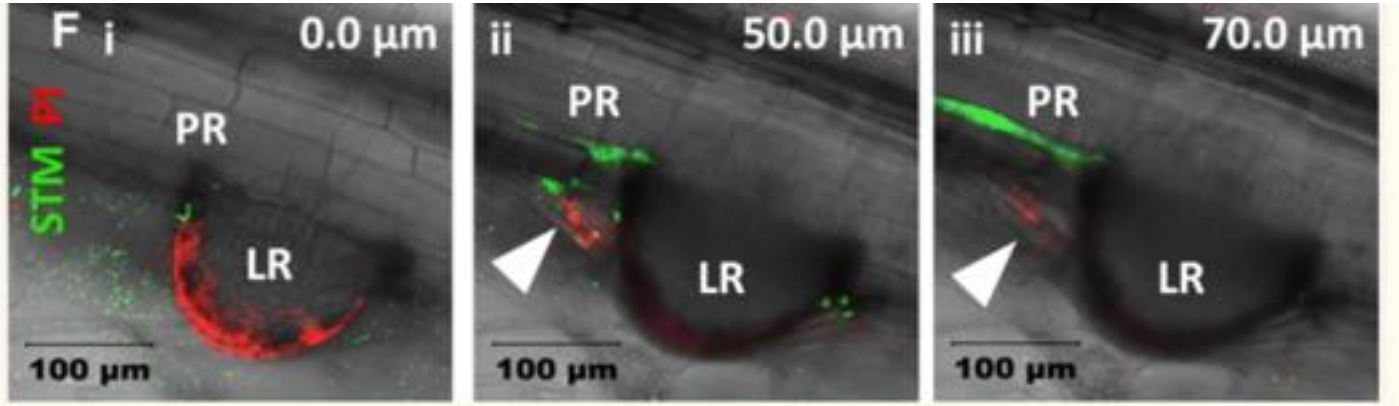
- Plant-pathogen specific interactions
- Cell surface moieties
- Inoculum load and pressure
- Active process?
  - *Salmonella* may use flagella to position near developing lateral root (chemotaxis?) increase potential for internalization (Cooley et al., 2003)

- Likely that surface moieties influence bacterial ability to interact with plant roots but mechanisms that promote internalization remain unclear

In-situ colonization of *Salmonella* is dependent on lateral roots

- *Salmonella* internalization was found higher in the plants with more lateral roots. However, the epiphytic colonization in both these plants remained unaltered.
- Tomato roots inoculated with these organisms were observed to study patterns of colonization inside the root tissue. Determined that phytopathogens can cause tissue degradation, but *Salmonella* cannot.



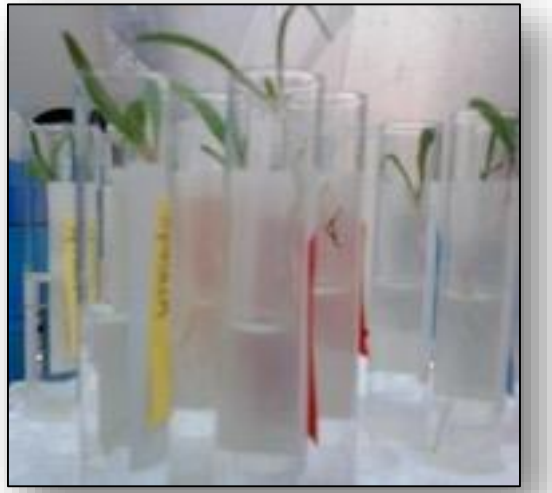


Internalization of viruses

- Viral persistence and motility in soil and hydroponic solution affects internalization to root systems
- Growth substrate plays the biggest role
  - Hydroponic solutions as growth substrate shows greater viral uptake
    - Affected by wounded roots
    - Water turbidity (viral interactions with colloidal suspensions)

What about in soil compared to hydroponics?

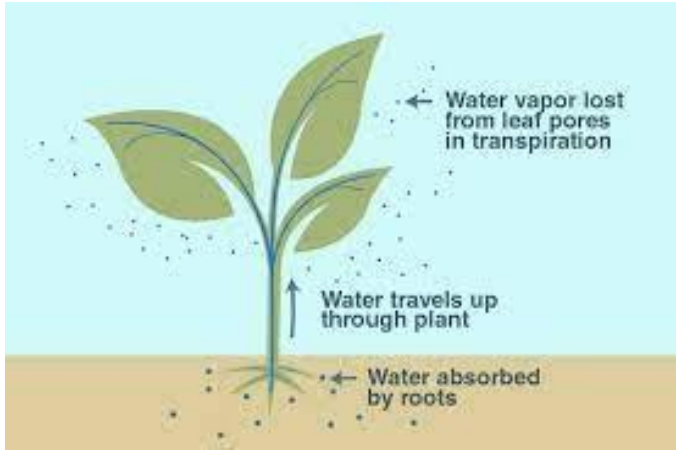
Virus survival and internalization determined for soil, small-scale hydroponics, and nutrient-film technique hydroponics



Only 1 with 2.43 genomic copies HAV

Internalization of viruses

- Viruses may be taken up in a passive manner by transpiration in plants
  - Transpiration is the driving force of water absorption by the plant from the growing substrate, and humidity is a major factor controlling plant transpiration, as high humidity will reduce the diffusion of water out of the leaf and slow the transpiration rate
  - Lettuce grown in 70% humidity resulted in a 10-fold higher transpiration rate and significantly greater internalization of murine norovirus as compared to plants grown in 99% humidity



Conclusions

- Uptake through internalization is a plant-pathogen specific interaction
- The plant growth substrate used plays a large role in the uptake of both bacterial and viral pathogens in plants
- Intact, healthy, non-injured roots seem to discourage the uptake of bacteria cells and viruses into plants
- Presence of internalized pathogens in roots of plants does not directly correlate with internalized pathogens in the edible or foliar tissues of crops

Generalizations…

- In review of the literature, contaminated soil resulted in little to no observed internalization as compared to hydroponic solutions
- In soil-grown crops, internalization was sporadic and at low levels

Thank You

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International Association for Food Protection WEBINAR

Questions?

Acknowledgments

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Upcoming Webinars

International Association for Food Protection WEBINAR

October 24, 2023	9:00 AM	Food Safety and Quality under the Auspices of Data Science
October 24, 2023	2:00 PM	Managing Meat Shelf Life and Spoilage to Ensure Food Security
November 1, 2023	10:00 AM	Plant-Based Meat Analogues: How Far of an Analogue in Microflora
November 17, 2023	1:00 PM	Matrix Additions Part 2: Alternative Approaches for Rapid Pathogen Detection Methods
December 14, 2023	9:00 AM	Impact of Water Use and Reuse in Food Production and Processing on Food Safety at the Consumer Phase: Focus on the Fresh Fruit and Vegetable Products Sector

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