Apple Horticulture/Postharvest Research Review
Wednesday, January 27, 2021

Time	Page	Presenter	Project Title	Yrs
8:00		Schell/Miles	Welcome	
8:10		Hanrahan	Introduction and meeting etiquette	
			Continuing Projects: 10 minutes each	
8:20	1	Kalcsits	N, Mg, and K guidelines to control disorders for Honeycrisp & WA38	20-22
8:30	8	Mendoza	Phase 3 evaluation of apple breeding selections	20-22
8:40	13	Rudell	Non-destructive detection of sun stress compromised apples	19-21
8:50	20	Rudell	Reducing carbon dioxide-related postharvest disorders	19-21
9:00	27	Torres	Postharvest system optimization for organic apple storage **(O)**	19-21
9:10	33	Musacchi	Understanding green spot origin, timeline and development	20-21
9:20	40	Honaas	Apple genomes for postharvest fruit quality biomarkers	19-21
9:30	48	Critzer	Critical limits for antimicrobials in dump tank systems	19-21
9:40	55	Ganjyal	Increasing the efficacy of antimicrobial chemicals with surfactants: ***NCE***	19-20
9:50	62	Critzer	Systems-based approach for improved packinghouse sanitation: ***NCE***	18-20
	70	Mendoza	Improving apple fruit quality & postharvest performance ***written only***	20-22
	75	Zhu	Fate of Listeria on apples at ozone and controlled atmosphere storage: ***NCE written only***	18-20
	83	Zhu	Control of Listeria on processing surfaces in apple packing facilities: ***NCE written only***	17-19
10:00			**Break**	
			Final Reports: No-Cost Extensions 10 mins	
10:30	91	Kalcsits	How does fruit acclimation to sunburn affect sunburn management?	18-19
10:40	104	DuPont	How do we measure and manage soil health for productive orchards?	17-19
10:50	114	Critzer	Utility of rapid tools to assess cleanliness in apple packinghouses	18-19
	128	Ganjyal	Complying with the FSMA preventative controls for human food rule: ***written report only***	17-18
			Final Reports: 15 mins	
11:00	135	Musacchi	Optimizing harvest time for WA38 **(O)**	19
11:15	147	Sallato	Calcium fertilization efficacy	20
11:30	161	Serra	Pollination, flower biology and fruit development in WA38 apples	19-20
11:45	173	Schmidt	Crop load and canopy management of WA tree fruit	18-20

CONTINUING PROJECT REPORT **YEAR**: 1 of 3

Project Title: N, Mg, and K guidelines to control disorders for Honeycrisp and WA 38

PI:	Lee Kalcsits	**Co-PI:**	Bernardita Sallato
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Cooperators:	Washington State Apple Growers		

Total Project Request: $252,156 **Year 1:** $81,270 **Year 2:** $84,015 **Year 3: $86,871**

Other funding sources: **None**

WTFRC Budget: ***none***

Budget 1
Organization Name: WSU **Contract Administrator: Shelli Tompkins/Katy Roberts**
Telephone: 509-293-8803 **Email Address: shelli.tompkins@wsu.edu/arcgrants@wsu.edu**
Station manager: Chad Kruger **Email address:** cekruger@wsu.edu

Item	2020	2021	2022
Salaries	43,200[1]	44,928	46,726
Benefits	15,895[2]	16,530	17,192
Wages	7,800[3]	8,112	8,436
Benefits	1,735[4]	1,805	1,877
Equipment			
Supplies	6,900[5]	6,900	6,900
Travel	3,240	3,240	3,240
Miscellaneous			
Plot Fees	2,500	2,500	2,500
Total	$81,270	$84,015	**$86,871**

Footnotes:

OBJECTIVES:

1. **Test how varying rates of N, K, and Mg affects fruit quality traits, disorder incidence, return bloom and tree vigor in 5th-7th leaf Honeycrisp and WA 38 orchards.**

Despite issues surrounding COVID-19, we were able to prioritize application of fertilizer treatments. Crop load was light in our Honeycrisp block but heavier in our WA 38 block. We have the information to account for crop load in our statistical model that adds variability in disorder incidence to our results. When corrected for, we should be able to identify key nutrient thresholds that promote both bitter pit and green spot incidence. We still need to send samples for nitrogen analysis.

2. **Identify the relation between shoot growth, crop load, and nutrient concentration with disorder incidence at harvest and after storage for commercial orchards in WA State.**

In 2020, we sampled from 24 Honeycrisp sites and 15 WA 38 orchards. This approximately matched our goals for the year. In 2021, we will pursue a similar sampling number from the same or alternative orchards. We will also add in historical data from 2015-2019 where we have crop load, shoot vigor, nutrient composition and fruit quality metrics. We will also assess other postharvest disorders in this dataset in response to comments from industry members.

3. **Develop clear thresholds for N, K, and Mg fertilization based on fruit and leaf elemental concentrations for Honeycrisp and WA 38 orchards in WA State.**

We will start to develop vigor and nutrient thresholds for both WA 38 and Honeycrisp. These thresholds will create more accurate targets for growers to aim for and to assess risk in their orchards. These will likely fall in line with similar observations made to develop the Pennsylvania model.

SIGNIFICANT FINDINGS

1. For commercial sampling, green spot in WA 38 demonstrated the same risk indicators (high vigor, low crop load, and high K: Ca ratios) as bitter pit in Honeycrisp.
2. Green spot is fully developed at harvest and as such, the relationships between horticultural and nutrient-related factors were clearer.
3. Bitter pit was low in some orchards because these assessments were made within one month of harvest. We are expecting higher incidence in some orchards when we pull fruit for evaluations in early January.
4. What we plan to answer in the next two years is whether these nutrient ratios are affected by N, Mg, or K fertilizer applications and what applications rates are needed to avoid green spot or bitter pit incidence.
5. Non-trivial green spot averaged about 10% across all orchards. This may decrease in the next two years of the project as the trees reach maximum size and productivity increases. All of the sampled trees were third and fourth leaf.
6. Rootstock heavily contributed to green spot and bitter pit incidence through its effect on vigor. We will continue on compiling data on rootstock and bitter pit to develop extension material for the industry to make informed rootstock decisions for new Honeycrisp plantings.

Table 1. Timeline for project activities and 2020 progress

Activity	2020		2021		2022		2023
	Spring/ Summer	Fall/ Winter	Spring/ Summer	Fall/ Winter	Spring/ Summer	Fall/ Winter	Winter/ Spring
Obj. 1 - Sunrise Experiment 1							
Bud sampling	March ☒		March		March		
Fertilizer Treatments	May ✓		May				
Crop load management	June ✓		June		June		
Leaf and fruitlet nutrient sampling	June ✓		June		June		
Gas Exchange Measurements		July-Aug. ✓		July-Aug.			
Harvest and quality		Sept-Oct. ✓		Sept-Oct.		Sept-Oct.	
Nutrient Analysis		Oct.-Nov. ✓		Oct.-Nov.		Oct.-Nov.	
Poststorage Evaluations		December ✓	January	December	January	December	January
Obj. 1 - Sunrise Experiment 2							
Fertilizer Treatments	May ✓		May		May		
Crop load management	June ✓		June		June		
Leaf and fruitlet nutrient sampling	June ✓		June		June		
Gas Exchange Measurements		July-Aug. ✓		July-Aug.		July-Aug.	
Harvest and quality		Sept-Oct. ✓		Sept-Oct.		Sept-Oct.	
Nutrient Analysis		Oct.-Nov. ✓		Oct.-Nov.		Oct.-Nov.	
Poststorage Evaluations		December ✓	January	December	January	December	January
Obj. 2 - Commercial Sampling							
Site selection and grower practice documentation	Mar.-Apr. ✓		Mar.-Apr.		Mar.-Apr.		
Tree selection	June ✓		June		June		
Soil Sampling	June ✓		June		June		
Fruitlet and leaf sampling	June ✓		June		June		
Harvest and quality		Sept-Oct. ✓		Sept-Oct.		Sept-Oct.	
Shoot growth measurements and leaf sampling		September ✓		September		September	
Nutrient Analysis		Oct.-Nov. ✓		Oct.-Nov.		Oct.-Nov.	
Poststorage Evaluations		December ✓	January	December	January	December	January
Obj. 3 - Threshold Development							
Data analysis of critical factors controlling bitter pit and green spot			Jan-Mar.		Jan-Mar.		Jan-Mar.
Development of key thresholds for soil, bud, fruitlet, leaf, and fruit nutrient levels for K, N, and Mg					Jan-Mar.		
Publishing key bulletins and factsheets to help inform grower fertilizer decisions				Oct.-Nov.			
Project updates			Jan-Mar.		Jan-Mar.		Jan-Mar.

METHODS

The first objective is being conducted at Sunrise Research Orchard. In response to reviewer comments, in 2020, treatments were applied every two weeks over three applications in liquid form in May and June. For both cultivars, a second experiment was used to measure seasonal response of N, Mg, and K rates on growth, physiology, and fruit quality of both Honeycrisp and WA 38 trees. These experiments were conducted on untreated trees each year to determine seasonal responses of post-bloom applications of each of N, Mg, and K to WA 38 and Honeycrisp. For Honeycrisp, crop load was carefully regulated using the combination of bloom and fruitlet thinning strategies and hand clean-up to target crop loads by June 1. The Honeycrisp orchard was on an 'off' year and had a lighter crop load than usual. WA 38 was not thinned. Shoot growth was measured at harvest.

Table 2. Rates for nitrogen, potassium, and magnesium at low, medium, and high applications rates for single-year and multi-year experiments.

Lbs/acre applied	Nitrogen (N)	Potassium (K)	Magnesium (Mg)
Low	20	50	25
Medium	40	125	50
High	80	200	100

Physiological measurements and leaf elemental analysis

Gas exchange measurements were made on one sun-exposed leaf per tree once per month to identify whether fertilizer rates affect tree gas exchange and carbon balance. Leaves were sampled for nutrient analysis at the same time. For experiment 1 where applications will occur every year, stem and bud samples will be collected in November and March of each year to provide information on how fertilization affects nutrient storage in stem and reproductive tissues.

Fruit quality

At harvest (early September for Honeycrisp and early October for WA 38), all fruit was completely removed from each sample tree (two trees per replicate) and weighed to provide total yield (kg). Then, 48 fruit was randomly selected from each tree. Half of the fruit will be used for fruit quality at harvest and the other fruit will be stored in regular atmosphere for three months at 1° C and used for fruit quality analysis post storage. Elemental analysis will be performed using a pooled sample consisting of a peel sample collected from the calyx end of eight fruit from each replicate. Samples will be dried, ground, and acid digested then analyzed using an Agilent 4200 MP-AES elemental analyzer. Then, after 3 months of storage, bitter pit and green spot incidence and severity along with fruit firmness will be assessed again for fruit from each replicate.

1. Identify the relationship between shoot growth, crop load, and nutrient concentration with disorder incidence at harvest and after storage for commercial orchards.

Experiments conducted in objective 1 are valuable for determining thresholds and impacts of fertilization on fruit and tree physiology along with disorder incidence. However, commercial orchards span a larger range of environments, soil types, ages, training system, management strategies, and rootstocks that

underscore the importance of including a thorough sampling approach to capture the range in factors that affect disorder incidence for both Honeycrisp and WA 38.

As suggested from the preproposal stage, we will also seek to split the sampling between orchards with M9-T337 and G41 as a rootstock but will also include other rootstocks as appropriate. In 2020, there were a total of 42 orchards sampled for Honeycrisp and WA 38 in total. Management information will also be collected that will include soil type, physical and chemical conditions, location and management practices to better help understand key factors on the disorder development.

In all sampled commercial orchards, three representative trees were chosen and diameter measured. Fruitlet and leaf samples were collected at this time for nutrient analysis. Fruit was harvested within three days of commercial harvest for all sites. At harvest, fruit counts were determined for selected trees and a subsample of 32-48 fruit per tree was collected. Half will be placed in cold storage for three months and fruit quality will be measured using the parameters described in objective 1. Shoot growth will also be measured on 20 terminal shoots per tree. Fruit peel elemental analysis will be performed as described in objective 1 including N, Ca, Mg, and K concentrations along with $\delta^{13}C$ analysis as an indicator of irrigation management relative to soil type.

2. Develop clear thresholds for N, K, and Mg fertilization based on fruit and leaf elemental concentrations for Honeycrisp and WA 38 orchards in WA State.

This work will begin in January 2021 and continue until the end of the project. This will include Extension deliverable prepared by both Lee Kalcsits and Bernardita Sallato. We will communicate information via Fruit Matters, Extension factsheets, winter meeting talks, field grower visits, and social media. Rapid communication of results will enable growers to adjust their practices quickly to reduce the incidence of both bitter pit in Honeycrisp and green spot for WA 38.

RESULTS & DISCUSSION

Nutrient composition of fruitlet were positively correlated with at harvest fruit nutrient composition (This information will be part of a Fruit Matters article published in early 2020). This indicates that early-season conditions are critical for setting up adequate nutrient uptake into the fruit, similar to what has been reported elsewhere. As we get more data, we are anticipating that crop load and bloom numbers will contribute to those early season values because of its effect on the leaf: fruit balance. WA 38 had fruit calcium content that was almost two times higher than Honeycrisp. It also had slightly higher potassium content but overall K/Ca ratios were much lower.

Consistent with previous literature, (K+Mg)/Ca ratios were correlated with bitter pit in Honeycrisp for commercial orchards across the state (Figure 1). Similarly, green spot was also strongly correlated with these nutrient ratios (Figure 2). We used “non-trivial” to indicate green spot that was not colored over at harvest. Nitrogen content is still being measured and will be included in the 2021 report and in subsequent Extension publications. The sampling that occurred from commercial orchards included a range in crop loads spanning from 2 fruit cm^{-2} TCSA to as high as 14 fruit cm^{-2} TCSA. There was a significant relationship between fruit peel (K+Mg)/Ca ratios at harvest and crop load (Figure 3 and 4). Although not shown here, there were also significant relationships between fruit peel nutrient content and shoot length. These results will be communicated in winter meetings and a fruit matters article in 2021.

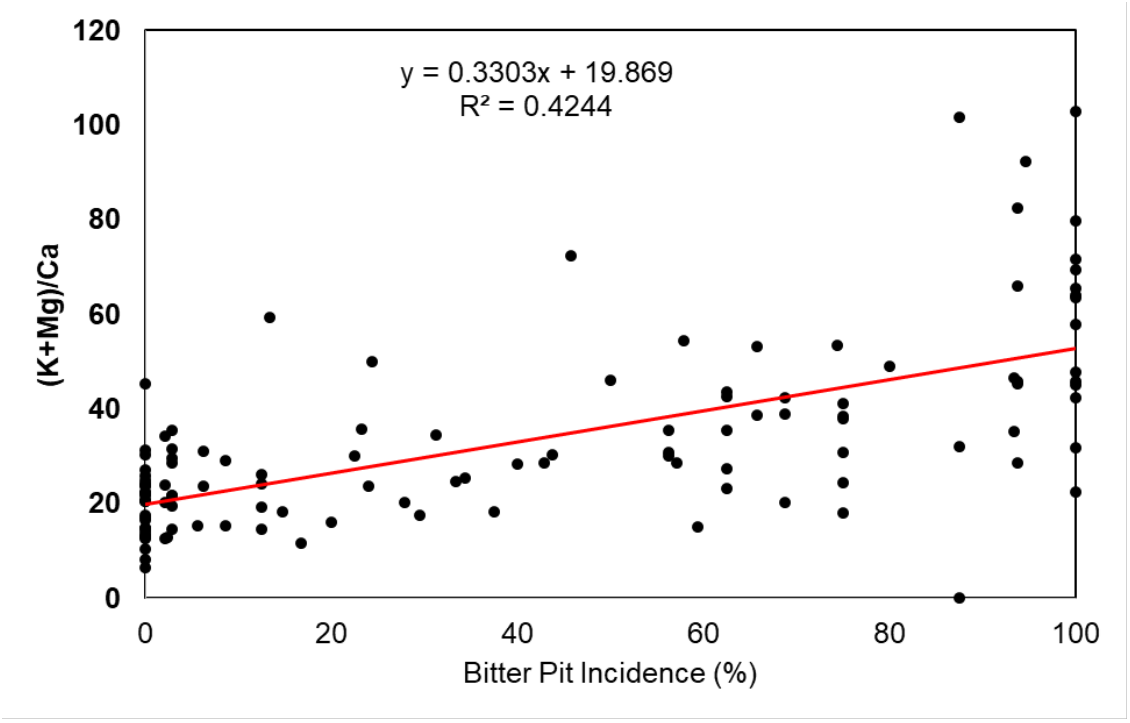

Figure 1. The relationship between (K+Mg)/Ca peel ratios at harvest and bitter pit incidence one month after harvest for Honeycrisp sampled from three trees at each of 22 commercial orchards across WA state spanning 8 rootstocks.

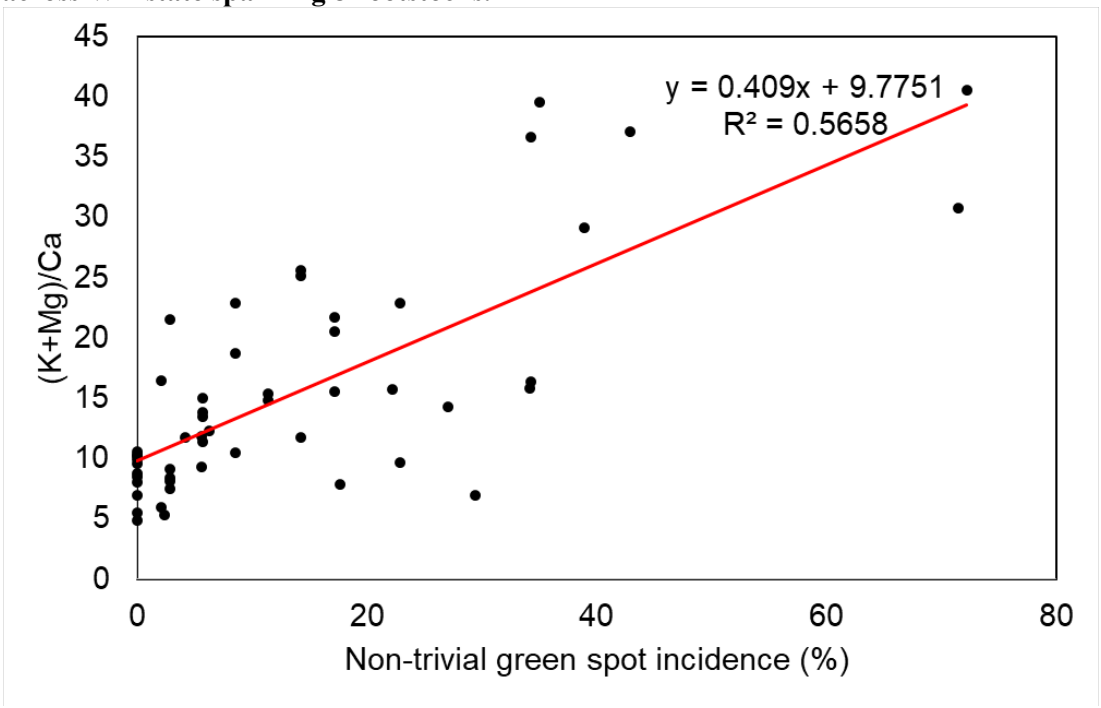

Figure 2. The relationship between (K+Mg)/Ca peel ratios at harvest and bitter pit incidence one month after harvest for WA 38 sampled from three trees at each of 15 commercial orchards across WA state spanning 6 rootstocks.

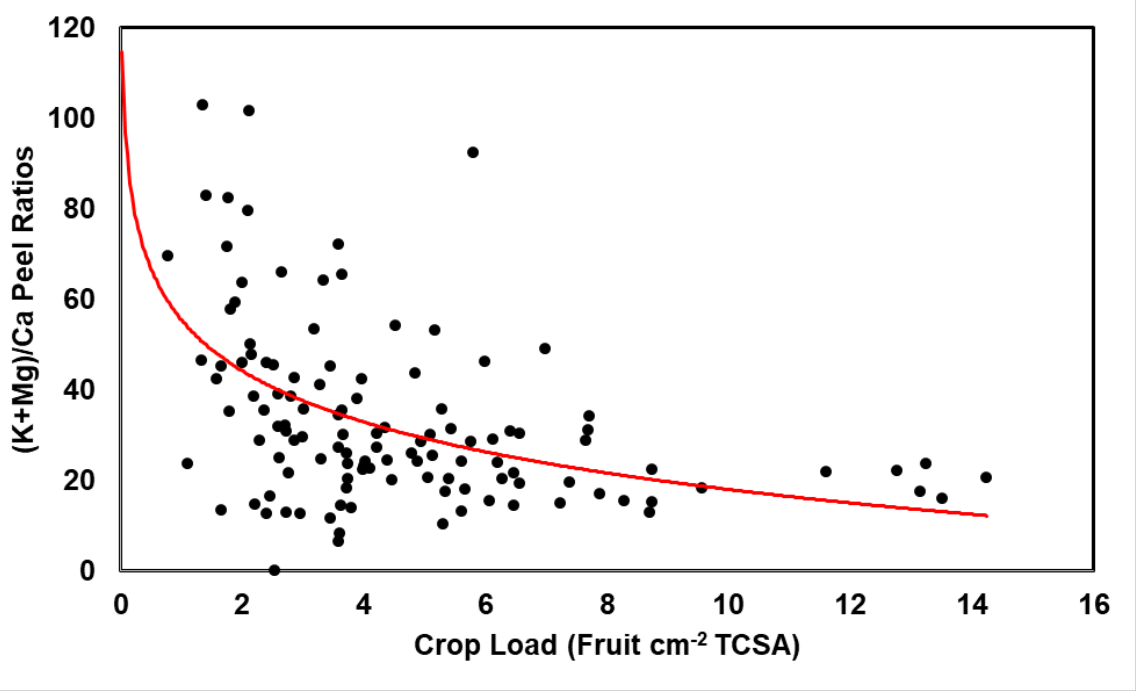

Figure 3. The relationship between crop load and (K+Mg)/Ca fruit peel ratios measured at harvest for Honeycrisp. Rootstock appear to be causing variability in this relationship but will be examined after another year of data.

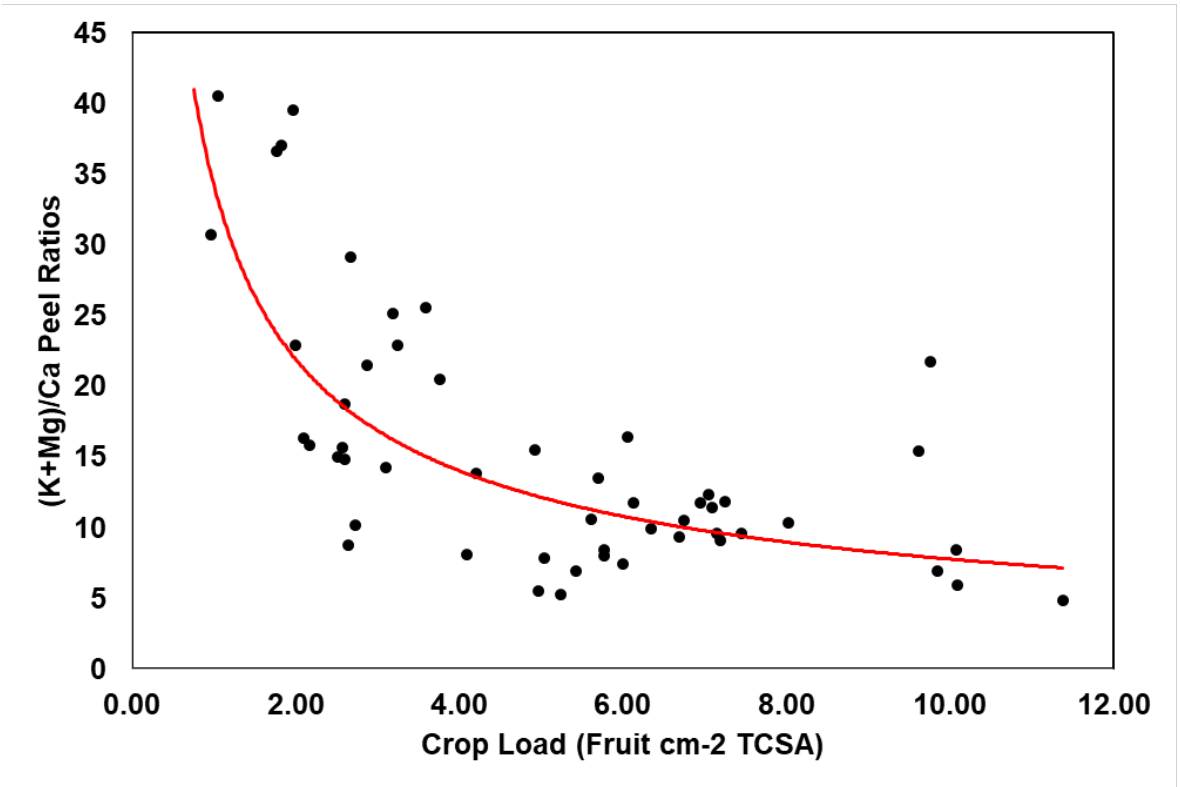

Figure 3. The relationship between crop load and (K+Mg)/Ca fruit peel ratios measured at harvest for WA 38. Rootstock appear to be causing variability in this relationship but will be examined after another year of data.

CONTINUING PROJECT REPORT **YEAR**: 1 of 3

Project Title: Phase 3 evaluations of apple breeding program selections

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Cooperators:

- Breeding Program Advisory Committee: Aylin Moreno (McDougall), Brent Milne (McDougall), Bruce Allen (Columbia Reach), Dale Goldy (Gold Crown), Dave Allan (Allan Bros.), Dave Gleason (Kershaw), Dena Ybarra (WTFRC commissioner), Hans Groenke , Harold Schell (Chelan Fruit), Jeff Cleveringa (Starr Ranch), Jeff LaPorte (Chelan Fruit), Jim Mattheis (USDA-ARS), Lauren Gonzalez (GS Long), Mike Robinson (Double Diamond), Paul Cathcart (Columbia Reach), Sarah Franco (Allan Bros.), Suzanne Bishop (Allan Bros.), Tim Welsh (Columbia Fruit), Carolina Torres (WSU)
- Researchers: Stefano Musacchi (WSU), Bernardita Sallato (WSU)
- Companies: Stemilt Inc., Allan Bros., Agrofresh Inc., Legacy Fruit

Total Project Request: **Year 1:** 50,813 **Year 2: $51,702** **Year 3:** $52,559

Other funding sources: None

WTFRC Budget:

Item	2020	**2021**	2022
Salaries			
Benefits			
Wages[1]	24,938	25,401	25,831
Benefits	11,375	11,396	11,407
RCA Room Rental[2]	13,500	13,905	14,321
Shipping			
Supplies[3]	500	500	500
Travel[4]	500	500	500
Total	50,813	**51,702**	52,559

Footnotes:

[1]Wages/Benefits: Calculated based on expected staff wage adjustments.

[2] RCA room rental: 1.5 rooms @ $6500/room plus $2500/room warehouse fees, adjusted yearly

[3] Supplies: consumables for fruit quality lab (KOH, distilled water, iodine solution, etc.)

4 In-state travel

OBJECTIVES

1. Evaluate and determine commercial potential of elite selections of the WSU Apple Breeding Program (WABP)

SIGNIFICANT FINDINGS

Currently there are five selections in the WABP Phase 3 (P3). Only selections L and P are in their production stage.

1. Three new selection were grafted in the Quincy site (Q, R, S)
2. Selection O, grafted in 2016 at the Quincy site, was discontinued
3. Selection L, grafted in 2015 at both Quincy and Prosser sites, is in the advanced P3 phase
4. P3 planting in Prosser will be transitioned to an orchard near Wapato

METHODS

General Procedures

Bud and Bloom observation: Field observations started as the trees began to bloom, occurring at least twice a week, taking into consideration the weather pattern and its influence on blooming. The full bloom date was determined in accordance with the WSU definition (60% of the king blooms are open on the north side of the trees) and "grower current practices" (80% of blooms open and first petal fall). Full bloom date information of standard varieties near the Phase 3 (P3) plots were collected for comparison. Starting at this stage, every field visit included general observations on disease incidence, tree growth habit and health. Standard management practices (rodent activity monitoring, powdery mildew sprays, row mowing, etc.) were discussed with field managers. Pest and disease incidence and monitoring is documented and carried out during the entire season.

Fruitlet development and pre-harvest: Field activities for this stage start after June drop. Orchard visits occurred bi-weekly until a month prior to predicted harvest. Observations on fruit set and self-thinning were documented. Hand-thinning and summer pruning were performed, when appropriate, by orchard crew, as if that selection were being produced commercially.

Harvest: To determine harvest date, starch degradation is assessed in combination with color development and flavor. Once harvest date was established, harvest was conducted in one to three picks, depending on selection and crop load. In 2020 all apple selections were strip picked. Apples were harvested using picking bags and placed in blue crates. Fruit was weighed in the field and separated in to two or three storage conditions: Refrigerated air (RA, 33°F), RA 37°F, and controlled atmosphere (CA, 34°F 1% CO_2, 2% O_2), with and without 1-MCP treatment.

Culls were sorted during harvest and weighed separately; reason for cullage was assessed on individual fruit. Storage samples were drenched with the fungicide Scholar at a Stemilt drencher location and stored at the Research CA rooms (RCA rooms) at Stemilt. 1-MCP treatment was administered within one week after harvest.

Quality at harvest was assessed within one day of harvest. The specific quality parameters tested at harvest were starch degradation (Cornell 1-8), firmness (lb.), soluble solids (% brix), titratable acidity (% m.a.), color (% of red coverage and background color), size (in.), weight (gr.) and presence/absence of internal and external defects/disorders.

Post-harvest: Quality assessment takes place after 3, 6 and 8 months of storage for apples in RA, and 6 and 9 months for apples in CA. Apples with and without 1-MCP treatment will be evaluated at the same timepoint. Quality analysis is conducted after 7 days at room temperature to determine the potential quality for consumers after shipping, handling and purchase. All apples handled in the lab will have the weight recorded to generate box size distribution data. Based on total amount of apples harvested, fruit will be distributed at meetings and events. This serves as industry taste panel and informal consumer acceptance evaluation.

Advanced Phase 3

When an elite selection is considered a good contender for commercialization (typically after at least three years in P3), it will receive the following additional evaluations:

- handling on commercial packing lines
- formal consumer taste panels

Due to the COVID-19 pandemic, we will most likely not be able to perform activities in packing house facilities and a consumer panel in the 2020-21 storage season.

Selection specific evaluations for 2020 harvest season

Selection P (Honeycrisp × Southern Snap):

- Evaluate late harvest effect on maturity parameters, field cullage and storage disorder incidence

Selection L (Honeycrisp × Cripps Pink):

- assess potential to be single picked, by improving light penetration into the canopy to promote even color development
- establish optimum harvest window based on maturity parameters, field cullage and storage disorders incidence
- evaluate storage performance in long term RA and with higher storage temperature
- evaluate packing line handling (waxing and bruising)
- assess consumer acceptance

RESULTS AND DISCUSSION

New apple selections: Three apple selections were top worked in Quincy in spring 2020:
Q (Cripps Pink × Honeycrisp), R (Cripps Pink × WSU 3), and S (Honeycrisp × WA 2). Selection evaluations will start in the Spring of 2021, according to the methods previously stated. Pruning techniques will be discussed with a specialist and conducted by the farm crew at the appropriate timing. The plot map was updated to reflect the inclusion of new, and removal of discontinued selections. Tree tags and row labeling are planned for spring 2021.

Discontinuation of selection O: This selection was in P3 for four years. Fatal flaws, such as alternate bearing and poor storage performance make this selection unfit to be produced commercially. Discontinuation was recommended by the Breeding Program Advisory Committee (BPAC) and confirmed by Dr. Evans.

Selection P: This selection was grafted in Quincy and Prosser in 2017 and 2018, respectively. It is a bi-colored apple that develops good color coverage (dark red). The trees are vigorous, capable of reaching the top wire in the first year. This selection is typically harvested mid to late-September. Apples have great texture and a unique tart tangy flavor profile, good shelf-life potential and develop very few storage disorders.

- *Evaluate late harvest effect on maturity parameters, field cullage and storage disorder incidence:* One unique characteristic of this selection is the high titratable acidity values at harvest (between 0.9 to 1.2) that remain high throughout storage (0.6 to 1.0). Data collected on previous years indicated this selection can be prone to greasiness if fruit is harvested late and not treated with 1-MCP. Multiple picks were performed in Quincy in 2020 to observe the effects of advanced maturity at harvest on titratable acidity and firmness degradation over time, incidence of stem bowl splitting in the field, and greasiness incidence in long term storage.

Selection L: This selection was grafted in Quincy and Prosser in 2015. This bi-colored selection develops a bright pink cheek when exposed to light, is self-thinning, slow to brown, and has excellent shelf-life potential. This selection is typically harvested late-September to early October. Some concerns are sensitivity to sunburn and powdery mildew (on the leaves). Because of its desirable characteristics, this selection was moved to an advanced P3 in 2019.

- *assess potential to be single picked by improving light incidence in the canopy to promote even color development:* Due to differences in tree training systems, fruit from Prosser (vertical fruiting wall) has developed better color than fruit from Quincy (spindle). Trees in Prosser were summer pruned in 2020 two weeks before first harvest, allowing more light to reach the apples in the mid and low canopy.
- *establish optimum harvest window based on maturity parameters, field cullage and storage disorder incidence:* Selection L can maintain high levels of fruit firmness (above 20 lb.) throughout storage, sometimes resulting in apples that are perceived by some consumers as too hard to eat. In 2020, we monitored fruit firmness as well as starch levels to establish optimum harvest timing. Multiple picks occurred in both locations to evaluate firmness over time during cold storage. Differences in maturity

parameters at harvest will be assessed to observe if advanced maturity will promote stem bowl splitting in the field, or soft scald development and greasiness incidence on long term storage.

- *evaluate storage performance in long term RA (8 months) and higher storage temperature:* Our typical storage protocol is to sample fruit stored at 33°F for 3 months in RA storage, and 6 and 9 months in CA storage, with and without 1-MCP. Based on preliminary results from previous years this selection has shown good results when kept in RA storage for three to six months. Some samples from the 2020 harvest (with and without 1-MCP) were stored in RA at 37°F; RA storage will be extended to 8 months.
- *evaluate packing line handling (waxing and bruising):* one packing line handling evaluation was conducted in early 2020. Data collected shows that fruit can hold wax well, losing some of the gloss when held at room temperature for 7 days. Apples show very little bruising susceptibility when run over a commercial packing line.
- assess consumer acceptance: Due to the COVID-19 pandemic, two taste panel events were cancelled in spring 2020.

Phase 3 planting in Prosser will be transitioned to Wapato: Starting in 2021, new P3 selections will be planted in an orchard near Wapato, and the P3 block in Prosser will be phased out.

BPAC meetings and field visits: The goal of these events is to receive input on any field practices that should be taken into consideration, based on growth habits and crop load characteristics of each selection, to keep industry representatives aware of the current state of each P3 selection, and to keep this phase moving forward, based on industry-oriented recommendations. Due to the pandemic, no field day events were held this year, and the BPAC meeting was held via ZOOM in July. Upcoming events will be planned accordingly.

CONTINUING PROJECT REPORT **YEAR:** 2 of 3
WTFRC Project Number: AP-19-104

Project Title: Non-destructive detection of sun stress compromised apples

PI:	David Rudell	**Co-PI**:	Carolina Torres
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Collaborators: Dr. Rene Mogollon, Manoella Mendoza, Dr. Lorenzo León

Budget: **Year 1**: $88,947 **Year 2**: $91,545 **Year 3**: **$94,246**

Other funding sources

Agency Name: USDA-ARS, In-house project
Amt. awarded/requested: $61,313/3 yrs.
Notes: In-house project with complimentary objectives. Funds for storage maintenance and costs ($8000/yr), supplies and materials ($3000/yr), travel ($5000/yr), and 0.1 FTE (co-PI).

Budget
Organization Name: USDA-ARS **Contract Administrator:** Chuck Myers
Telephone: (510) 559-5769 **Email address:** Chuck.Myers@ars.usda.gov

Item	2019	2020	2021
Salaries (WSU post-doc)	47,500	49,400	51,376
Benefits (WSU)	17,447	18,145	18,870
Wages (ARS part time)	10,000	10,000	10,000
Benefits			
Equipment			
Supplies			
Travel (for Lorenzo León)	2500	2500	2500
Miscellaneous*	11,500	11,500	11,500
Plot Fees			
Total	88,947	91,545	**94,246**

Footnotes: One-third instrument service contract (TFRL, USDA-ARS)

Objectives:
1. Determine best non-destructive methods to segregate sun stress compromised fruit.
2. Validate accuracy of non-destructive method for detecting chemistries associated with solar stress.
3. Test if non-destructive sorting improves storage outcome for different sun stress related disorders.

Goals and Activities for the next year:
Repeat Years 1 and 2 sunscald prediction and sorting according to cumulative sun exposure. In Year 3, a pilot study will use these indexes as a means of sorting 'Granny Smith' apples from bins picked from multiple orchards and, then, assess external and internal quality over a long period following CA storage. We will continue to dissect tissue to confirm sun exposure assessment and sunscald prediction using hyperspectral imaging matches expected levels of peel chemicals associated with spectral changes and cumulative sun exposure. We will focus on those images that indicate the presence of yet unknown differences of peel chemistry. Other work will continue determining the best configuration of our UV-Vis hyperspectral imaging system to improve the sorting accuracy and resolution over our indexes with the addition of Vis-NIR imaging.

SIGNIFICANT FINDINGS:

1. An accurate system was developed to detect sunburn and sunscald associated with excessive sun stress using Vis-NIR hyperspectral imaging.
2. Additional indexes were developed to be used for sorting 'Granny Smith' fruit not already damaged by sun according to cumulative sun exposure.

METHODS
Hyperspectral imaging
Apples were scanned monthly using a Nano-Hyperspec VNIR imager (400-1000 nm), tungsten light source, and scanning bed (Headwall Photonics, Bolton, MA) for all experiments. Composite hyperspectral images (data cubes) have been used for Vis-NIR predictive model development based on multiple spectra. We are testing multiple models/indexes we developed as a means to sort fruit according to cumulative sun exposure. This system is being compared and images added to a model we expect to develop using targets in the ultraviolet (UV-Vis) (250-500 nm).

Objective 1: Determine best non-destructive methods to segregate sun stress compromised fruit.

In years 1 and 2 of the project, we are imaging fruit from 2 different sun sensitive cultivars at harvest and then storing them in air for up to 6 months and assessing fruit finish and appearance defects monthly. Ongoing analysis of hyperspectral images and spectra has been used to develop multiple indexes that could be used to sort sunburn, predict risk for sunscald and other sun-related disorders, and sort apples according to relative sun exposure. We are continuing to refine these models by improving their accuracy by extending the reference spectrum used for imaging as well as employing the indexes using commercial sorting equipment. The following details the current season's harvest and storage activities: Granny Smith apples were harvested from Sunrise orchard in Rock Island, WA. Sun exposed fruit was selected from the periphery of the trees. Fruit were sorted into 3 categories according to sunburn severity: clean (no sunburn), mild, and moderate yielding 288, 128 and 160 apples, respectively, for a total of 576. The sun exposed side of each fruit was marked using a indelible marker. Honeycrisp apples were collected from a commercial orchard located in near Quincy, WA. The same harvest procedure was followed. Rather than Fuji as in year 1, a total of 576 Honeycrisp apples were harvested and segregated in clean (288), mild (128) and moderate (160).

Objective 2: Validate accuracy of non-destructive method for detecting chemistries associated with solar stress.

The following details the current season's activities: In addition, a subset of Granny Smith apples were sorted into the same three categories above mentioned: clean (48), mild (38) and moderate (48). All apples were scanned with both VNIR and UV-Vis cameras. Afterwards, the sun-exposed and non-exposed sides of 10 of these apples per each category were peeled and stored in liquid nitrogen for further analysis. The remaining fruit will be scanned with both VNIR and UV-Vis after 3 and 6 months of air storage, followed by apple peel sampling.

Objective 3: Test if non-destructive sorting improves storage outcome for different sun stress related disorders.

Once a method for non-destructive sorting is established, larger numbers of fruit from different orchards will be harvested sorted, and stored in both air and CA to determine how accurately and reliably sun stress fruit are segregated and if sorting fruit actually does improve storage outcome with respect to sun-stress associated postharvest disorders.

RESULTS AND DISCUSSION

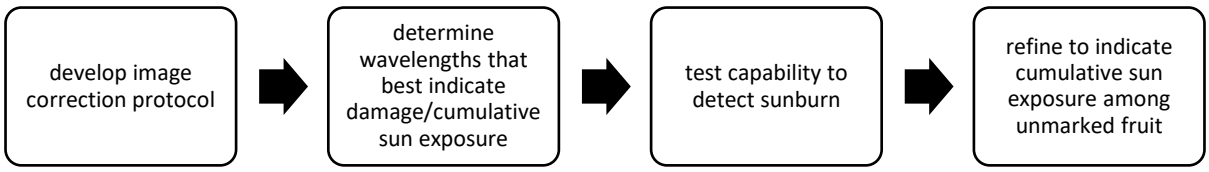

Figure 1. Steps used in developing a protocol for sorting apples at harvest according to cumulative sun exposure.

Image correction protocol and determining spectral regions indicating cumulative sun exposure (Figure 1)

A hyperspectral camera uses spectra from every pixel within the image. As not all information within the image is useful, adjustments must be made to ensure only good information is incorporated in the prediction (not shown). This was accomplished by manual annotation using the software SuperAnnotate (Sunnyvale, CA) to remove all glare pixels and those that were too dark. The remaining information was used to construct a model (first) that detects sunburn and, consequently, sunscald. This was completed using a population of 'Granny Smith' apples containing severely, moderately, and lightly sunburned fruit as well as exposed, but healthy fruit. Unusable regions were removed and models generated to best detect sunburn and predict sunscald using a process called Convolutional Neural Networks (CNN) analysis. Four models were generated that indicated sunburn in the images based on differences in 2 main regions in the visible-near infrared spectrum (for 'Granny Smith') using 91 equidistant wavelengths in the range from 600 to 800 nm. Based on 2 of the models, apples could be sorted for sunburn with very low false positive (0.87-1.6%) and false negative (0.29-0.49%) rates.

While this may be useful and would remove all sunburned apples, including those with nearly unnoticeable levels, as well as those most likely to sunscald, our goal was to determine cumulative sun exposure, even among the "clean" fruit. We chose to base this analysis on differences in levels of

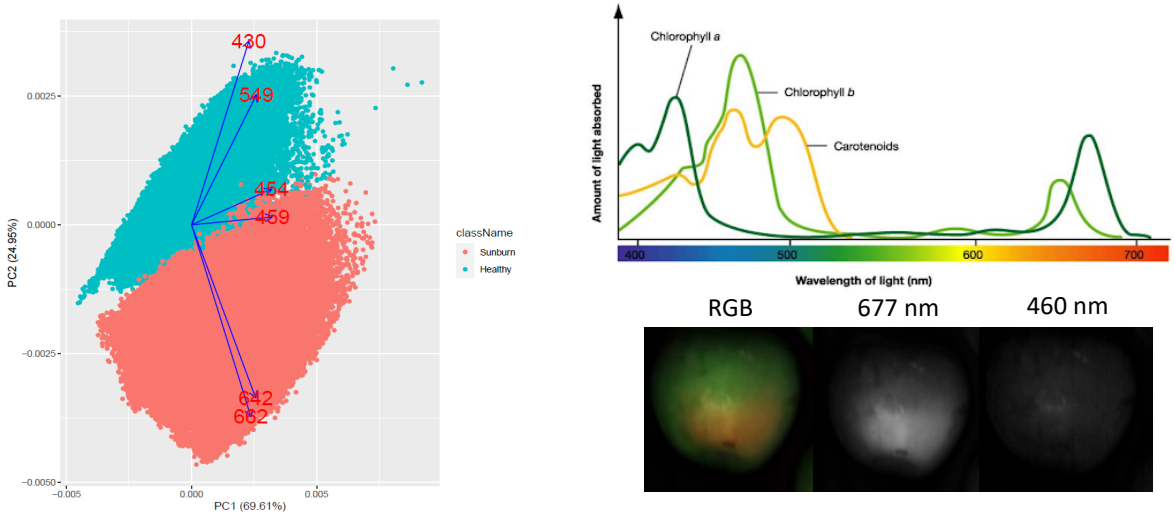

Figure 2. Determination of spectral regions most indicative of tree position. A principal component loading plot indicating spectral bands associated with chlorophyll and carotenoids are most responsible for indicating sunburn compared to unmarked fruit (left). Differences of absorbance in these spectral bands are illustrated in the upper right graph. To demonstrate in an image, in the 430-500 nm band, the increased levels of carotenoids in sunburned tissue counter the diminished chlorophyll levels leaving us with absorbance in both areas and a solid dark picture (bottom right). However, at 642 and 662 nm, chlorophyll content can be estimated without interference from the carotenoids (top right). Consequently, the ratio between reflectance at 642 and/or 662 nm and 549 nm (chlorophyll/carotenoid) provides a good estimation of sun exposure in 'Granny Smith'.

metabolites that we know are associated with tree position (Musacchi et al, PR14-108A) and sun exposure (AP-16-102, Racsko and Schrader, 2012; Grandón et al., 2019). This was validated by an evaluation of readable regions from the sunburn gradient population mentioned above. In this way, we are using a non-destructive imaging system to analyze levels of chemicals that we destructively analyzed in prior work. Spectral bands resulting from peel chemicals that absorb light at 430 nm (chlorophyll a–green pigment), 459 nm (chlorophyll b–green pigment), 454,549 nm (carotenoids–orange and yellow pigments), and 642

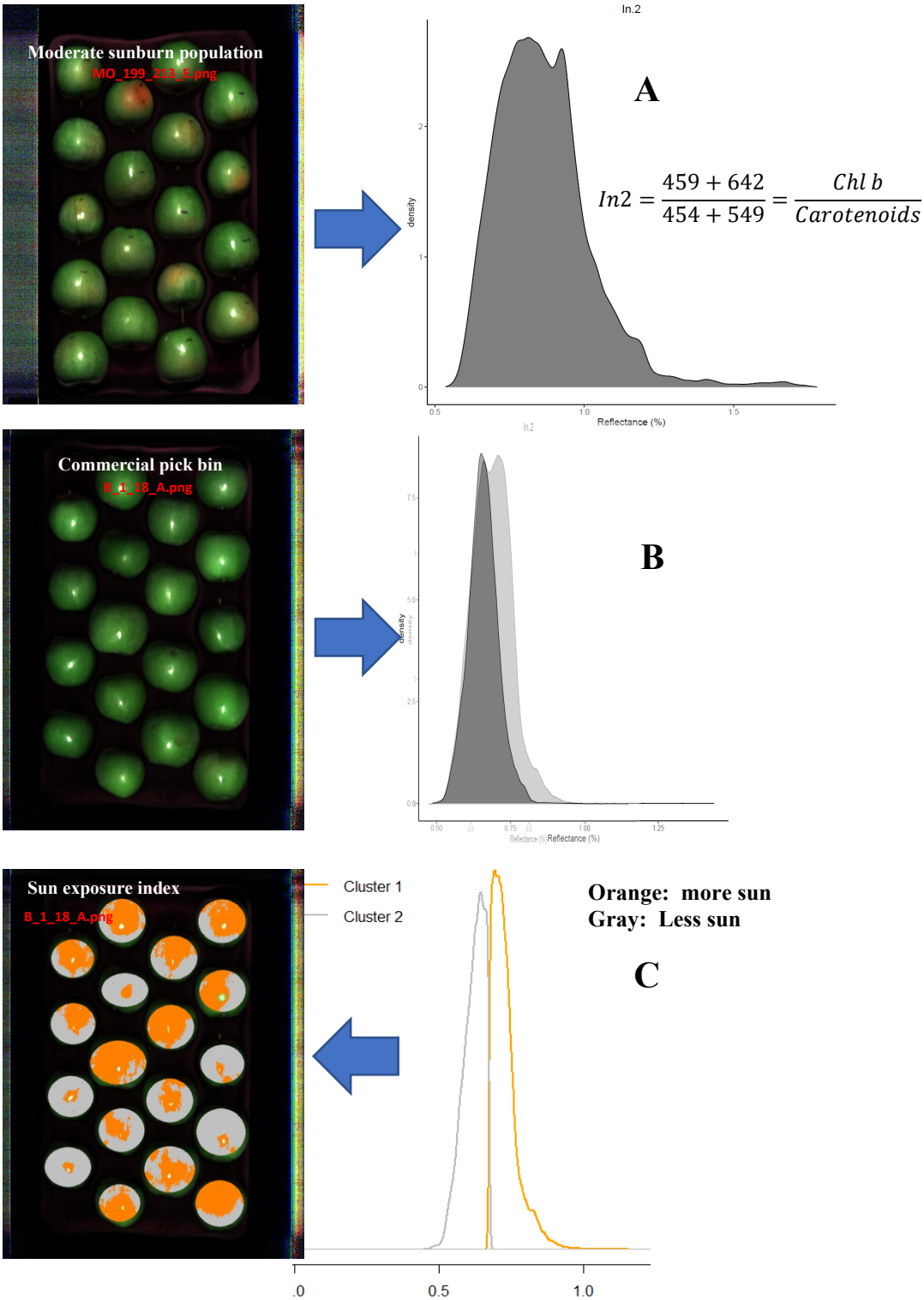

Figure 3. Populations of non-sunburned fruit can be non-destructively sorted according to cumulative sun exposure using 2 potential indexes based on chlorophyll to carotenoid ratio. The top image and figure (A) shows a 'Granny Smith' population selected for moderate sunburn and the consequent wide range of reflectance (Index 2) of the total image area indicating diverse values in this index in this population. The image and reflectance profile of In2 from a healthy population have a narrower range on the exposed side (B) and even narrower on the unexposed side (darkened area in the curve) indicating greater consistency as sun exposure diminishes. The In2 reflection on the exposed side is statistically separable (C) into groups representing each of 2 reflectance peaks and levels can be used to indicate where unmarked fruit have received more light.

nm/662 nm (chla/chlb–upper bands, green pigments) provided a good separation of sunburn and healthy peel (Figure 2). This indicates we can expect carotenoid (orange color) levels to be high and chlorophyll (green color) levels to be relatively lower as peel is progressively more sunburned while healthy tissue has a higher ratio of either or both of the chlorophylls to carotenoids (Figure 2). Consequently, the chlorophyll to carotenoid ratio may be expected to be lower, even in entirely unmarked peel, with greater cumulative sun exposure.

To test this, 'Granny Smith' apples from the progressively sunburned population were compared with the front and back side of apples from a commercially picked bin that were then labeled and imaged monthly (front and back) as well as photographed for appearance rating. Usable pixels from each of the populations were analyzed according to three different indexes to create logistic models testing the accuracy of the characterization and, then, actually characterizing each pixel. From this analysis, we could determine the range of reflectance values and the relative area of peel represented in each range with index 2 (In2) providing the greatest range of values and, possibly, the most capacity to categorize fruit according to cumulative sun exposure. Comparison of the In2 curves from all 3 populations highlights this factor with sunburned population having the highest range (Figure 3A), and the exposed and unexposed sides of the random population from the bin having relatively intermediate and narrow ranges of values, respectively (Figure 3B). The diminishing ranges of In2 values within these populations with sun exposure indicate the utility of characterizing a random population, such as the apples from the bin, according to relative sun exposure using this index. A "cluster" analysis of only exposed side images confirms the former observation (Figure 3C).

Sorting fruit according to cumulative sun exposure

One of our current activities is to finish characterizing each apple in the random (bin) population according to the first sunscald prediction model and In2 from already acquired images from Year 1. We are placing fruit into 3-4 catagories, at least to include sunburn, external, and internal fruit. We will compare category with sunscald, superficial scald, and other peel defect incidence. In the current year, we are repeating both experiments. In addition to appearance defects, we will be determining relative fruit internal quality differences among different sun exposure catagories after storage for each fruit from half of a bin. In Year 3 of the project, we expect to characterize and sort multiple populations (bins from different orchards) using these indexes, potentially using the WSU test sorting line which has the capacity to perform this pilot test. Storage outcome will be assessed to determine benefits of sorting according to light exposure. This is also expected to determine how to adapt these sorting criteria among orchards.

Improving capacity and accuracy of sorting apples according to cumulative light exposure.

One way of increasing the accuracy and capacity, beyond using In2, to sort into more catagories is to find and incorporate more accurate indexes based on additional sun exposure-associated metabolites into the indexing model. To do this, we are using a novel ultraviolet (UV) hyperspectral camera alongside a powerful UV light source to image in spectral regions where other chemicals linked with sun exposure absorb light as well as potentially identify new spectral bands that indicate risk for peel defects. Spectral regions within the higher wavelengths (400-500 nm), detected using this imager, differentiate sunburned from healthy peel. This difference is based on contrasting levels of carotenoids detected as also detected by the Vis-NIR sensor and, potentially, other components visible outside the sunburned region (Figure 4A). Differences in our target region, below 400 nm, were not detectable when imaging whole fruit.

This system also potentially detects other key chemicals that accumulate more in sun exposed peel. These natural peel chemicals absorb UV light between 350 and 360 nm but have been challenging to image in whole fruit using the vis-NIR system. Consequently, we are performing a series of tests to determine whether detection is possible by altering our light source or camera/light source configuration. In the first test, solutions containing pure target compounds were drawn onto white filter paper and imaged indicating most of the compounds that may be visualized in this spectral region were detectable and our principal targets, the flavonol glycosides (rutin), were not interfered with from other common metabolites (Figure 4B). Another test of different concentrations of rutin painted onto filter paper indicated that the imaging could indicate amount of this class of chemicals within a range typically found in apple peel (Figure 4C). Finally, a test of ethanolic extract from the sun exposed and shaded side of

Figure 4. Ultraviolet-visible hyperspectral imaging may improve accuracy of non-destructive assessment of cumulative sun exposure. Images of apple between 400 and 500 nm using a UV-vis hyperspectral camera coupled with a high intensity UV light show pattern on the peel not visible to the naked eye (A). Pure natural chemicals painted onto paper cannot be seen with the naked eye but can be imaged at different wavelengths in the UV range (B). Rutin is a target chemical and spectra of other chemicals in that range do not interfere with imagining. The reflectance intensity of rutin solutions painted onto paper ("full concentration", 50:50, 1:10, and 1:100) diminishes with dilution indicating the images are quantitative (C). 'Granny Smith' peel extracted with ethanol from the "unexposed" and "exposed" sides and imaged at the wavelength that rutin and related compounds (flavonol glycosides) absorb most indicates more absorbance on the exposed side and, therefore, more of these compounds.

apple peel, painted onto filter paper, and then imaged indicated that levels of flavonol glycosides were far lower on the unexposed than the exposed side. Given these results our earlier success on whole apples with weaker source light and a simpler camera, we expect interference in the target wavelengths may result from absorption of light by chemicals in cell layers below the peel, confounding differential absorption by target compounds in the peel. Consequently, we are working to change our set up to optimize peel imaging.

CONTINUING PROJECT REPORT **YEAR:** 2 of 3
WTFRC Project Number: AP-19-100

Project Title: Reducing carbon dioxide-related postharvest disorders

PI: David Rudell
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Telephone: 509 664 2280 (ext. 245)
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Collaborators: Dr. Ines Hanrahan, Christine McTavish, Erin Tudor, Shae Milne

Budget: **Year 1**: $79,314 **Year 2**: $92,893 **Year 3**: **$95,036**

Other funding sources

Agency Name: USDA-ARS, In-house project
Amt. awarded/requested: $174,719/3 yrs.
Notes: In-house project with complimentary objectives. Funds for storage maintenance and costs ($8000/yr), supplies and materials ($3000/yr), travel ($5000/yr), and 0.2 FTE (PI, co-PI) and 0.1 FTE (technical).

Agency Name: USDA-NIFA
Amt. requested: $540,888/4 yrs.
Notes: Pre-proposal with complimentary objectives submitted to SCRI program.

Budget
Organization Name: USDA-ARS **Contract Administrator:** Chuck Myers
Telephone: (510) 559-5769 **Email address:** Chuck.Myers@usda.gov

Item	2019	2020	2021
Salaries (GS-9 step 1)	52,116	53,679	55,290
Benefits (33.3%)	17,198	17,714	18,246
Wages (part-time employee)	10,000	10,000	10,000
Benefits			
Equipment			
Supplies			
Travel			
Miscellaneous*		11,500	11,500
Plot Fees			
Total	79,314	92,893	**95,036**

Footnotes: One-third instrument service contract

Objectives:
1. Develop methods to consistently identify CO_2 sensitivity.
2. Determine best cold chain practices when CO_2 sensitivity is indicated.
3. Identify fruit chemistry associated with CO_2 sensitivity.

SIGNIFICANT FINDINGS

1. A variety of internal and external browning symptoms may be attributable to CO_2 sensitivity in many of the cultivars tested.
2. Incidence of symptoms related to CO_2 sensitivity were reduced or eliminated by DPA drenching.
3. Peel chemistry of typically CO_2 sensitivity related symptoms is different from other peel defects.

METHODS

Equipment and Cooperative Summary: Storage experiments, fruit quality assessment, fruit chemistry analyses using analytical instrumentation (gas and liquid chromatography-mass spectrometry), and tissue cryopreservation will be performed using facilities currently in place at ARS-TFRL, Wenatchee. Storage experiments will be conducted in our in-house CA chambers capable of maintaining both O_2 and CO_2 CA environments accurately.

Outreach (Deliverables are summarized under "Anticipated Products" Table 2): Aside from reports to the WTFRC, new information will be disseminated through presentations at industry meetings and at professional conferences, and by publications in industry publications and peer-reviewed journals. We will cooperate with WTFRC (Lead: Ines Hanrahan) to document symptoms of injury not already covered by the new WSU Apple Defect guide. Symptomatic fruit will be photographed, defect notes assembled, and associated descriptive text created. These updates will be incorporated into the existing guide as needed.

Objective 1: Develop methods to consistently identify CO_2 sensitivity

In year 1, 15 apple cultivars were harvested at approximately 2-4 weeks prior to commercial harvest and 7 days after commercial harvest. Harvest maturity (starch index and internal ethylene concentration) and external/internal appearance were evaluated, and fruit was imaged with a digital camera. Two trays of apples were drenched with an emulsion containing DPA (2000 ppm), and 2 other trays were treated with a solution containing only the inactive ingredients from the DPA emulsion (referred to as control trays). The DPA and control trays were put in separate CA chambers to avoid DPA cross contamination and set at 0.6 % O_2: 5% CO_2. After 4 months, apples were evaluated for internal and external defects. Fruit along with the external and internal defects were imaged. Damaged tissue was sampled, flash frozen, and cryo-preserved for chemical analysis where defects were found. Cultivars that did not develop CO_2-related symptoms in Year 1 will be re-evaluated in Years 2 and 3.

Objective 2: Determine best cold chain practices when CO_2 sensitivity is indicated

Year 2 activities under this objective include 1) determining thresholds for O_2:CO_2 storage atmosphere combinations and 2) developing strategies for managing CO_2-related disorders in higher risk apples in any cold chain. An extra activity 3) focused on determining the relationship between maturity and CO_2 sensitivity for WA38.

For activity 1, 'Golden Delicious' was harvested 2 weeks before commercial maturity while 'Fuji', WA38, 'Braeburn', 'Honeycrisp', 'Scilate', 'Pazazz', and JUICI were harvested 7 days after commercial maturity and stored in 1 of 4 CA settings at 33 °F: 0.6% O_2, 1% CO_2; 0.6% O_2, 5% CO_2; 1% O_2, 1% CO_2; 1% O_2, 5% CO_2 (36 apples per CA environment). These will be stored for 4 months, removed and internal and external injury evaluated and documented. The remainder of cultivars determined to be CO_2 sensitive under Objective 1 will be tested in Year 3. For activity 2, 'Pazazz', Juici, 'Scilate', 'Honeycrisp', and WA38 were harvested at commercial maturity. Apples were treated with 1 ppm 1-MCP for 12 h. Following 1-MCP treatment, apples were placed into one of the following regimes: immediate CA, 2 weeks air (33 °F) then CA, or 4 weeks air (33 °F) then CA, with two CA conditions (0.6% O_2, 1% CO_2 OR 0.6% O_2, 5% CO_2) at 33 °F utilized. Each cultivar had 36 apples per treatment combination. At 3 months, external and internal disorder incidence as well as fruit quality will be evaluated. The remainder of cultivars determined to be CO_2 sensitive under Objective 1 will be tested in Year 3. For activity 3, WA38 was harvested at 15, 6, and 4 days prior to commercial harvest, commercial harvest, and 7 days following commercial harvest. Apples were immediately placed in 33 °F CA with elevated CO_2 (0.6% O_2, 5% CO2) and stored for up to 6 months, evaluating external condition at 3 months and internal and external condition at 6 months.

Objective 3: Identify chemistry associated with CO_2 sensitivity

Our broad analysis of peel and cortex chemistry is ongoing. To develop a system for diagnosing peel and cortex browning caused by CO_2 sensitivity, browned peel and cortex from activities outlined under objective 1 continue to be sampled regularly with adjacent healthy tissue and healthy tissue from DPA treated fruit as control. Any browned tissue in or on DPA treated fruit will be sampled as a control to reveal any similarities or differences of chemistry caused by non-CO_2 related browning. This is expected to improve our accuracy of discerning browning injuries caused by CO_2 sensitivity from browning caused by other factors.

We are determining how increasing CO_2 levels in storage influence symptom development alongside changes in levels of chemicals linked with CO_2 sensitivity. By doing this, we are confirming the chemistries that are specific to CO_2 sensitivity. 'Pazazz' was one of the most CO_2 sensitive cultivars and, consequently, was chosen for this activity. At harvest, apples were drenched with 2000 ppm DPA or a solution containing the inactive ingredients. Apples were, then, stored at 33 °F CA at 0.6% O_2 and different levels of CO_2 (0, 1, 2.5, or 5%). Peel and cortex have been sampled at harvest, 0, 2, 4, and 8 weeks, with the last sample point at 16 weeks.

RESULTS AND DISCUSSION

Table 1. Percent incidence of different internal and external disorders in Year 1 (see Figure 1). Radial browning and rough or "orange peel" peel texture symptoms are typically associated with CO_2 sensitivity. DPA drenches typically reduce or eliminate CO_2-related disorders.

Cultivar	harvest	Treatment	Cavities	Non-radial browning	Radial browning	External browning	Orange peel
Golden Delicious	early	no DPA					
	early	DPA					
	late	no DPA	3				3
	late	DPA					
Gala	early	no DPA					
	early	DPA					
	late	no DPA					
	late	DPA					
Cripps Pink	early	no DPA					
	early	DPA					
	late	no DPA					
	late	DPA		3			
Ambrosia	early	no DPA					3
	early	DPA					
	late	no DPA					
	late	DPA					
Red Delicious	early	no DPA	6				
	early	DPA					
	late	no DPA					
	late	DPA					
Fuji	early	no DPA	**11**	3			**14**
	early	DPA	3				
	late	no DPA	3	**86**	**86**		3
	late	DPA		**28**	**28**		
Autumn Glory	early	no DPA					
	early	DPA					
	late	no DPA					
	late	DPA					
Plumac	early	no DPA	**11**				
	early	DPA					
	late	no DPA	3	6			
	late	DPA					
Braeburn	early	no DPA	**14**	**14**			
	early	DPA					
	late	no DPA	**58**		**89**	6	6
	late	DPA		6			
Smitten	early	no DPA					
	early	DPA					
	late	no DPA	6				
	late	DPA					
Scilate	early	no DPA					
	early	DPA					
	late	no DPA			**86**		**17**
	late	DPA					
JUICI	early	no DPA	**14**				
	early	DPA	3				
	late	no DPA	**33**		**67**		
	late	DPA	**19**				
Honeycrisp	early	no DPA	6				
	early	DPA					
	late	no DPA			**69**	**33**	
	late	DPA		**30**		**30**	
Pazazz	early	no DPA	3		**72**		**78**
	early	DPA					
	late	no DPA	6		**56**	**47**	**72**
	late	DPA					
WA38	early†	no DPA		**67**		**11**	
	early†	DPA		3			
	late†	no DPA					
	late†	DPA					

†Early and late samples were harvested from different orchards. Bold text indicates significant incidence (pooled z-test, n=36, $p<0.05$).

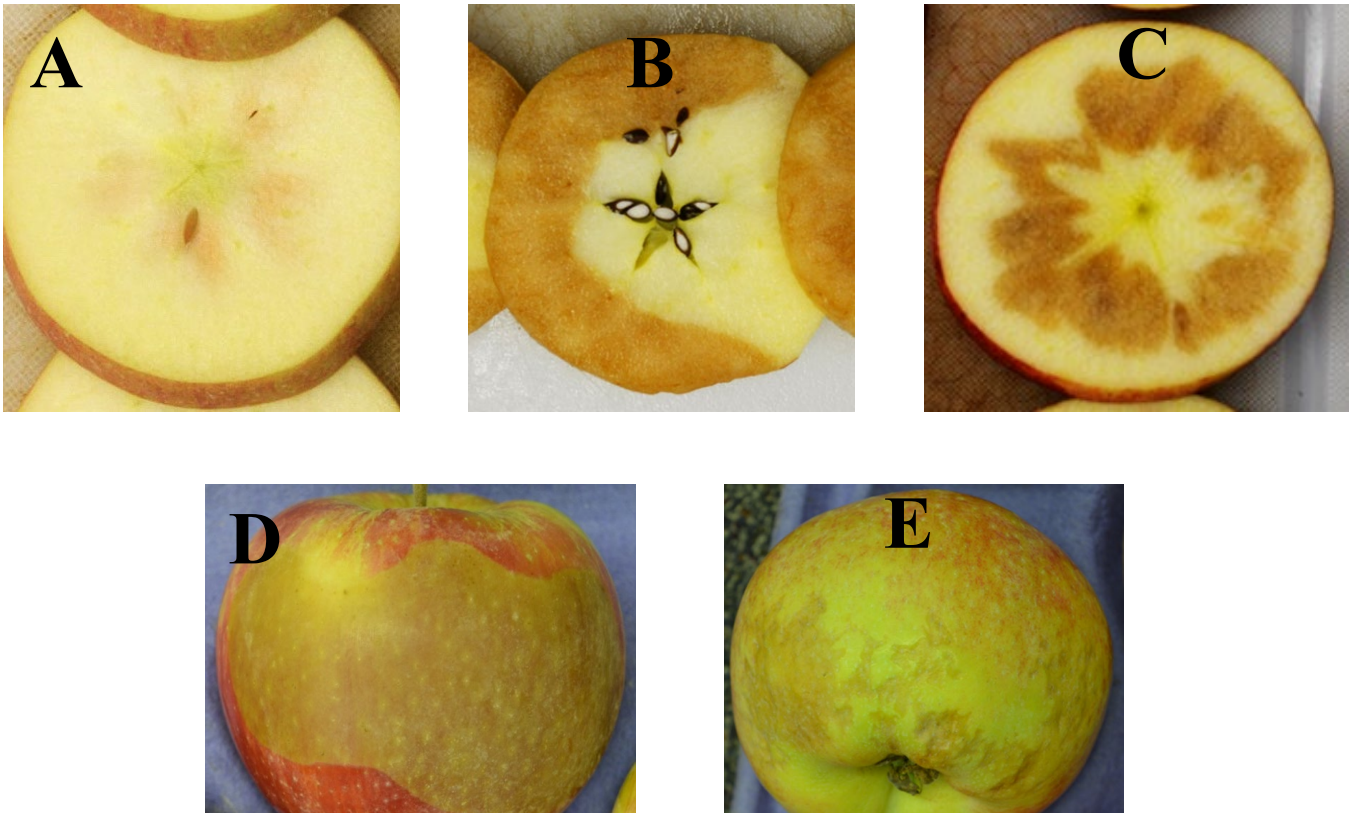

Figure 1. General categories (types) of observed disorders. (A) Lens shaped cavities ('Braeburn'), (B) non-radial browning (soggy breakdown 'Honeycrisp'), (C) radial browning ('Honeycrisp'), (D) Non-radial browning (soft scald, 'Honeycrisp'), and (E) orange ("rugose scald") peel ('Pazazz'). C and E are typically associated with CO_2 sensitivity.

Triggering external and internal CO_2 sensitivity and distinguishing symptoms typically attributed to CO_2 sensitivity from other disorders.

A variety of internal and external symptoms were observed and recorded across many cultivars in the test. These ranged from soft scald of the peel and soggy breakdown in the cortex to the typical symptoms attributed to CO_2 sensitivity, such as orange peel ("rugose") scald on peel and lens-shaped cavities and/or radial browning in the cortex with an asymptomatic barrier immediately under the peel (Figure 1). Symptoms were presented and discussed at a scientific roundtable to amend WSU online disorder databases.

All cultivars, other than 'Gala' and 'Autumn Glory', developed some sort of disorder, although incidence was insignificant in many cases (Table 1). Cultivars with significant symptom development of any type on any of the treatments included 'Fuji', 'Braeburn', 'Smitten', 'Scilate', JUICI, 'Honeycrisp', 'Pazazz', and WA38. Harvest maturity impacted disorder development. 'Fuji' orange peel symptoms were more prevalent on earlier harvested fruit while most cortex disorders were either more prominent or only found in cortex of the later harvest. Pitting symptoms were the least impacted by harvest maturity. We observed some symptoms that were less recognizable such as severe core and peel browning of 'Fuji' and WA38 (Figure 2).

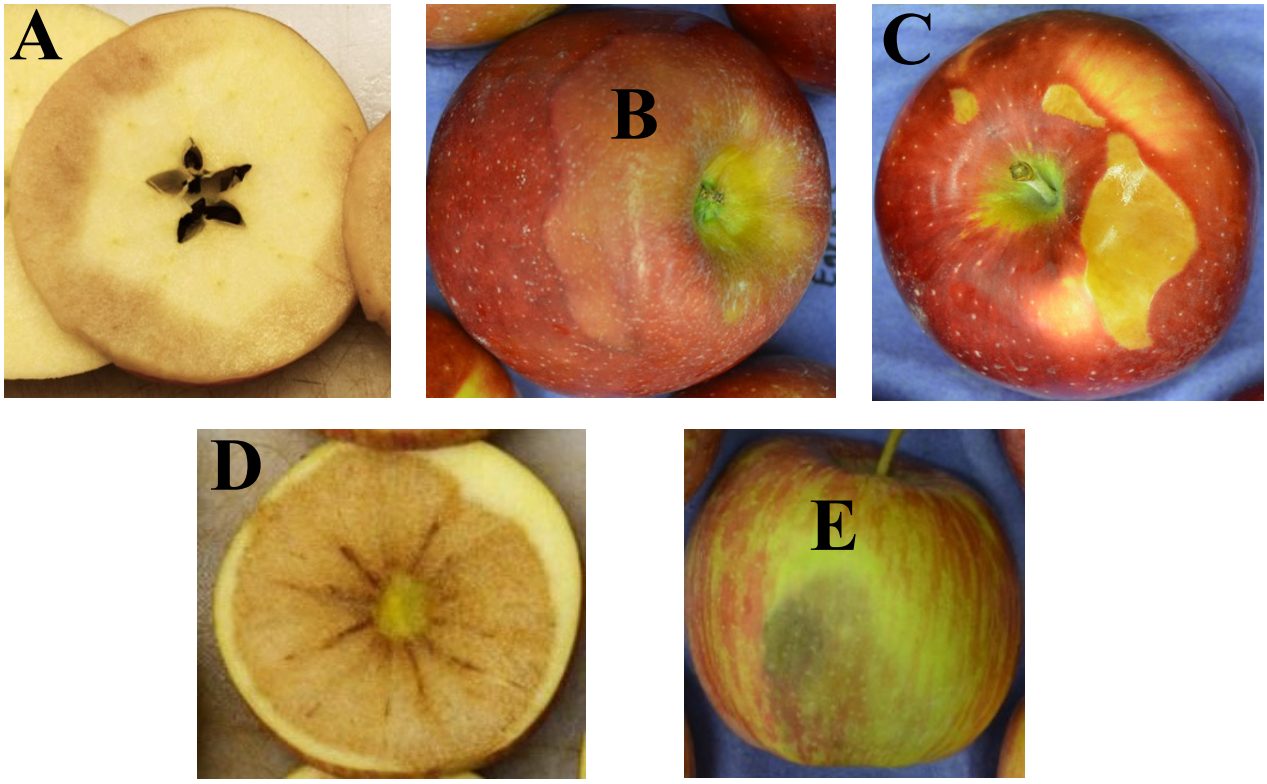

Figure 2. Unique symptoms found on WA38 (A-C) and 'Fuji' (D,E). All of the symptoms on WA38 were not present on fruit treated with a DPA drench. Incidence of these specific symptoms on 'Fuji' was not impacted by DPA drench.

As apples used for this activity were all stored in high CO_2 and low O_2, we expected DPA treatment to indicate disorders that were associated with CO_2 sensitivity, as DPA typically reduces or eliminates both internal and external symptoms of these disorders. Given this criterion, disorders symptomatically attributable to CO_2 sensitivity were observed in 'Golden Delicious', 'Delicious', 'Plumac', 'Braeburn', 'Smitten', 'Scilate', JUICI, WA38, 'Honeycrisp', and 'Pazazz' that were not drenched at harvest with DPA emulsion. 'Fuji' developed severe browning that had "radial" appearance, cavities, and softened solid brown cortex and incidence was not altered by DPA treatment (Figure 2 D,E). Predictably, 'Honeycrisp', as well as its progeny, 'Pazazz', developed both CO_2 sensitivity-related and soft scald/soggy breakdown. In 'Honeycrisp', these disorders could be segregated using DPA treatment which eliminated the radial browning symptoms but not soggy breakdown (Figure 1). In 'Pazazz', disorders were not present in DPA drenched fruit. Similarly, another 'Honeycrisp' progeny, WA38, developed all of these disorders and another, more severe, cortex browning symptom all of which were not present in or on the DPA-treated fruit (Figure 2 A-C).

Different chemistries are linked with different symptoms and causes of symptoms

Our preliminary screening of peel chemistry among symptomatic and periphery tissue from all cultivars and all peel and flesh defects is ongoing. To date, we have completed a partial peel chemistry analysis of 173 compounds. Even with less than half of the compounds analyzed, we can already distinguish tissue with "typical" CO_2 sensitivity-related peel symptoms such as dimpled or "orange peel" from healthy (Fig. 3A) peel or peel with other defects such as soft scald (Fig. 3B) or novel CO_2 sensitivity-related symptoms (Fig. 3C). Further refinement of the search identified chemicals whose levels are higher (Fig. 3E) or lower (Fig.3F) depending upon whether the symptoms are related to CO_2 sensitivity. For the current

storage season, chemical studies of the different symptoms continue this year as well as an activity using 'Pazazz' in different CO_2 levels in CA that is expected to confirm whether peel chemistry is only related to CO_2 sensitivity.

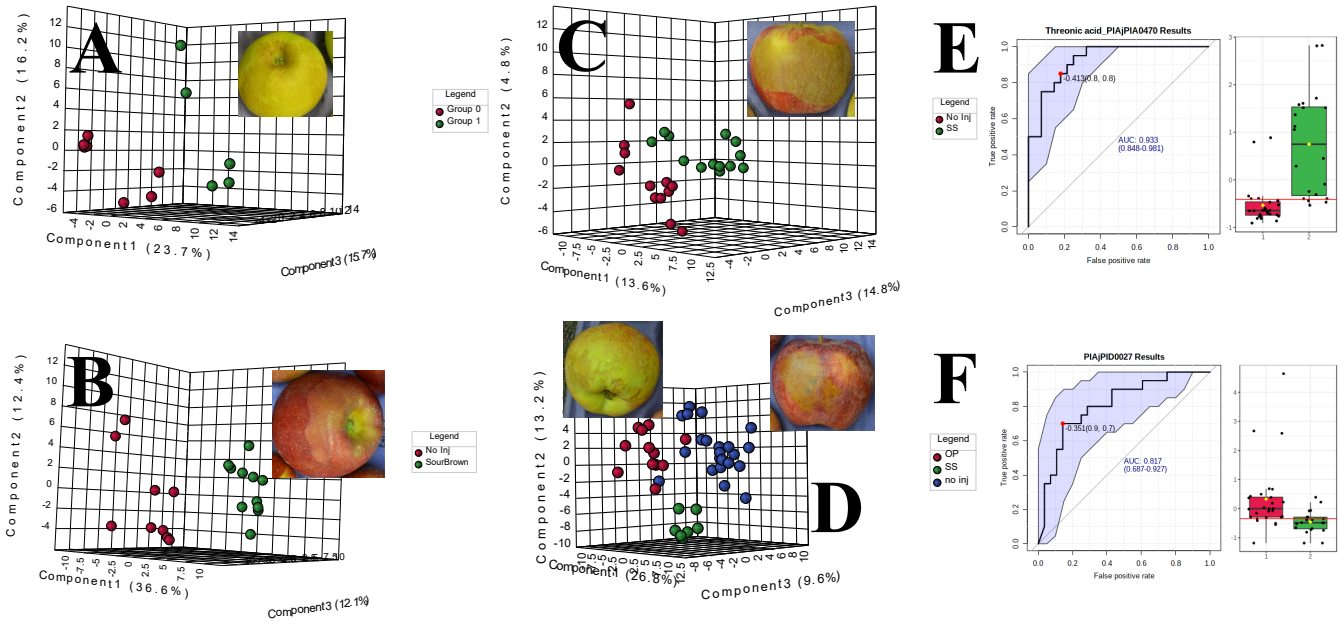

Figure 3. Peel chemistry analysis of CO_2 sensitivity related and other symptoms. Peel chemistry of CO_2 sensitivity-related symptoms including orange peel (rugose scald) of 'Golden Delicious' (A), novel "sour" browning on WA38 (B) compared to a non-CO2 sensitivity-related disorder, soft scald of 'Honeycrisp' (C), were different from periphery tissue. Chemistry of orange peel and soft scald was different in 'Pazazz' which developed both (D). Examples of multiple natural peel chemicals of chemicals that are lower (E) or higher (F) in peel from multiple cultivars with CO_2 sensitivity-related symptoms (red) compared to other similar peel browning symptoms (green).

To summarize, in the first year, we recorded a wide variety of symptoms, many of which may, with further validation, be attributable to CO_2 sensitivity and point to CO_2 mitigation as a focus for disorder control in any cold chain using CA storage. Year 2 work under this objective focuses on re-testing those cultivars that did not develop any significant symptoms in Year 1 to confirm any CO_2 sensitivity. Other work in Year 2 is directed towards finding storage management strategies that can be applied to reduce CO_2-related disorders in any cold chain for those cultivars found to be CO_2 sensitive (objective 2) and continued chemical analysis to find chemical signatures linked specifically with disorders caused by CO_2 sensitivity.

CONTINUING PROJECT REPORT **YEAR**: 2 of 3

Project Title: Postharvest system optimization for organic apple storage

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Cooperators: David Granatstein (granats@wsu.edu), Lee Kalcsits (lee.kalcsits@wsu.edu), Stemilt.

Total project Request: Year 1: $50,000 **Year 2**: $50,600 **Year 3: $50,600**

Other funding sources

Agency Name: Valent Biosciences, RipeLocker, WSU, USDA-ARS.
Cost-sharing: $150,000
Notes: Funds for technical support ($30,000/yr), travel expenses ($3,000/yr), and 0.1 FTE (P.I) from start-up funds.

Budget
Organization Name: Washington State University **Contract Administrator 1**: Katy Roberts
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Contract Administrator 2 (TFREC): Shelli Tompkins
Telephone: 509 293-8803 **Email address:** shelli.tompkins@wsu.edu
Station manager: Chad Kruger **Email address:** cekruger@wsu.edu

Item	2019	2020	2021
Salaries			
Benefits			
Wages	20,000	16,000	16,000
Benefits	7,000	5,600	5,600
Equipment [1]	13,000	13,000	13,000
Supplies[2]	3,500	3,000	3,000
Travel			
RCA rental	6,500	13,000	13,000
Plot Fees			
Total	50,000	50,600	**50,600**

[1]Three LabPods (Storage Control Systems Inc) leasing for DCA-RQ.
[2]Fruit, laboratory consumables, boxes

OBJECTIVES:

1. Evaluate the combination of DCA systems and RA storage on fruit quality postharvest.
2. Evaluate the effect of organic Retain OL in combination with different storage systems on fruit maturity and quality postharvest.
3. Evaluate the performance of vacuum storage (RipeLocker) under different temperatures regimes on fruit quality and physiological disorder development.

SIGNIFICANT FINDINGS 2019-2020:

- The effect of storage regime over maturity indices was block-dependent. For the most part, there no major differences between them in any of the cultivars tested.
-There were no statistical differences in soft scald or soggy breakdown incidence between storage regimes, but there were between blocks. Bitter pit (+lenticel blotch pit) was significantly reduced by vacuum RL storage in most sites, regardless of differences in lot susceptibility.
- Weeks in RA after DCA and RL bins, simulating a potential cold-chain scenario, reduced the benefits of low-oxygen storage in some fruit quality attributes.
- Fruit maturity progression preharvest during Year 2 in Honeycrisp apples showed that flesh firmness decrease had a steeper slope in fruit from warm sites (W42, W25) compared to those from cool ones (C802, C21). The rest of the maturity indices did not follow this clear pattern. In Fuji, differences in maturity indices between sites were less consistent.
-Retain OL-treated fruit with 20 f oz/acre showed higher flesh firmness from harvest until 9m of CA storage than the other treatments, but not always statistically different. Both, T2 and T3 significantly affected fruit's red blush coverage but mostly in Honeycrisp apples.

METHODS

Obj. 1. Evaluate the combination of DCA systems and RA storage on fruit quality postharvest.

Activities 2020-2021

During Year 2, temperature and relative humidity sensors were placed in every orchard in spring, and data collected at harvest. Maturity progression was monitored in fruit from all sites for both Fuji and Honeycrisp by sampling every week for four weeks before harvest (WBH). At commercial harvest, fruit quality (flesh firmness, SSC, IEC, Starch Index, I_{AD}, Respiration) was performed in 18 fruit per orchard, and peel samples were collected for further mineral analysis. After conditioning, fruit was placed in different dynamic storage regimes established in year 1 and air at 37°F for Honeycrisp and 34°F for Fuji (Table 1) in addition to regular air at the same temperature. Postharvest evaluations are currently being carried out and will end in July 2021.

Table 1.

Cultivar	Block	Harvest date Year 1	Year 2	Conditioning 50°F	DCA
Honeycrisp	W25 (warm)	8/31/2019	8/27/2020	7 days	CF: (LOL< 0.4%O_2)- 3.0% O_2/ 0.5% CO_2 ILOS: 0.5% O_2/ 0.5% CO_2-10d; 1.0% O_2/0.7% CO_2 RQ: 3.0% O_2/0.5% CO_2
	W42 (warm)	9/02/2019	9/04/2020	7 days	
	C21 (cool)	9/10/2019	9/03/2020	7 days	
	C802 (cool)	9/06/2019	9/09/2020	7 days	
Fuji	W40 (warm)	10/03/2019	10/07/2020	Delayed CA-34°F-20d	CF: (LOL< 0.4%O_2)- 5-2-0.8% O_2 in 7 days, 0.8% CO_2; 0.8% O_2/0.8% CO_2 ILOS: 0.6% O_2, 0.8% CO_2-10 d; 0.8% O_2/0.8% CO_2 RQ: 0.8% O_2/0.8% CO_2
	W18 (warm)	10/04/2019	10/07/2020	Delayed CA-34°F-20d	
	C4 (cool)	10/04/2019	10/07/2020	Delayed CA-34°F-20d	
	C902 (cool)	10/09/2019	10/07/2020	Delayed CA-34°F-20d	

Obj. 3. Evaluate the performance of vacuum storage (RipeLocker) under different temperatures regimes on fruit quality and physiological disorder development.

Activities 2020-2021

After commercial harvest, fruit from both cultivars and sites were also placed in vacuum storage bins at 33°F. Postharvest evaluations are currently being carried out and will finish in July 2021.

RESULTS & DISCUSSION 2019-2020 (Objective 1 and 3):

Fruit Maturity & Physiological disorders

Honeycrisp:

- Overall fruit maturity was mostly similar between DCA systems after 6m+4 wk RA. After 9 months, there were no major differences in maturity between vacuum RL storage and DCA systems.
- Fungal infections in vacuum RLs (from different apple lots stored in the units), especially at 37°F, dramatically increased rots in all fruit, decreasing 10-30% clean fruit compared to DCA systems.
- There were no statistical differences in soft scald or soggy breakdown incidence between storage regimes, but there were differences between blocks, with the highest being for W42. Bitter pit (+lenticel blotch pit) was significantly reduced by vacuum RL storage in most sites, regardless of differences in lot susceptibility (Table 2).

Table 2. Soft scald, soggy breakdown and bitter pit+lenticel blotch pit incidences (%) indices after 6 and 9 months in DCA/Vacuum RL plus 4 weeks in RA at 37◦F otherwise indicated, and 1 and 7 days at 68◦F (shelf-life). Honeycrisp apples. Season 2019-2020.

			Soft Scald (% incidence)			
Block (A)	6m	6m+4wk+1d	6m+4wk+7d	9m	9m+4wk+1d	9m+4k+7d
W42	8.0 a [Y]	8.0 a	10.6 a	2.5 ab	5.4	10.9 ab
W25	0.9 b	3.1 ab	4.4 ab	0.5 ab	1.8	0.9 a
C21	11.1 a	12.4 a	20.0 a	4.6 b	5.6	12.1 b
C802	0.0 b	0.4 b	0.6 b	0.3 a	3.7	3.7 a
P value [Z]	**	**	*	*	*	**
Treatment (B)						
DCA1	7.3	9.0	11.3	3.3	3.7	5.8
DCA2	4.3	5.7	11.3	3.0	2.7	4.6
DCA3	3.3	3.3	4.2	1.3	3.7	5.4
RL 33	n/a	n/a	n/a	1.4	6.5	10.3
RL 37	n/a	n/a	n/a	0.8	4.0	8.3
P value	ns	ns	ns	ns	ns	ns
A x B	**	**	**	*	**	**
			Soggy Breakdown (% incidence)			
Block (A)	6m	6m+4wk+1d	6m+4wk+7d	9m	9m+4wk+1d	9m+4k+7d
W42	0.0	0.0	8.9 a	0.0	5.3 b	12.6
W25	0.0	0.0	1.7 ab	0.0	0.2 a	1.4
C21	0.0	0.0	0.0 b	0.0	0.0 a	1.3
W802	0.0	0.0	0.0 b	0.0	0.5 a	0.5
P value		-	**	-	**	*
Treatment (B)						
DCA1	0.0	0.0	6.3	0.0	2.3	7.5
DCA2	0.0	0.0	0.4	0.0	4.0	6.7
DCA3	0.0	0.0	1.3	0.0	0.7	2.5
RL 33	-	-	-	0.0	0.7	2.1
RL 37	-	-	-	0.0	0.0	1.1
P value	-	-	ns	-	ns	ns
A x B	-	-	*	-	**	ns
			Bitter pit+LB (% incidence)			
Block (A)	6m	6m+4wk+1d	6m+4wk+7d	9m	9m+4wk+1d	9m+4k+7d
W42	8.9	12.9	19.4	0.2	22.1	20.3 a

W25	0.4	1.8	4.4	0.2	8.0	4.2 b
C21	7.6	10.7	15.0	0.0	6.1	9.3 ab
W802	6.2	2.2	7.8	1.5	7.3	3.3 b
P value	*	ns	*	ns	ns	**
Treatment (B)						
DCA1	4.0	5.7	9.7	0.3	17.3	9.2 ab
DCA2	5.7	8.7	12.5	0.3	12.7	16.7 b
DCA3	7.7	6.3	13.3	0.0	7.3	10.8 b
RL 33	n/a	-	-	0.8	7.9	5.2 ab
RL 37	n/a	-	-	1.0	9.2	4.5 a
P value	ns	ns	ns	ns	ns	*
A x B	**	ns	*	*	ns	**

[Z]Kruskal-Wallis (P≤0.05); [Y]Different letters within columns indicate statistically significant differences (Dunn test).

Fuji:

-Fruit maturity at harvest and during the storage season was mostly similar between treatments (Block x Storage regime), with some exceptions where the maturity index was block-dependent, especially after 9 months of storage (Table 3).

-Superficial scald appeared after 9m+4w+7d (shelf-life) with incidences between 2.8 and 11.8% between blocks. Similarly, Internal Browning had the highest incidences at this time for fruit from warm sites (10.3 and 0.5%). Lenticel breakdown was mainly observed in W40 block, except after 9m+4w+7d were all blocks had between 2.8-6.9 % incidence and mainly in vacuum RL but only significantly different in RL37 compared to the rest of the treatments.

Table 3. Fruit maturity indices postharvest after 6 and 9 months in DCA/Vacuum RL plus 4 weeks in RA at 37◦F otherwise indicated, and 1 and 7 days at 68◦F (shelf-life). Fuji apples. Season 2019-2020.

	Weight (g)	Red coverage (%)	Bkgd color (1-4)	I_{AD}	Firmness (lb)	SS (°Brix)	IEC (ppm)	Respiration ($mLCO_2/kg/h$)	TA (mg malic/mL)
				6m+4w+1d					
Block (A)									
W40	203.3	83.2 bc[Y]	3.2	0.67	15.1 a	13.5	0.6	31.5 a	0.37 a
W18	212.0	91.4 a	3.1	0.98	14.0 b	14.9	0.0	20.3 b	0.37 a
C4	221.8	87.9 ab	3.0	0.68	14.8 a	14.6	0.1	25.6 ab	0.29 b
C902A	227.6	80.9 c	3.1	0.84	15.2 a	13.4	0.5	22.4 ab	0.38 a
Sign.[Z]	**	**	ns	**	**	**	ns	*	**
Treatment (B)									
DCA1	205.8	87.2	3.0	0.75	14.6	13.9	0.0	23.4	0.37 a
DCA2	219.1	84.9	3.1	0.89	15.0	14.3	0.8	25.0	0.33 b
DCA3	223.6	85.4	3.3	0.74	14.7	14.1	0.0	26.5	0.36 ab
Sign.	*	ns	ns	**	ns	ns	**	ns	*
A x B	**	ns	ns	**	ns	ns	ns	ns	ns
				6m+4w+7d					
Block (A)									
W40	221.4	78.7 b	3.1	0.61 c	14.7	14.1 b	0.0	19.0 a	0.35
W18	191.1	90.4 a	3.0	0.99 a	14.9	15.1 a	0.0	7.1 b	0.35
C4	245.3	87.8 a	3.0	0.83 b	15.1	14.6 b	1.8	15.0 a	0.28
C902	216.9	79.0 b	3.1	0.84 b	15.1	13.4 c	1.2	14.6 a	0.37
Sign.	**	**	ns	**	ns	**	ns	**	*
Treatment (B)									
DCA1	224.6	84.8	3.1	0.86 a	14.6 b	14.0 b	0.0	9.4 b	0.34
DCA2	209.0	83.7	3.1	0.83 ab	15.0 a	14.5 a	0.0	14.8 ab	0.36
DCA3	222.5	83.4	3.0	0.75 b	15.3 a	14.4 a	2.3	17.7 a	0.31
Sign.	*	ns	ns	*	**	**	ns	**	ns
A x B	**	ns	ns	ns	ns	ns	ns	ns	ns
				9m+4w+1d					

Block (A)									
W40	209.0	3.0	82.7 b	0.58	14.6	13.5	0.7	28.9	0.30
W18	214.1	3.2	91.6 a	0.84	14.8	15.0	0.1	16.7	0.29
C4	236.0	3.1	83.0 b	0.69	14.4	14.3	1.3	21.2	0.23
C902	213.3	3.1	80.9 b	0.75	14.8	13.1	1.3	22.0	0.32
Sign.	**	ns	**	**	**	**	**	**	**
Treatment (B)									
DCA1	221.1	3.0	84.7	0.71	15.1	14.2	1.4	20.1	0.31
DCA2	223.1	3.0	82.7	0.78	14.8	13.9	1.1	21.4	0.26
DCA3	204.9	3.0	82.8	0.64	14.8	14.0	1.7	20.2	0.31
RL 33	220.3	3.5	89.3	0.69	14.2	13.9	0.0	24.8	0.27
RL 37	221.1	3.1	83.4	0.76	14.2	13.8	0.0	24.4	0.27
Sign.	*	**	ns	*	**	ns	**	ns	ns
A x B	*	**	ns	**	**	*	**	*	ns
				9m+4w+7d					
Block (A)									
W40	201.2	3.1	73.9	0.51	14.6 ab	13.6 b	17.1	24.0	0.24
W18	216.6	3.1	84.1	0.63	14.9 a	15.4	11.8	23.7	0.26
C4	210.9	3.5	78.3	0.61	14.3 b	14.6	31.6	27.2	0.19
C902	210.5	3.2	74.5	0.65	14.6 ab	13.2 b	14.8	26.3	0.27
Sign.	*	**	**	**	*	**	**	ns	**
Treatment (B)									
DCA1	203.3	3.2	78.8	0.63	15.4 a	14.4	3.2	16.8	0.25
DCA2	208.0	3.2	81.9	0.65	15.3 a	14.2	5.9	18.6	0.22
DCA3	208.8	3.3	72.2	0.66	14.9 a	14.5	2.6	23.5	0.23
RL 33	215.5	3.1	78.2	0.55	13.7 b	14.0	32.1	32.1	0.27
RL 37	213.2	3.3	77.5	0.51	13.6 b	13.8	50.3	35.6	0.22
Sign.	ns	ns	**	**	**	*	**	**	**
A x B	*	**	**	**	ns	ns	**	**	*
				9m					
Block (A)									
W40	217.7 b	71.5	3.2	0.57 b	14.7	14.0	0.0	19.1	0.24
W18	217.6 b	83.7	3.0	0.76 a	14.3	14.6	0.0	15.1	0.30
C4	241.4 a	77.8	3.0	0.63 b	14.4	14.4	0.0	18.9	0.32
C902	227.1 ab	79.7	3.0	0.87 a	15.1	14.0	0.0	19.3	0.32
Sign.	*	*	ns	*	ns	ns	-	ns	*
Treatment (B)									
RL 33	227.0	79.0	3.0	0.66	14.7	14.2	0.0	22.1	0.29
RL 37	224.8	77.3	3.1	0.75	14.6	14.3	0.0	14.1	0.31
Sign.	ns	ns	ns	ns	ns	ns	-	**	ns
A x B	ns	*	ns	ns	ns	ns	-	**	ns

[Z]ANOVA (P≤0.05); [Y]Different letters within columns indicate statistically significant differences (Tukey, HSD, P≤0.05).

Obj. 2. Evaluate the effect of organic Retain OL in combination with different storage systems on fruit maturity and quality postharvest.

Activities 2020-2021

During Year 2, five treatments were applied in a commercial orchard of Gala apples (Manson, WA). These are shown in Table 3). Treatments were arranged in a complete randomized design with 3 replicates of 4 trees each. Fruit was harvested twice: at commercial harvest (H1; 9/3/2020) and 7 days later (H2:9/11/2020). Maturity indices were evaluated from 27 days before harvest (DBH) until harvest by collecting 18 fruit per treatment (similar size and canopy position). These measurements included flesh firmness, soluble solids content, starch degradation index, skin and background color, ethylene

production, respiration rate, and I_{AD} value (DA meter). At harvest, 400 fruit per treatment were stored in CA, which will be evaluated after 3, 6, and 9 months after storage plus 7 days at room temperature.

Table 3. Retain OL treatments. Gala, Season 2020-2021.

Treatment 1	Untreated Control (UTC)
Treatment 2	21d before normal harvest
Treatment 3	7d before normal harvest
Treatment 4	3d before normal harvest
Treatment 5	1d before normal harvest

RESULTS & DISCUSSION 2019-2020

In Gala and Honeycrisp, T3 (T3: 20 fl oz/acre, 1 week before harvest)-treated fruit showed higher flesh firmness from harvest until 9m of CA than that from T1 (Untreated Control) or T2 (10 fl oz/acre, 4 and 1 week before harvest), but not always statically different. Both, T2 and T3 significantly affected fruit's red blush but mostly in Honeycrisp apples. No consistent differences between treatments were found in IEC, SI, SSC, I_{AD} in Gala. Similar results were obtained in Honeycrisp, but I_{AD} were usually higher (less ripen), but not always statistically different, in Retain OL-treated fruit compared to the Untreated control. Physiological disorder's incidences were not statistically different between treatments.

CONTINUING PROJECT REPORT **PERIOD:** 1 year of 2 years

Project Title: WA38: understanding green spot origin, timeline, and development

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Cooperators: Ryan Sheick and Stefan Roeder (WSU-TFREC)

Total Project Request: Year 1: $108,875 **Year 2: $111,790**

Budget 1
Organization Name: WSU
Contract Administrator: Katy Roberts
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Organization Name: WSU-TFREC
Contract Administrator: Shelli Tompkins
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Supervisor or Station Manager name and email address: Chad Kruger, cekruger@wsu.edu

Item	2020	2021
Salaries	$54,000	$56,160
Benefits	$18,875	$19,630
Plot Fees	$3,000	$3,000
Consumables	$14,000	$14,000
Travel	$4,000	$4,000
Total	$93,875	**$96,790**

Budget 2
Organization Name: USDA
Contract Administrator: Chuck Myers
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Telephone: 510-559-5769

Item	(Year 1 (2020)	Year 2 (2021)
Good and Service (LCMS annual service)	$12,000	$12,000
Consumables	$3,000	$3,000
Total	**$15,000**	**$15,000**

RE-CAP OBJECTIVES:

1. Determine the effect of bagging fruit on the intensity of green spot appearance.
2. Verifying the effect of netting on green spot appearance along the season.

SIGNIFICANT FINDINGS:

1. Determine the effect of bagging fruit on the intensity of green spot appearance.

- Green spot started to appear at the end of July and became more in the first 2 weeks of August.
- Chemistry of sunken green spot revealed signs of localized osmotic stress in the peel.
- Differences in temperature and humidity were found inside the bags (higher avr. %RH and lower max temperature in July and August).
- Significantly higher green spot incidence in the WA38/G41 control trees (61%) with respect to WA38/G41 bagged trees (28%).
- All apples, regardless of the rootstock, if bagged early (between 06/01/20 and 06/18/20), did not show any green spot at all at harvest.

2. Verifying the effect of netting on green spot appearance along the season.

- Comparison of green spot incidence between "netted" and control apples within each rootstock did not reveal any significant differences.
- WA38-netted/G41 and WA38-ctrl/G41 treatments were affected in a higher proportion by green spot in comparison to both combinations with NIC29.

METHODS:

1. Determine the effect of bagging fruit on the intensity of green spot appearance.

Experimental Design

An experimental WA38 block planted in 2013 [Sunrise Research Orchard (SRO), Rock Island, WA] was used for this experiment. WA38 trees grafted on G41 or M9-NIC29 were planted in rows consisting of four plots of eleven trees in which the two rootstock combinations were randomized by plot in each row. Trees selected for this trial were trained to spindle, and their planting density is 10 ft × 3 ft (=1499 trees/A). Starting from June 1, 2020, experimental bagging was imposed twice per month, approximately two weeks apart, until the end of August. Time points were named from T1 to T6.

Bagging

In 2020, the selected trees were hand-thinned to one fruit per cluster (if necessary) prior to bagging at each time point. Two-layer commercial fruit bags were used to enclose fruitlets and were secured at the spur by an additional twist-tie to mitigate the bag's chances of falling off over the course of the growing season. In each bagged tree, 3-10 apples were left not-bagged to observe and check the onset of the GS.

Metabolomics sampling

In 2019, a pilot study was conducted to study the effects of time point bagging on green spot incidence. Visual assessment at harvest showed some green spot mitigation in bagged treatments, but this effect was diminished at later bagging time points. The most frequent and severe of the "bagged" green spot symptoms were found in the time point 10 (T10) bagging set; T10 apples were left without bags throughout the growing season until (08/19/19). We collected peel and cortex tissue for LCMS analysis to understand the interplay of the metabolic changes between healthy and green spot-affected tissue, bagged (at T10) or without bags. Green spot-affected tissue areas were sampled along with healthy tissue areas from the same apples in bagged and not-bagged treatments. Because WA38 fruit grown on G41 rootstocks have generally presented more severe green spot symptoms in the experimental block in SRO compared to M9-NIC29, we focused on collecting samples from treatment combinations on G41 as a primary goal, including investigating both "sunken" type green spot and "superficial" type green spot. For a rootstock comparison, green spot fruit grown on NIC29 (without bags) was sampled for the "superficial"-type green spot and

compared to their respective "healthy" areas. In the present report, we will show preliminary metabolomic results for polar compounds related to not-bagged fruit at harvest 2019 (regardless of the rootstocks, Figures 1 and 2).
In 2020, due to the reduction in available tree replications, the original plan to dedicate one tree replicates for destructive metabolomics sampling was modified. Approximately two weeks after each time point treatment was imposed (coinciding with each successive bagging time point), we sampled five apples across three tree replicates in each experimental group. Fruits were transported to the lab where they were weighed, measured, photographed, and rinsed with deionized water prior to peel sampling ("whole apple" peel approach). Peels were submerged in liquid nitrogen immediately upon peeling, and snap-frozen tissues were stored at -80ºC.

Fruit tracking

The experimental block was scouted weekly between June and September 2020 for signs of green spot onset. Examples of apples with emerging signs of the green spot were photographed and tagged for development tracking. On-tree fruit size was tracked weekly throughout the growing season with digital calipers by recording the fruit diameter at its widest points until harvest (09/24/20).

Green spot assessment

Harvest and grading

At harvest, all fruit from selected trees were harvested and transported to the lab, where they were graded. For each rootstock, the production of 3 entire "bagged" trees per time point (T1-T6; 18 trees) and 9 control trees (without bags) were graded.
Fruit size was measured and categorized into 5 mm increments ranging from <65 to 105 mm. Fruits were visually assessed and scored for color and green spot symptoms. Green spot (GS) was graded by increasing severity on a scale from 1 – 4, with GS 1 including fruit with few green spots, small in diameter (less than the diameter of a pencil eraser), or pronounced green halos around lenticels, and GS 4 consisting of severe green spot symptoms with cracking. Fruits were scored GS 5 if the green spots appeared to be a darker brown color, even if cracking was not present. Finally, GS 6 was reserved for fruit showing the superficial "flecking" green spot symptoms, regardless of severity.

Microclimate monitoring

On June 1, 2020, twelve dataloggers (iButton®, Maxim Integrated) were deployed throughout the experimental block to monitor temperature and humidity over the course of the growing season. Six spindle trees on G41 rootstocks were selected for microclimate monitoring: three trees that doubled as the first bagging time point in the G41 treatment and three trees that doubled as the control (no bags) for the first time point in the G41 treatment. Dataloggers were placed inside the fruit bags in the experimental treatment to collect temperature and relative humidity data throughout the season. In the control trees, dataloggers were secured to a branch or spur. Two dataloggers were added to each of the six trees: one datalogger located in the "upper canopy" and one in the "lower canopy." Dataloggers were collected, and data was retrieved on 09/22/20.

2. Verifying the effect of netting on green spot appearance along the season.

Sunlight is a major environmental factor affected by bagging; however, because bagging is not an economically feasible solution to green spot mitigation in our growing region, we wanted to implement an alternative netting approach as a secondary means of studying the effects of light exposure on green spot incidence. In 2020, drape netting (Diamond V5® Monorang, 10% shading, 2.8 mm x 4.0 mm weave, Helios® anti-hail systems, Bergamo, Italy) was deployed after blooming on June 1. We monitored fruit weekly and first observed green spot symptoms on July 29. At harvest (09/28/20), fruit from each tree replicate was harvested, and green spot incidence was visually assessed and graded.
At harvest 2020, fruit with green spot symptoms and fruit without green spot symptoms grown under the nets were sampled for metabolomics analysis.

RESULTS AND DISCUSSION:

1. Determine the effect of bagging fruit on the intensity of green spot appearance.

Chemistry of green spot

Our ongoing, broad chemical analysis of green spot has already indicated key differences among different symptomatic peel and cortex and surrounding healthy tissue categories. As may be expected, given differences in appearance, the chemistry of both cortex and peel differed depending upon the symptoms (sunken or superficial) or which symptoms the tissue was surrounding (Fig.1). This may point to differences in chemistry resulting from maturity, genetics, or interaction with an environmental factor that triggers the more severe necrosis of the cortex underlying the GS. Different amounts of natural chemicals elevated in different tissues can often tell us about interactions between the fruit and the environment that may lead to (or protect from) symptom development and the mechanisms the fruit uses to cope with the stress.

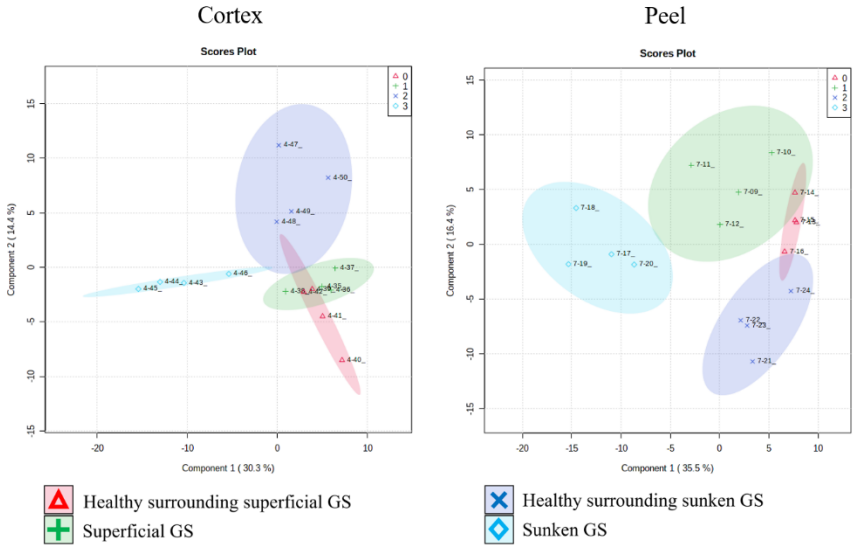

Figure 1. WA38 cortex and peel chemistry are different depending upon green spot (GS) symptom category. An analysis of 198 natural peel and cortex chemicals from symptomatic and surrounding tissue reveals different amounts of many chemicals. Different colored circles represent the degree of these differences as these circles' position indicates how closely chemistries of each tissue are related to each other. This analysis can be used to rank each natural chemical difference with respect to GS severity.

Often "symptoms" are merely the physical manifestations of plant defenses. For instance, levels of 2 compounds, glucose, and sorbitol, that can accumulate in tissues to relieve osmotic stress, were elevated in sunken tissue and tissue surrounding sunken tissue, indicating a response triggered by light, water, or heat stress associated with the development of the more severe symptoms (Fig. 2). Other evidence of stress includes elevated levels of chemicals associated with photoprotection, barrier formation, nitrogen metabolism, and cell wall chemistry. While other photoprotective pigments accumulate, related compounds responsible for red peel color disappear. It is also interesting to note that these typically light-related peel compounds are also elevated in the sunken symptomatic cortex. Our ongoing analysis points to a transformation of cortex (or "parenchyma") cells to produce chemicals typically only found on the peel surface (epidermis). A common plant defense mechanism is to form a barrier to prevent pathogen invasion or water loss. This is what appears to be happening in the case of sunken green spot—localized osmotic stress in the peel, eventually leading to a barrier-forming defense response in deeper tissue (Fig. 2). Other pathways related to nitrogen metabolism are also implicated and may be associated with differential energy production. Determining precisely when these chemical changes occur, if they are pre-symptomatic changes leading to the disorder, and what environmental cues provoke them could lead to defining the disorder's causes. Experiments in the 2020 growing season were designed and performed using multiple sampling points with just that in mind.

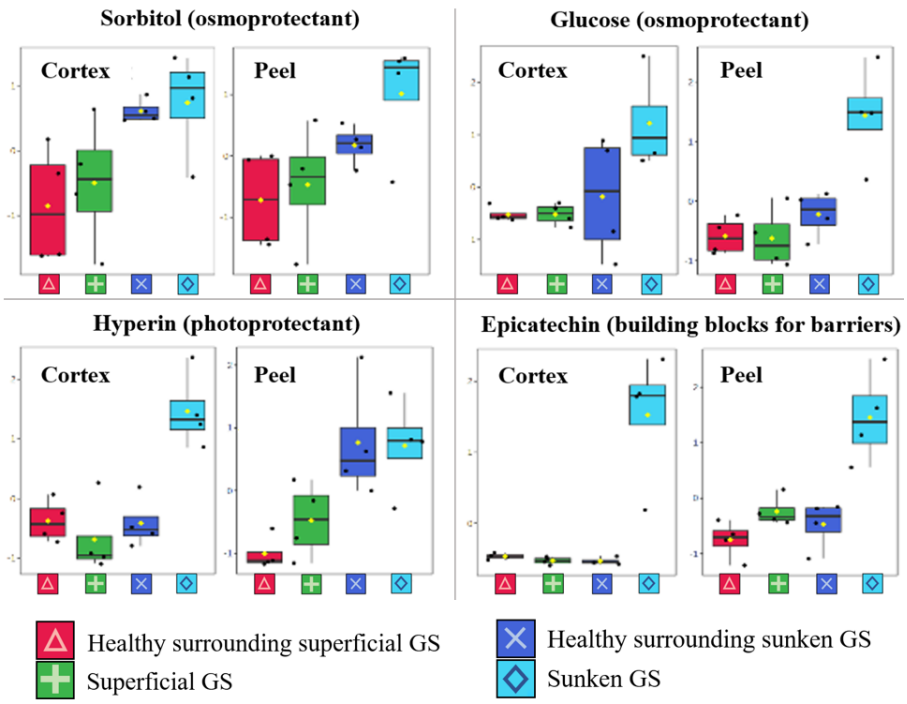

Figure 2. Examples (out of 198 screened to date) of natural peel and cortex chemicals associated with different types of green spot (GS) symptoms and tissue immediately surrounding the disorder at harvest 2019. These examples appear to outline a process by which osmotic stress may provoke a multifaceted defense response, including photoprotection and barrier formation and, eventually, more severe, sunken symptoms. In 2020 we sampled at multiple time points during the season to help determine which cultural and environmental events trigger these chemical changes to detect the event before symptoms occur.

Fruit tracking for size

Throughout the 2020 growing season, fruit size was tracked in both WA38/M9-NIC29 and WA38/G41 by selecting and measuring ten apples per tree at their widest diameter with digital calipers. Fruit size was not statistically different between rootstocks after August 19 until harvest (data not showed).

Green spot scouting

Our first observations of suspected green spot in 2020 occurred in late July and were photographed on July 29. In July, symptoms appearing were typically minor discolorations that became more easily visible by contrast to the developing overcolor in the following weeks. Green spot symptoms typically worsened until September. In some cases, superficial green spot symptoms diminished as the overcolor development intensified in the weeks leading up to harvest; however, this was not the case for apples with sunken-type green spot symptoms.

Microclimate monitoring

Dataloggers were arranged in both bagged and control WA38/G41 trees, and daily average temperatures, minimum daily temperatures, maximum daily temperatures, average daily percent relative humidity (%RH), minimum daily %RH, and maximum daily %RH were averaged by month and analyzed in SAS 9.4 using proc GLM and SNK test (Table 1). The analysis indicated significant differences between the bagged and control treatments in average %RH in June, July, and August (but not September), with average %RH values always higher in the bagged treatment (Table 1). Daily lows averaged by month also differed significantly, with minimum %RH in the control treatment always lower than the bagged treatments. Maximum %RH values were generally not statistically significant, although we did see differences in September at $p<0.05$ (bagged=75.5%; control=78.5%), perhaps due to precipitation on some days that led to saturated humidity levels in the no-bag control treatments compared to somewhat lower %RH values recorded inside the bags (protected from direct precipitation). Temperature differences were generally not statistically significant, although maximum daily temperatures averaged by month were statistically lower in the bagged trees in July and August than in the control (Table 1). These results show measurable differences in temperature and humidity that may be attributable to a "buffering" effect of the bag that helps

protect the fruit from extreme daily high temperatures in the hottest summer months as well as retain humidity throughout the season.

Table 1. Comparison of temperature and relative humidity measurements inside fruit bags versus outside fruit bags in 2020. Average daily temperatures, minimum daily temperatures, maximum daily temperatures, average daily percent relative humidity (%RH), minimum daily %RH, and maximum daily %RH were averaged by month and statistically analyzed using SAS 9.4 proc GLM/SNK test.

Months	Treatment	N	Average Temperature (°C)	Min Temperature (°C)	Max Temperature (°C)		Average RH (%)		Min RH (%)		Max RH (%)	
Jun	bagged	180	20.7	14.5	28.4		54.0	A	38.3	A	71.3	
Jun	CONTROL	180	20.9	14.4	29.0		47.6	B	28.6	B	69.1	
Significance			NS	NS	NS		***		***		NS	
Jul	bagged	186	24.6	17.4	32.8	B	45.1	A	29.7	A	62.9	
Jul	CONTROL	186	24.8	17.1	33.7	A	41.7	B	24.0	B	62.4	
Significance			NS	NS	*		***		***		NS	
Aug	bagged	186	24.3	17.3	32.2	B	45.2	A	30.3	A	62.5	
Aug	CONTROL	186	24.5	16.8	33.5	A	41.7	B	23.6	B	62.8	
Significance			NS	NS	***		***		***		NS	
Sep	bagged	132	20.1	13.9	26.9		58.8		42.3	A	75.5	B
Sep	CONTROL	132	20.2	13.5	28.1		57.8		37.4	B	78.5	A
Significance			NS	NS	NS (0.059)		NS		**		*	

N= 2 positions in the canopy *n days recorded in the month * 3 experimental tree per each trt. Significance: NS=not significant, *= p<0.05, ** p<0.01, ***p<0.001. Different letters on the right of the means indicate significant discrimination by SNK (p<0.05) in column.

Fruit grading

Green spot incidence

Green spot incidence at harvest in 2020 was determined by visually inspecting each fruit based on the methodology reported in the section above and expressing the incidence for each experimental treatment as the number of apples presenting the disorder (regardless of the type and severity of the disorder) over the total apples/tree in percentage. The first comparison was made by analyzing green spot incidence in bagged trees versus control ones within each rootstock combination and reported in Table 2. Results reported a significantly higher green spot incidence in the WA38/G41 control trees (61%) with respect to WA38/G41 bagged trees (28%). The same comparison run in WA38/NIC29 combination did not show significant differences between bagged and control treatment, with about 21-27% of green spot incidence (Table 2).

Rootstock	trt 2020	*N trees=*	*GS incidence (%)* ✧	
G41	bagged	*18*	*27.9*	*B*
G41	control	*9*	*60.6*	*A*
Significance			**	
NIC29	bagged	*18*	*27.2*	
NIC29	control	*9*	*21.8*	
Significance			*NS*	

✧ Each apple presenting 1 type of GS or more was counted just once (like presence/absence of GS regardless of the type)

*Table 2: WA38 green spot incidence (%) in 2020: comparison between apples inside the bags and apples grown in control trees (no fruit bags) within each rootstock. Significance by treatment (2020) within each rootstock: **=p<0.01 and NS=no significant difference.*

The "bagged" treatment in Table 2 represents 6 different time points (06/01/20 to 08/28/20) averaged together. Because the time point treatments influenced green spot development, we cannot understand the effect of time point bagging unless we compare the incidence of green spot for each rootstock along with the time points (Fig. 3).

Figure 3 represents the incidence of green spot (%) on WA38 for each of the times of bagging; all apples, regardless of the rootstock, if closed in the bags on 06/01/20 or on 06/18/20, did not show any green spot at all. Starting from T3 (07/06/20), but mainly at T5 (08/11/20) the disorder also started to appear in the

bagged fruit (mainly as type GS1), suggesting that only early bagging strategy will be able to mitigate green spot development.

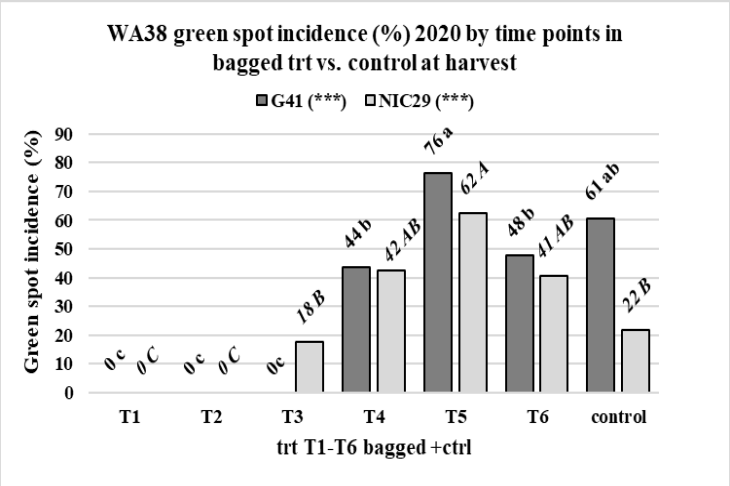

*Figure 3: Comparison between green spot incidence (%) in WA38 apples inside the bags at each time point and apples grown in control trees (all fruit not bagged) within each rootstock: G41 and Nic29 after harvest 2020. Significance by time points within each rootstock: ***=p<0.001.*

To better understand the effect of the bagging on the green spot incidence, a direct comparison of the apples inside and outside the bags was conducted for each time point independently. As a control, in this case, we utilized the apples left not-bagged inside the trees assigned for the bagging treatment. For the early time points, T1 and T2, the use of bagging zeroed the onset of green spot on apples inside the bags until harvest, while the (internal) controls hanging on the same trees showed at harvest GS incidences equal to 41% and 50% in the WA38/G41 and 40% and 37% in the WA38/NIC29 respectively (data not shown). Comparing bagged apples with respect to internal-control apples (no bag), we found a meaningful difference in the percentage of culled fruit between those two treatments equally significant for WA38/G41 as for WA38/NIC29. Indeed, bagging reduced by 27% the incidence of cull in WA38/G41 apples, while just 18% in WA38/NIC29 (data not shown).

2. Verifying the effect of netting on green spot appearance along the season.

Fruit grading

Color

The color grading of WA38 apples grown under the net from June 1 to harvest (09/28/2020) showed differences across the combinations on the two different rootstocks, confirming results obtained in previous studies on this variety. The significantly higher proportions of apples (99%) in the most colored category (color1=50-100% red overcolor) was found in WA38/NIC29 control trees, followed by WA38/NIC29 under nets (94%), WA38/G41 control (91%), and then WA38/G41 netted (84%; data not shown). The latter combination reported the highest proportion of fruit in the color category 2 (red overcolor covering 30-50%). These data confirm that the combinations on NIC29 registered the best pigmentation.

Green spot incidence

The incidence of green spot in 2020 was also assessed on the production picked under the net for both rootstocks and compared to fruit harvested from the un-netted control (no bag). When analyzed independently, there were no statistically significant effects on green spot incidence between netted and control trees; however, when the four combinations were analyzed together, we found apples grown on G41 had elevated and statistically different GS incidence compared to apples grown on NIC29, regardless of whether or not they were grown under the net. The difference in green spot incidence between WA38 netted/G41 and WA38 netted/NIC29 is 38%.

CONTINUING PROJECT REPORT YEAR: 2 of 3

Project Title: Apple genomes for postharvest fruit quality biomarkers AP-19-103

PI: Dr. Loren Honaas
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City/State/Zip: Pullman, WA 99164

Co-PI (3): Dr. Jim Mattheis
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Address: 1104 North Western Ave
City/State/Zip: Wenatchee, WA 98801

Cooperators: Dr. Claude dePamphilis (Penn State Dep. of Biology), Dr. Dave Rudell (USDA ARS), Dr. Alex Harkess (HudsonAlpha Institute for Biotechnology)

Total Project Request: Year 1: $87,142 **Year 2:** $96,692 **Year 3: $97,991**

Other funding sources

Agency Name: USDA ARS base funding
Amount: $220,000
Notes: personnel $100,000, RNA-Seq $90,000, consumables $30,000

Agency Name: WSU Ficklin Start-Up Funds
Amt. awarded: $86,000
Notes: These funds were used to purchase high-performance computing resources on WSU's Kamiak computing cluster. These resources will be used to perform data analysis for this project.

Agency Name: US National Science Foundation (NSF) Award #1659300
Amt. awarded: $150,000
Notes: A portion of this award was used to fund almost 1 Petabyte of storage for execution of scientific workflows and storage of results. We will use that infrastructure for this project.

Budget 1
Organization: USDA-ARS **Contract Admin:** Chuck Meyers & Sharon Blanchard
Telephone: 510.559.5769, 509.664.2280 **Email address:** chuck.myers@ars.usda.gov, sharon.blanchard@ars.usda.gov

Item	2019	2020	2021
Salaries	33,000		
Benefits			
Wages			
Benefits			
Equipment			
Supplies	5,000	5,000	5,000
Travel			
Miscellaneous[1]	49,142		
Plot Fees			
Total	87,142	5,000	**5,000**

Footnotes: [1]Miscellaneous expenses category is genome sequencing for 3 apple varieties

Budget 2
Organization Name: WSU **Contract Admin:** Ian McDonald, Katy Roberts
Telephone: 509-335-3943 **Email address:** grants.bc.johnson@wsu.edu

Item	2019	2020	2021
Salaries[1]		70,326	71,339
Benefits[1]		20,121	20,357
Wages[1]		1,245	1,295
Benefits			
Equipment			
Supplies			
Travel			
Plot Fees			
Miscellaneous			
Total		91,692	**92,991**

Footnotes: [1]Salaries, wages, and benefits will support a fulltime postdoc for 2 years and will provide partial support to a graduate student in Co-PI Ficklin's lab

Objectives:

1. Sequence genomes to build variety-specific genomes for 'Honeycrisp,' 'WA 38' (Cosmic Crisp®), and 'Gala'

   NOTE: The 'Gala' genome has been published by another group, so we will divert resources from the 'Gala' genome to the 'Granny Smith' genome, allowing us to add 'Granny Smith' to the project (see more details below)
2. Refine biomarker discovery pipeline using machine learning algorithms, comparative network analyses, and comparative genomics
3. Begin validation of biomarkers via PCR gene tests in multi-lot, multi-year surveys

Year 3 goals:

In year 3 we will sequence and assemble the 3 apple genomes. We will analyze the gene activity data from 4 apple cultivars towards defining a list of candidate biomarkers. We will gather a third set of maturity marker validation samples and test the activity of candidate biomarkers in this set to assess cross-cultivar utility.

Significant findings:

- Obtained gene activity data from 'WA 38' and 'Granny Smith'
- Preliminary analysis of 'Gala' gene activity data shows structure that differentiates treatments
- Year 2 validation fruit samples obtained, adding 14 sample sets
- Improvements made to biomarker discovery pipeline
- Added 'Granny Smith' to the project at no additional cost to the WTFRC

Methods:

Gene activity analysis via transcriptome sequencing

Cryopreserved RNA samples for all project cultivars (including a fine scale maturity time course from 2018 for 'Granny Smith' that matches the data for 'WA 38' and 'Honeycrisp') have been sent to the genomics core facility at Penn State for transcriptome sequencing (global scale gene activity assessment called RNA-Seq). We aim to sequence project samples (in biological triplicate) to a volume of ~8-9 million reads each. We ran a preliminary analysis of the 'Gala' data that included raw data quality analysis (% data that passed filtering and trimming), read processing (including usage statistics), and a principle components analysis (PCA). We compared mapping rates using the new 'Gala' genome from the Boyce Thompson Institute (https://doi.org/10.1038/s41588-020-00723-9), and all 3 available public *Malus x domestica* genomes from the Genome Database for Rosaceae (rosaceae.org). We are in the process of modifying a gene activity toolset called GEMmaker (github/SystemsGenetics/GEMmaker) for efficient processing of the special, high-efficiency data type we are using for this project.

Year 2 validation samples

We collected year 2 validation samples which consisted of a 3 time point sampling scheme centered on the approximate commercial harvest date. Year 1 samples were processed to a fine powder using a Spex® Cryogenic Grinder Mill and cryopreserved in -112˚F freezers. We added 14 cultivar/locations in 2020 (See Table 1 for current project total).

Improvements to biomarker discovery methods

We improved our biomarker discovery toolkit by analyzing 'd'Anjou' pear data during the SARS-CoV-2 pandemic shutdown of the Penn State genomics core facility. We used a gene activity data set from previous WTFRC-funded work to execute a series of standard statistical analyses (as described by Honaas et al., 2019 - https://doi.org/10.1016/j.postharvbio.2018.09.016). We also used a toolset called GSForge (github.com/SystemsGEnetics/GSForge) that is under development in Co-PI Stephen Ficklin's lab. This

toolset includes a machine learning step to select genes associated with variables we choose - in the case of these pear data we selected tissue type and fruit maturity. We added a function to another tool set from the Ficklin lab called KINC (https://doi.org/10.1109/ACCESS.2019.2951284) that builds in a test for differential co-expression.

Genome of 'Granny Smith'

Young leaf tissue from 'Granny Smith' (that was in cryogenic storage in the Honaas lab) was sent to Penn State cooperator dePamphilis for cryogenic storage until extraction for high molecular weight DNA and cleanup prior to sequencing. Genomic DNA isolations methods have been developed and tested, and extraction of DNA from frozen tissue samples is underway. The DNA from these samples will be used for genome sequencing for 'Granny Smith' following the strategy we have defined for other project cultivars. The sequencing will be facilitated by new project cooperator Dr. Alex Harkess at the HudsonAlpha Institute for Biotechnology (hudsonalpha.org).

Construction of a comparative framework for apple comparative genomics

We have sorted the available apple and pear genomes (available at the Genome Database for Rosaceae – www.rosaceae.org), plus the WTFRC-funded 'd'Anjou' genome, into a comparative framework built by the dePamphilis lab called PlantTribes3.0 (https://github.com/dePamphilis/PlantTribes). This framework is used to find genes that correspond between pome fruit genomes and helps us understand groups of genes that are active in coordinated ways.

Results and Discussion:

Gene activity analysis – RNA-Seq

All fine scale gene activity data should be in hand by the time of the WTFRC review. We have 'Gala,' 'WA 38,' and 'Granny Smith' data in hand at the writing of this report. Our initial assessment of the 'Gala' gene activity data indicated that it was of very high quality, with average error rates well below 0.1%. We generated on average 8 million measurements for each biological replicate for a total of >1.1 billion measurements across 147 RNA samples from 'Gala' fruit. The average rates of data usage (% data matched to the genome) to the recently published 'Gala' genome were higher than mapping rates to other published genomes by up to ~7%. This is consistent with Honaas' previous work and supports our hypothesis regarding the advantages of using a matching genome to analyze gene activity data. Further, these gene activity data show significant structure in a PCA (Figure 1). This indicates, like the pear data we analyzed in 2020 (Honaas et al., in review), that there are gene activity signatures we can relate to fruit quality changes, and also the various storage regimes used in the 'Gala' storage experiment. *This is important for biomarker development as we need to search for gene activity we can relate to outcomes and the various aspects of the postharvest environment.*

Validation samples

We continued to gather validation samples from research orchards and industry partners. We have currently over 350 validation samples that we can analyze for gene activity towards building a candidate biomarker list. We will use this sample set to see if candidate biomarker genes could be deployed across additional apple cultivars beyond the cultivars used for biomarker candidate discovery.

A multi-step gene activity analysis reveals maturity-linked gene activity

In a study completed this year, which is under consideration for a special issue at Frontiers in Plant Science, we discovered gene activity signatures that distinguish pear fruit of different maturity. We pivoted to this data set to improve our biomarker discovery methods during the closure at the Penn State Genomics Core Facility due to SARS-CoV-2. This closure interrupted genome and transcriptome sequencing for this project, though it has since resumed at partial capacity.

In the pear paper we report that standard approaches for statistical analysis of gene activity data indicated >15,000 genes showed changes through time, or differences between treatments, in the

postharvest period. We searched for clues about what types of biological processes may be influencing maturity and while we did find interesting patterns, the lists of gene functions were far too large to parse in a meaningful way. This was probably due to gene activity changes in fruit during the postharvest period that occur regardless of maturity, resulting in noisy gene activity data. To attempt to parse out changes linked to maturity we used a machine learning approach (via GSForge) to select genes that had activity specifically related to the factors in the experiment: ***tissue type*** and ***relative maturity***. This reduced the list of genes, but failed to readily identify gene activity related exclusively to maturity.

Then, from the output list of genes from GSForge, we manually examined many instances of correlated gene activity. We observed that gene activity was skewed based on maturity, but very strongly correlated in both samples thereby masking the maturity effect. For hundreds of genes in our list the relationship was shifted enough that we could differentiate maturity-linked patterns of expression based on a statistical test (see example in Figure 2). When we checked these results against the initial standard tests, we found that a majority of the genes with shifted expression were also strongly significantly different between treatments. This indicates we were able to reduce gene activity noise unrelated to maturity, while still capturing strongly significant gene activity differences related to maturity. *This is relevant to the search for biomarkers because we need to find gene activity signatures that are clearly linked to maturity, strongly significant, and distinct from background noise.*

Building a comparative genomics framework for apple

The funding for this project included salary for personnel working on fruit quality biomarkers – we hired Dr. Huiting Zhang in August 2020, a comparative genomics expert who received her Ph.D. in 2020 from Penn State. She has built a comparative framework for all genes in pome fruits. This allows us to see the genome context of genes related to fruit quality, like those that encode polygalacturonase proteins (PG) that degrade cell walls and contribute to fruit softening. There are many genes in apple that encode polygalacturonases. Indeed, the PG from apple that is part of a Quantitative Trait Locus (QTL) for fruit firmness is different from the PG in peach that is also part of a QTL for firmness. It is therefore possible that these genes are part of different co-expression groups, and this comparative context will help us understand how plant genes work together to specify complex traits like fruit texture.

This information provides important context for the development of biomarkers that we aim to deploy across cultivars – we need to make sure we are targeting corresponding genes between cultivars, not different versions or copies of the genes. This information can also help us understand cultivar differences. It allows us to know if a cultivar has extra, missing, rearranged, or broken copies of a gene leading to insight about the genetics of important fruit quality traits.

Figure 1. PCA plot of 'Gala' postharvest data shows structure that separates each postharvest treatment. In this experiment we stored fruit in various regimes, ranging from room temprature air, to 1-MCP treated fruit in CA at 33˚F. After an initial week at 33˚F in air to normalize the fruit response to chilling, we moved the fruit to the various storage conditions (that lasted up to 9 months). This PCA plot shows that each treatment elicited different patterns of gene activity that we can mine for biomarkers, as well as clues about how the fruit respond to the postharvest environment. **1-MCP 33** = air storage with 1-MCP at 33˚F, **1-MCP CA 33** = controlled atmosphere storage plus 1-MCP at 33˚F, **A 33** = air storage at 33˚F, **A 50** = air storage at 50˚F, **A 68** = air storage at 68˚F, **CA 33** = controlled atmosphere storage at 33˚F, **Pre-Treat** = brief conditioning to storage in air at 33˚F.

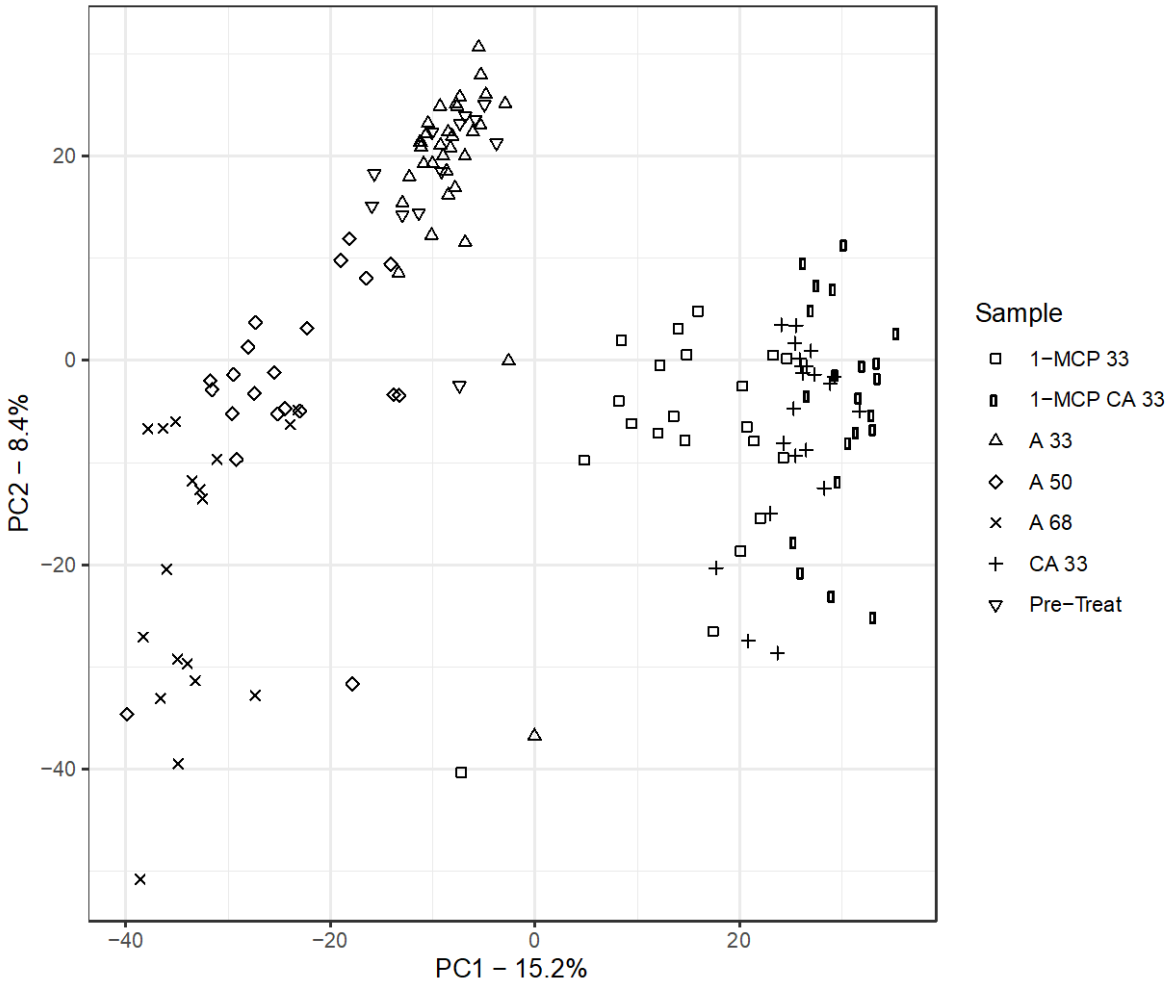

Figure 2. Correlated gene activity shows maturity dependent shifts. Our new method for finding differential co-expression reveals maturity-linked gene expression subclusters. In the search for biomarkers, we are likely to encounter highly similar patterns of gene activity between fruit of different maturity, so the development of sensitive techniques like this one improve our chances of finding meaningful activity signatures. In this experiment, we imposed a maturity contrast by harvesting fruit from different canopy positions. **A** shows highly peel-specific co-expression for two pear genes. When we highlight the peel gene activity signals from each canopy position (i.e. maturity class) in **B,** we can see the relationship is shifted based on maturity, creating sub-clusters that are distinct (P=8.008e-07). This is one of hundreds of statistically significant, maturity-linked subclusters reported in Honaas et al. (in review).

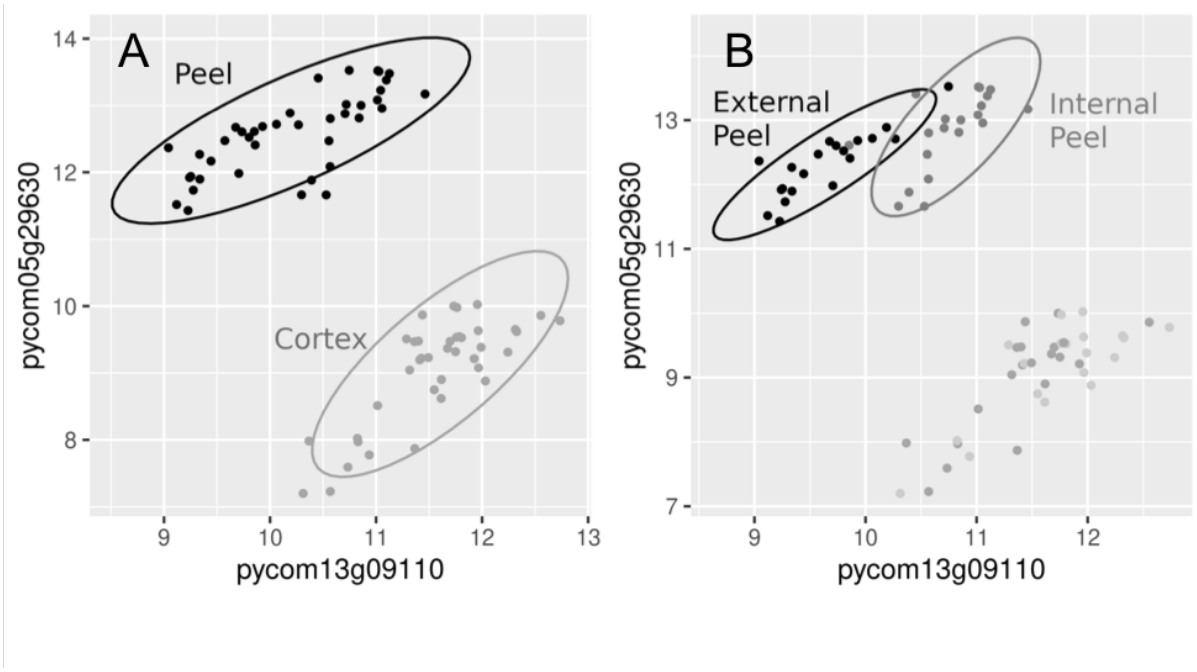

Table1. Summary of cryopreserved apple fruit peel samples. **High granularity samples for maturity biomarker discovery are bold**, validation samples are in normal font.

Cultivar	Location	2018 #picks	2019 #picks	2020 #picks
WA 38	WSU Sunrise Orchard Block 9 (Rock Island, WA)	-	**11**	3
Honey Crisp	WSU Sunrise Orchard Block 9 (Rock Island, WA)	-	**10**	3
Braeburn	Mattawa, WA	-	2	-
WA 38 #2	George, WA	-	1	3
Fuji	WSU Sunrise Orchard Block 10 (Rock Island, WA)	-	2	3
Gala	WSU Sunrise Orchard Block 5 (Rock Island, WA)	-	3	3
Golden Delicious	WSU Sunrise Orchard Block 5 (Rock Island, WA)	-	3	3
Granny Smith	WSU Sunrise Orchard Block 5 (Rock Island, WA)	**12**	3	3
Honeycrisp #2	Quincy, WA	-	2	3
Jonagold	WSU Sunrise Orchard Block 5 (Rock Island, WA)	-	3	3
Juici	Quincy, WA	-	2	3
Pazazz	Brewster, WA	-	2	3
Pink Lady	Mattawa, WA	-	2	3
Autumn Glory	Quincy, WA	-	6	3
Red Delicious	WSU Sunrise Orchard Block 11 (Rock Island, WA)	9	-	3

CONTINUING PROJECT REPORT **YEAR:** 2 of 3

Project Title: Critical limits for antimicrobials in dump tank systems

PI: Faith Critzer
Organization: Washington State University
Telephone: 509 786 9203
Email: faith.critzer@wsu.edu

Cooperators: WA packinghouses (TBD)

Total Project Request: **Year 1**: $86,183 **Year 2**: $93,414 **Year 3**: **$8,660**

Other funding sources: None

Budget 1
Organization Name: Washington State University
Contract Administrator: Samantha Bridger
Telephone: (509)786-9204
Email address: prosser.grants@wsu.edu

Item	2019	2020	2021
Salaries	38,245	39,775	
Benefits	2,538	2,639	
Wages			
Benefits			
Equipment			
Supplies	42,000	50,000	8,660
Travel	3,400	1,000	
Miscellaneous			
Plot Fees			
Total	86,183	93,414	**8,660**

Footnotes:

Salaries: In year 1, $38,245, and year 2, $39,775, is requested for a Graduate Research Assistantship for a MS student to work on all objectives.

Benefits: $2,538 and $2,639 are requested for benefits tied to the Graduate Research Assistantship for a MS student to work on all objectives for years 1 and 2, respectively.

Supplies: Supply costs of $42,000 in year 1, $50,000 in year 2 and $8,660 in year 3 are requested to pay for disposable supplies such as glassware, microbiological media, pipettes, water attribute measurement instrumentation and calibration standards, and water makeup analysis.

Travel: $3,400 and $1,000 is requested in years 1 and 2, respectively, for mileage and associated travel costs at a rate of $0.535/mi and adhering to all university policies for per diem associated with overnight travel. Increased travel costs in year 1 are associated with cost of traveling to participating facilities to collect water samples associated with objective 1.

Objectives:

1. Establish the carbohydrate, protein, and mineral makeup of dump tank water during production in addition to the attributes of chemical oxygen demand (COD), temperature, pH, oxidation reduction potential (ORP), turbidity, and conductivity.
2. Determine the impact of free chlorine, peroxyacetic acid, chlorine dioxide or ozone concentration on the survival of Shiga toxigenic *E. coli, Salmonella,* or *Listeria monocytogenes* over time in water which has the similar composition as water evaluated in objective 1 and is representative of water chemistries observed throughout production in dump tank systems.

Significant Findings

- Chemical Oxygen Demand (COD) has been shown to be very effective parameter for predicting sanitizer efficacy in postharvest wash systems in other commodities.
- Mean COD value (preliminary data) was 592 mg/L, with considerable variation amongst sites and over time.
- COD significantly impacts the efficacy of chlorine and PAA against *Salmonella* and Shiga-toxigenic *E. coli*
- Greater injury was observed with PAA as compared to chlorine, with

Methods

Objective 1. Establish the carbohydrate, protein, and mineral makeup of dump tank water during production in addition to the attributes of chemical oxygen demand (COD), temperature, pH, oxidation reduction potential (ORP), turbidity, and conductivity.

Packinghouse selection and descriptions. Three commercial apple packinghouses were recruited into the study which encompass different industry management practices for managing flumes. One packinghouse has a single flume which is used up to 68 hr or until water changeover is needed (e.g. conventional to organic break). There is no filtration within the system. The second packinghouse has a single flume up to 68 hr or until water changeover is needed (e.g. conventional to organic break). There is a flocculation system installed. The third packinghouse utilizes two flumes, the first is used for the same duration as the first two packinghouses and has a filtration system installed. The secondary downstream flume is utilized for up to 10 days and also has a filtration system installed. The initial plan of work scoped data collection for only the 24hr of production. To encompass the full period water will be used within the flumes, sampling periods were changed as shown in figure 1. Timing was set to occur throughout the packing season to encompass natural differences which occur as apples are held in storage. The first sampling event for all packinghouses occurred November-December of 2019. There was a slight delay due to COVID in the spring, but sampling resumed in the summer. Production variables such as additives to the flume system (e.g. acid, antimicrobials), flume capacity, varieties packed, storage conditions, % culls, line speed, was provided by the packinghouse and noted for each data collection period.

Water sample collection. Two 500 mL water samples were taken at 0, 4, 8, 12, 18, 24, 36, 48, 60, 72, 84 h at a consistent location from the flume. Once samples was shipped to a third party lab to deteremine carbohydrate, protein, and mineral content. The other sample was used for in real-time water quality parameters of chemical oxygen demand (COD), oxidation reduction potential (ORP), conductivity, pH, turbidity, temperature and amount of aniticmicrobial/acid present. All samples were held at 4°C (39.2°F) if not analyzed in real-time.

Establishing carbohydrate, protein, and mineral makeup of dump tank. Samples were shipped overnight for analysis with Merieux Nutrisciences. Target analytes were as follows: carbohydrates

[simple sugars (fructose, glucose, maltose, sucrose), starch, and fiber (pectin, cellulose, and hemicellulose)], protein, and minerals (calcium, iron, magnesium, phosphorus, potassium, and sodium). Based upon outcomes from the first replication, certain analytes may be discontinued if they consistently are below the limit of detection for the analyses.

Quantifying water chemistry attributes of dump tanks. Chemical oxygen demand was calculated using a reactor digestion method with colorimetric quantification *(4)* using the Hach DRB200 Reactor and DR900 multiparameter colorimeter. The colorimeter was also used to measure sample turbidity. A multiparameter meter (Hach probe model 5048) determined pH, ORP, conductivity, and temperature during real time during collection.

Statistical analysis. A completely randomized design with repeated measures will be used to evaluate significant differences of water attributes and nutritional compounds.

Objective 2. Determine the impact of free chlorine, peroxyacetic acid, chlorine dioxide or ozone concentration on the survival of Shiga toxigenic *E. coli* (STEC)*, Salmonella,* or *Listeria monocytogenes* over time in water which ha similar composition as water evaluated in objective 1 (year 2).

Water composition. Water quality measurements used in this part of the study will be developed to represent standard features of washwater used in packinghouses in Washington. Three variations of dump tank water quality will be used to represent postharvest water quality features which will be inclusive of real-life conditions as determined by objective 1. The parameters described in objective one will also be determined for this objective.

Microbial cultures. A five-strain cocktail of STEC, *Salmonella,* and *L. monocytogenes* associated with an outbreak will be used for this objective. Bacterial strains to be used are as follows: STEC cocktail [O104 (2011 European outbreak), O111 (apple juice outbreak), O103 (venison outbreak), O157 F4546 (alfalfa sprout outbreak) and O157 321 (spinach outbreak)]; *Salmonella* cocktail [Agona (alfalfa sprout outbreak), Montevideo (tomato outbreak), Gaminara (orange juice outbreak), Michigan (cantaloupe outbreak), and Saint Paul (pepper outbreak)]; *L. monocytogenes* cocktail [390-1 (cantaloupe outbreak), 390-2 (cantaloupe outbreak), 1452 (caramel apple outbreak), 108 (hard salami outbreak), 310 (goat cheese outbreak)]. Each strain of Shiga-toxigenic *E. coli* and *Salmonella* will be individually grown in Tryptic Soy Broth (TSB) at 37°C (98.6°F) for 24 h with three successive transfers prior to inoculation of Tryptic Soy Agar (TSA) plates with each individual strain. TSA will be incubated at 37°C (98.6°F) for 24 h to achieve a lawn of each strain. Each plate will be flooded with 10 ml of Buffered Peptone Water (BPW) to harvest cells. *E. coli* and *Salmonella* strains will be combined in equal volumes to create the five-species cocktail for inoculation. The same process will be used for *L. monocytogenes*, with the exception that each strain will be individually grown in Tryptic Soy Broth with Yeast Extract (TSBYE) at 32°C (89.6°F).

Sanitizer concentration. Three concentrations plus a no sanitizer control will be evaluated for chlorine and PAA, while two concentrations plus no sanitizer will be evaluated for chlorine dioxide and ozone. The upper limit will be based upon EPA label (chlorine, PAA or chlorine dioxide) or 1 ppm for ozone (which does not have an EPA label as it is an EPA registered device). To determine the efficacy of chlorine, as per industry practice, the pH of the system is maintained at 6.5 with the addition of a 1 in 10 dilution of 50% (v/v) of phosphoric acid, and PAA at approximately 4.00

Determining impact of sanitizers on pathogen survival. Simulated washwater treatments are inoculated and bacteria enumerated to estimate survival after 15, 30 and 60 seconds of exposure. All samples are neutralized with sodium thiosulphate to arrest sanitizer activity, then are serially diluted and

plated onto both TSA or TSYE and selective media and incubated at 37°C (98.6°F; STEC and *Salmonella*) and 32°C (89.6°F; *L. monocytogenes*) for 48 h to enumerate surviving bacteria.

Statistical analysis. Each experiment is being independently replicated three times with three technical replicates (n=9) for reach sanitizer concentration evaluated. A completely randomized design with analysis of variance (ANOVA) will be conducted. Post-hoc analyses will also be conducted to determine significant differences between survival rates between and within treatments. Additionally, mean inactivation rates will be calculated using the formula shown below:

$$\%\ inactivation = \left(\frac{original\ population - population\ at\ 30\ s}{original\ population}\right) x\ 100$$

Expected outcomes. Concentrations for each sanitizer have been determined which result in rapid inactivation of pathogens in water with similar properties to that observed during production. This will provide supporting documentation for apple packinghouses to substantiate minimum concentrations of each compound. This is especially important with the focus of HACCP-approaches for managing food safety risks which require critical limits (minimum concentrations of sanitizers) to be specified for dump tank systems to mitigate the risk of cross-contamination.

Results and Discussion

Mean, minimum and maximum values obtained for real-time physicochemical measurements for all replicates of objective 1 are presented in Table 1. Given the natural variation within and between the data set, it is important not to over analyze any values given that they may vary considerably. Based upon the significant amount of variation, no significant correlations were observed amongst any parameters over time ($p>0.05$). Replication amongst sites helped determine mean values for the parameter COD over production time. These values were used to determine the water quality parameters in objective 2.

Table 1. Observed phiscochemical attributes for flume water chemistry (n=104).

	pH	ORP (mV)	Conductivity (µS/cm)	Temperature °C (°F)	Turbidity (FAU)	COD (mg/L)	PAA (ppm)	Free Chlorine (ppm)
Mean	5.21	562.99	386.30	20.33 (68.6)	72.57	592.37	62.42	11.46
Min	2.46	194.30	2.41	11.70 (53.1)	0.00	10.00	2.00	0.50
Max	7.46	969.00	1574.00	34.30 (93.7)	250.00	2510.00	150.00	65.00

The first replicate complex chemical analyses were returned below the limit of detection for the assay, with the exception of ICP-MS, which had several minerals above the limit of detection. Therefore, the research team determined it is most cost effective to continue with only the ICP-MS and forgo carbohydrates [simple sugars (fructose, glucose, maltose, sucrose), starch, and fiber (pectin, cellulose, and hemicellulose)], and protein analysis.From the data analysis we have found a lack of correlation to any analyte and production time, but have reported mean, minimum and maxium values in Table 2.

Table 2. ICP mineral analysis for flume water (n=72).

	Mean (std. dev.)	Min	Max
Alumium	0.78 (2.82)	0.01	21.1
Barium	0.06 (0.05)	0.01	0.28
Calcium	52.94 (49.53)	15.3	306.0
Chromium	0.01 (0.05)	0.00	0.20
Copper	0.05 (0.12)	0.00	0.49
Iron	0.67 (1.72)	0.00	7.40
Magnesium	7.25 (3.98)	1.37	22.9
Manganese	0.09 (1.09)	0.00	8.79
Phosphorous	13.94 (132.42)	0.08	757.0
Potassium	10.53 (47.81)	0.82	398.0
Sodium	24.88 (19.38)	6.75	87.0
Stronium	0.29 (0.43)	0.04	2.8
Zinc	0.28 (4.36)	0.00	22.30

COD parameters for objective 2 were determined based upon observations in objective 1 and were set at 30, 500, and 2500 ppm for low, medium and high COD categories. Thus far, we have completed data collection for chlorine and PAA in all COD conditions for *Salmonella* and Shiga-toxigenic *E. coli* as shown in Figure 1 and 2.

While statistical analysis has not been completed as of yet, results do demonstrate that microbial inactivation is dependent upon COD level, sanitizer concentration, and exporsure time for pathogenic microorganisms. The coming year will be focused on completing objective 2 (Jan-March) and reporting findings and results (April-July).

Citations

1. Allende, A., M. V. Selma, F. Lopez-Galvez, R. Villaescusa, and M. I. Gil. 2008. Impact of wash water quality on sensory and microbial quality, including Escherichia coli cross-contamination, of fresh-cut escarole. *Journal of Food Protection*. 71:2514-2518.

2. Buchanan, R., S. Edelson, R. Miller, and G. Sapers. 1999. Contamination of intact apples after immersion in an aqueous environment containing Escherichia coli O157: H7. *Journal of Food Protection*. 62:444-450.

3. Goverd, K., F. Beech, R. Hobbs, and R. Shannon. 1979. The occurrence and survival of coliforms and salmonellas in apple juice and cider. *Journal of Applied Bacteriology*. 46:521-530.

4. Jirka, A. M., and M. J. Carter. 1975. Micro semiautomated analysis of surface and waste waters for chemical oxygen demand. *Analytical chemistry*. 47:1397-1402.

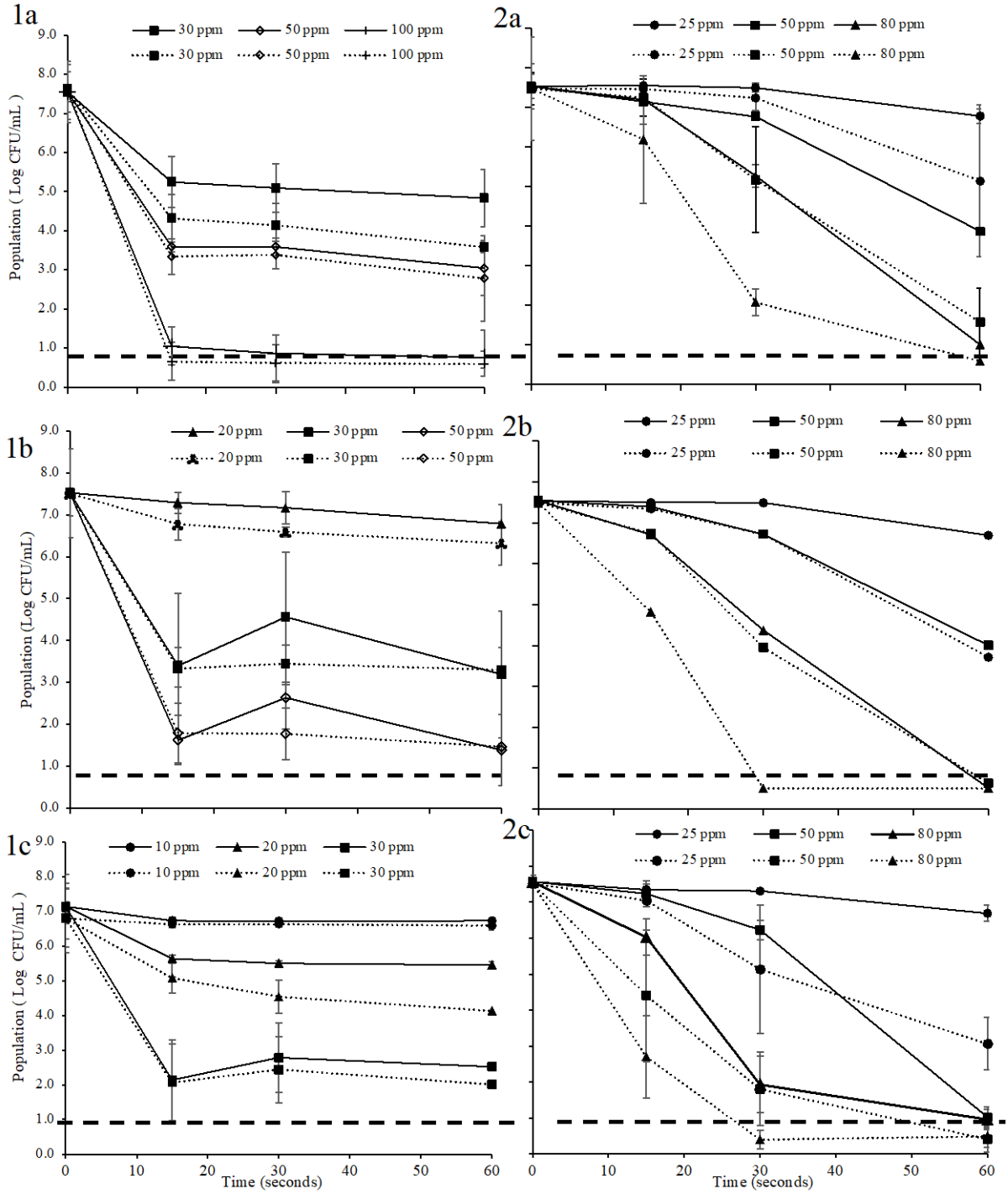

Figure 1. Bacterial survival (Log CFU/mL) of a five-strain Shiga-toxigenic *E. coli* cocktail against exposure time for 0-60 seconds in simulated processing water with different concentrations (ppm) of either Chlorine (**1**) or Peroxyacetic Acid (**2**) and with varying levels of COD (a) 2500 mg/L, (b) 500 mg/L and (c) 30 mg/L. Data points represent the means of log transformed *E.coli* populations from triplicate replications (n=9).Solid lines represent populations enumerated on Tryptic Soy agar and dotted lines represent populations enumerated on MacConkey's agar. Error bars represent the standard deviations from the mean. The dashed line represents the detection limit of 1 Log CFU/mL

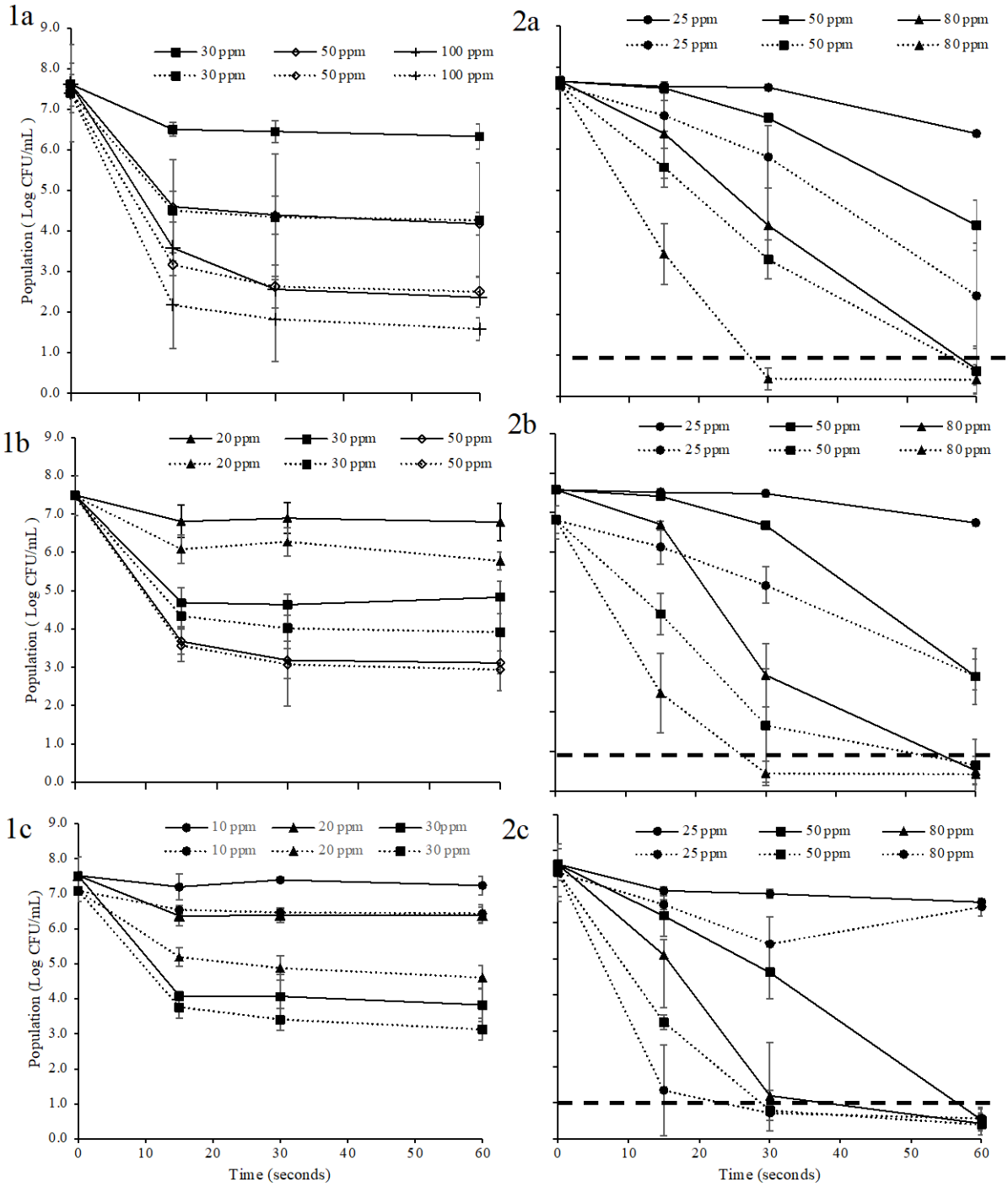

Figure 2. Bacterial survival (Log CFU/mL) of a five-strain *Salmonella* cocktail against exposure time for 0-60 seconds in simulated processing water with different sanitizer concentrations (ppm) of either (**1**) Chlorine or Peroxyacetic Acid (**2**) and with varying levels of COD (**a**) 2500 mg/L, (**b**) 500 mg/L and (**c**) 30 mg/L. Data points represent the means of log transformed *E.coli* populations from triplicate replications (n=9).Solid lines represent populations enumerated on Tryptic Soy Agar and dotted lines represent populations enumerated on XLT-4 agar. Error bars represent the standard deviations from the mean. Dashed line represent the detection limit of 1 Log CFU/mL.

CONTINUING PROJECT REPORT **YEAR**: No-Cost Extension

Project Title: Increasing the efficacy of antimicrobial chemicals with surfactants

PI: Dr. Girish Ganjyal
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City/State/Zip: Pullman, WA, 99164

Cooperators: Various apple packing houses in Yakima and Wenatchee (Borton Fruit, Double Diamond), Wesmar, Inc., Decco, CleanLogix, Inc., ZEP, and others

Total Project Request: **Year 1:** $47,148 **Year 2:** 53,575 **Year 3: 0**

Other funding sources: **None**
Amount:
Agency Name:
Notes: Donation of apples, sanitizer, and surfactants will be requested to decrease the cost of this research.

WTFRC Budget: ***None***

Organization Name: WSU **Contract Administrator:** Katy Roberts
Telephone: 509-335-2885 **Email address:** arcgrants@wsu.edu

Item	2019	2020	2021
Salaries	$17,098	$17,782	
Benefits[1]	$2,172	$2,270	
Wages[1]	$14,880	$15,475	
Benefits[1]	$1,488	$1,548	
RCA Room Rental	0	0	
Shipping	0	0	
Supplies[2]	$10,000	$15,000	
Travel[3]	$1,500	$1,500	
Plot Fees	0	0	
Miscellaneous	0	0	
Total	$47,148	$53,575	0

Footnotes:
[1] Salaries, Wages and Benefits for technical and student support
[2] Supplies and analysis fees, including for microbial testing
[3] Travel costs of trips to the packing facilities in Wenatchee and Yakima.

OBJECTIVES

In this project, we proposed to evaluate the efficiency of surfactants (with different chemical properties), combined with sanitizers, for the removal of *Listeria* from fresh apples.

The objectives for the next year are as follows:

1. Explore how different PAA formulations can influence the efficacy of the cleaning treatments.
2. Assess the impact of optimum treatment on the quality of the most significant apple varieties, including Gala, Fuji, Granny Smith, Honeycrisp, and Cosmic Crisp.

SIGNIFICANT FINDINGS

1st year of study

- Combining three selected surfactants, including Tween20 (T-20), sodium dodecyl sulfate (SDS), and lauric arginate (LAE) with peracetic acid (PAA), decreased the population of *L. innocua* on apples.
- Treating apples with PAA-T20 was most effective and reduced the load of *L. innocua* by 2.2 log.
- Stem bowl and calyx cavity are difficult to reach areas during the cleaning operation.
- Cleaning treatments were not completely effective in removing all *L. innocua* from apples.
- Additional commercially available surfactants will be examined on their cleaning efficacy.

2nd year of study

- Significant differences in bacterial reduction were observed between Gala and Granny Smith apples when subjected to the same cleaning treatments.
- Additional five commercially available surfactants combined with PAA were examined.
- The addition of new surfactants to PAA increased the bacterial reduction in Granny Smith apples, but it did not have the same effect on Gala apples.
- The new type of PAA was used in the 2nd year of study, showing greater antimicrobial potential than PAA used during the 1st year of study.
- The formulations of commercial PAA solutions can have a significant impact on their effectiveness.
- The cleaning solutions resulting in the greatest *L. innocua* reductions were: PAA in Gala apples (2.77 log) and 0.2% Norfox 90 with PAA in Granny Smith apples (2.76 log).
- Extending the dipping time from 1 min to 4 and 7 minutes did not significantly increase the bacteria reduction on apples.
- Our test results so far have led us to continue the evaluation of a few different commercially available PAA solutions.

METHODS

Inoculum preparation. *L. innocua* 51742 (ATCC) isolate was used as a non-pathogenic surrogate for *L. monocytogenes*. The inoculum was later diluted with room temperature water to reach the proper concentration.

Apple inoculation. Fifteen apples were placed into 5L of 10^7 CFU/mL *L. innocua* solution and gently agitated for 10 minutes. Apples were then removed and allowed to dry at room temperature until visibly dry under a chemical hood.

Preparation of cleaning solutions. Surfactant solutions used included DP081901 (Decco US Postharvest, Inc., Monrovia, CA), Barlox 12 (Wesmar Company, Inc., Lynnwood, WA), Barlox 10S (Wesmar Company, Inc.), Stepanol EHS (Wesmar Company, Inc.), and Norfox 90 (Wesmar Company, Inc.). The surfactants were used at high (H) and low (L) concentrations delineated in Table 1, and all were combined with 80ppm peracetic acid (PAA) (Tsunami ™ 200, EcoLab Saint Paul, MN, U.S.A). PAA concentration was measured using a titration kit (LaMotte, Chestertown, MD, U.S.).

Table 1. List of surfactants used in the 2nd year of study.

Name	Company	Composition	Final Concentration	Disassociation in H_2O
DP081901 (DP)	Decco	D-glucopyranose, oligomeric, C10-16, alkyl glycosides (% by weight: 20-25%); D-glucopyranose, oligomers, decyl octyl glycosides (% by weight: 25-50)	0.05% [DP-L] 0.2% [DP-H]	nonionic
Barlox 12 (B12)	Wesmar	Amines, cocoalkyldimethyl, n-oxides 30%	0.05% [B12-L] 0.2% [B12-H]	nonionic
Barlox 10S (B10)	Wesmar	N,N-dimethyldecylamine N-oxide 30%	0.05% [B10-L] 0.2% [B10-H]	nonionic
Stepanol EHS (ST)	Wesmar	Sodium 2-ethylhexyl sulfate 40-50%	0.02% [ST-L] 0.2% [ST-H]	anionic
Norfox 90 (N90)	Wesmar	Sodium dodecylbenzene sulfonate 90%	0.05% [N90-L] 0.2% [N90-H]	anionic

Cleaning procedure. The cleaning procedure is summarized in Figure 1. Briefly, each apple was dipped into 250 mL of cleaning solution and kept submerged for 1, 4, and 7 minutes. Once removed from the cleaning solution, the apple was gently rubbed with gloved hands for one minute to replicate the brush bed during the apple packing process. Then, the apple was sprayed with approximately 4.2 mL of an 80 ppm PAA solution. The apples were allowed kept under a chemical hood until visibly dry. Nine apples were used for each treatment. Inoculated, untreated apples were subjected to microbial enumeration as a control.

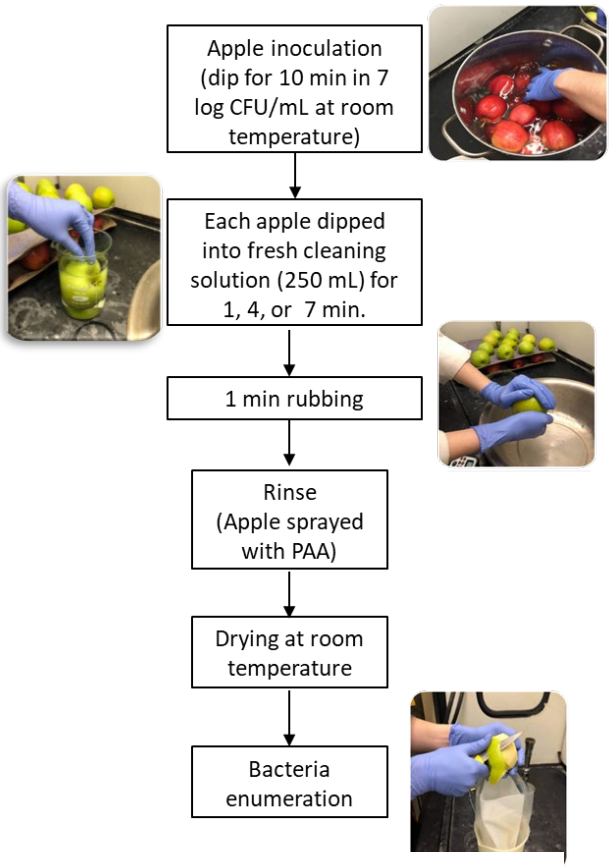

Figure 1. Cleaning treatment process.

Enumeration of *L. innocua* after cleaning. Dry apples were peeled using a sterile knife. The apple peels from each apple were placed into separate sterile filtered stomacher bags, weighed, and diluted with 25 mL D/E N broth (Dey-Engley Neutralizing Broth) (Hardy Diagnostics, Lacey, WA). Samples were homogenized and serially diluted in 9 mL PBS and plated on TSAYE. Plates were then inverted and incubated for three to four hours at 35°C (95˚F) before being overlaid with approximately 10 mL of Modified oxford agar (MOX) (Criterion, Hardy Diagnostics, Santa Maris, CA) at 40˚C (104˚F). The overlay solidified after 30 minutes, then inverted and incubated at 35˚C (95˚F) for 48 hours before being counted.

Statistical analysis. Results were presented as means with standard deviations. Data were analyzed using a one-way analysis of variance (ANOVA). The least significant difference test, LSD Fisher, was completed with Minitab 19 (Minitab Inc., State College, PA, U.S.).

RESULTS & DISCUSSION

Enumeration of *L. innocua* after cleaning. Preliminary studies on Gala and Granny Smith varieties determined that cleaning apples with surfactants yielded lower log reductions than treatments using a surfactant combined with PAA. Following this conclusion, all experiments with data shown in Figures 2, 3, and 4 were performed using EcoLab Tsunami 200 PAA at 80 ppm.

The PAA and DP-L-PAA treatments on Gala's yielded the greatest bacteria reductions, as shown by the lowest bacteria counts in Figure 2. Several surfactant treatments, ST-H & L, and DP-H, did not significantly reduce *L. innocua* compared to treatment of only water. Using only PAA resulted in the highest log reduction of 2.72. Overall, the results from Figure 2 indicate that *Listeria* on Gala apples can be removed and deactivated effectively without the addition of a surfactant to the cleaning treatment.

These results are not consistent with the results from the 1st year of this study (Pietrysiak et al., 2019), where the addition of surfactants consistently performed better than PAA alone. This difference might be due to a change in the brand of peracetic acid. In the 1st year of study, we used PAA provided by Pace International, whereas in the 2nd year we used PAA provided by EcoLab. Although the concentration of PAA in the cleaning solution was the same in years 1 and 2, significant differences in the pH were observed. To investigate this further, we requested additional commercially available PAA, and we are planning to test their efficacy against *Listeria* on apple.

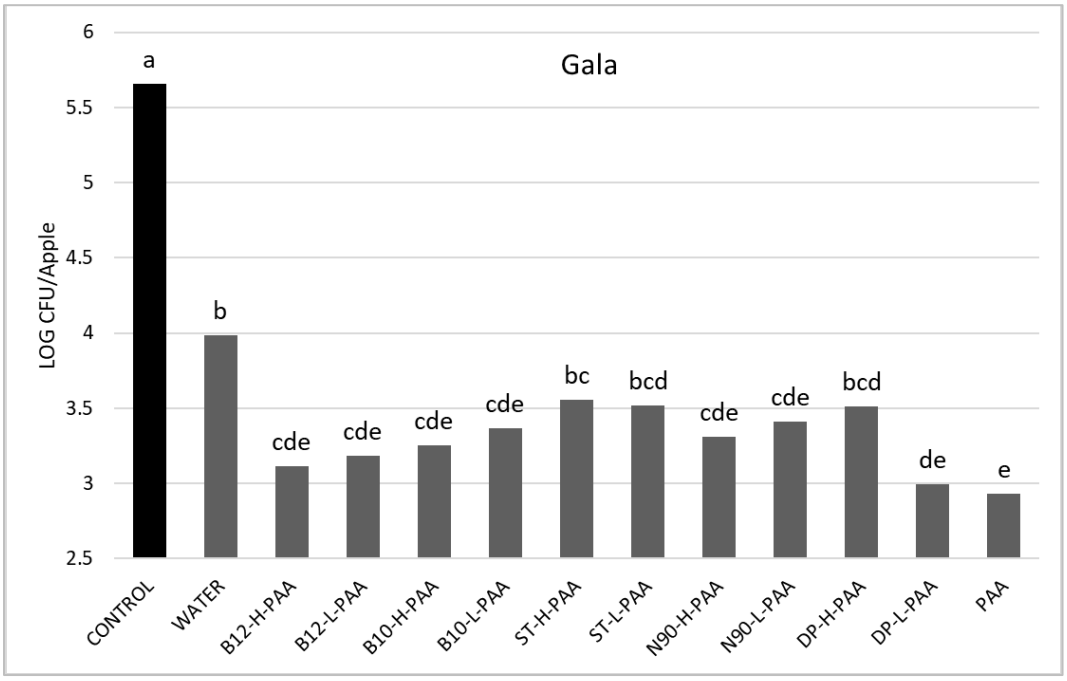

Figure 2. Antimicrobial activity of cleaning solutions against *L. innocua* on Granny Smith apples (n=9). Bars labelled with different letters indicate a significant difference (P<0.05).

All surfactant/PAA combination treatments on Granny Smith apples were significantly different than the water-only treatment (Figure 3), which had a bacteria reduction of 1.02 log. *L. innocua* on Granny Smith apples appeared to be more securely attached compared to that on Galas, as a water-only treatment on Galas had a reduction of 1.67 logs. The best treatment on Granny Smith apples was N90-H, an anionic surfactant, with a reduction of 2.76 log CFU/apple.

The PAA treatment with Granny Smiths was also less effective, resulting in a reduction of 1.97 logs compared to 2.72 on Galas. This could indicate that the addition of surfactants is more important in cleaning Granny Smiths, the opposite of what was concluded from the results on Galas in Figure 2. When developing cleaning treatments for apple packing lines, it can be concluded that the variety of the apples being treated is an important factor to consider and can change which type of treatment will be most effective at enhancing food safety.

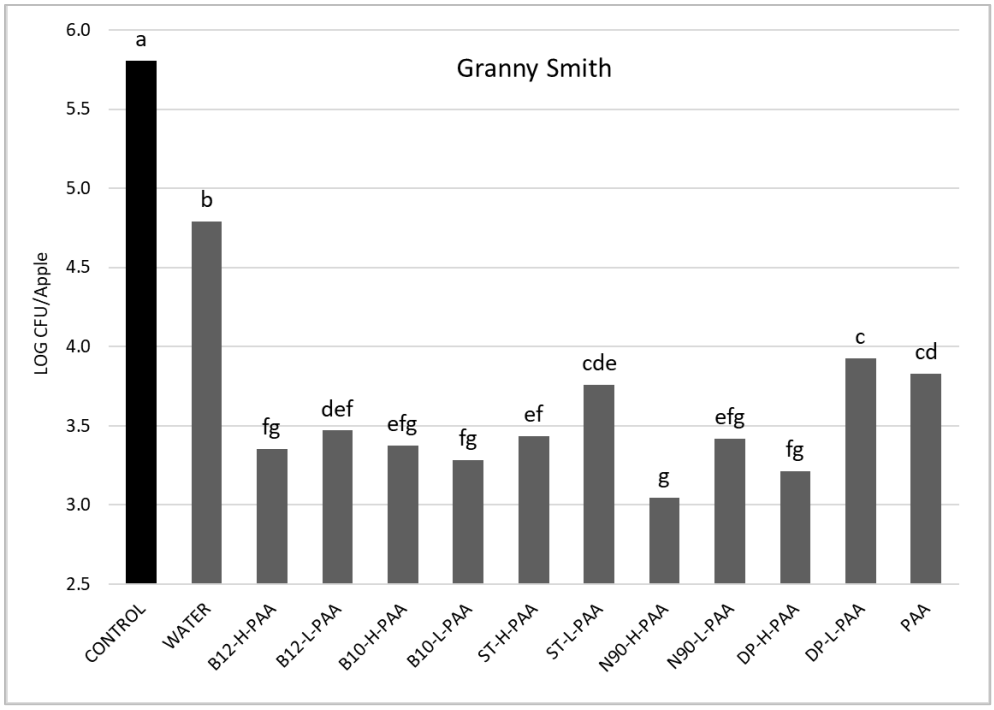

Figure 3. Antimicrobial activity of cleaning solutions against *L. innocua* on Granny Smith apples (n=9). Bars labelled with different letters indicate a significant difference (P<0.05).

When the dipping time was increased from 1 minute to 4 and 7 minutes, shown in Figure 4, the log reductions for both N90-H-PAA and ST-H-PAA increased. With N90-H-PAA yielding log reductions of 3.00 and 3.26 at 4 minutes and 7 minutes, respectively. Using the ST-H-PAA treatment log reductions of 2.52 and 2.63 were recorded at 4 and 7 minutes, respectively. While this is an increase in log reduction, the amount of time needed for an incremental log reduction is impractical for application purposes. Approximately 3 logs of bacteria have remained stubbornly attached to the apple surface, regardless of changes in the dipping time

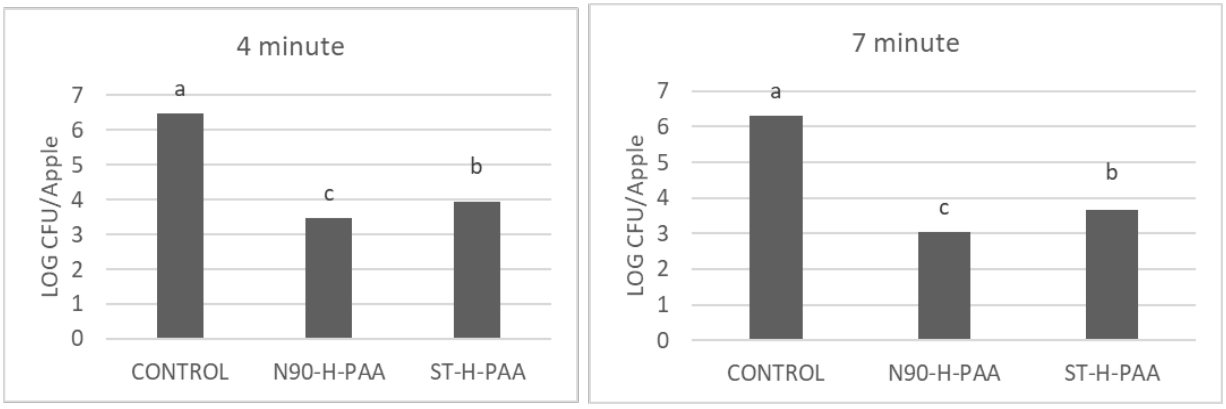

Figure 4. Antimicrobial activity of cleaning solutions against *L. innocua* on Granny Smith apples dipped for 4 and 7 minutes (n=9). Bars labelled with different letters indicate a significant difference (P<0.05).

In summary, the experiments indicate the potential for PAA used with or without surfactants in the apple cleaning process and how the activity of certain surfactants are more effective than others. The results further highlight the challenges that different apple varieties present in developing the most effective cleaning treatments, as well as the difficulty of removing bacteria that are sheltered by the apple structure. Further research is necessary to assess the quality impact the cleaning treatments have on several apple varieties, as well as explore how different PAA formulations can influence the efficacy of the cleaning treatments.

REFERENCES:

Pietrysiak, E., Kummer, J. M., Hanrahan, I., & Ganjyal, G. M. (2019). Efficacy of Surfactant Combined with Peracetic Acid in Removing Listeria innocua from Fresh Apples. Journal of Food Protection, 82(11), 1965-1972.

CONTINUING PROJECT REPORT **YEAR**: No-Cost Extension

Project Title: Systems-based approach for improved packinghouse sanitation

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Co-PI: Girish Ganjyal
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Cooperators: Washington apple packinghouses and Jacqui Gordon (WSTFA)

Total Project Request: **Year 1:** 67,369 **Year 2:** 71,399 **Year 3:** 58,209

WTFRC Budget:

Item	2018	2019	2020
Salaries	4,050	4,131	
Benefits	1,337	1,363	
Wages	4,500	4,703	
Benefits	1,485	1,552	
RCA Room Rental			
Shipping			
Supplies			
Travel	500	500	
Plot Fees			
Miscellaneous			
Total	11,872	12,249	0

Footnotes:

Salaries/Benefits: estimate of percent of time spent for Mendoza (5%) and Hanrahan (1%), a 33% benefit rate and 2% annual increases.

Wages/Benefits: calculated based on expected staff wage adjustments proportional to the WA state minimum wage increases (2018=$11.50, 2019=$12.00), approx. 350 hours

Travel: in state travel for Hanrahan (lodging in Wenatchee)

Budget 1
Organization Name: Washington State University
Contract Administrator: Samantha Bridger
Telephone: (509)786-9204 **Email address:** prosser.grants@wsu.edu

Item	2018	2019	2020
Salaries	26,274	27,509	28,807
Benefits	2,373	2,468	2,566
Wages	6,000	8,112	5,192
Benefits	600	811	519
Equipment			
Supplies	19,250	19,250	21,125
Travel	1,000	1,000	
Miscellaneous			
Plot Fees			
Total	55,497	59,150	58,209

Footnotes: Salaries: $26,274, $27,509, and $28,807 is requested in years 1, 2 and 3 , respectively, for a Graduate Research Assistantship for a MS student to work on all objectives.

Benefits: $2,373, $2,468, and $2,566 is requested in years 1, 2 and 3, respectively, for benefits tied to the Graduate Research Assistantship for a MS student to work on all objectives.

Wages: $6,000 in year 1, $8,112 in year 2 and $5,192 in year three are requested for hourly wages for student employee to conduct experiments as relating to the surface characteristics of the different types of materials used on packing lines from an engineering point of view.

Benefits: $600 in year 1, $811in year 2 and $519 in year three are requested for benefits of the student employee.

Supplies: Supply costs of $19,250 in year 1, 19,250 in year 2 and 21,125 in year 3 are requested to purchase disposable supplies such as glassware, microbiological media, Petri dishes, pipettes, and PCR reagents tied to objectives 1 and 3.

Travel: $1,000 is requested in years 1 and 2 for mileage and associated travel costs at a rate of $0.535/mi and adhering to all university policies for per diem associated with overnight travel.

Objectives

3. Identify harborage points and niches for *Listeria monocytogenes* indicator organism (*Listeria* spp.) on food contact surfaces in produce packinghouses (complete).
4. Rank surfaces based upon prevalence of indicator organisms to identify material types and design features with the greatest likelihood of harborage (complete).
5. Evaluate standard design features from a microbiological and engineering perspective to determine if alternative sanitation practices can compensate for less than ideal hygienic design.

Significant Findings

- Among 2,988 samples tested, 4.6% (n=136) were positive for *Listeria* spp
- Wax coating was the unit operation from which *Listeria* spp. were most frequently isolated.
- The FCS that showed the greatest prevalence of *Listeria* spp. were polishing brushes, stainless steel dividers and brushes under fans/blowers, and dryer rollers
- The prevalence of *Listeria* spp. on FCS increased throughout apple storage time.

Methods

Objective 1. Identify harborage points and niches for *Listeria monocytogenes* indicator organism (*Listeria* spp.) on food contact surfaces in produce packinghouses (years 1-3).

Packinghouse selection. Five packinghouses were enlisted into the study and have been sampled once quarterly during packing season for a total of eight data collection points per facility (Figure 1).

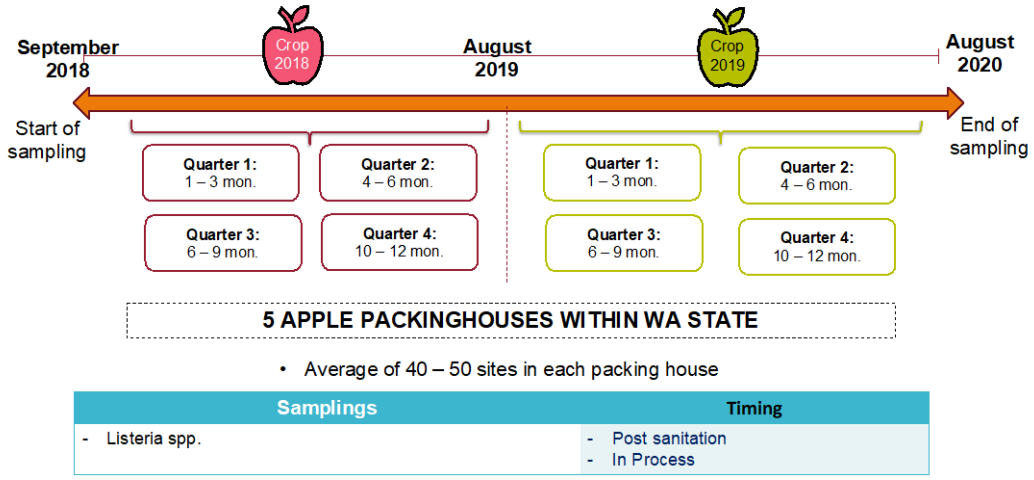


Figure 1. *Listeria* species sampling overview of apple packinghouses for the 2018 and 2019 apple crop.

Surface sampling methods. Sampling was coordinated to occur both after a sanitation (post sanitation) event and within 4 hrs of startup (in-process) to align with current FDA guidance. A pre-moistened sterile sponge is being utilized to sample a 100 cm^2-area or as large a space as is permissible for smaller surfaces.

Isolation of *Listeria* species. Bacteria are eluted in D/E neutralizing buffer, enriched in Buffered Listeria Enrichment Broth (BLEB) with antibiotic supplements, and confirmed through polymerase chain reaction (PCR) targeting the *iap* gene (Figure 2). This approach identified only *Listeria* sensu strictu as a group (*Listeria* species including: *L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri,* and *L. welshimeri*) and did not identify *Listeria monocytogenes* specifically.

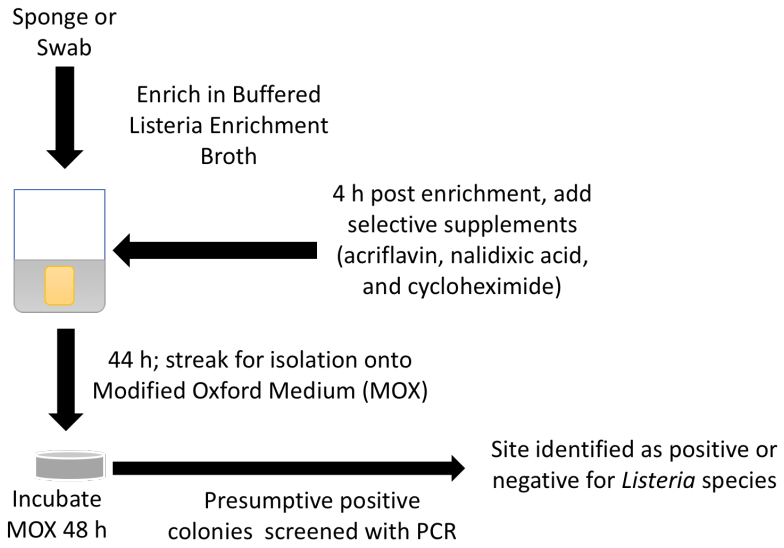


Figure 2**.** Sample processing to determine presence or absence of *Listeria* species (environmental indicator for *L. monocytogenes*).

Statistical analysis. Non-parametric methods were utilized to analyze the categorical data of presence or absence of *Listeria* spp. recovered based upon the surface type (e.g. stainless steel, painted steel, hard plastic, PVC belting, vinyl-like belting, cloth belting, PVC rollers, brush rollers, and foam rollers), unit operation, and any significant differences between facilities where similar surfaces are found at specific unit operations.

Objective 2. Rank surfaces based upon prevalence of indicator organisms to identify material types and design features with the greatest likelihood of harborage (year 3).

Review for hygienic design features. Outcomes from the statistical analysis in objective 1, combined with pictures of sampling locations and measurements taken from surfaces within packinghouses were analyzed to evaluate hygienic design features of equipment with significantly more prevalence of *Listeria* spp. on food contact surfaces. Surfaces were ranked by type and unit operation based upon likelihood of *Listeria* spp. presence.

Objective 3. Evaluate standard design features from a microbiological and engineering perspective to determine if alternative sanitation practices can compensate for less-than-ideal sanitary design (Year 4).

Objectives one and two have been completed. The following surfaces were associated with >5% frequency of *Listeria* spp. isolation and will the focus of this objective in the coming year: polishing brushes, stainless steel dividers used on brush line, dryer rollers, brushes, and plastic interlocking belts. These surfaces will be soiled with microorganisms and wax where appropriate, and various sanitation practices evaluated to determine if they can mitigate less than ideal hygienic design. This will be extremely beneficial given that the cost of design improvements may be prohibitive in the short-term, but alternate sanitation strategies could prove to be effective.

Selection of surfaces for further evaluation. The surfaces will be characterized for roughness, contact angle for water drops on the surface and surface morphology using microcopy techniques. Each surface will be purchased new from suppliers and sterilized to remove background microflora prior to inoculation. When possible, used surfaces of the same material will be included also.

Inoculation of surfaces with *Listeria* species. *L. seeligeri*, *L. marthii*, *L. ivanovii*, *L. welshimeri*, and *L. innocua* will be individually grown in Tryptic Soy Broth with Yeast Extract (TSBYE) at 32°C (89.6°F) for 24 h with three successive transfers prior to inoculation of Tryptic Soy Agar (TSA) plates with each individual strain. TSA will be incubated at 32°C (89.6°F) for 24 h to achieve a lawn of each species of *Listeria*. Each plate will be flooded with 10 ml of Buffered Peptone Water (BPW) to harvest cells. Each *Listeria* species will be combined to create a five-species cocktail for inoculation. Surfaces will be spot inoculated with 100 10μL spots.

Incubation of inoculated surfaces. To allow for bacterial attachment, each surface will be incubated in an environmental chamber for 24 h at two temperatures and humidity levels determined from packinghouse conditions observed in objective 1.

Treatment of surfaces. Surfaces will be exposed to three treatments in addition to a no treatment control and one commonly used sanitation protocol that will be determined by the research team based upon outcomes of objective 1 and 2, knowledge of current industry practices, and other research projects also focusing on this topic (e.g. Blakey brush bed sanitation). Example treatments include the use of alternative sanitizers (e.g. steam or quaternary ammonia compounds), use of alternating sanitizers, or extended exposure to sanitizers. All experiments will be replicated three times with three samples evaluated per replicate (n=9).

Enumeration/isolation of *Listeria* species. After treatment, surfaces will be hand massaged for 30 s in 50 mL BPW with 1% Tween 20 to remove attached *Listeria* species. The rinsate will be serially diluted and direct plated in duplicate on MOX. Plates will be incubated for 24 h at 32°C (89.6°F) prior to enumeration.

For instances where the population of attached *Listeria* species are below the level of detection, the remaining BPW Tween 20 rinsate will be enriched as described in objective one to determine the presence or absence of *Listeria* species.

Statistical analysis. A mixed model with mean separation will be used to determine where significant differences in populations exist to infer if alternate sanitation practices result in significantly fewer *Listeria* species. Additionally, a non-parametric procedure will be utilized to determine which treatments are significantly less likely to result in *Listeria* species positive surfaces.

Results and Discussion

Prevalence of *Listeria* spp. in apple packinghouses. *Listeria* spp. were isolated from all five packinghouses during both packing seasons. Among all tested samples (n=2,988), 136 (4.6%) were confirmed positive for *Listeria* spp. The prevalence of *Listeria* spp. was compared neither between packinghouses nor across packing seasons.

Occurrence of *Listeria* spp. in different unit operations. The prevalence of *Listeria* spp. in each unit operation is displayed in Table 1. *Listeria* spp. were most frequently isolated from the wax coating unit operation (17.3%; n=110), followed by both the first drying (fan/blower) (9.4%; n=394, and the second drying (tunnel dryer) (8.2%; n=304) unit operations. The lowest prevalence of *Listeria* spp. was obtained from the washing, washing/sanitizing/rinsing, and packing unit operations (<1.2%).

Table 1. Prevalence of *Listeria* spp. (%) by unit operation and timing of sampling.

Unit operation	Examples of surfaces tested	N[a]	Timing of sampling		Total prevalence
			Post- sanitation (n=1,497)	In-process (n=1,491)	
Washing (Dump tank/flume)	Dump tank, flumes, PVC[b] rollers, traction belting.	285	0 (a)[c]	1.4 (a)	0.7 (a)
Washing/Sanitizing/Rinsing(Spray bars)	Brush rollers, plastic flaps, side edges.	331	0.6 (a)	1.8 (a)	1.2 (a)
First drying (Fan and/or blower)	Brush rollers, dividers.	394	4.6 (b)	14.2 (cd)	9.4 (c)
Wax coating	Polishing brushes, plastic flaps, transfer points.	110	10.9 (b)	23.6 (d)	17.3 (d)
Second drying (Tunnel dryer)	Dryer rollers, bristle rollers, transfer points.	304	4.6 (b)	11.8 (bc)	8.2 (c)
Sorting	Sorter cups, interlocking conveyor belts, solid conveyor belts, plastic guide rails, side edges, Teflon tape, transfer points.	1,254	0.8 (a)	6.9 (b)	3.8 (b)
Packing	Packing tables, solid conveyor belts, plastic crates, plastic flaps.	310	0 (a)	0.7 (a)	0.3 (a)
Total		2,988	1.9	7.2	4.6

[a] Number of samples tested.
[b] Polyvinylchloride
[c] Values within a column that are not followed by the same letter are significantly different ($p \leq 0.05$).

Prevalence of *Listeria* spp. by timing of sampling (Post-sanitation, in-process). Of the 1,497 post-sanitation samples, 1.9% were positive for *Listeria* spp., compared to 7.2% of the 1,491 in-process samples (Table 1). Among all the positive *Listeria* spp. samples 21% (n=28) were detected during the post-sanitation sampling, whereas 79% (n=108) were detected during the in-process sampling. In addition, timing of *Listeria* spp. isolation was also evaluated for each site amongst the cohort which were positive during a sampling event based upon three scenarios, 1) the location testing positive post-sanitation and negative in-process, 2) negative post-sanitation and positive in-process, or 3) positive during both post-sanitation and in-process, to determine the frequency of each (Table 2). The outcomes of each scenario were significantly different from each other ($p \leq 0.05$), with *Listeria* spp. positive sites most frequently positive only for the in-process sample (75.9%), and 17.2% of sites positive for both.

Table 2. Frequency of *Listeria* spp. isolation for a specific sampling location based on timing of sampling during a sampling event

	Timing of sampling		**Frequency (%) (n=136)**
	Post-sanitation	**In-process**	
Scenario 1	Positive	Negative	6.9 (a)[a]
Scenario 2	Negative	Positive	75.9 (c)
Scenario 3	Positive	Positive	17.2 (b)

[a] Values within a column that are not followed by the same letter are significantly different ($p \leq 0.05$).

Prevalence of *Listeria* spp. by FCS type. The FCS that showed the greatest prevalence of *Listeria* spp. were polishing brushes (19.6%), dividers under fans/blowers (17.4%), dryer rollers (10.5%), and brushes under fans/blowers (9.7%) (Table 3). Sites which were exposed to sanitizers throughout production [brushes under spray bars (0.9%), dump tank/flume (0.9%)], as well as side edges (3.3%), sorter cups (2.6%), solid conveyor belts (1.6%), sorting guide rails (2.1%), traction belting (1.5%), PVC rollers (0.8%), packing tables and plastic crates (0.0%), sorting brushes (0.0%), and cup droppers (0.0%) had the lowest occurrence of *Listeria* spp.

Prevalence of *Listeria* spp. by sampling periods (quarters). The highest prevalence of *Listeria* spp. was obtained during the last quarter of sampling (Q_4) in the in-process sampling (38.2%; $p \leq 0.05$). The prevalence of *Listeria* spp. increased throughout crop storage time (quarters) but differed by unit-operation. The only unit-operation where the prevalence of *Listeria* spp. increased during the post-sanitation sampling was the tunnel drying (from Q_1=0% to Q_3=13.9%; $p \leq 0.05$). The three unit operations that accounted for the increase of the in-process prevalence of *Listeria* spp. over storage time were fan drying, tunnel drying, and sorting. These unit-operations showed significantly higher frequencies of isolation after the first quarter of sampling.

Table 3. Frequency of *Listeria* spp. by food contact surface

Food contact surfaces	N[a]	Frequency (%)
Polishing brushes (e.g., polyethylene, polypropylene, nylon, horsehair mix)	92	19.6 (a) [b]
Stainless steel dividers under fan/blowers	46	17.4 (ab)
Dryer rollers (e.g., stainless steel roller wrapped with vinyl or Teflon)	143	10.5 (abc)
Brushes under fan/blower (e.g., polyethylene, polypropylene)	206	9.7 (abc)
Bristle rollers (e.g., polyethylene, polypropylene)	160	8.8 (bcd)
Plastic interlocking chain conveyor belts (e.g., polypropylene, polyethylene)	256	5.1 (cde)
Teflon transfer points and tape	304	4.6 (cde)
Plastic flaps and transfer points (e.g., polyvinylchloride (PVC), polyurethane)	427	4.2 (de)
Side edges (e.g., Painted-steel or high-density polyethylene)	123	3.3 (cdef)
Sorter cups	76	2.6 (cdef)
Solid conveyor belts (e.g., PVC, polyurethane, polyester nylon)	186	1.6 (ef)
Sorting plastic guide rails	128	1.6 (ef)
Traction belting (e.g., polyurethane, polyester nylon)	66	1.5 (cdef)
Brushes under spray bars (e.g., polyethylene, polypropylene)	227	0.9 (f)
Stainless steel dump tank and flume	108	0.9 (ef)
PVC rollers	123	0.8 (ef)
Packing tables and plastic crates	64	0.0 (ef)
Cup droppers (e.g., painted steel)	60	0.0 (ef)
Sorting brushes (e.g., polyethylene, polypropylene)	193	0.0 (f)

[a] Number of samples tested.

[b] Values within a column that are not followed by the same letter are significantly different ($p \leq 0.05$

CONTINUING PROJECT REPORT **YEAR:** 2 of 3

Project Title: Improving apple fruit quality and postharvest performance

PI: Manoella Mendoza
Organization: Washington Tree Fruit Research Commission
Telephone: 509-665 8271
Email: manoella@treefruitresearch.com
Address: 1719 Springwater Ave
City/State/Zip: Wenatchee, WA, 98801

Cooperators:

- WTFRC internal program: Mackenzie Perrault, Ines Hanrahan, Marcella Galeni, Federico Grignaffini, Francisco Sarmiento-Torres, Gerardo Garcia
- Stemilt: Rob Blakey, Hannah Walters, Enrique Garcia
- Other: misc. grower collaborators
- WA 38 folder distribution: WSU Tree Fruit Extension Team, Agrofresh, GS Long, Storage Control Systems
- WA 38 Defect guide: WSU: Carolina Torres, Stefano Musacchi, Sara Serra, Kate Evans, Karen Lewis, USDA-ARS: David Rudell, WTFRC: Ines Hanrahan, Manoella Mendoza, Mackenzie Perrault, PVM: Jill Burberry
- Multistate FreshCloud technology validation: Chris Watkins & Al Shoffe (Cornell), Renae Moran (Main), Randy Beaudry (MSU), Jennifer De Ell (Ontario), Carolina Torres (WSU), Tara Baugher & Daniel Weber (PA)

Other funding sources
Majority of supplies and fruit donated by industry cooperators (approx. value: $5,000). WA 38 information folder printing and assembly was covered by WSU Extension. All costs for re-printing of the WA 38 starch scale are covered by Storage Control Systems.

Organization Name: WTFRC **Contract Administrator: Kathy Coffey**
Telephone: 509 665 8271 **Email address: Kathy@treefruitresearch.com**

Item	2019	2020	2021*
Salaries[1]			
Salary benefits[1]			
Wages[2]	15000	15450	15900
Wage benefits[2]	7950	8189	8427
RCA rental	0	0	0
Equipment + supplies	500	500	500
Travel	500	500	500
Total net costs	**23,950**	**24,639**	**25,327**

Footnotes:
[1]Salaries and benefits: Does not include time commitment of Mendoza (8%), Hanrahan (6%) and Schmidt (1%)
[2]Wages and benefits calculated at a yearly increase rate of 3%

***Note: This is an internal program report. Budget for 2021 is an estimation based on the assumption that the same amount of work will be performed as of in 2020. Activities for 2021 will be based on need and are subject to WTFRC board approval.**

OBJECTIVES

1. WA 38 outreach material
    a. Development of an WA 38 apple defect guide
    b. Distribution of WA 38 starch scale (1-6)
    c. Develop harvest criteria information for commercial WA 38 storage in 2020

2. Multistate validation of Fresh Cloud technology to predict bitter pit and soft scald in Honeycrisp

3. WA 38 Collaborative efforts
    a. Participation in three WSU virtual field days
    b. Coordination of fruit sampling for Decco, Pace, Crunch Pak
    c. Assisted WSU extension with WA 38 projects
    d. Lead scientific input to PVM

SIGNIFICANT FINDINGS

Objective 1: WA 38 outreach material

a. A variety-specific defect guide was developed and is available at WSU Tree Fruit Extension website (http://treefruit.wsu.edu/wa-38-defects-guide/)
b. The WA 38 starch scale was finalized in 2019 and distributed at no cost to the industry. In 2020, industry training was continued via extension events and distribution of printed copies.
c. The harvest criteria for commercial WA 38 Storage in 2020 document is available at the WSU Tree Fruit Extension website (http://treefruit.wsu.edu/) under WA 38 resources.

Objective 2: Multistate validation of Fresh Cloud technology to predict bitter pit and soft scald in Honeycrisp

Evaluations are ongoing

Objective 3: WA 38 Collaborative efforts

a. The virtual meetings held by the WSU extension team had a wide range of participants including growers, packers, retailers, and researchers.
b. Commercial companies received WA 38 samples to accelerate work on wax and greasiness issues. Tests are ongoing.

c. No significant findings to report.
d. The WA 38 2020 Marketing and Quality Standards is available at the WSU Tree Fruit Extension webpage (http://treefruit.wsu.edu/) under WA 38 resources. WTFRC facilitated scientific input to PVM.

METHODS

Objective 2. Multistate validation of Fresh Cloud technology to predict bitter pit and soft scald in Honeycrisp

This year 6 orchards were chosen, and 7 sampling sets collected (one orchard was samples twice). The orchards were located in the upper Yakima Valley region. While choosing the sampling sites, the preference was given to orchards with known historical information for a range of bitter pit susceptibilities. A minimum of 10 representative trees were selected in each block. If crop load required more trees to be labeled, the tree count was recorded. Horticultural information is recorded if available, including but not limited to:

- Orchard details (HC strain, rootstock, tree age, training system, preharvest sprays including ReTain/Harvista/fungicide, yields
- use of preharvest growth regulators

The following activities were conducted per each sampling site:

Pre-harvest – A sample of 10 representative apples per tree (total of 100 per site) were taken 3 weeks before anticipated first harvest and kept at 68°F for 3 weeks. Bitterpit Incidence (absent/present) was evaluated weekly (when possible).

At harvest – A total of 380 apples were harvested per sampling site, from 20 representative trees. Fruit was defect-free and of similar size. Shoot growth measurement were recorded from 5 one-year shoots per each tree harvested.

Quality analysis for 20 apples were conducted in the laboratory within 24 hours of harvest. The following parameters were evaluated: Fruit weight (gr.), Firmness (lb.), Soluble solids concentration (SSC, %Brix), Titratable acidity (TA, % malic acid), Starch degradation (Cornell 1-8; Honeycrisp 1-6) and DA meter (I_{AD}).

Storage – A total of 360 apples per orchard were equally divided between three treatments: 33°F, 38°F + conditioning (7 days at 50°F), and 38°F without conditioning. Evaluation of bitterpit incidence will occur at 2 months, 4 months and 4 months + 7 days at room temperature (68°F).

Additional Trials

- Two orchards were sampled 5 weeks before harvest. A sample of 100 fruit was collected weekly and keep at 68°F until harvest. Bitterpit incidence was recorded weekly.
- For one orchard that was harvested in two consecutive weekly picks, samples were collected at 3 weeks pre-harvest and at harvest. Bitterpit incidence was recorded weekly.

RESULTS & DISCUSSION

The WTFRC internal program has continued to focus part of its effort on Honeycrisp fruit quality. Due to the change in leadership at WTFRC in 2018, Manoella Mendoza assumed the role of staff lead for this internal program area. Dr. Torres (WSU, Endowed Chair Postharvest Systems) will lead the WA 38 postharvest program and extension efforts with full support of the WTFRC internal Program in 2021.

Development of an WA 38 apple defect guide

A variety-specific defect guide was developed and is available at WSU Tree Fruit Extension website (http://treefruit.wsu.edu/wa-38-defects-guide/). This effort was led by Carolina Torres (WSU) and Ines Hanrahan (WTFRC). The defect guide was developed with a focus on defects typically observed in WA 38 to date and includes three modules: defects visible during the growing season and at harvest, defects visible after storage, and unique characteristics to WA 38. The guide will be updates regularly.

Distribution of WA 38 starch scale (1-6)

A starch scale for WA 38 and a detailed description was developed, distributed and industry wide training was performed in 2019. In 2020, industry training was continued through extension events and distribution of 700 folders containing the starch scales and other relevant materials, such as 2020 Marketing and Quality Standards and Recommended Harvest Criteria for commercial WA-38 Storage in 2020.

Starch scales can be downloaded from WSU Tree Fruit Extension Team website (http://treefruit.wsu.edu/wa38-starch-scale/). Printed material can be requested from WTFRC, PVM or WSU Tree Fruit Extension and will be provided to industry at no cost.

Developed harvest criteria information for commercial WA 38 storage in 2020

This effort was led by Ines Hanrahan and completed in collaboration with Carolina Torres and input of WSU Extension team members. The 2020 recommendations are available at the WSU Tree

Fruit Extension website (http://treefruit.wsu.edu/) under WA 38 resources. The document will be updated for 2021.

Multistate validation of Fresh Cloud technology to predict bitter pit and soft scald in Honeycrisp

This project is led by Chis Watkins (Cornell University). Carolina Torres (WSU) is the principal investigator in a State level. The WTFRC supported Dr. Torres with trail set up, data collection and quality analysis, under the leadership of Mackenzie Perrault (WTFRC internal Program). Data analysis is ongoing and will be completed by Dr. Torres.

Participation in WSU virtual field days

There were three virtual events: WA 38 grower/field staff pre-harvest Q and A web meeting, WA 38 Packer/Shipper Q&A web meeting, and WA 38 Virtual Pre-Harvest Field Day. This effort was led by the WSU Extension Team. Ines Hanrahan was a presenter in these webinars, focusing on apple quality and postharvest issues.

Coordination of WA 38 fruit sampling for Decco, Pace and Crunch Pak

WTFRC coordinated with Lee Kalcsits (WSU) and Bernardita Sallato (WSU) to make fruit samples (bins) available to allied industry partners to accelerate work on wax and greasiness issues.

Assisted WSU Extension with WA 38 projects

Helped Bernardita Sallato (WSU) and Karen Lewis (WSU) with harvest of several bins of WA 38 in Prosser. WTFRC crew transported and stored the bins at a Stemilt RCA in Wenatchee. WTFRC further assisted conducting titratable acidity analysis for a WA 38 experiment.

Led Scientific input to PVM

PVM has published the Marketing & Quality Standard 2020 Crop Year, based on scientific input provided by a group of researchers under the leadership of Ines Hanrahan. It includes updated starch specifications for harvest and shipping, stem clipping recommendation, grading criteria for defects and color, and compliance actions. The general release date was updated and set as no earlier than Monday, November 23, 2020 at 8 am (PT).

The document will be reviewed and updated annually by the Quality Standards Advisory Committee. It can be found at the WSU Tree Fruit and Extension webpage (http://s3-us-west-2.amazonaws.com/treefruit.wsu.edu/wp-content/uploads/2020/07/22140927/Cosmic_CrispR_Quality_Standards_2020-Crop-Year_Final.pdf)

For more information on industry guidance refer to https://quality.cosmiccrisp.com/

CONTINUING PROJECT REPORT **YEAR**: No-Cost Extension

WTFRC Project Number: AP-18-104A

Project Title: Fate of *Listeria* on apples at ozone and controlled atmosphere storage

PI:	Meijun Zhu	**Co-PI**:	Ines Hanrahan
Organization:	Washington State University	**Organization**:	WTFRC
Telephone:	509-335-4016	**Telephone**:	509-669-0267
Email:	meijun.zhu@wsu.edu	**Email**:	hanrahan@treefruitresearch.com
Address:	100 Dairy Road, 106 FSHN	**Address**:	2403 S. 18th St., Suite 100
City/State/Zip:	Pullman/WA/99164	**City/State/Zip**:	Yakima, WA 98903

Cooperators: Allan Brothers. Inc., Stemilt Growers LLC., Guardian Manufacturing, Inc. AgroFresh Inc.

Total Project Request: **Year 1:** 118,779 **Year 2:** 121,797 Year 3: 125,404

Other funding sources: None

WTFRC Budget:

Item	2018	2019	**2020**	2021
Salaries[1]	4,141	4,224	**4,308**	
Benefits[1]	1,367	1,394	**1,422**	
Wages[2]	4,500	4,703	**5,267**	
Benefits[2]	1,485	1,552	**1,738**	
RCA Room Rental	8,316	8,316	**8,316**	
Travel	500	500	**500**	
Total	20,309	20,689	**21,551**	0

Footnotes:
[1]Salaries/Benefit for WTFRC staff support.
[2]Wages/Benefits for research intern support
[3]RCA room sharing with Stemilt
[4]Travel cost for transferring of fruit from Wenatchee to Pullman

Budget: Meijun Zhu

Organization Name: WSU-Pullman **Contract Administrator:** Katy Roberts
Telephone: (509) 335-2885 **Email address:** arcgrants@wsu.edu

Item	2018	2019	**2020**	2021
Salaries[1]	37,124	38,609	**40,154**	
Benefits[1]	12,412	12,909	**13,424**	
Wages[1]	15,340	15,953	**16,592**	
Benefits[1]	1,094	1,137	**1,183**	
Equipment	0	0	**0**	
Supplies[2]	25,500	25,500	**25,500**	
Travel[3]	2,000	2,000	**2,000**	
Miscellaneous [4]	5,000	5,000	**5,000**	
Plot Fees	0	0	**0**	
Total	98,470	101,108	**103,853**	0

Footnotes:
[1]Researchers'salaries plus benefits.
[2]Bacteria culture media, reagents and consumable supply cost
[3]Travel funds for industrial sampling and experiments.
[4]Funds are requested to partially cover the Biosafety Level 2 facility and equipment maintaining fees.

OBJECTIVES

1. Assess fate of *Listeria* on apple surfaces stored under RA and CA with continuous low doses of ozone.
2. Examine survival of natural microorganisms on apple surfaces stored under RA and CA with continuous low doses of ozone.
3. Evaluate impacts of ozone in the storage environment on final fruit quality.

SIGNIFICANT FINDINGS

1. There were 2.9-3.5 and 2.2-2.7 Log_{10} CFU/apple reduction of *Listeria innocua* on Granny Smith apples (GSA) after 36 weeks of cold storage under a commercial RA and CA storage environment, respectively. A 2.2 Log_{10} CFU/apple reduction of *L. innocua* on Red delicious apples was obtained at the same storage condition and duration.
2. Continuous low dose ozone gas application in CA storage provided an additional 2 Log_{10} CFU/apple of *L. innocua* on GSA at ozone gas concentration between 51-87ppb. However, for GSA, MCP-1 application in CA room slightly decreased antimicrobial efficacy of ozone gas.

   Compared to GSA, ozone gas was more effective against *L. innocua* on Red Delicious apples. A 60-80ppb ozone gas application resulted in an additional 3.3-3.4 Log_{10} CFU/apple reduction compared to those at RA or CA storage. MCP-1 treatment prior to storage had no effects on *L. innocua* survival on Red Delicious apples ($P > 0.05$).
3. The resident bacteria on GSA apples remained stable (3.5 – 4.0 Log_{10} CFU/apple) during the 36-week storage at RA or CA at 33°F. Continuous low dose ozone gas application in CA room significantly decreased resident bacteria on GSA after 24 weeks of storage.

   Red Delicious apples had similar initial bacterial count (~ 3.8 Log_{10} CFU/apple). It increased by ~ one Log_{10} CFU/apples after 12-week storage at RA or CA at 33°F and maintained this high level throughout 36-week storage. Ozone gas application in CA storage decreased resident bacteria in Red Delicious apples by 1.2-1.3 Log_{10} CFU/apple after 36 weeks of storage.
4. The initial population level of indigenous yeast/mold counts of non-inoculated GSA apples was 4.5-5.0 Log_{10} CFU/apple. The yeast/mold counts of GSA remained stable during the first 12 weeks of RA and CA. By 24-week of storage and beyond, the yeast/mold count of GSA stored under RA was significantly more than that of CA room. The yeast/mold counts in GSA of CA with different doses of ozone gas decreased during the first 24 weeks of storage. Nevertheless, the inhibitory effect of ozone was attenuated with prolonged storage time.

   Red Delicious apples had a similar intial yeast/mold counts, which was increased by more than one Log_{10} CFU/apple at the end of 36-week storage at RA and CA regardless of MCP-1 treatment. The yeast/mold counts on Red Delicious apples gradually decreased under CA with 60-80ppb ozone gas; there was ~ 0 .7 Log_{10} CFU/apple reduction of yeast/mold at the end of cold storage.
5. During 36-week CA storage, continuous low dose ozone gas at 50-87 ppb had no negative influence on fruit firmness, total soluble solids (TSS) and titratable acidity (TA), as well as internal disorders. However, prolonged continuous low dose ozone gas in CA storage had some impacts on the visual quality of GSA pretreated with MCP-1.

   For Red Delicious apples, low dose ozone gas application has no impact on TSS while improved TA and firmness of apples compared to RA and CA storage. Neither 6-month nor 9-month ozone gas application at 60-80ppb caused ozone burn in Red Delicious apples. Ozone application had no effects on superficial scale, lenticel decay, Russet, CO_2 damage compared to CA. Furthermore, ozone application improved internal browning and visual appearance of Red Delicious apples.

METHODS

We have established methods for proposed objective 1-3 studies as detailed in the following.

Objective 1: Assess fate of *Listeria* on apple surfaces stored under RA and CA with continuous low doses of ozone.

1. *3-strain Listeria inoculum preparation, inoculation, and establishment on apple surface*

A 3-strain *L. innocua* cocktail was prepared via mixing equal numbers of each respective strain into a suspension. *Unwaxed and unbruised a*pples of the selected varieties at commercial maturity were individually and separately inoculated to establish 1×10^6 CFU/apple of 3-strain *Listeria* cocktail through dipping inoculation and held at room temperature for 24 h prior to different storages.

2. *Cold storage treatments in a commercial packing facility*

Apples of the selected varieties inoculated with ~1×10^6 CFU/apple of *L. innocua* were randomly separated into six groups and subjected to three different storages: refrigerated air (RA, 1 °C/ 33 °F), controlled atmosphere (1 °C/ 33 °F, 2 % O_2, 1 % CO_2) treated with (CAMCP) or without 1-methycyclopropene (CA), CA with a low dose gaseous ozone and MCP-1 treatment (CAMCPLowPO_3), CA with high dose gaseous ozone with (CAMCPHigh O_3) or without MCP-1 treatment (CAHighO_3) for up to 36 weeks. Apples under different storage conditions were sampled at 0, 3-, 6-, 12-, 18-, 24-, 30-week and 36-week of storage, when the counts of *L. innocua* survived on apples were enumerated.

3. *Microbial analysis*

At each sampling day, apples under the respective storage condition were sampled and transferred to sterile whirl-pak bags with 10 ml of 0.1% buffered peptone water, hand rubbed to release attached microorganisms, then serial diluted. Appropriate dilutions were plated on agar plates. Plates were incubated at 35˚C (95˚F) for 48h and enumerated manually. Enrichments were done when *L. innocua* levels were under the detection limit of 10 CFU/apple following our previous publication (Sheng et al., 2018).

Objective 2. Examine fate of natural microorganisms on apple fruit surfaces when stored in refrigerated air, controlled atmosphere in the presence or absence of ozone.

1. *Cold storage treatments in a commercial packing facility*

Non-waxed, non-inoculated GSA apples were subjected to different storage conditions (RA, CA, CAMCP, CAMCPLowO_3, CAMCPHingO_3, CAHingO_3) as described previously. Apples were sampled at 0-, 6-, 12-, 24, and 36-week of storage for total plate count and yeast and mold enumeration.

2. *Survival microorganism analysis*

At each sampling day, apples were sampled and transferred to a sterile Whirl-Pak bag with 10 ml of 0.1% buffered peptone water bag, rubbed to release attached microorganisms, then serial diluted. The appropriate dilution was plated onto TSAYE plates for total plate count (TPC) and potato dextrose agar (PDA) plates for yeasts and molds, respectively per our established methods(Shen et al., 2019; Sheng et al., 2018; Sheng et al., 2020). TPC colonies were counted manually after incubation at 35°C (95˚F) for 48h, and PDA plates were counted after incubation at room temperature for 5 days.

Objective 3: Examine the effect of ozone in the storage environment on final fruit quality.

1. Fruit quality analysis

Fruit maturity and quality measurements such as firmness, total soluble solids (TSS) and titratable acidity (TA) were performed at harvest, after 6-month and 9-month storage and following an additional week of storage at room temperature per our established methods (Sheng et al., 2018). Briefly, fruit

firmness was assessed with a fruit texture analyzer using a 1 cm diameter probe on a peeled area of ~3 cm^2 on both sun and shade side of the apples. Total soluble solids were evaluated using Atago PR-32 digital brix refractometer. Titratable acidity of fruit juice was measured with a potentiometric titrator. Measurements of each parameter were repeated four times independently with a sample size of 10 apples per replication per storage regimen.

2. Disorder analysis

The incidence of disorders was assessed after cold storage followed by one day at room temperature (RT) for external disorders and 7 days at RT for both internal and external disorders. The absence or presence of the following external disorders was visually inspected and recorded: ozone burn, superficial scald, lenticel decay, visible decay, sunburn, russet, and CO_2 damage. Apples were sliced 3 times to determine the presence of any internal disorders including watercore, internal browning, or cavities. Sample size for both external and internal disorder analyses were 50 apples per replication per storage regimen, with 4 replicates for each analysis.

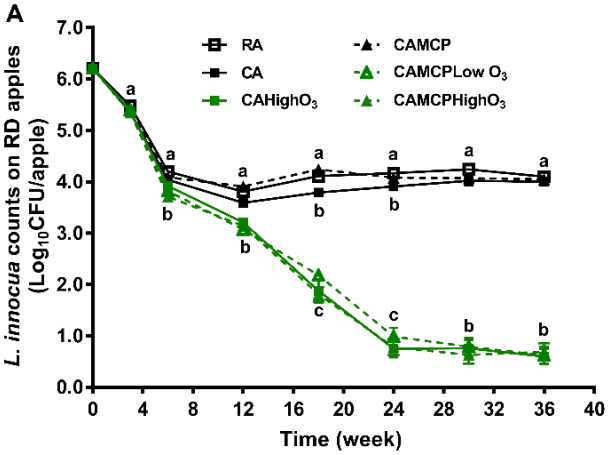
RESULTS AND DISCUSSION

In the past years, we reported that continuous low dose ozone gas application in CA cold storage is an effective in eliminating or controlling *L. innocua* on GSA, which generated an additional 2 Log_{10} CFU/apple reduction. However, different doses of ozone gas at the tested range, 51-87ppb, showed a similar anti-*Listeria* effects. MCP-1 application in CA room slightly decreased antimicrobial efficacy of ozone gas, and prolonged continuous low dose ozone gas in CA storage had some impacts on the visual quality of GSA pretreated with MCP-1. We further evaluated efficacy of low dose ozone gas at different concentration range against *L. innocua*, resident bacteria and decay microorganism on Red Delicious apples, as well as their potential impacts on fruit quality attributes. The new findings were summarized below.

1. Survival of *L. innocua* on Red Delicious under commercial cold storage.

During 3 weeks of cold storage, *L. innocua* was reduced by 0.7-0.9 Log_{10} CFU/apple on Red delicious apples stored in RA, CA, and CA plus different doses of O_3 with a die-off rate of 0.24-0.29

A



B

Reduction of *L. innocua* during storage (log_{10}CFU/apples)

		CA		CA+1-MCP		
Week	RA	0	High O_3	0	Low O_3	High O_3
0	0.00	0.00	0.00	0.00	0.00	0.00
3	0.73	0.86	0.83	0.80	0.81	0.86
6	2.01	2.17	2.29	2.12	2.39	2.50
12	2.40	2.62	3.00	2.30	3.14	3.07
18	2.10	2.42	4.33	1.97	4.03	4.40
24	2.04	2.30	5.46	2.13	5.22	5.43
30	1.97	2.19	5.45	2.14	5.42	5.58
36	2.15	2.21	5.61	2.18	5.59	5.52

Figure 1. Survival of *L. innocua* on Red Delicious apples during 36-week of commercial cold storage. A. *L. innocua* count on apples over cold storage period. Mean ± SEM, n = 32-40. [a-c] Mean at each sampling point without common letter differ significantly ($P < 0.05$).; B. Reduction of *L. innocua* on apples under different storages. Mean averaged from 32-40 apples. RA: refrigerated atmosphere; CA: controlled atmosphere; MCP: apples were treated with 1-methycyclopropene before subjecting to cold storage; $CAHighO_3$: CA storage with continuous gaseous O_3 application at 78.7 ± 13.2 ppb; $CAMCPLowO_3$: CA storage with continuous gaseous O_3 application at 60.2 ± 5.7 ppb, where apples were treated with 1-methycyclopropene treatment before subjecting to storage.

Log_{10} CFU/apple/week (Figure 1), which is smaller than that obtained on GSA. There was ~2.2 Log_{10} CFU/apple reduction of *Listeria innocua* on Red Delicious apples (GSA) over 36 weeks of cold storage under a commercial RA and CA storage environment. This reduction was smaller especially in RA storage, compared to that in GSA apples. Compared to GSA, low dose ozone gas at similar dose (60-80 ppb for Red Delicious apples vs 51-87 ppb for GSA) was more effective against *L. innocua* on Red Delicious apples. There were an additional 3.3-3.4 Log_{10} CFU/apple reduction compared to those at RA or CA storage. MCP-1 treatment prior to storage had no effects on *L. innocua* survival on Red Delicious apples ($P > 0.05$) (Figure 1).

2. Fates of resident microbiota on Red Delicious apples stored in refrigerated air, controlled atmosphere, and controlled atmosphere with different doses of ozone gas.

Resident bacteria, mold and yeast cause postharvest decay of apples (Janisiewicz and Korsten, 2002), which were further assessed during storage. Non-waxed and uninoculated Red Delicious apples were subjected to different storage conditions (RA, CA and CA with different dose of ozone gas) in the same condition as inoculated apples. Total plate count (TPC) and yeasts/molds (Y/M) count were evaluated at the selected storage durations.

Red Delicious apples had similar initial bacterial count (~ 3.8 Log_{10} CFU/apple). It increased by one Log_{10} CFU/apples after 12-week storage at RA or CA at 33°F and maintained this high level throughout 36-week storage (Figure 2). Ozone gas application at different doses in CA storage decreased resident bacteria in Red Delicious apples by 1.2-1.3 Log_{10} CFU/apple after 36 weeks of storage (Figure 2).

The initial level of indigenous yeast/mold counts of non-inoculated Red Delicious apples was 4.7 Log_{10} CFU/apple, which was similar to that of non-inoculated GSA apples. The yeast/mold count gradually increased in apples under RA and CA storages (Figure 3). By 36-week of storage, the yeast/mold counts of Red Delicious apples stored under RA or CA room were increased by 1.1-1.3 Log_{10} CFU/apple (Figure 3). On the other hand, the yeast/mold count on Red Delicious apples gradually decreased under CA with 60-80ppb ozone gas; there was ~ 0 .7 Log_{10} CFU/apple reduction of yeast/mold at the end of cold storage (Figure 3).

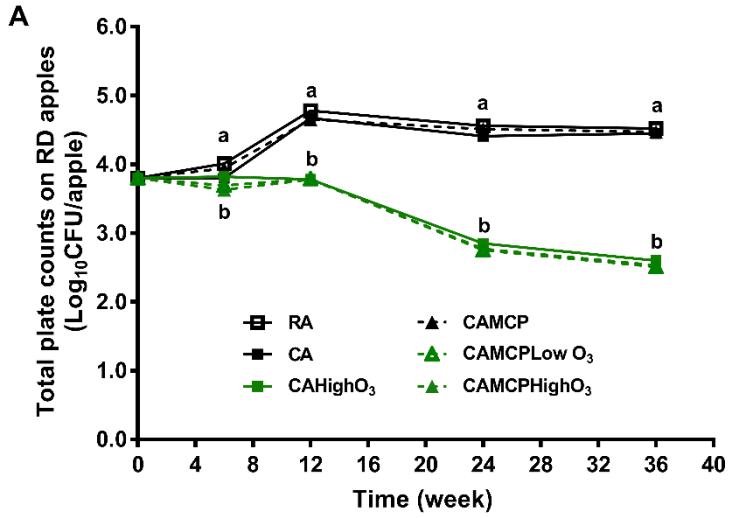
3. **Effects of continuous low dose ozone in cold storage environment on final fruit quality**

Quality attributes of Red Delicious apple fruits under different storage conditions were assessed both at harvest and after 6-month or 9-month storage. TSS of Red Delicious apples did not differ among storage treatments and over 9 months storage (Table 1). Red Delicious apples subjected to RA storage had a significantly lower firmness and TA compared with CA with MCP-1 pretreatment or without gaseous ozone at 6-month and 9-month storages (Table 1). Ozone gas application significantly improved the firmness and increased TA of apples comparted to RA and CA storage (Table1).

Table 1. Fruit quality attributes of Red Delicious apples over cold storage under different conditions

Treatment	Firmness (kg)			TSS (% Brix)			TA (% malic acid)		
	At harvest	6-month	9-month	At harvest	6-month	9-month	At harvest	6-month	9-month
RA	6.61 ± 0.81^{A}	3.75 ± 0.14^{aB}	3.90 ± 0.12^{aB}	12.94 ± 0.21^{A}	12.53 ± 0.15^{aA}	12.50 ± 0.15^{aA}	0.31 ± 0.01^{A}	0.11 ± 0.02^{aB}	0.10 ± 0.00^{aB}
CA		4.37 ± 0.27^{aB}	4.19 ± 0.06^{aB}		14.18 ± 0.28^{bA}	13.23 ± 0.24^{abA}		0.16 ± 0.01^{abB}	0.15 ± 0.01^{abB}
CAMCP		6.25 ± 0.19^{bcA}	5.82 ± 0.24^{bcA}		12.95 ± 0.78^{abA}	13.83 ± 0.08^{abA}		0.14 ± 0.02^{abB}	0.17 ± 0.01^{bB}
$CAMCPLowO_3$		6.46 ± 0.15^{bcA}	5.66 ± 0.24^{bcA}		13.85 ± 0.38^{abA}	13.93 ± 0.19^{abA}		0.18 ± 0.01^{bB}	0.19 ± 0.01^{bB}
$CAMCPHighO_3$		6.46 ± 0.15^{bcA}	6.79 ± 0.10^{bA}		13.70 ± 0.07^{abA}	14.00 ± 0.17^{bA}		0.18 ± 0.01^{bB}	0.18 ± 0.02^{bB}
$CAHighO_3$		5.51 ± 0.15^{cB}	5.23 ± 0.15^{cB}		13.95 ± 0.17^{bA}	13.40 ± 0.13^{abA}		0.17 ± 0.01^{bB}	0.18 ± 0.02^{bB}

TSS: Total soluble solids; TA: titratable acidity. $^{a-c}$ Mean within a column without common letter differ significantly ($P < 0.05$). $^{A-B}$ Mean the comparison of individual quality parameter at harvest, 6-month and 9-month storage within each storge treatment without common letter differ significantly ($P < 0.05$). RA: refrigerated atmosphere; CA: controlled atmosphere; CAMCPHighO3/ CAHighO3: CA with 78ppb O_3 with or without MCP-1 pre-treatment. $CAMCPLowO_3$: CA with 60 ppb O_3 and MCP-1 treatment.

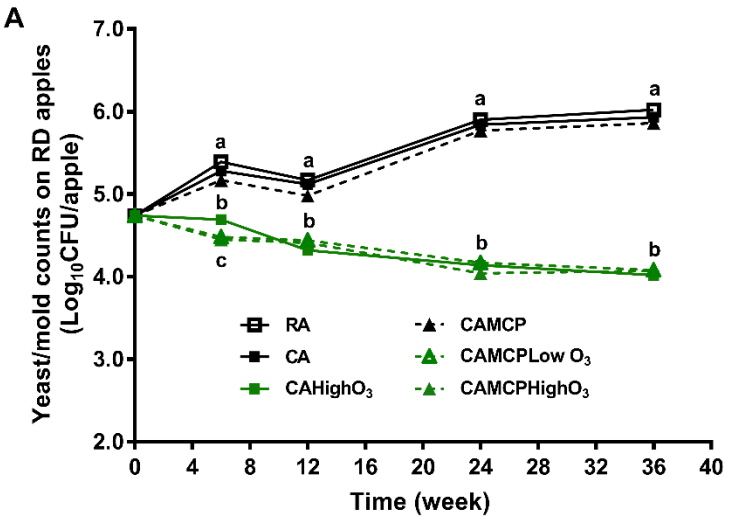


B Change of total plate counts during storage (log_{10}CFU/apples)

		CA		CA+1-MCP		
Week	RA	0	High O_3	0	Low O_3	High O_3
0	0.00	0.00	0.00	0.00	0.00	0.00
6	0.21	0.00	0.02	0.14	-0.11	-0.17
12	0.98	0.87	-0.02	0.85	-0.01	-0.02
24	0.76	0.61	-0.95	0.71	-1.05	-1.03
36	0.72	0.65	-1.20	0.67	-1.29	-1.27

Negative values indicate a reduction of microbial counts.

Figure 2. Apple resident bacteria on Red Delicious apples during 36-week of commercial cold storage. A. Total plate count on apples during storage; Mean ± SEM, n = 40. [a-b] Mean at each sampling point without common letter differ significantly ($P < 0.05$). B. Alteration of resident bacteria on apple surfaces compared to counts before storage. RA: refrigerated atmosphere; CA: controlled atmosphere; MCP: apples were treated with 1-methycyclopropene prior to cold storage; CAHighO3: CA storage with continuous gaseous O_3 application at 78.7 ± 13.2 ppb; CAMCPHighO3: CA storage with continuous gaseous O_3 application at 78.7 ± 13.2 ppb, where apples were treated with MCP-1 prior to different storages;CAMCPLowO3: CA storage with continuous gaseous O_3 application at 60.2 ± 5.7 ppb, where apples were treated with 1-MCP prior to cold storage.



B Change of yeast/mold counts during storage (log_{10}CFU/apples)

		CA		CA+1-MCP		
Week	RA	0	High O_3	0	Low O_3	High O_3
0	0.00	0.00	0.00	0.00	0.00	0.00
6	0.65	0.54	-0.05	0.43	-0.26	-0.29
12	0.43	0.38	-0.42	0.24	-0.30	-0.33
24	1.16	1.10	-0.60	1.03	-0.57	-0.70
36	1.28	1.19	-0.72	1.12	-0.66	-0.67

Negative values indicate a reduction of microbial counts.

Figure 3. Apple natural decay microorganisms on Red Delicious apples during 36-week of commercial cold storage. A. Yeast and mold count on apples during storage; Mean ± SEM, n = 40. [a-b] Mean at each sampling point without common letter differ significantly ($P < 0.05$). B. Alteration of yeast and mold counts on apple surfaces compared to counts before storage RA: refrigerated atmosphere; CA: controlled atmosphere; MCP: apples were treated with 1-methycyclopropene prior to cold storage; CAHighO3: CA storage with continuous gaseous O_3 application at 78.7 ± 13.2 ppb; CAMCPHighO3: CA storage with continuous gaseous O_3 application at 78.7 ± 13.2 ppb, where apples were treated with MCP-1 prior to different storages;CAMCPLowO3: CA storage with continuous gaseous O_3 application at 60.2 ± 5.7 ppb, where apples were treated with 1-MCP prior to cold storage.

Table 2. External disorders (%) of Red Delicious apples under different conditions.

	Treatment	Ozone burn	Superficial scald	Lenticel decay	Russet	CO_2 damage
6-month	RA	0^{aA}	14.2 ± 0.1^{aB}	0^{aA}	0^{a}	0^{aA}
	CA	0^{aA}	0^{bA}	0^{aA}	0^{a}	0.5 ± 0.0^{aA}
	CAMCP	0^{aA}	2.0 ± 0.0^{abA}	0^{aA}	1.0 ± 0.0^{a}	1.0 ± 0.0^{aA}
	$CAMCPLowO_3$	0^{aA}	1.0 ± 0.0^{bA}	0^{aA}	0^{a}	0^{aA}
	$CAMCPHighO_3$	0^{aA}	0^{bA}	0^{aA}	1.0 ± 0.0^{a}	1.0 ± 0.0^{aA}
	$CAHighO_3$	0^{aA}	0^{bA}	0^{aA}	4.8 ± 0.0^{a}	0^{aA}
9-month	RA	0^{aA}	38.0 ± 0.1^{aB}	0^{aA}	0^{a}	0^{aA}
	CA	0^{aA}	7.5 ± 0.0^{bA}	3.0 ± 0.0^{aA}	2.0 ± 0.0^{a}	0^{aA}
	CAMCP	0^{aA}	1.0 ± 0.0^{bA}	1.0 ± 0.0^{aA}	2.1 ± 0.0^{a}	0^{aA}
	$CAMCPLowO_3$	0^{aA}	2.0 ± 0.0^{bA}	3.0 ± 0.0^{aA}	4.0 ± 0.0^{a}	1.0 ± 0.0^{aA}
	$CAMCPHighO_3$	0^{aA}	6.6 ± 0.0^{bA}	3.8 ± 0.0^{aA}	2.7 ± 0.0^{a}	0^{aA}
	$CAHighO_3$	0^{aA}	5.5 ± 0.0^{bA}	4.0 ± 0.0^{aA}	1.8 ± 0.0^{a}	0^{aA}

All disorder measurements at harvest were 0%. $^{a-b}$ Mean within a column without common letter differ significantly ($P < 0.05$). $^{A-B}$ Mean within a column, each external quality parameter was compared through at harvest, 6-month, and 9-month storage, and without common letter differ significantly ($P < 0.05$). CA: controlled atmosphere; CAMCPHighO3/ CAHighO3: CA with 78 ppb O_3 and with or without MCP-1 pre-treatment. $CAMCPLowO_3$: CA with 60 ppb O_3 and MCP-1 treatment.

Both ozone gas application at 60-80ppb did not cause ozone burn in Red Delicious apples at both 6-month and 9-month storage (Table 2). Neither of ozone application had effects on superficial scale, lenticel decay, Russet, CO_2 damage compared to CA, which were all significantly better than those at RA storage (Table 2). Ozone applications further improved the visual appearance of apples (Figure 4).

Figure 4. Appearance of Red Delicious apples after 30 weeks cold storage under different storage conditions. RA: refrigerated atmosphere; CA: controlled atmosphere; 1-MCP: 1-methycyclopropene; $CAHighO_3$: CA storage with continuous gaseous O_3 application at 78.7 ± 13.2 ppb; $CAMCPHighO_3$/$CAHighO_3$: CA storage with 78.7 ± 13.2 ppb O_3 and with or without MCP-1 pretreatment: $CAMCPLowO_3$: CA storage with application at 60.2 ± 5.7 ppb O_3 and MCP-1 pretreatment.

CONCLUSION

Continuous low doses of ozone gas application delayed decay microbial growth and provided additional antimicrobial efficacy against *Listeria* on fresh GSA and Red Delicious apple surfaces over 9-month of CA storage and had no negative influence on the apple fruit quality. Ozone 60-80 ppb showed similar antimicrobial efficacies against *Listeria* as well as resident microbiota; however, its antimicrobial effectiveness was variety dependent.

CONTINUING PROJECT REPORT **YEAR**: No-Cost Extension

WTFRC Project Number: AP-17-102

Project Title: Control of *Listeria* on processing surfaces in apple packing facilities

PI: Meijun Zhu
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Address: 2403 S. 18th St., Suite 100
City/State/Zip: Yakima, WA 98903

Cooperators: Stemilt Growers LLC.; McDougall & Sons; Hansen Fruit; Washington Fruit; Allan Bros Fruit; Josh Tucker, Pace International; Guardian Manufacturing, Inc.

Budget: **Year 1**: 98,447 **Year 2**: 101,752 **Year 3:** 105,882

Year 3: No request

Other funding sources None

WTFRC collaborative expenses:

Item	2017	2018	2019	2020
Salaries	1,573	2,172	2,172	
Benefits	1,049	1,305	1,305	
Wages	2,750	2,750	2,750	
Benefits	825	825	825	
Total	6,197	7,052	7,052	0

Footnotes:

Dr. Hanrahan is committing to spend 2%/year of her time on this project. Mendoza will supervise Wenatchee based team members (0.5% of her time in year 1, 2% in years 2&3). Timeslip wages are calculated at $11/hr. plus benefits for 250 hours/year.

Budget 1: Meijun Zhu

Organization Name: WSU-Pullman **Contract Administrator:** Katy Roberts
Telephone: (509) 335-2885 **Email address:** arcgrants@wsu.edu

Item	2017	2018	2019	2020
Salaries[1]	$13,562	$19,889	$20,685	
Benefits[1]	$4,386	$6,094	$6,338	
Wages[2]	$38,054	$30,773	$32,003	
Benefits[2]	$3,248	$3,300	$3,432	
Supplies[3]	$26,000	$26,644	$27,872	
Travel[4]	$2,000	$3,000	$3,500	
Miscellaneous[5]	$5,000	$5,000	$5,000	
Total	$92,250	$94,700	$98,830	0

Footnotes:

[1]Postdoc research associate and professor's salaries plus benefits.

[2]PhD graduate student stipends and undergraduate assistant wages plus benefits.

[3]Bacteria culture media, reagents and consumable supply cost

[4]Travel funds for industrial sampling and experiments.

[5]Funds are requested to partially cover the Biosafety Level 2 facility and equipment maintaining fees.

OBJECTIVES

4. Assess antimicrobial efficacies of different commonly used chemical sanitizers against *L. monocytogenes* biofilm on the main food-contact surfaces.
5. Examine antimicrobial efficacies of steam against *Listeria* biofilm on different food-contact surfaces.
6. Evaluate antimicrobial efficacies of steam and selected sanitizers against biofilm on common food-contact surface using optimized parameters.

SIGNIFICANT FINDINGS

6. Efficacies of all tested sanitizers against aged (7-day-old) *Listeria* biofilm were reduced when compared 2-day-old biofilm.
7. In general, efficacies against *L. monocytogenes* biofilms on major food-contact surfaces including stainless steel (SS), low-density polyethylene (LDPE), polyvinyl chloride (PVC), polyester (PET) and rubber were enhanced by increasing concentrations of quaternary ammonium compound (QAC), chlorine, and chlorine dioxide, or extending treatment time from 1 min to 5 min.
8. A 5 min treatment of 400 ppm QAC, 5.0 ppm chlorine dioxide, or 200 ppm chlorine reduced 3.0-3.7, 2.4-2.7, and 2.6-3.8 $\log_{10}$ CFU/coupon *L. monocytogenes* biofilms depending on surfaces.
9. Peroxyacetic acid (PAA) at 160 - 200 ppm and 1-5 min contact showed similar antimicrobial efficacies against *L. monocytogenes* biofilms on all tested food-contact surfaces, causing in 4.0-4.6 $\log_{10}$ CFU/coupon reduction of *L. monocytogenes* biofilms on tested surfaces.
10. Food-contact surfaces had more impact on the efficacies of QAC and chlorine, less influence on those of PAA and chlorine dioxide.
11. Saturated steam caused a rapid kill of *L. innocua* biofilms on food contact surfaces. A 6-sec steam treatment attained a 2.4 - 3.2 $\log_{10}$ CFU/coupon (1.5 × 1.5 cm^2) reduction depending on the type of surface.
12. Effectiveness of steam in eliminating *L. innocua* biofilms decreased dramatically during prolonged steam treatment.
13. Bactericidal activity of steam against *Listeria* biofilms was most effective for stainless steel surface, while least effective for rubber surfaces.
14. Organic matter soiling, regardless of sources, impaired sanitizer efficacies against *L. monocytogenes* biofilms independent of food-contact surfaces but did not negatively impacted efficacy of steam against *Listeria* biofilm on different surface.
15. Steam exposure had no impact on the hydrophobicity and surface roughness of SS, PET and rubber surfaces.

METHODS

We have established methods for proposed studies as detailed in the following.

Objective 1: Assess antimicrobial efficacies commonly used chemical sanitizers against *L. monocytogenes* biofilm on the main food-contact surfaces.

1. *Strain selection*

To elucidate the impact of strain variability on biofilm formation and sanitizer's antimicrobial efficacy, six strains of *L. monocytogenes* were evaluated. These *L. monocytogenes* strains were either outbreak strains or processing plant/food isolates. They have been stored at -80°C until used.

2. *Selection and preparation of food-contact surfaces*

Surface: SS, PVC, PET, LDPE and rubber along with polyester were selected.

Organic matter conditioning: The above surfaces were cleaned and exposed with diluted apple juice before being subjected to *Listeria* biofilm growth and sanitizer treatments.

3. *Listeria biofilm formation on different surface materials*

Inoculum preparation: Before inoculation, respective strains were twice activated in Tryptic Soy broth (TSB) with yeast extract (TSBYE)*, washed,* and re-suspended in nutrient broth to achieve the target population density.

Biofilm formation on different surfaces: All surface coupons (conditioned with/without organic matter) were transferred to 6- strain *Listeria* suspension in culture media prepared as described above and incubated at room temperature (22°C/72°F) for 2 or 7 days statically to form biofilm.

4. *Sanitizer* intervention against *Listeria* biofilm on different surfaces.

Wells of polystyrene plates or coupons of the selected surface bearing *Listeria* biofilm cells were rinsed with sterile distilled water, then subjected to respective sanitizer treatments (2.0/4.0 ppm ozonated water, 200/400 ppm quaternary ammonium compound (QAC), 100/200ppm chlorine, 2.0/5.0 ppm chlorine dioxide or 160/200ppm peroxyacetic acid (PAA)) at appropriate concentrations for 1- or 5-min. Untreated control wells with biofilm were subjected to distilled water instead of sanitizer solution treatments.

5. *Microbiological* analysis.

The biofilm on respective surfaces was detached from surface per our established method. The detached cell suspensions were serially diluted in sterile PBS and plated in duplicate Tryptic Soy Agar (TSA) with yeast extract (TSAYE) agar plates. Colonies that had formed on the plates were counted after 48 h of incubation at 37°C (98°F).

Objective 2: **Examine antimicrobial efficacies of steam against *L. monocytogenes* biofilm on different food-contact surfaces**

1. *Strain selection*

Three *L. innocua* isolates from produce packing facility/ processing plants were used to prepare 3-strain cocktail *Listeria* inoculum per our well-established method.

2. *Food-contact surface selection and conditioning*

The surface selection and condition were the same as the objective 1 studies.

3. *Biofilm formation*

Biofilm formation on different food-contact surfaces were conducted as described in the Objective 1 studies. To mimic harsh conditions in the apple packing facilities, the aged (7-day-old) multi-strain *Listeria* biofilm were used in the Objective 2 studies.

4. *Steam* generator and temperature monitoring

The steam generator was located at Washington State University pilot plant due to power requirements. A stainless-steel chamber with three steam pipes and 25 steam nozzles was used to treat *L. innocua* biofilms formed on different food-contact surfaces. The temperature profile of food-contact coupons inside the steam brancher was monitored using a T-type self-adhesive thermocouple (OMEGA, Norwalk, USA). Three-wire thermocouples were used to monitor the temperature profiles of steam at three different sites of the chamber (Fig. 1AB).

5. *Steam intervention against biofilms*

The 7-day-old *L. innocua* biofilms on food-contact surfaces were treated with steam for 0-180 seconds. The treated surface coupons were immediately transferred to 50 ml Falcon tubes containing 2 ml sterile PBS immediately after treatments.

6. *Microbiological analysis.*

Detachment and enumeration of *Listeria* biofilm were conducted as described in Objective 1.

Objective 3: Evaluate antimicrobial efficacies of steam and selected sanitizers against biofilm on common food-contact surface using optimized parameters.

Methods developed in Objective 1 and 2 studies are used for Objective 3 studies. The outcomes of Objective 1 & 2 studies guide standardization of sanitizer concentrations in relation to the variable residence time.

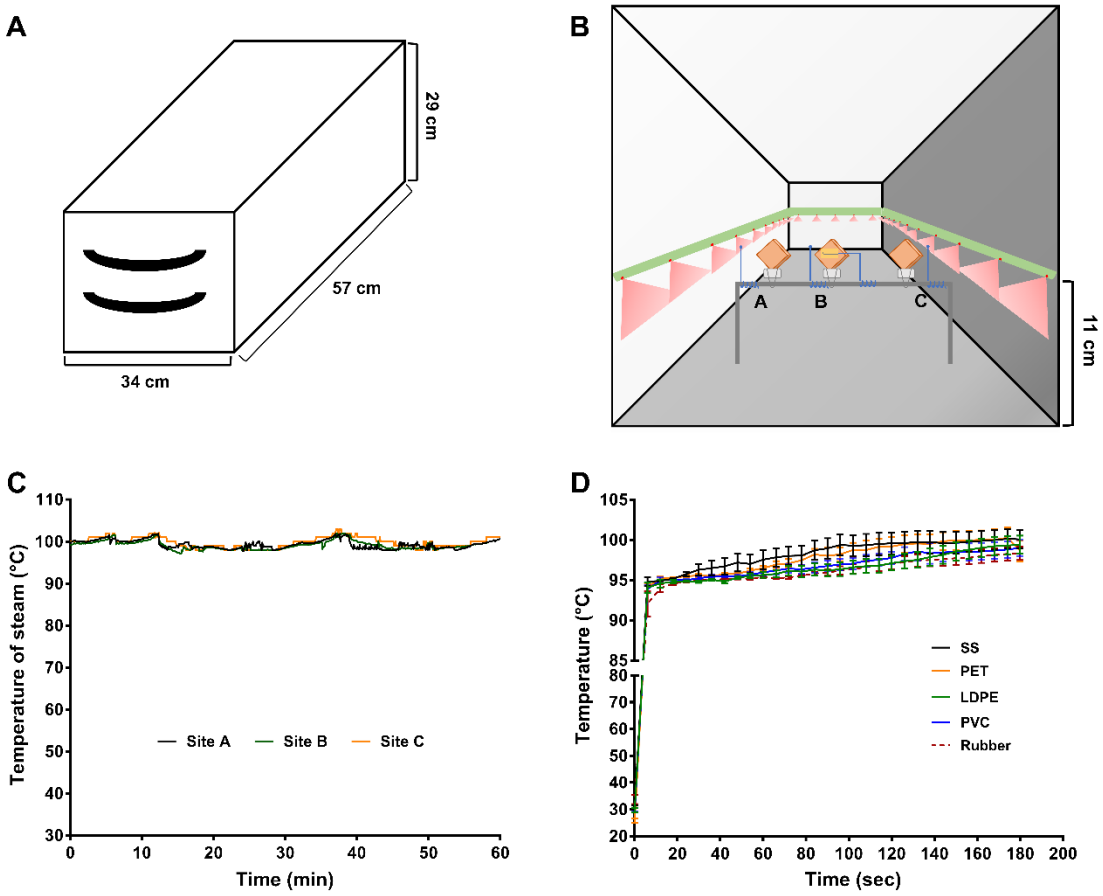


Fig. 1 Steam blancher apparatus and temperature profiles. A. The dimension of stainless-steel chamber. B. Interior view of the steam blancher. Green tubes: steam pipelines; red dots: steam nozzles, 25 in total. C. Typical temperature profile of saturated steam at site A, site B and site C during 60 min duration. D. Typical temperature profile of different surface coupons (1.5 x 1.5 cm) during the 180-sec treatment. SS: stainless steel, LDPE: low-density polyethylene, PET: polyester, PVC: polyvinyl chloride. Surface temperature of each surface was averaged from six independent measurements.

RESULTS AND DISCUSSION

The antimicrobial efficacies of sanitizers against *L. monocytogenes* biofilms were dramatically impacted by biofilm stage, strains present and cleaniess of surfaces (Korany et al., 2018). The antimicrobial efficacies of the tested sanitizers against *L. monocytogenes* biofilms on common food-contact surfaces were enhanced by increasing concentrations of QAC, chlorine, chlorine dioxide, or extending treating time from 1 min to 5 min. The 5 min treatments of 400 ppm QAC, 5.0 ppm chlorine dioxide, or 200 ppm chlorine reduced 3.0-3.7, 2.4-2.7, and 2.6-3.8 $\log_{10}$ CFU/coupon *L. monocytogenes* biofilms depending on surfaces. PAA is the most effective sanitizer among those tested; a 5-min treatment of 160/200 ppm PAA caused 4.0-4.6 $\log_{10}$ CFU/coupon reduction of *L. monocytogenes* biofilms on tested surfaces. Surface material had more impact on the efficacies of QAC and chlorine, less influence on those of PAA and chlorine dioxide, while organic matter soiling impaired anti-*Listeria* efficacies of test sanitizers independent of surface (Hua et al., 2019). We further evaluated and

compared antimicrobial efficacy of steam against *L. innocua* biofilm on the selected food-contact surfaces using a pilot-scale steam intervention system; investigated the impacts of diluted apple juice on steam disinfection efficacy against *L. innocua* biofilms on surfaces; and examined the potential links between surface properties and steam inactivation efficacies.

1. Steam inactivation of *L. innocua* biofilms on different food-contact surfaces

Steam temperature was maintained at 100 °C with a minor fluctuation (Fig. 1C). The temperature of the treated surface coupons rapidly reached 92 °C/197.6 °F within 6 sec (Fig. 1D). The mean surface temperatures of treated surface coupons ranged from 96-98 °C/205-208 °F (Fig. 1D). Steam had a quick bactericidal effect against 7-day-old *L. innocua* biofilms on all surfaces. A 6-sec exposure of steam provided a 3.2, 2.6, 2.4, 2.5 and 2.6 $\log_{10}$ CFU/coupon reductions of *L. innocua* biofilm on SS, PET, LDPE, PVC and rubber surface coupons, respectively (Fig. 2). Fig 2C showed a representative image of Live/Dead staining of *L. innocua* cells in 7-day-old biofilms on SS surface before and after a 6-sec steam treatment, which further showed a rapid bactericidal effect of steam. The inactivation rate of steam against *L. innocua* biofilm on all surfaces declined with increasing treatment time, especially on rubber surfaces. Among all surfaces treated, steam pasteurization was most effective against *L. innocua* biofilm on SS, followed by PET. A 30-, 60-, 120- and 180- sec steam treatment resulted in 4.0, 4.6, 5.7 and 6.4 $\log_{10}$ CFU/coupon reductions on SS, and 3.1, 3.3, 4.6 and 4.8 $\log_{10}$ CFU/coupon reductions on PET surface coupons, respectively (Fig. 2). To understand the declined killing rate of steam against aged biofilm, we further evaluated the inactivation of steam against 1-day or 2-day *L. innocua* cells attached on SS and rubber surface coupons. As seen in 7-day-old biofilms, a rapid *L. innocua* reduction was achieved within a 6-sec of steam treatment (Fig. 3). A 6-sec steam pasteurization at 100 °C resulted in a 5.5- or 5.1- $\log_{10}$ CFU/coupon reduction of 1-day or 2-day *L. innocua* cells attached on SS (Fig. 3); the steam was less effective against 1-day or 2-day *L. innocua* cells attached on rubber than that on SS surface. However, unlike the 7-day-old biofilm, increasing the contact time gradually enhanced the bactericidal effects of steam against *L. innocua* on the rubber surface (Fig. 3), indicating the role of biofilm architecture in conferring *Listeria* resistance to intervention.

2. Impact of organic matter on efficacy of steam pasteurization against *L. innocua* biofilm

Organic soiling with diluted apples did not decreased steam antimicrobial efficacies against 7-day-old biofilms formed on different surface coupons (Table 1). Like clean surfaces, steam caused a rapid kill of *L. innocua* biofilms on soiled surfaces with a 6-sec of exposure, reducing cell counts by 2.5 - 4.1 $\log_{10}$ CFU/coupon on all surfaces. Increasing the treatment time from 6 sec to 30 sec enhanced inactivation efficacies on SS and PET surfaces only (Table 1).

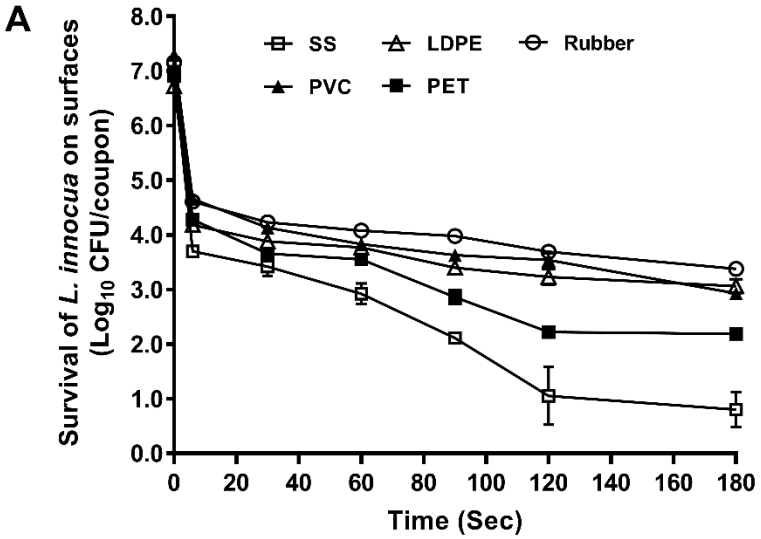
3. Surface properties before and after steam treatments

The hydrophobicity of SS, PET, LDPE or PVC was smaller than the rubber surface. The PET surface had the smallest R_a value, an indicator of the roughness, followed by LDPE, SS and PVC, while rubber had the largest R_a value. Repeated steam exposure had no effects on the hydrophobicity and roughness of SS, PET and rubber surfaces, but negatively impacted PVC and LDPE surfaces.

CONCLUSIONS

Steam exhibited a fast killing kinetic against *L. innocua* biofilm on different food-contact surfaces; a 6-sec steam treatment attained a 2.4 - 3.2 $\log_{10}$ CFU/coupon reduction depending on type of surface materials. However, the killing rate of steam decreased dramatically during subsequently steam treatment and exhibited a tailing effect which was more pronounced on rubbers, PVC, and LDPE surfaces. Organic matter soils did not compromise bactericidal effects of steam against *L. innocua* biofilm on tested surfaces. Our data suggested that a short duration of steam exposure alone or in combination with chemical disinfection is a promising sanitization strategy in removing *Listeria* biofilm or other foodborne pathogens on food contact surfaces, especially for SS, PET and rubber surfaces, which warrants further studies.

A



B

The reduction of 7-day-old *L. innocua* biofilm

Time (sec)	SS	PET	LDPE	PVC	Rubber
6	3.15 ± 0.09^{aB}	2.55 ± 0.05^{aAB}	2.41 ± 0.13^{aA}	2.48 ± 0.08^{aA}	2.59 ± 0.17^{aAB}
30	3.99 ± 0.50^{bB}	3.05 ± 0.21^{abA}	2.85 ± 0.12^{abA}	2.74 ± 0.22^{abA}	2.61 ± 0.20^{aA}
60	4.63 ± 0.32^{cC}	3.33 ± 0.04^{bB}	3.06 ± 0.13^{bAB}	3.26 ± 0.34^{bcAB}	2.68 ± 0.20^{abA}
90	5.05 ± 0.11^{cD}	4.15 ± 0.35^{cC}	3.34 ± 0.09^{bcAB}	3.63 ± 0.13^{cBC}	2.83 ± 0.24^{abA}
120	5.71 ± 0.19^{dD}	4.55 ± 0.29^{cdC}	3.87 ± 0.19^{cdB}	3.82 ± 0.11^{cB}	3.00 ± 0.24^{abA}
180	6.44 ± 0.23^{eC}	4.79 ± 0.15^{dB}	4.23 ± 0.40^{dB}	4.53 ± 0.11^{dB}	3.26 ± 0.27^{bA}

The initial *L. innocua* level was 6.8-7.3 $\log_{10}$ CFU/coupons. $^{a\text{-}e}$Mean within a column without common letter differ significantly ($P < 0.05$). $^{A\text{-}D}$Mean within a row without common letter differ significantly ($P < 0.05$).

C

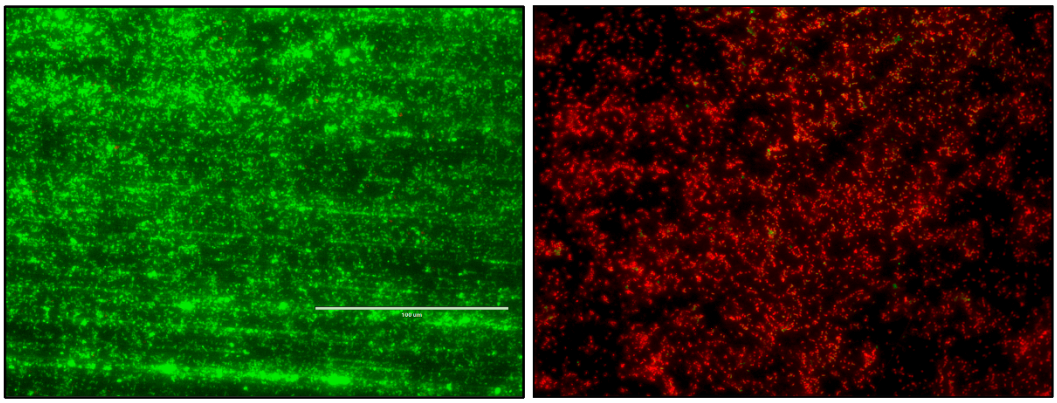
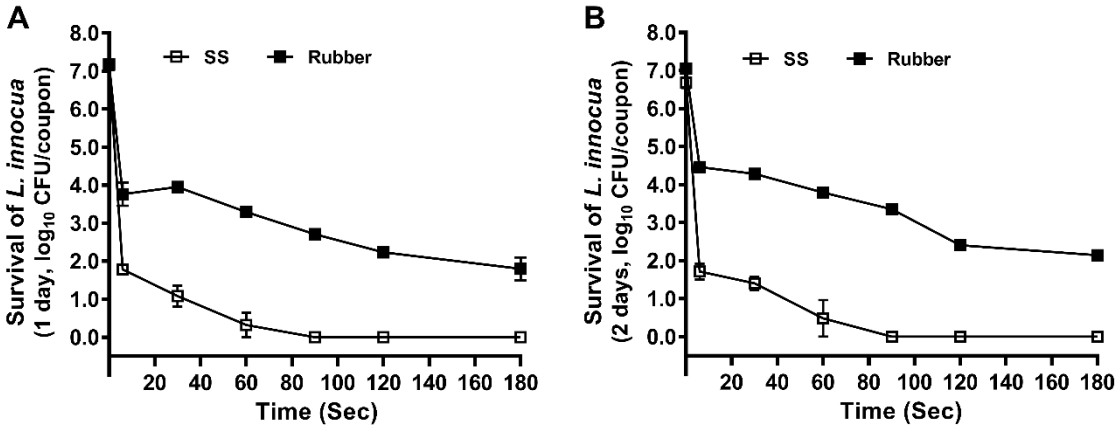


Fig. 2 Steam efficacy against *L. innocua* cells in biofilm on food-contact surfaces. The 7-day-old *L. innocua* biofilm on different food-contact coupons was subjected to 100 °C steam for 0-180 sec and surviving bacteria was analyzed. A. Representative survival of *L. innocua* biofilm on different food-contact surface coupons (1.5 cm × 1.5 cm). B. Log-reduction of *L. innocua* biofilm on each food-contact surface. C. Live/Dead staining of *L. innocua* cells in 7-day-old biofilm on SS surface. Left; *L. innocua* cells before steam treatment; right: *L. innocua* cells after a 6-sec steam treatment; Green: live cells; Red: dead cells; bar: 100 µm. SS: stainless steel, PET: polyester (polyethylene terephthalate), LDPE: low-density polyethylene, PVC: polyvinyl chloride. Mean ± SEM were averaged from three independent studies where three replicates were used for per time point and surface coupons within each independent study.



C

The reduction of *L. innocua* on surfaces

Time (sec)	1 day		2 days	
	Stainless steel	Rubber	Stainless steel	Rubber
6	5.47 ± 0.13^{aB}	2.65 ± 0.39^{aA}	5.09 ± 0.15^{aB}	2.56 ± 0.17^{aA}
30	6.10 ± 0.15^{abB}	2.91 ± 0.18^{abA}	5.64 ± 0.36^{aB}	2.46 ± 0.26^{aA}
60	6.69 ± 0.17^{bcB}	3.45 ± 0.24^{bA}	6.49 ± 0.15^{bB}	3.03 ± 0.15^{abA}
90	$>6.68^{cC}$	4.28 ± 0.31^{cB}	$>6.60^{bC}$	3.37 ± 0.43^{bA}
120	$>6.68^{cB}$	4.73 ± 0.12^{cA}	$>6.60^{bB}$	4.36 ± 0.19^{cA}
180	$>6.68^{cB}$	5.73 ± 0.45^{dA}	$>6.60^{bB}$	5.55 ± 0.48^{dA}

Mean ± SEM were averaged from three independent studies where three replicates were used for per treatment. $^{a-d}$ Mean within a column without common letter differ significantly ($P < 0.05$). $^{A-C}$ Mean within a row without common letter differ significantly ($P < 0.05$).

Fig. 3 Steam efficacy against cells in *L. innocua* on food-contact surfaces. The 1-day/2-day attachment/biofilm on stainless steel and rubber coupons were subjected to 100°C steam for 0-180 sec and surviving bacteria was analyzed. A. Representative survival of *L. innocua* post-1 day or 2 days attachment on stainless steel (SS) and rubber coupons (1.5 cm × 1.5 cm). B. Log reduction of *L. innocua* attachment/biofilm on SS and rubber. Initial inoculation level was ~7.0 log CFU/coupon. The detection limit was 0.3 $\log_{10}$ CFU/coupon.

Table 1 Efficacy of steam against *L. innocua* biofilms on apple juice soiled food-contact surfaces

Surface	Steam (sec)	Log reduction (CFU/coupon)	
		Clean	Soiled
SS	6	3.2 ± 0.1^{aA}	4.1 ± 0.1^{aB}
	30	3.8 ± 0.2^{bA}	4.4 ± 0.1^{aB}
PET	6	2.5 ± 0.1^{aA}	2.8 ± 0.1^{aA}
	30	2.8 ± 0.1^{aA}	3.5 ± 0.1^{bB}
LDPE	6	2.4 ± 0.1^{aA}	2.9 ± 0.2^{aA}
	30	2.9 ± 0.1^{bA}	3.0 ± 0.1^{aA}
PVC	6	2.5 ± 0.1^{aA}	2.8 ± 0.1^{aA}
	30	2.7 ± 0.1^{aA}	2.8 ± 0.1^{aA}
Rubber	6	2.6 ± 0.1^{aA}	2.5 ± 0.1^{aA}
	30	2.6 ± 0.1^{aA}	2.6 ± 0.1^{aA}

The 7-day-old *L. innocua* biofilms formed on clean or 1:10 diluted apple juice soiled food-contact surfaces were treated with 100°C steam for 6 sec or 30 sec, respectively, and numbers of survivors was analyzed. SS: stainless steel, PVC: polyvinyl chloride, LDPE: low density polyethylene, PET: polyester. $^{a-b}$ Means within a column with no common letter differ significantly ($P < 0.05$) for the same surface coupons under different steam exposure. $^{A-B}$ Means within a row with no common letter differ significantly ($P < 0.05$).

FINAL PROJECT REPORT

Project Title: How does fruit acclimation to sunburn affect sunburn management?

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Cooperators: Brenda Castaneda, WSU TFREC; Hector Camargo-Alvarez, WSU TFREC; Cameron Burt, WSU TFREC; Alexander Haase, WSU TFREC.

Total Project Request: **Year 1:** 86,621 **Year 2:** 87,846

Other funding sources

None

Budget: Kalcsits, Waliullah, Waite
Organization Name: WSU Contract Administrator: Katy Roberts/Kim Rains
Telephone: 509-335-2885/509-293-8803
Email: arcgrants@wsu.edu/kim.rains@wsu.edu

Item	2018	2019
Salaries[1]	49,920	51,917
Benefits[2]	18,201	18,929
Travel[3]	1,500	1,500
Goods and Services[4]	17,000	15,500
Total	86,621	87,846

Footnotes:
[1,2] Salaries and 36.5% benefits for Post-Doctoral Research Associate (Dr. Sumyya Waliullah, and Dr. Jessica Waite)
[3]For frequent travel to orchard site (Quincy) where trials are being conducted
[4]Goods and services include irrigation supplies, fruit respiration chamber, basic physiological and molecular lab supplies including molecular biological enzymes and chemicals for gene expression analysis and pigment analysis, liquid nitrogen tank rental and lab consumables.

OBJECTIVES

1. **Identify how acclimation to high light and near sunburn threshold temperatures influences fruit susceptibility to sunburn**

Two postdoctoral researchers worked on this project. In 2018, Dr. Sumyya Waliullah left for a new position at the University of Georgia after completing the summer experiments. Since then, Dr. Jessica Waite has joined the Kalcsits lab and took over responsibilities for this project under the guidance of Lee Kalcsits. Jessica has since taken a position at the USDA-ARS Tree Fruit labs in Wenatchee focused on pear molecular biology and genetics.

2. **Link physiological and biochemical changes in the fruit to sunburn development.**

In 2018 and 2019, we completed a set of experiments that included controlling fruit surface temperature and examining the impact of fruit surface temperatures on susceptibility to future sunburn inducing events. These controlled experiments will form the foundation of future lines of research into understanding the genetic and physiological controls underlying susceptibility to fruit sunburn. These experiments are important for better understanding sunburn risk for current and emerging cultivars and eventually contributing to the development of future cultivars that are less susceptible to sunburn.

3. **Use information provided on temperature and light conditions that stimulate natural resistance to guide evaporative cooling and sunburn protectant applications and reduce losses to sunburn**

Here, we tested the use of automated evaporative cooling triggers when air temperature is either 85 or 90 °F. This experiment was completed in 2020 in collaboration with Cameron Burt.

SIGNIFICANT FINDINGS

1. **Sunburn Management:** Cooling applied when temperatures exceeded 85°F had significantly less sunburn than when cooling began when air temperatures exceeded 90°F

2. **Sunburn Management:** Honeycrisp has elevated fruit surface temperatures compared to Granny Smith, WA 38, and Cripps Pink under the same light and heat conditions. This contributes to a greater sunburn risk compared to the other three cultivars tested.

3. **Understanding Risk:** Fruit are less susceptible to sunburn early in the season due to differences in stomatal conductance (transpiration) and pigmentation.

4. **Understanding Risk:** Exposure to near threshold temperatures in June increased sunburn resistance in July and August for experiments completed in 2018.

5. **Scientific Knowledge:** Anthocyanins were found to increase in response to higher heat treatments in fruit that had received no priming stimulus, and did not respond to heat in fruit that had been previously primed.

6. **Scientific Knowledge:** Candidate genes were selected based on acclimation studied in a variety of plant species. At three days after fruit were heated, no differences were detected between treatments, suggesting either changes in gene expression occur earlier, or these genes are not involved in apple acclimation to sunburn.

RESULTS & DISCUSSION

Honeycrisp are less able to keep fruit cool under high light and heat conditions

Fruit surface temperatures monitored throughout the 2018 growing season indicate that there are cultivar level differences in response to light and air temperature (Figures. 1-3). This was further supported by research comparing Cripps Pink and Honeycrisp in 2020 (Figure 4). Honeycrisp apples maintain greater fruit surface temperatures under similar conditions, and under the most extreme temperature and light conditions can vary by as much as 10°F more than Cripps Pink, Granny Smith, or WA 38. Approximately three-quarters of the variation in fruit surface temperature can be explained by two variables: air temperature and light intensity. Wind speed and unknown physiological factors contribute to the other 25% of variation. For experiments conducted to determine whether fruit can physiologically acclimate to elevated fruit surface temperatures, fruit that was exposed to near threshold fruit surface temperatures in June when temperatures were relatively cool showed reduced sunburn compared to fruit that was exposed to normal conditions (Figure 5). When the temperature rapidly increased from highs in the mid-70s to about 100 °F in early July, sunburn incidence and severity was greater for unexposed fruit (Figure 5). This was true for both Honeycrisp and Granny Smith; although overall incidence was lower for Granny Smith than Honeycrisp, which further supports the observations that Honeycrisp, with elevated fruit surface temperatures, is more susceptible to sunburn. Fig. 6 shows two images of sunburn development in untreated fruit but no sunburn development in adjacent fruit that was exposed to near threshold temperatures. These conditions did not exist in 2019 and temperatures gradually increased rather than suddenly increased like in 2018. Sunburn pressure was relatively low in 2019 and a small number of days in August were conducive to the development of fruit sunburn.

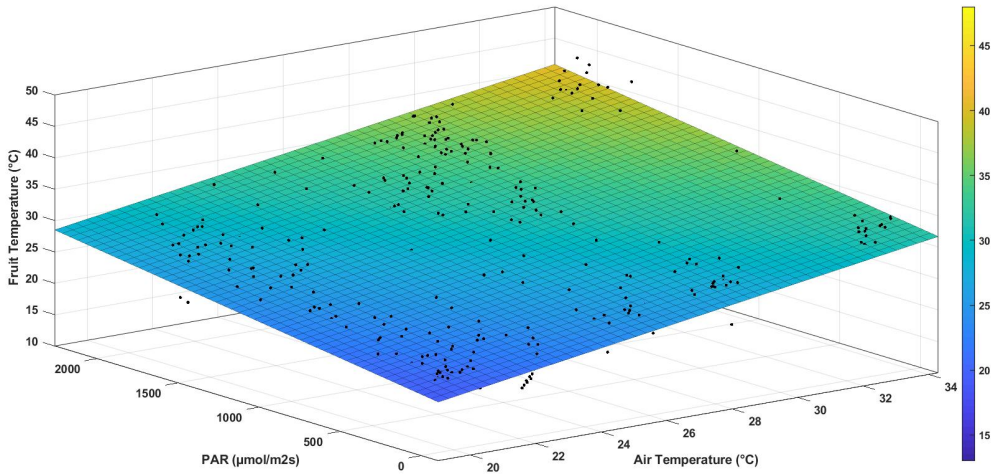

Figure 1. Fruit surface temperature of WA 38 fruit as a function of light intensity at the fruit surface and air temperature. Each point represents one fruit measured from June 1 through to August 24, 2018. The surface represents the best fit model using air temperature and light intensity to explain fruit surface temperature

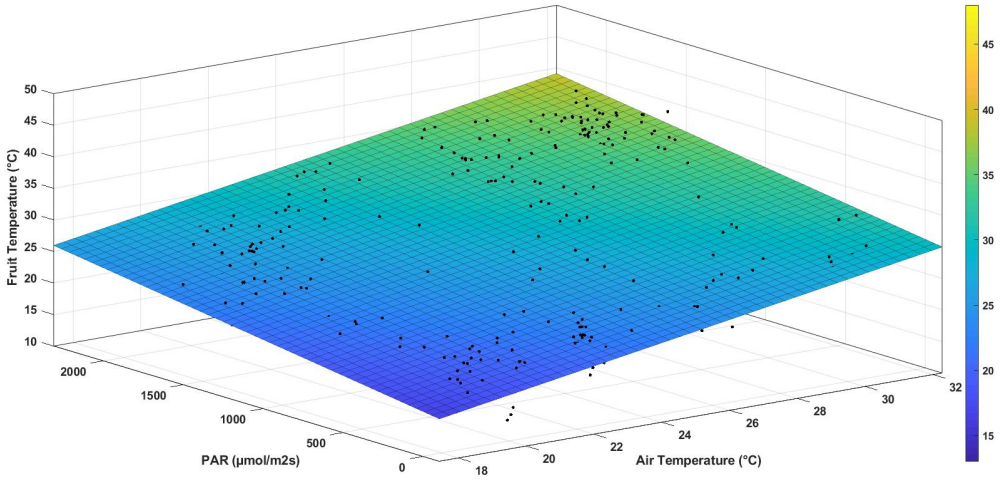

Figure 2. Fruit surface temperature of Granny Smith fruit as a function of light intensity at the fruit surface and air temperature. Each point represents one fruit measured from June 1 through to August 24, 2018. The surface represents the best fit model using air temperature and light intensity to explain fruit surface temperature

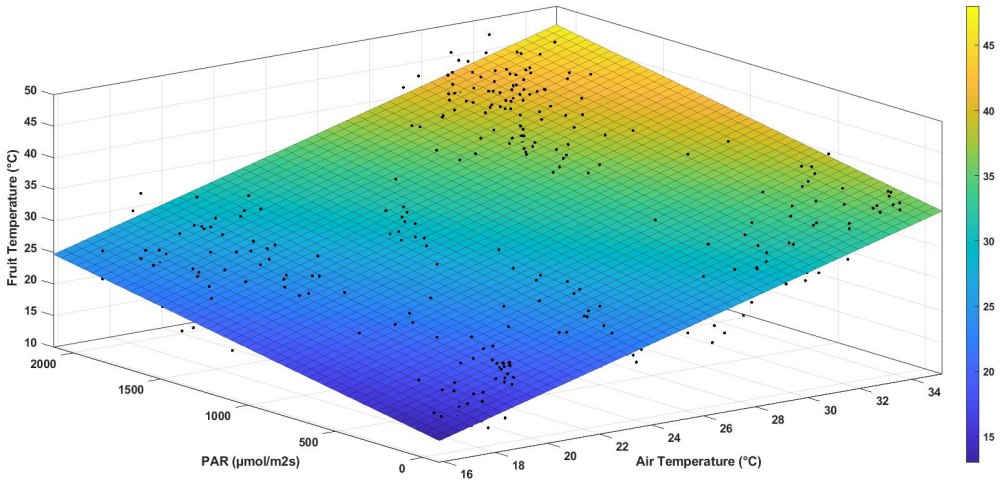

Figure 3. Fruit surface temperature of Honeycrisp fruit as a function of light intensity at the fruit surface and air temperature. Each point represents one fruit measured from June 1 through to August 24, 2018. The surface represents the best fit model using air temperature and light intensity to explain fruit surface temperature

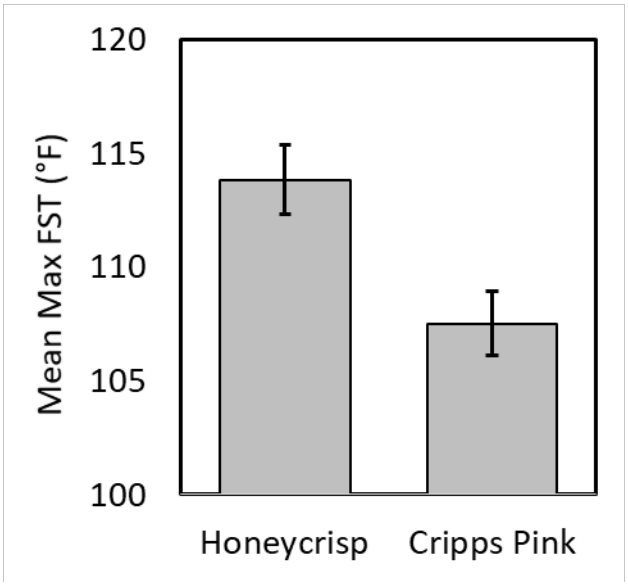

Figure 4. Mean daily maximum fruit surface temperature (FST; °F) for Honeycrisp and Cripps Pink during the period of July 3- August 10, 2020.

Evaporative cooling for Honeycrisp is best initiated at 85 °F

In 2020, solar-powered, temperature-activated solenoids were set up for a replicated experiment at the WSU Sunrise Research Orchard in Wenatchee. The orchard used was a Honeycrisp orchard that was top-worked in 2016 from Granny Smith on M9-T337. There were three replicates of each treatment; evaporative cooling activated when air temperatures reached either 85°F or 90°F compared to an uncooled control. It was set to cycle between on and off for 15 minutes on and 45 minutes off. Cooling sprinklers were standard Nelson R10's with an output rate of 45 gallons per acre per minute. Over the entire period of the middle of June to harvest, there were 402 hours when temperatures exceeded 85°F (Figure 5). That equals approximately 0.88 acre feet of water. That represents approximately 20% of the yearly irrigation needs just applied through evaporative cooling. The amount reaching the soil is limited because of cycling but the added irrigation is substantial and needs to be accounted for when making irrigation decisions. When evaporative cooling was activated when air temperatures reached 90°F, the amount of water applied was approximately 60% that of the 85°F activation temperatures. Early July had some of the highest cooling requirements but cooling was required at least one day per week for June through August. With small amounts of elevated temperatures required for sunburn damage on fruit, conservative systems are most frequently adopted. Fruit was harvested August 31, 2020. 100 pounds of fruit was picked at random from each replication. Each fruit was individually assessed for sunburn severity and incidence. Further quality metrics and bitter pit development will be assessed in January 2021. In these experiments, trees that were cooled starting at 85 had less overall sunburn and lower severity than fruit from trees where cooling started at 90 F or were uncooled overall. Cooling started at 90 F reduced the amount of severe sunburn compared to the uncovered control. While these results suggest that EC should be started at 85, other cultivars may be less susceptible compared to Honeycrisp similar to what we observed in elevated fruit surface temperatures compared to other cultivars.

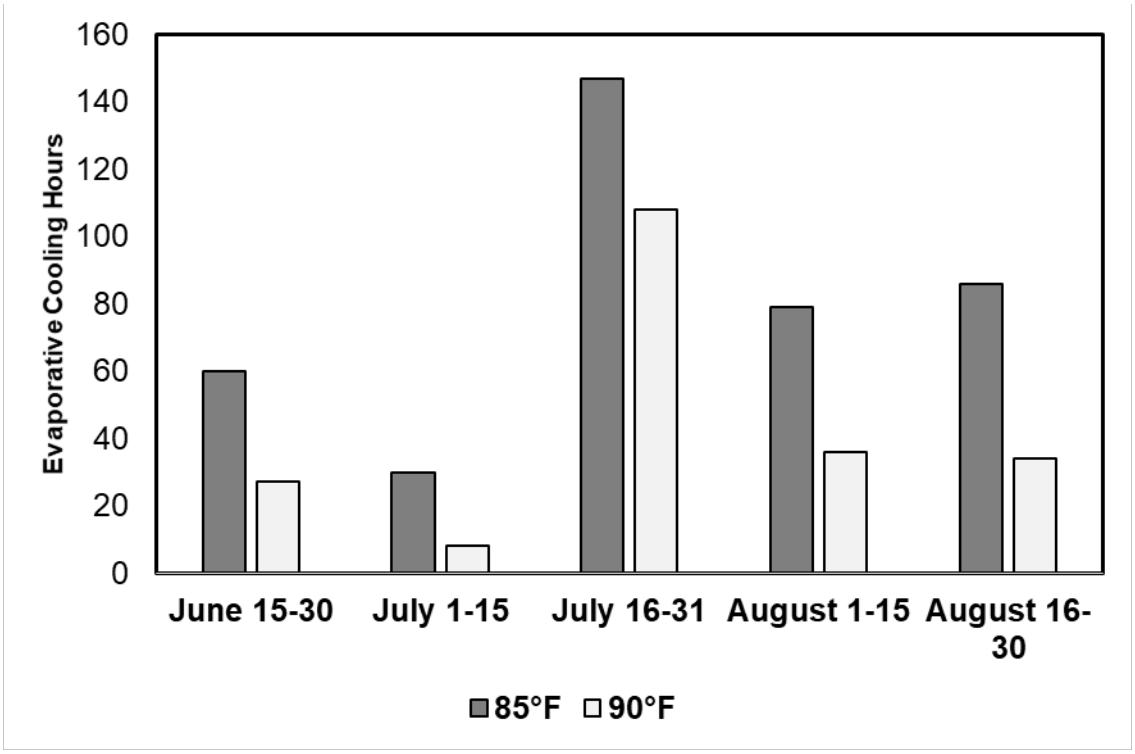

Figure 5. Total evaporative cooling hours for late June, July, and August of 2020 when cooling was initiated at air temperatures of either 85°F or 90°F

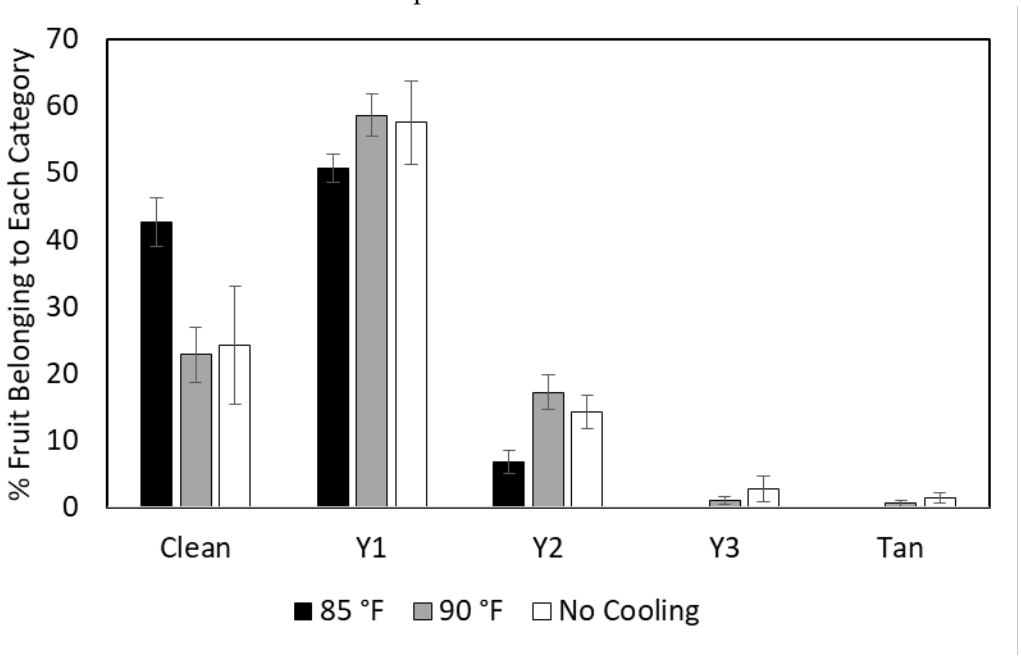

Figure 6. The proportion of fruit with either no sunburn (Clean) or belonging to three classes of sunburn browning (Y1-Y3) or showing leathery sunburn tanning of the fruit peel harvested from trees that were either cooled when temperatures exceeded 85 °F or 90 °F compared to an uncooled control.

Fruit can acclimate to high temperatures to resist sunburn development

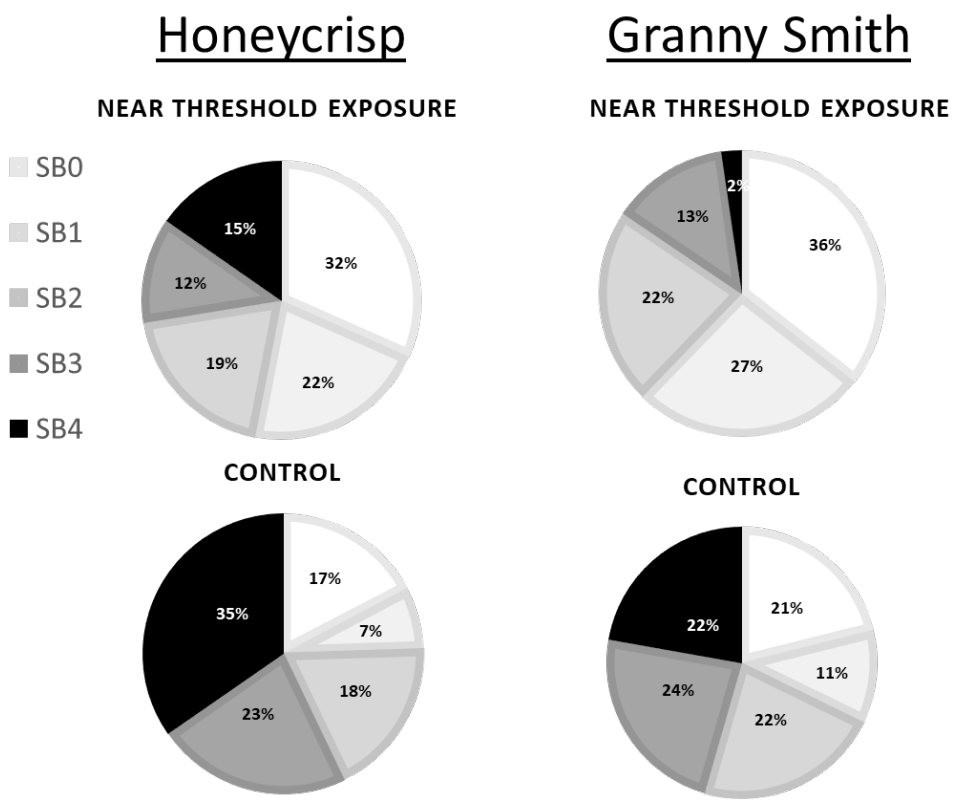

Figure 7 Fruit sunburn severity of Honeycrisp or Granny Smith apples (N=90) after being exposed to sunburn inducing temperatures in July that were either exposed to near sunburn threshold temperatures in June or only exposed to cooler ambient conditions that were present in June, 2018. The sunburn scale used was a 5-point scale where SB0 is where there is no sunburn present and SB5 is where there is browning formed on the fruit surface.

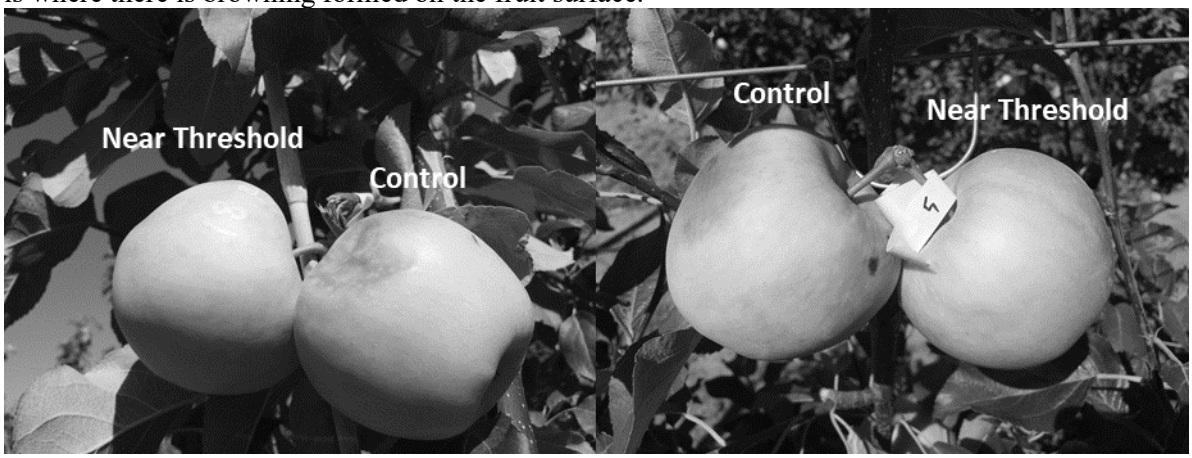

Figure 8. Fruit sunburn development after sudden increased in temperature during early July 2018. Adjacent fruit was either left untreated or exposed to elevated, near sunburn threshold fruit surface temperatures for one hour in June 2018. Fruit that was part of the untreated control suffered more severe sunburn than fruit that was exposed to near-threshold temperatures.

From measurements of stomatal function during fruit development, we observed that transpiration rapidly decreased in fruit during the month of June and slowly began to decrease as fruit continued to grow and mature. While changes in pigmentation during sunburn have been well documented, the changes in pigmentation that provide further protection for fruit have been less documented. Figure 7 shows changes in reflectance during fruit development where reflectance increases as fruit matures meaning that it absorbs less energy. Specifically, more green and red light is reflected. There were also differences between interior or exterior fruit where exposed fruit reflects more red light and non-exposed fruit reflects more green light. However, this will be confirmed based on a larger dataset that is being processed in December.

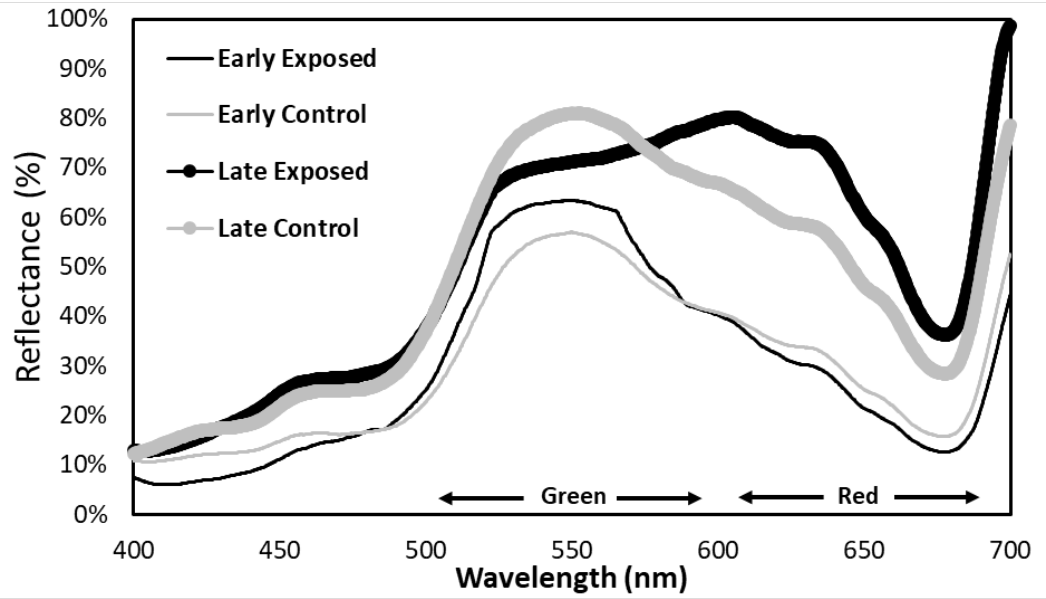

Figure 9. Spectral reflectance (% of incoming energy) for Honeycrisp before and after a rapid heating event for fruit that was either exposed to high radiation pressure (exposed) versus low radiation pressure (control).

For experiments conducted to determine whether fruit can physiologically acclimate to elevated fruit surface temperatures, fruit was exposed to near threshold fruit surface temperatures in June of 2018 when temperatures were relatively cool. These experiments were repeated in 2019. In addition to showing reduced sunburn compared to fruit that was exposed to normal conditions, peel tissue from these fruit also showed no increase in anthocyanin production, while fruit under normal conditions showed increased production with increased heat (Figure 13). To understand the genes involved in sunburn acclimation, we selected five candidate genes from the thermotolerance literature shown to be involved in heat stress and acclimation (Table 1). Tissue from heat-primed fruit and untreated fruit showed no significant difference in gene expression of these candidate genes three days after heat treatments (Figure 12). This could suggest that these genes are either not involved in apple heat stress acclimation, or that we did not have enough temporal resolution to see changes in expression. These experiments were repeated in 2019, with the addition of collecting tissue 24, 48, and 72 hours post-

treatment to capture the dynamics of pigment accumulation and gene expression, as changes in expression may occur sooner than 3 days. During the 2019 season however, with the exception of a few days in late July and early August, temperatures throughout much of the season were not sufficient to produce much sunburn pressure on fruit, thus acclimation could not take place and control fruit were not highly stressed as in the previous season. However, due to our experimental design (Fig 10), we obtained samples from fruit that had been treated with near-threshold and above threshold temperatures, both at the beginning and late in the season, which can be used to perform an RNA-sequencing experiment in the winter of 2020 to address questions about the molecular players and pathways underlying sunburn development in apple (Fig 11), which is a largely unanswered question from our field experiments.

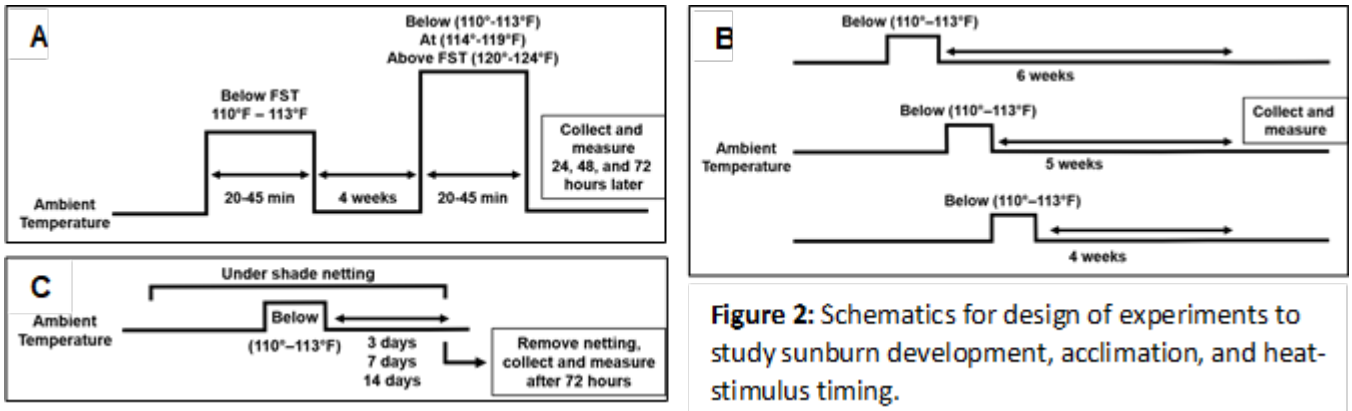

Figure 2: Schematics for design of experiments to study sunburn development, acclimation, and heat-stimulus timing.

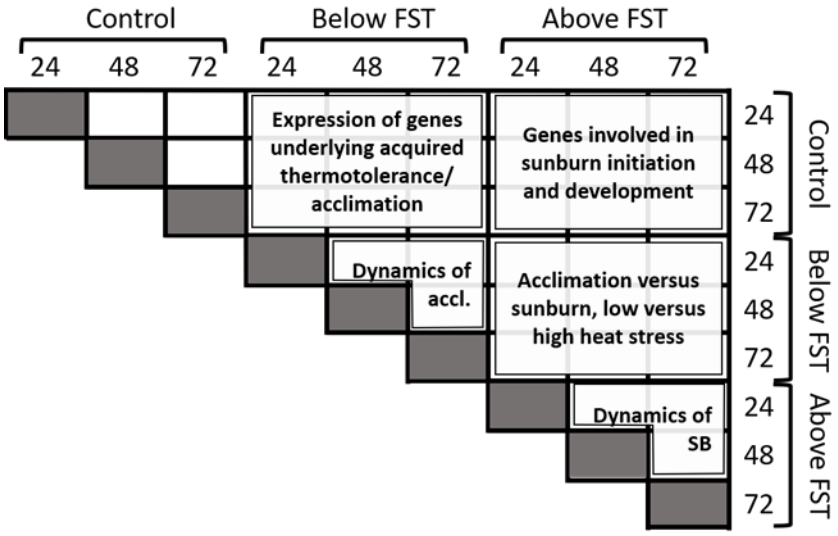

Figure 11: Potential comparisons to be made using RNA-sequencing. The outlined comparisons would highlight differentially expressed genes involved in acclimation, sunburn initiation and development, and useful information of the dynamics of these genes after heat treatments.

Additional experiments were designed in 2019 to understand the importance of the timing of the priming heat stimulus (Fig 10B and C). From the literature on acquired thermotolerance

across plant species, the amount of time that a heat stimulus confers priming to heat stress can vary. In addition, mechanisms for short-term acquired thermotolerance (SAT), long-term acquired thermotolerance (LAT), and thermotolerance to moderately high temperatures (TMHT) involve distinct molecular pathways, and our initial experiments were not designed to tease apart which are involved in apple sunburn acclimation. These samples are currently being measured and will shed light on heat acclimation pathways that will have the potential to better inform risk in current cultivars and then, as new cultivars are released heat tolerance should also be well understood for those cultivars.

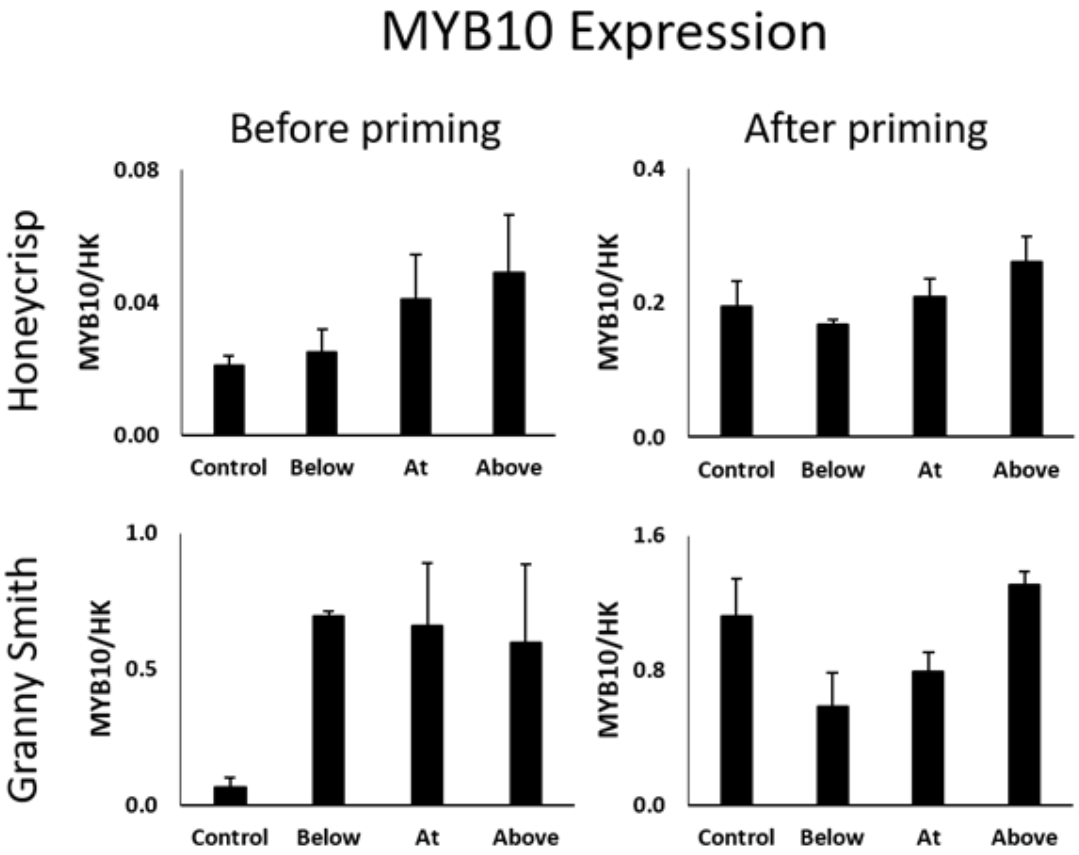

Figure 12: MYB10 expression in peel tissue from heat-primed fruit and fruit grown under normal conditions prior to being challenged with below-, at- and above-FST thresholds for sunburn. Similar to other genes observed, MYB10 showed no significant differences between treatment categories. This gene is involved in resistance to abiotic stress and anthocyanin production (red color)

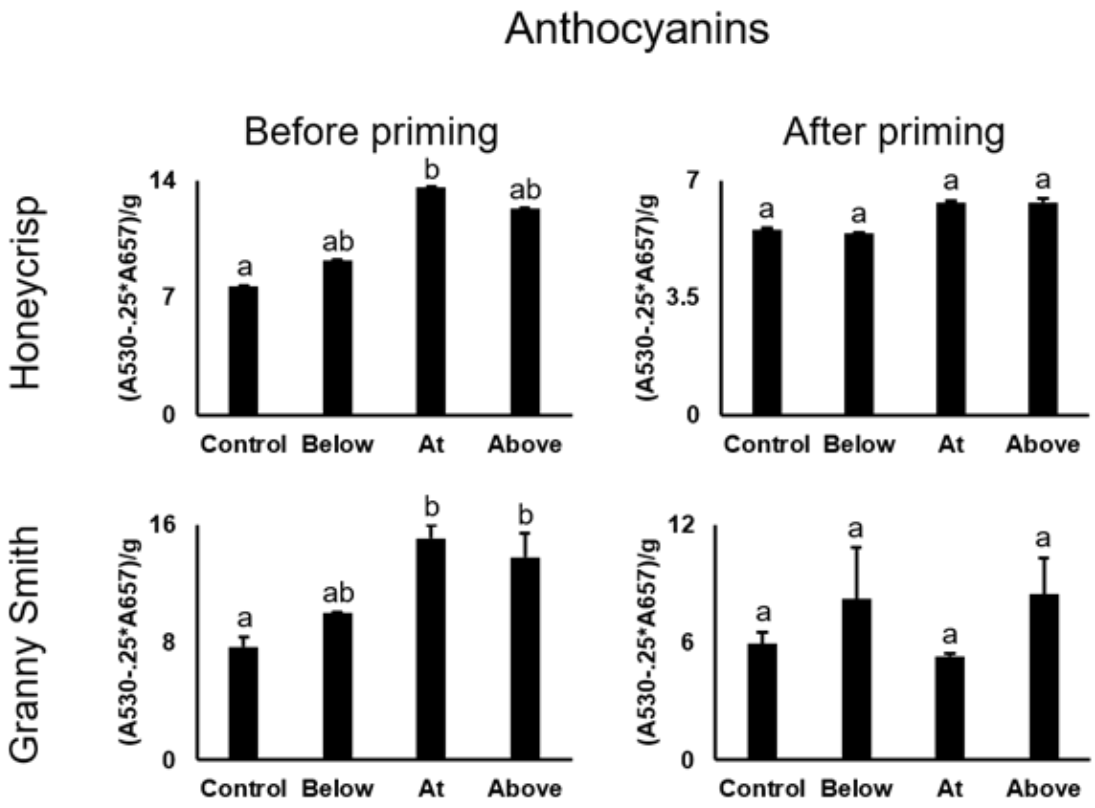

Figure 13: Anthocyanin levels in peel tissue from heat-primed fruit and fruit grown under normal conditions prior to being challenged with below-, at- and above-FST thresholds for sunburn. Non-primed fruit showed an increase in anthocyanins with increased heat treatments, while fruit that were primed did not.

Table 1. Candidate genes involved in heat stress and acquired thermotolerance

Gene name	Candidate Gene ID	Processes involved	References
LDOX	MD06G1071600	Phenylpropanoid/flavonoid pathway. Showed higher expression in sunned fruit peels.	Feng et al. Plant Phys Biochem 2013
MYB10	MD09G1278600	Anthocyanin biosynthesis. Peels of shaded fruit showed lower MYB10 expression and anthocyanin levels compared to sunned fruit.	Feng et al. Plant Phys Biochem 2013
APX2	MD12G1125600	Ascorbate peroxidase. Dependent on HSFA2, a gene required for heat shock memory after an acclimatizing stimulus.	Friedrich et al. Plant Cell Env 2018, Lamke et al. EMBO 2015, Charng et al. Plant Phys 2007.
DFR1	MD15G1024100	Phenylpropanoid/flavonoid pathway. Showed higher expression in sunned fruit peels.	Feng et al. Plant Phys Biochem 2013
HSP17.6/ HSFA2-Like	MD15G1209400	Heat shock protein, similar to HSFA2, a gene required for heat shock memory after an acclimatizing stimulus.	Friedrich et al. Plant Cell Env 2018, Lamke et al. EMBO 2015, Charng et al. Plant Phys 2007.
HK (reference)	MDP0000274900	Housekeeping gene, used as a reference for expression.	Perini et al. Mol Breed 2014.

Research and Extension Outputs

Kalcsits L. **2019.** Developing Resilient Orchards. BC Agricultural Climate Adaptation Research Workshop. December 2, 2019. Kelowna, BC. **Keynote Presentation.**

Kalcsits L, Mupambi G, Waite J, Waliullah S, Reid M, Rajopalan K, Noorazar H, Jones V, and Jones M. **2019.** Impact and Mitigation of Shifting Seasons and Elevated Summer Temperatures for Apple Production in the United States. Workshop: Effects of Climate Change on Fruit Production. American Society for Horticultural Sciences Annual Meeting. Las Vegas, NV. July 25, 2019. **Invited Presentation**

Kalcsits L, Waliullah S, Mupambi G. **2018.** Taking Advantage of Climate Extremes to Grow High Quality Tree Fruit. Washington State Tree Fruit Association Horticultural Show. Yakima, WA. December 3-5, 2018. **Invited Presentation.**

Kalcsits L. **2019**. Climate Change Brings New Challenges for the Pacific Northwest Tree Fruit. October 2, 2019. Washington State STEM Education Innovation Alliance. Wenatchee, WA.

Kalcsits L. **2019**. Bitter pit and sunburn mitigation in apple. August 7, 2019. WSU Sunrise Research Orchard Field Day. Wenatchee, WA

Waite J, Waliullah S, Kalcsits L. **2019.** Physiological changes associated with heat stress acclimation for developing apple fruit. American Society for Plant Biologists Annual Meeting. The Environmental and Ecological Plant Physiology Section Meeting. San Jose, CA. August 3-7, 2019.

Waite J, Waliullah S, Kalcsits L. **2019.** Physiological changes associated with heat stress acclimation for developing apple fruit. American Society for Plant Biologists Annual Meeting. San Jose, CA. August 3-7, 2019.

In 2021, Extension programming will be developed to guide sunburn mitigation practices and the use of evaporative cooling in orchards. One component will be communicating when sunburn risk is the highest. Another section will include mitigation practices and key cultivars that are important to carefully control fruit surface temperature to limit sunburn losses.

Leveraged Funding and future grant applications

FUNDED - 2020-2022 'Modeling Orchard Effects on Meteorological Measurements' (Co-PI; PI – Dr. Dave Brown) WTFRC Technology Review. ($206,100)
FUNDED - 2018-2021 'Risk modelling for a future climate for growing tree fruit in WA State.' (Co-PI; PI- Dr. Kirti Rajagopalan). Washington State Department of Agriculture Specialty Crop Block Grant. ($249,502).

PENDING - 2021-2022 'Sunburn Risk Management Strategies for the Pacific Northwest Apple Producers' Western SARE ($50,000)
PENDING - 2021-2022 'Is netting removal prior to harvest to improve color a risk for sunburn' WTFRC Apple Review ($75,882)
PENDING - 2021-2026 'Enhancing resilience of U.S. pome fruit production to extreme temperatures in a changing climate' USDA-NIFA SCRI. Project Director. ($4,700,000)

EXECUTIVE SUMMARY

Project title: How does fruit acclimation to sunburn affect sunburn management?

Key words: WA 38, heat, evaporative cooling, Honeycrisp, Granny Smith

Abstract: Sunburn is the leading cause of losses across all apple cultivars grown in Washington State. As fruit develops during the growing season, its susceptibility to sunburn browning changes depending on internal physiological factors as well as the environment. This project sought to determine how apple cultivars differ in their susceptibility to sunburn and what changes occur in response to environmental conditions that lead to varying degrees of sunburn severity across different growing seasons. This information will help contribute to enhanced sunburn risk modelling, more efficient mitigation practices as well as information that will eventually lead to the identification of cultivars that are less susceptible to sunburn. During fruit developments, transpiration is elevated during the 6 weeks following petal fall. Then, transpiration dramatically decreases as fruit expands and develops thicker cuticle layers that limit cooling from transpiration. Here, we report that Honeycrisp has elevated sunburn risk compared to other cultivars. This is because the fruit surface temperatures of Honeycrisp fruit are higher than fruit from WA 38, Granny Smith, and Cripps Pink under the same environmental conditions. Through experiments conducted in 2019 and 2020, we identified the anthocyanin pathway as a potential contributor to acclimation to high temperatures. This pathway is important in plants for heat dissipation under high energy inputs which is what may help keep fruit surfaces from developing sunburn symptoms under high light or temperature conditions. We will be continuing this research in other projects and look forward to developing physiological knowledge in this area in the future. Furthermore, as fruit ripens and light harvesting chlorophyll content is reduced in the apple peel, fruit becomes more susceptible to sunburn even as red color continues to develop. The applied side of this project proposed to identify whether sunburn incidence is affected in Honeycrisp apple when evaporative cooling is initiated at either 85 °F or 90 °F. We found that cooling initiated at 85 °F was more effective at reducing sunburn although both cooling treatments reduced sever sunburn compared to the uncooled control. Water use for R10s cycled every 15 minutes and off for 45 minutes applied a total of 0.8 acre feet of water in June, July, and August. This accounts for approximately 20% of the water demand for an orchard in an average year and needs to be accounted for in irrigation decisions, especially for Honeycrisp apple. Switching to low water volume fogging or evaporative cooling systems could substantially reduce the amount of water applied to an orchard and keep most of it in the tree canopy where it has the greatest impact on fruit surface temperatures. Overall, we identified key differences in susceptibility in commercially important cultivars grown in Washington State. We determined that susceptibility changes as fruit develops and in response to the environment. Lastly, we confirmed that the conservative practice of turning on evaporative cooling at 85 °F is important for reducing sunburn losses in Honeycrisp apple and that activation at higher temperatures lead to higher losses from sunburn. Ongoing work will focus on better understanding fruit acclimation to heat and the pathways responsible. These efforts will enhance sunburn risk models and forecasting to better protect fruit from sunburn in Washington State.

FINAL PROJECT REPORT

Project Title: How do we measure and manage soil health for productive orchards?

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Cooperators: Clark Kogan, Washington State University, Department of Mathematics and Statistics

Orchardist site hosts: Hannah Walters, Bernardo Reyes, Stemilt; Tony Mena, Joe Gabriel, Rob Mc Graw, David Keller, Daniel Canales, Gilbert Orchards; Michael Burns, Zirkle Fruit; Keith Valeska, Harrison Ranch; Craig Obrien; Orlin Knutsen, Alamo Orchard; Keith Oliver, Olsen Bro; Lauren Gonzalez, Kershaw; Rolando Martin, WA Fruit; Jake Robison, Robison Orchards, Mark Gores; Rory Otte; Brian McMillon, Red Top Orchard; Steve Scheib, Highland Partnership; Suzanne Neiman, Alan Brothers; Chris and Paul Williams; Sam Godwin, Box Canyon Fruit; Geoff Thorton; Ray Fuller; Mike Brownfield; Jeff Cleveringa, Starr Ranch; Lee Pobst; Jim Baird; Mike Robinson; Columbia Fruit.

Total Project Request: Year 1: $48,884 **Year 2**: $51,258 **Year 3**: $51,686

Budget 1
Organization Name: WSU-TFREC **Contract Administrator:** Kim Rains/Katie Roberts
Telephone: 509.293.8803/509.335.2885 **Email:** kim.rains@wsu.edu/arcgrants@wsu.edu

Item	2017	2018	2019
Salaries[1]	$24,600	$25,584	$26,607
Benefits[2]	$9,740	$10,130	$10,535
Wages	0	0	0
Benefits	0	0	0
Equipment	0	0	0
Supplies[3]	$10,272	$11,272	$10,272
Travel[4]	$4,272	$4,272	$4,272
Miscellaneous	0	0	0
Plot Fees	0	0	0
Total	$48,884	$51,258	$51,686

Footnotes:
[1]Salaries for a 25% scientific assistant (Kalcsits) and a 33% scientific assistant (DuPont).
[2]Benefits at 44.1% for scientific assistant (Kalcsits) and 37% for scientific assistant (DuPont).
[3]Goods and services include soil nutrient analysis, soil quality analysis, plant tissues tests, fruit quality analysis, sampling and lab materials.
[4]Travel to collect soil, yield, and fruit quality samples from farm sites.

Acknowledgements: Thank you to technical support from Abigail Kowalski, Jared Dean and Hayley Mendez.

OBJECTIVES

1. Test the relationship between soil quality and fruit productivity.
2. Determine which of a suite of 21 soil quality indicators are appropriate for tree fruit production systems in the irrigated west.
3. Increase grower understanding of soil quality indicators, what they mean, and how to use the information they provide to improve management.

SIGNIFICANT FINDINGS

Indicators measured in Washington orchards had a wide range but with generally lower organic matter, lower available water capacity, higher % sand and lower wet aggregate stability than Midwest, Mid-Atlantic and Northeastern soils measured in other studies. Water related factors available water capacity and % sand had significant yield models, and root health factors *Pratylenchus* nematode and bean root health rating had consistent but not significant relationships with yield according to linear mixed models. The minimum dataset of soil health indicators for Central Washington orchards should include measurements of water availability (AWC, % sand) and of root health (bean root health rating, *Pratylenchus* nematodes*)* as well as fertility indicators to meet stakeholder management goals. High levels of mineralizable N in some orchards indicate the need to include a measurement of organic N availability in the minimum data set. With more than 25% of surveyed orchards with high subsurface penetration resistance values, a measurement of compaction should be included. While OM and active carbon (POXC) were not correlated with the stakeholder management goal of productivity, soil organic matter influences multiple soil functions including microbial activity, nutrient cycling, soil carbon accumulation and water relations, and as such should be included in the minimum dataset as indicators of environmental health.

METHODS

Site description: To date **101 orchard plots have been soil sampled. Of these plots 60 plots (30 matched pairs) were well matched with available/measured yield data**. A subset of 32 plots (16 matched pairs) were sampled for fruit yield and fruit quality. Matched plots were on the same general soil type with matching variety, tree age and training system. One plot in each pair was high performing based on grower description and the other site in the matched pair was underperforming.

Soil sampling: Fifty to one hundred soil probe subsamples to an 8-inch depth just inside the drip line of the canopy were taken for nutrient, soil health and nematode analysis. Four four-inch deep intact soil cores were taken for bulk density analysis. Five intact cores two inches deep by two-inch diameter were taken for micro-arthropod analysis. Water infiltration was measured by timing the length of time for water to fully infiltrate when one inch of water was added to a 10-inch diameter ring pounded 2 inches into the ground.

Soil health analysis: Soil health indicators measured included water availability: available water capacity (AWC), water infiltration, and % sand; indicators of root health: apple root health rating, bean root health rating, *Pratylenchus spp.* nematodes; indicators of soil structure: surface and subsurface penetration resistance (PR), bulk density (BD), and wet aggregate stability (WAS); chemical fertility factors: P, K, Mg, Ca, Fe, Mn, Zn, pH; microbially available fertility factors: autoclaved citrate-available protein (ACE), potentially mineralizable N (PMN); and OM and biological activity indicators: organic matter (OM), permanganate oxidizable active carbon (POXC), microarthropods, soil food web structure and enrichment indices (SI, EI), and respiration.

Fruit yield and quality: Fruit yield and quality were determined by collecting grower reported packing house yield data for the previous two to four years where possible. For orchards where packing house data was not collected, a subset of five representative trees were selected for each orchard. At harvest, fruit per tree were counted and 20 fruit per tree collected to determine mean fruit weight and to estimate total yield. To assess the proportion of high-quality fruit free of sunburn, bitter pit, or poor color, 60 fruit were collected from the three representative trees in each orchard. Fruit quality assessments included sunburn analysis following the Washington Tree Fruit Research Commission sunburn scale for bi-color fruit based on Schraeder et al. (2003). Red overcolor was graded based on <25% coverage, 25-50% coverage, 50-75% coverage, or 75-100% coverage. Bitter pit, lenticel breakdown or other external disorders were also assessed on all fruit. If fruit contained less than 50% red over color, bitter pit, or sunburn incidence that was greater than YII, fruit was classified as a cull. From this, packout % and total packout (packed boxes per acre) was calculated for each orchard.

RESULTS

Indicators of water availability varied widely across Central Washington orchards where 11 sites had limited water availability. Available water capacity ranged from 0.2 g g^{-1} in coarse and medium texture soils to 0.3 g g^{-1} in fine textured soils. Almost half of the soils sampled had coarse soil texture with an average of 66% sand. Water infiltration varied by site with a range of 10 seconds to 5 minutes for 1-inch of water to infiltrate.

Indicators of root health function showed disease potential in 50% of Central Washington apple orchard fields sampled according to apple root health ratings (values <50%), 29% of fields according to bean root health ratings (values 5-9), and 15% based on *Pratylenchus* nematode counts. Twenty-nine percent of orchard fields showed moderate damage to advanced decay in bean root health ratings used to detect disease potential for common plant pathogens.

Central Washington orchards surveyed generally had optimum macro and micronutrient levels. pH levels were generally within the optimum range of 6.0 to 7.5 with one site at a limiting level of 5.5 where macronutrients would be less available and 28 sites above 7.5 but below 8.0. Only 12% of sites had P levels below 10 ppm considered limiting for tree fruit and two sites had excessive levels (>50 ppm). Potassium levels generally were equal to, or greater than, optimum (150-250 ppm) with the exception of four sites that had soil K concentrations of less than 100 ppm and 11 sites were between 100 and 150 ppm. However, 47 sites had greater than 300 ppm K.

Measurements of microbially available N in Central Washington orchards showed a range of levels with many orchards where substantial organic N pools should be accounted for when nutrient applications are made. Washington orchards measured had average potentially mineralizable nitrogen of 21.1, 15.1 and 6.5 µ N g^{-1} $week^{-1}$ for coarse, medium and fine soils, respectively. ACE soil protein was relatively low with 6.7, 4.3 and 4.5 mg g^{-1} for coarse, medium and fine soils, respectively. The soil food web Enrichment Index varied widely with 82% of sites showing an EI rating of 50 or higher indicating soil fauna with a large capacity to respond to and mineralize N additions.

Soil structure in Central Washington orchards surveyed had moderate to low wet aggregate stability (average 19% medium, 27% fine and 30% coarse), bulk density averaging 1.1 to 1.3 g cm^{-3} for fine and medium-coarse soils and moderate surface and subsurface penetration resistance (PR) with the exception of 26 sites where subsurface PR exceeded 2070 kPa (300 psi) (Figure 4). On average, bulk density was 1.1 g cm^{-3} in fine texture soils and 1.3 g cm^{-3} in medium and coarse texture soils and lower than levels proposed to impact root growth and yield. Five orchard fields had bulk density of 1.5 g cm^{-3} indicating a potential limitation in some sites. Compaction measured as penetration resistance is considered to limit root growth as well as access to water and nutrients when levels exceed 2070 kPa (300 psi) Twenty-six of

the sites had subsurface PR higher than 2070 kPa with five sets of matched pairs where subsurface PR was higher in low yielding orchard sites compared to high yielding sites indicating a potential limiting effect.

Indicators of soil biological activity and food web structure were on average moderate too low in this study but were highly variable. Microbial activity measured by respiration varied from 0.01 to 1.25 mg CO^2 g^{-1}. These respiration levels were generally low with 78% of samples below the scoring curve average of 0.6 mg CO^2 g^{-1}. Soil food web structure was also low on average with 78% of sites scoring less than 50% as calculated by the soil food web Structure Index. Micro arthropods including fungal feeding and predatory mites and collembolan in the surface soils were highly variable from 0 to 90,000 counts m^{-2}.

Washington orchard soils surveyed had a wide range of organic matter, but levels were generally lower than those documented in other regional surveys. Organic matter in Central Washington orchards ranged from 1.0 to 5.5%, with active carbon (POXC) ranging from 191 to 1145 ppm. Fifty seven percent of soils had less than 2% OM and scored less than 50% on the scoring curve for active carbon indicating relatively low carbon availability.

In order to identify a minimum dataset for a soil quality index for Washington orchards several methods were employed to relate soil factors to management factors important to stakeholders: fruit yield and fruit quality. Statistical methods included lasso regression, linear mixed effects models, principal components analysis and nonlinear Bayesian modeling.

Using an integration of biological knowledge of the system we looked for trends to see at what thresholds yield trended to decrease between matched pairs (sets of two orchards with matching scion, rootstock and location) as a factor increased or decreased. *Pratylenchus* nematode has a known threshold where 20-70 *Pratylenchus* 500 g^{-1} may cause crop damage and 80 + is likely to damage young trees. All six matched pairs with values over 80 *Pratylenchus* 500 g^{-1} have a downward slope indicating potential reduced yield capacity at high *Pratylenchus* nematode densities. The bean root health rating is on a scale of 1 to 9 where 1 is healthy and values over 4 generally show root damage. In this dataset all the fields with values over 5 have a negative slope where percent yield goal decreases as bean root health rating values increase. Available water capacity (AWC) of 0.1-0.15 g g^{-1} is thought to create moderate water limitation with AWC less than 0.1 g g^{-1} severely limiting water availability. In exploratory analysis AWC showed negative slopes for matched pairs where low yielding sites had less than 0.15 g g^{-1} AWC. Additionally, in soils with over 70% sand, a downward trend for percent yield goal was apparent.

We then used a linear mixed effects model to characterize the association between yield (percent goal) and each of the selected soil health factors which showed strong trends in exploratory analysis. *Pratylenchus* nematode with a threshold of 80 and bean root health rating with a threshold of 4 had consistent but not significant yield (percent goal) models (P=0.24, P=0.08), with similar results for packout (P=0.19, P=0.17). AWC with a threshold of 0.15 and % sand with a threshold of 70% had significant yield (percent goal) models (P=0.009; P=0.03), with non significant results for packout (P=0.09; P=0.20).

A nonlinear Bayesian model was computed to discern association between soil health components and tree fruit productivity. The model coallates available water capacity and percent sand into one factor for water relations, pratylenchus nematode numbers and bean root health ratings into a second factor representing a root health function and macro and micronutrient levels to a factor for nutrient availability. We hypothesize that the Bayesian model will better serve to represent nonlinear interdependence of the soil health variables and outcomes.

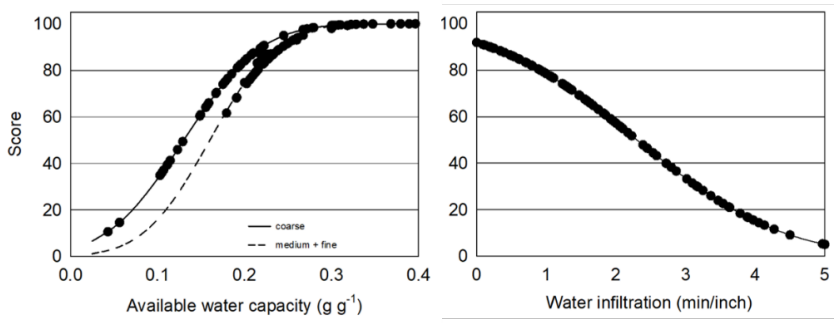

Figure 1 Soil water indicators: available water capacity (g g^{-1}) and water infiltration (min/inch).

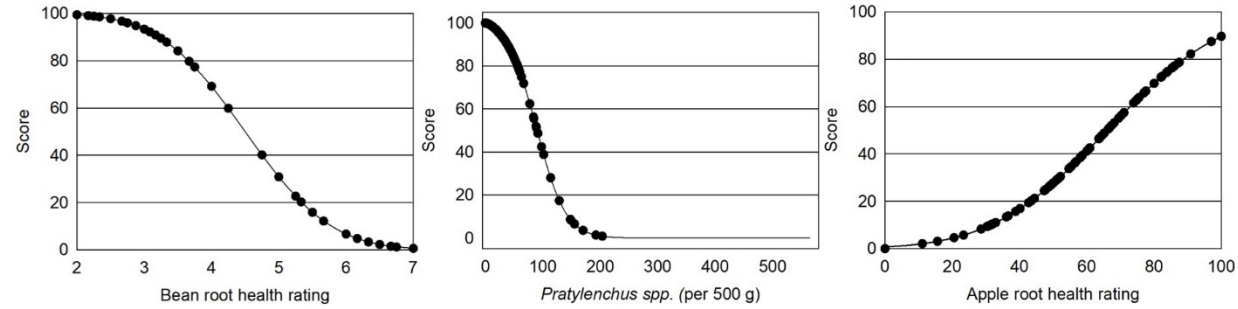

Figure 2 Indicators measuring the root health function of soil including bean root health rating, numbers of Pratylenchus spp. nematodes*, and apple root health rating.*

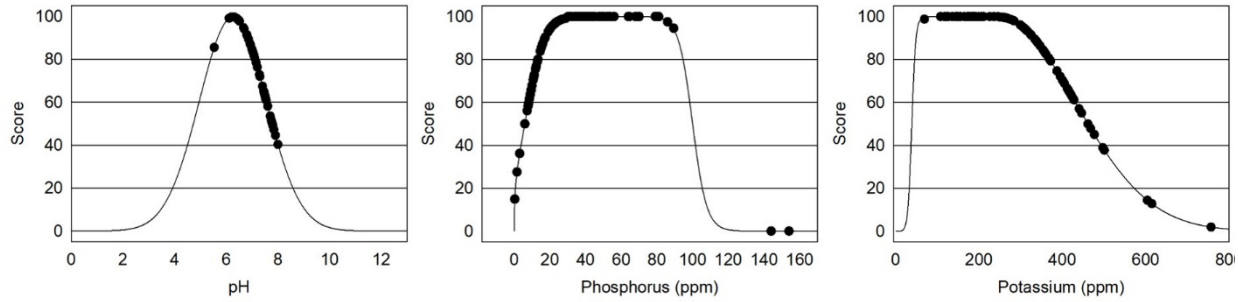

Figure 3 Soil fertility indicators: pH, P and K in 101 Central Washington orchards surveyed.

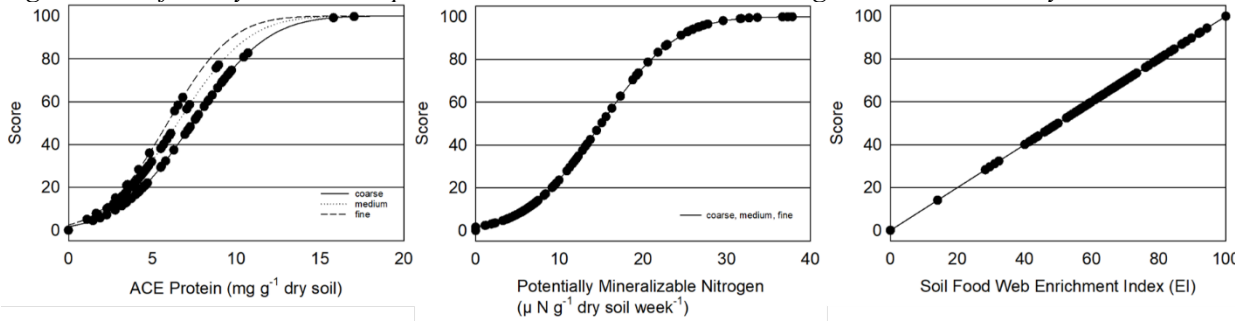

Figure 4. Indicators of microbially available N: ACE Protein (mg g^{-1} dry soil), Potentially Mineralizable N (μ N g^{-1} $week^{-1}$), and Soil Food Web Enrichment Index for 101 Central Washington orchard field soils.

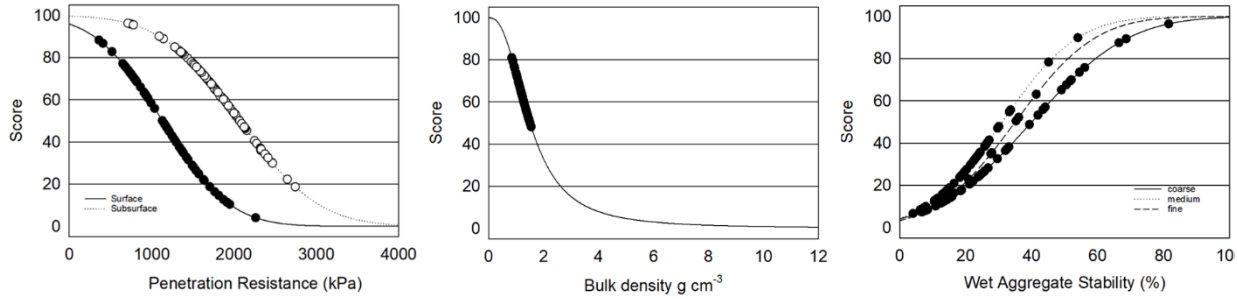

Figure 5. Indicators of soil structure: penetration resistance, bulk density, and wet aggregate stability in 101 Central Washington orchards field soils.

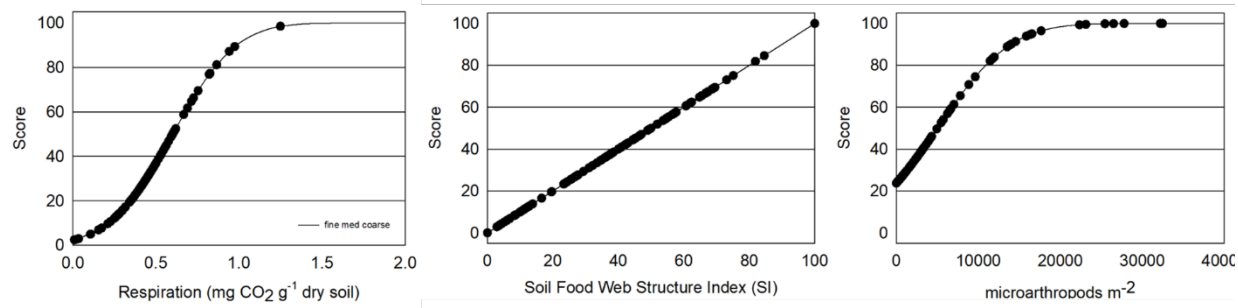

Figure 6. Indicators of soil biological activity and food web community structure: respiration (mg CO^2 g^{-1}), soil food web structure index (0-100 scale) and micro arthropods (m^{-2}) in 101 Central Washington orchards fields.

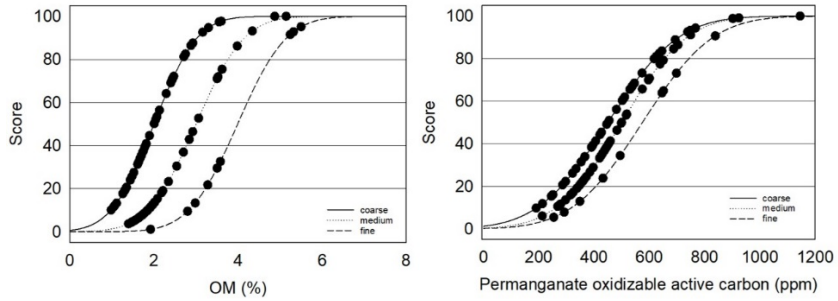

Figure 7. Soil organic matter (OM %) and permanganate oxidizable active carbon (POXC ppm) in 101 Central Washington orchards fields.

Table 1. Data from individual orchards for yield and packouts for 32 orchards sampled from 2017-2019.

Year	Orchard	Cultivar	Crop Load (fruit cm^{-2} TCSA)	Fruit $Tree^{-1}$	Mean Fruit Weight	Yield $Tree^{-1}$ (kg)	Trees Ha^{-1}	Yield (T Ha^{-1})	% cull	Packed boxes Ha^{-1}
2017	BR27+	Gala	1.76	270	176	47.4	1621	76.8	6.7	3271
2017	BR28-	Gala	3.51	49	175	8.7	2928	25.3	18.3	1043
2017	AB42+	Honeycrisp	3.77	87	161	14.0	3194	44.7	1.7	1358
2017	AB43-	Honeycrisp	5.47	60	179	10.7	3194	34.1	1.7	1928
2017	AB40+	Honeycrisp	2.80	421	126	53.0	651	34.5	5.0	3798
2017	AB41-	Honeycrisp	0.77	71	179	12.7	1505	19.1	1.7	4047
2017	KG48+	Granny Smith	6.06	46	260	12.0	2197	26.3	1.7	1806
2017	KG49-	Granny Smith	7.73	72	217	15.5	2197	34.1	1.7	1047
2017	H38+	Granny Smith	8.68	120	169	20.3	3758	76.2	0.0	2479
2017	H39-	Granny Smith	8.28	110	174	19.1	3758	72.0	16.7	1587
2018	z52+	Gala	6.40	441	144	63.5	1256	79.7	1.7	3951
2018	Z53-	Gala	4.45	137	153	20.9	1621	33.8	6.7	1500
2018	Z66+	Gala	3.16	332	141	46.8	1256	58.8	1.7	2976
2018	Z67-	Gala	2.10	133	151	20.0	1621	32.4	16.7	1433
2018	RB58+	Gala	2.61	143	163	23.4	2928	68.5	0.0	3722
2018	RB59-	Gala	3.52	91	204	18.6	2928	54.4	3.3	2283
2018	K54+	Gala	3.84	474	152	72.1	823	59.4	3.3	3318
2018	K55-	Gala	4.35	125	190	23.6	2197	51.9	3.3	2307
2018	WA56+	Honeycrisp	2.55	121	235	28.4	2928	83.1	10.4	3613
2018	WA57-	Honeycrisp	3.68	77	258	19.9	2928	58.2	29.2	1996
2018	O50+	Gala	2.98	237	178	42.1	1350	56.9	1.7	2518
2018	O51-	Gala	4.51	221	174	38.4	1350	51.9	1.7	2352
2019	Al88+	Gala	6.59	121	185	22.4	3514	78.8	1.7	3694
2019	Al89-	Gala	5.26	53	113	6.0	3514	21.1	20.0	913
2019	Gil76+	Gala	9.31	92	133	12.2	4392	53.7	26.7	1972
2019	Gil77-	Gala	7.99	44	139	6.1	4392	26.9	13.3	1116
2019	S70+	Honeycrisp	4.72	139	194	27.0	2928	79.0	0.0	5082
2019	S71-	Honeycrisp	5.00	27	232	6.3	2928	18.4	8.6	820
2019	Zi82+	Honeycrisp	8.74	75	226	16.9	4392	74.4	10.4	3685
2019	Zi83-	Honeycrisp	3.61	79	216	17.1	1505	25.7	27.3	981
2019	KMO68+	Pinata	5.65	352	202	71.2	968	68.9	10.0	3481
2019	KMO69-	Pinata	4.21	233	216	50.2	968	48.6	8.3	2198

DISCUSSION

Indicators measured in Washington orchards had a wide range but with generally lower organic matter, lower available water capacity, higher % sand and lower wet aggregate stability than Midwest, Mid-Atlantic and Northeastern soils measured in other studies. Water related factors available water capacity and % sand had significant yield models, and root health factors *Pratylenchus* nematode and bean root health rating had consistent but not significant relationships with yield according to linear mixed models. A high percentage of sites with subsurface compaction and high organic nitrogen content suggest these factors are important to track in Washington orchards.

Root health and available water were the most common limiting factors in the orchards we studied. Almost half of the soils sampled had coarse soil texture with an average of 66% sand. Available water capacity is a measure of the porosity of soil and indicates the amount of plant available water a soil can hold where below 0.15 g/g available water is considered moderately to severely limiting. Of sites surveyed 11% had available water capacity indicating moderate water limitation and 5% levels indicating severe water limitation. For example, consider matched granny smith on M9.337 blocks where the high productivity block yielded 64 bins per acre and the low yielded 34 bins per acre on average. Available water capacity was 19 g/g (56% sand) in the high yielding block compared to 15 g/g in the low yielding block (75% sand).

Root health was an important factor in Central Washington orchards. Plant pathogens *Phytophthora* and *Pythium, Ilyonectria robusta*, *Rhizoctonia solani* as well as the lesion nematode *Pratylenchus penetrans* are known to negatively impact growth and production in young apple trees. Root health ratings measured negative impacts in 29% of orchards surveyed according to bean root health ratings and 15% based on lesion (*Pratylenchus*) nematode counts. The impacts of poor root health can be significant. For example, in two matched Gala on M9 rootstock orchards the orchard with 33 bin/A average versus 60 bin/A in the productive block had high lesion nematodes numbers (129 per 500 cc) well over the 80 per 500 cc threshold.

Soils with high bulk density and compaction limit root growth and root access to water and nutrients. Twenty-six of the surveyed orchards had high subsurface penetration resistance indicating compaction and limited rooting area. Five of the matched sets of orchards had higher compaction in low-yielding compared to high-yielding sites. For example, in Ultima gala on Nic.29 rootstock orchards planted the same year with the same training system, the orchard yielding 15 bin/A less (55 bin/A vs 70 bin/A) had a deep compaction layer at an 18 inch-depth. While neither penetration resistance nor bulk density had significant effects on yield in mixed model analysis, trends indicate that this factor should continue to be tracked in order to measure potential effects that may be confounded by the limited number of sites analyzed.

Many orchards surveyed had high organic N content. Including a measurement of organic nitrogen in the minimum dataset for soil health assessment in orchards could be critical to avoid nitrogen over applications. For example, the average PMN for sites measured was 21 μ g^{-1} $week^{-1}$ which would supply 2.45 lb/A per week reducing N needs by 49 lbs/A over the 20-week season. Assuming an 80 bin/A yield goal and N recommendations of 70 lb/A per season the N needs may be only 21 lbs/A. Unfortunately, while extractable organic N fractions are generally positively correlated with mineralizable N, they often only partially explain the variation in mineralizable N and there is disagreement about which test provides more usable information.

The minimum dataset of soil health indicators for Central Washington orchards should include measurements of water availability (AWC, % sand) and of root health (bean root health rating, *Pratylenchus* nematodes*)* as well as fertility indicators to meet stakeholder management goals. High levels of mineralizable N in some orchards indicate the need to include a measurement of organic N availability in the minimum data set. With more than 25% of surveyed orchards with high subsurface PR values, a measurement of compaction should be included. While OM and POXC were not correlated with the stakeholder management goal of productivity, soil organic matter influences multiple soil functions including microbial activity, nutrient cycling, soil carbon accumulation and water relations, and as such should be included in the minimum dataset as indicators of environmental health.

EXECUTIVE SUMMARY

Project title: How do We Measure and Manage Soil Health for Productive Orchards?

Key words: soil health, organic matter, available water capacity

Abstract:

Soil health assessment has been recognized as a critical soil testing tool. But what does soil health mean in perennial orchards in the irrigated west? Our group set out to identify a set of soil health indicators that are useful to track in Central Washington orchards. Specifically, we were challenged to track which factors may be limiting to yield and fruit quality. This study measured twenty-one soil health indicators in 101 Central Washington apple orchards. To determine the relationship between soil health indicators and fruit yield and quality we used 30 sets of matched sites with high and low productivity orchards of the same or similar variety, rootstock, age, and training system. Fruit yield and packout were determined using two-to-four year grower averages and fruit measurements from five representative trees per orchard. The soil health indicators we measured had a wide range across Washington orchards surveyed but overall organic matter, available water capacity, and wet aggregate stability were lower, and % sand higher than soils measured in other Midwest, Mid-Atlantic and Northeastern studies. Water related factors (available water capacity and % sand) had a significant relationship with yield according to linear mixed model analysis and root health factors (*Pratylenchus* nematode and bean root health rating) had consistent but not significant relationships. A high percentage of sites with subsurface compaction and high organic nitrogen content suggest these factors are important to track Washington orchards. The minimum dataset of soil health indicators for Central Washington orchards should include measurements of water availability (AWC, % sand) and of root health (bean root health rating, *Pratylenchus* nematodes*)* in addition to standard fertility indicators to meet stakeholder management goals.

FINAL PROJECT REPORT

Project Title: Utility of rapid tools to assess cleanliness in apple packinghouses

PI:	Faith Critzer	**Co-PI**:	Ines Hanrahan
Organization:	Washington State University	**Organization**:	WTFRC
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Email:	faith.critzer@wsu.edu	**Email**:	hanrahan@treefruitresearch.com

Total Project Funding: **Year 1:** 55,956 **Year 2:** 56,525

WTFRC Budget:

Item	2018	2019
Salaries	3,900	3,978
Benefits	1,287	1,313
Wages	3,350	3,503
Benefits	1,106	1,156
RCA Room Rental		
Shipping		
Supplies		
Travel	500	500
Plot Fees		
Miscellaneous		
Total	10,143	10,450

Footnotes:

Salaries/Benefits: Estimate of percent of time spent for Mendoza (3%) and Hanrahan (2%), a 33% benefit rate and 2% annual increases.

Wages/Benefits: Calculated based on expected staff wage adjustments proportional to the WA state minimum wage increases (2018=$11.50, 2019=$12.00), approx. 250 hours

Travel: In state travel for Hanrahan (lodging in Wenatchee)

Budget 1
Organization Name: Washington State University
Contract Administrator: Samantha Bridger
Telephone: (509)786-9204 **Email address:** prosser.grants@wsu.edu

Item	2018	2019
Salaries	32,440	34,107
Benefits	2,373	2,468
Wages		
Benefits		
Equipment		
Supplies	10,000	8,500
Travel	1,000	1,000
Miscellaneous		
Plot Fees		
Total	45,813	46,075

Footnotes:
Salaries: In year 1, $32,440, and year 2, $34,107, is requested for a Graduate Research Assistantship for a PhD student to work on all objectives.

Benefits: $2,373 and $2, 468 are requested for benefits tied to the Graduate Research Assistantship for a PhD student to work on all objectives for years 1 and 2, respectively.

Supplies: Supply costs of $10,000 in year 1 and $8,500 in year 2 are requested to purchase disposable supplies such as swabs, sponges, glassware, microbiological media, Petrifilm, pipettes, and PCR reagents tied to objective 1.

Travel: $1,000 is requested in years 1 and 2 for mileage and associated travel costs at a rate of $0.535/mi and adhering to all university policies for per diem associated with overnight travel.

Objectives:

1. Determine the correlation of ATP or carbohydrate swabs to populations of indicator microorganisms (aerobic plate counts, total *Enterobacteriaceae,* coliforms, and *E.* coli) in typical packinghouse settings on zone 1 (food contact) surfaces.
2. Model thresholds for accepting and rejecting a surface cleanliness for ATP and carbohydrate residues and resulting populations of indicator microorganisms based upon material type.

Significant Findings

- Rapid tests are not suitable for predicting microbial loads on food contact surfaces.
- Rapid tests are useful to assess residual matter and allow for re-cleaning of equipment.
- Cleaning and sanitation practices should focus on both wet and dry areas of apple packinghouses.
- To validate sanitation practices, traditional microbiological methods are still needed.

Methods

Objective 1. Determine the correlation of ATP or carbohydrate swabs to populations of indicator microorganisms (aerobic plate counts, coliforms, and *E.* coli) in typical packinghouse settings on zones 1 and 2.

Packinghouse selection. Commercial apple packinghouses in Washington were recruited into the study. Five packinghouses were enlisted into the study and were sampled once a quarter during packing season (October 2018-August 2019). Table 1 describes the types of surfaces sampled within each unit operation.

Table 1. Examples of food contact surfaces tested at each unit operation

Area	Unit operation	Sample sites (Food contact surfaces)
Wet	Washing (Dump tank)	Dump tank, rollers, traction belting, brushes under the rot blaster
	Washing/Sanitizing/Rinsing (Brush beds)	Brush rollers, bristle rollers, Teflon tapes, plastic flaps
	First drying (Fan and/or blower)	Brush rollers, metal dividers, plastic flaps
	Wax coating	Brush rollers, rubber flaps
	Second drying (Tunnel drier)	Foam rollers, bristle rollers, Teflon tapes, rubber flaps
Dry	Sorting	Rollers, foam rollers, bristle rollers, brush rollers, sorter cups, cup-droppers, rubber flaps, interlocking belts, belts, Teflon tapes, guide rails
	Packing	Packing tables, belts, rubber flaps, plastic flaps, Teflon tape, guide rails

Surface sampling methods. Sampling has been coordinated to occur after a sanitation event. For microbiological analysis, a pre-moistened sterile sponge has been utilized to sample a 25 cm^2-area. For ATP and carbohydrate swabs, surfaces adjacent to those for microbiological sampling will be used to swab a 25 cm^2-area.

ATP determination. An ATP luminometer and accompanying swabs have been utilized to determine the ATP present in the given surface area expressed as reflective light units (RLU).

Glucose and lactose presence. The SpotCheck Plus Glucose and Lactose Residue swab (Hygiena) have been used to determine if there is presence of either of these sugars on the surface. The results will be categorized as pass (no color change=0), moderate fail (light green=1), and severe fail (dark green=2).

Microbiological isolation. Bacteria are eluted in D/E neutralizing buffer and surface plated onto Petrifilm E. coli/Coliform Count Plates (to enumerate *E. coli* and coliforms), Petrifilm Enterobacteriaceae Count Plates (to enumerate total Enterobacteriaceae), Petrifilm Aerobic Count Plates (to enumerate aerobic, mesophilic bacterial counts).

Statistical analysis. Data analysis was carried out using Minitab software (version 19). APC, *Enterobacteriaceae,* coliforms*, E. coli,* and ATP values were normalized using log transformation. To identify the correlation between populations of indicator organisms (APC, *Enterobacteriaceae,* coliforms , and *E. Coli*) with RLU values, Pearson correlation coefficient (r) was determined. A Student's *t* test was performed for pairwise mean comparisons of the different populations of indicator organisms with the scores of Glucose/Lactose residue swabs (Pass or Fail); populations of indicator organisms with the detection of *Listeria* spp. (Positive or Negative), and rapid tests with the detection of *Listeria* spp. (Positive or Negative). Tukey test was used for multiple mean comparisons of populations of indicator organisms (APC, *Enterobacteriaceae,* coliforms and *E. Coli*) and RLU values throughout unit operations with $\alpha = 0.05$.

Alterations to original design of experiments. Due to a high prevalence of Enterococci present on food contact surfaces, it was determined that the methodology for enumerating *Listeria* spp. would always overestimate the population as Enterococci (*Enterococcus faecalis* or *Enterococcus faecium*) cannot be differentiated on selective and differential media. Therefore, enumeration of listeria was abandoned as it is was not going to accurately reflect populations of *Listeria* spp.

Objective 2. Model thresholds for accepting and rejecting a surface cleanliness for ATP and carbohydrate residues and resulting populations of indicator microorganisms based upon material type.

Statistical analysis. Whenever indicators are utilized for making risk-based decisions, many firms wrestle with what thresholds should be established for action (e.g. re-clean surface). Based upon outcomes of objective 1, equations will be evaluated in year two for any moderate to highly correlated indicator to determine the threshold at which the likelihood of having *Listeria* spp. present significantly increases.

Alterations to original design of experiments. Unfortunately, no significant correlations were obtained for any indicator and rapid test, highlighting the fact that rapid tests cannot be utilized to supplant microbiological testing.

Results and Discussion

Populations of indicator organisms throughout unit operations. As shown in Table 2, the highest populations recovered were from APC, followed by, in order of population size, *Enterobacteriaceae*, coliforms, and *E. coli.* APC, *Enterobacteriaceae,* and coliforms populations were significantly

different at the different unit operations ($p \leq 0.05$). For APC, the wax coating and tunnel drying unit operations showed significantly higher mean values than the washing step. However, regarding *Enterobacteriaceae* and coliform populations, the highest mean populations tended to occur in unit operations associated with the wet area (Table 2). For all unit operations *E. coli* populations were relatively low (0.2 - 0.3 log CFU/100 cm^2) and not significantly different across unit operations ($p > 0.05$).

Table 2. Mean of populations of indicator organisms at each unit operation

Unit operation	n[A]	**Mean ± Std Dev of indicator organism populations** (Log CFU/100 cm^2)			
		Aerobic plate count [B]	*Enterobacteriaceae*	Coliforms	*E. coli*
Washing	70	2.7 ± 1.2 (b)[C]	1.7 ± 1.5 (a)	1.4 ± 1.3 (ab)	0.2 ± 0.5 (a)
Washing/sanitizing /rinsing	79	2.8 ± 1.2 (ab)	1.6 ± 1.3 (a)	1.4 ± 1.3 (a)	0.2 ± 0.4 (a)
Fan drying	75	2.9 ± 1.1 (ab)	1.3 ± 1.2 (ab)	0.9 ± 1.1 (bcd)	0.3 ± 0.5 (a)
Wax coating	50	3.3 ± 0.9 (a)	1.3 ± 1.3 (ab)	1.0 ± 1.1 (abcd)	0.2 ± 0.4 (a)
Tunnel drying	85	3.2 ± 0.8 (a)	1.5 ± 1.2 (a)	1.1 ± 1.1 (abc)	0.2 ± 0.4 (a)
Sorting	302	3.0 ± 0.8 (ab)	1.0 ± 1.0 (b)	0.6 ± 0.9 (d)	0.2 ± 0.4 (a)
Packing	80	3.0 ± 0.7 (ab)	1.0 ± 1.0 (b)	0.8 ± 0.9 (cd)	0.3 ± 0.6 (a)

[A] Number of samples

[B] Aerobic plate count (APC) included all the microorganisms that could grow in aerobic conditions and at 35°C

[C] Means within a column that are not followed by the same letter are significantly different ($p \leq 0.05$)

Association between RLU values of the ATP test with CFU values of populations of indicator organisms. Table 3 summarizes the Pearson correlation coefficients (r) of RLU values between the different populations of indicator organisms ($r < 0.01$). No statistically significant association was found.

Table 3. Pearson coefficient correlation between populations of indicator organisms (Log CFU/100 cm^2) with ATP test (Log RLU/100 cm^2)

INDICATOR oRGANISM	R^2 (PEARSON COEFFICIENT)	P-VALUE
Aerobic Plate Count	0.010	0.011
Enterobacteriaceae	0.003	0.158
Coliforms	0.001	0.373
E. coli	0.011	0.009

ATP and Glucose/Lactose residue swab readings throughout unit operations. The obtained readings for ATP and glucose/lactose residue swabs on the different food contact surfaces are described by unit operation in Table 4. Concerning the ATP rapid test, the sorting and packing steps, both part of the dry area, showed the lowest and highest RLU mean values respectively. The results for the glucose/lactose residue tests were expressed as percentages of "fail" or "pass" for hygiene surfaces. The unit operations that presented the greatest percentage of "failed" surface hygiene were sorting and packing. Unlike the ATP test, the wet area showed more "pass" results when Glucose/lactose swabs were tested.

Table 4. Rapid test readings at each unit operation

UNIT OPERATION	N[A]	ATP TEST	GLUCOSE/ LACTOSE RESIDUE TEST	
		Mean ± Std Dev (Log RLU/100 cm^2)	**% Pass**	**% Fail**
Washing	59	2.28 ± 0.83 (ab)[B]	66.1	33.9
Washing/sanitizing /rinsing	83	2.27 ± 0.70 (ab)	63.9	36.1
Fan drying	75	2.09 ± 0.69 (ab)	60.0	40.0
Wax coating	51	2.38 ± 0.81 (ab)	52.9	47.1
Tunnel drying	78	2.19 ± 0.78 (ab)	38.5	61.5
Sorting	236	2.08 ± 0.97 (b)	22.9	77.1
Packing	77	2.48 ± 0.86 (a)	27.3	72.7

[A] Number of samples
[B] Means within a column followed by different letters are significantly different ($p \leq 0.05$)

Association of the Glucose/Lactose residue test with different populations of indicator organisms. The APC population was significantly higher when the test for surface hygiene failed. The population dropped significantly to reach a passing level on this test (Table 5). However, the test did not detect significant differences in the populations of *Enterobacteriaceae*, coliforms, and *E. coli* populations with failing and passing scores.

Table 5. Association between indicator organism populations with Glucose/Lactose residue test

MEAN ± STD DEV OF INDICATOR ORGANISM POPULATIONS (LOG CFU/100 CM^2)			
Indicator organisms	Pass (n=269)	Fail (n=390)	p-value
Aerobic Plate Count	2.91 ± 1.06	3.08 ± 0.84	0.031*
Enterobacteriaceae	1.25 ± 1.26	1.13 ± 1.13	0.219
Coliforms	0.98 ± 1.15	0.89 ± 1.08	0.341
E. coli	0.20 ± 0.42	0.19 ± 0.42	0.865

*Significant difference ($\alpha < 0.05$)

Association between traditional detection of Listeria spp. and rapid tests. Table 6 shows that ATP test readings were not statistically different when comparing both positive and negative detections of *Listeria* spp. ($p > 0.05$). Regarding Glucose/Lactose swabs, the percentage of sites that presented a "pass" result was higher (66.7%) than the percentage of sites with a "failed" result (33.3%), where *Listeria* spp. were detected as positive. However, it is important to consider that the number of positive samples for *Listeria* spp. was low (n=7).

Association between traditional detection of Listeria spp. and populations of indicator organisms. Table 6 also shows that mean populations of APC, *Enterobacteriaceae,* coliforms, and *E. coli,* were not statistically different when comparing both positive and negative detections of *Listeria* spp. ($p > 0.05$). However, it is important to consider that the number of positive samples for *Listeria* spp. was low (n=7).

Table 6. Association between indicator organism populations, and rapid tests with the detection of *Listeria* spp.

		Detection of *Listeria* spp.		p-value
		Positive (n=7) [A]	Negative (n=740)	
Indicator organisms (Log CFU/100 cm²) Mean ± Std Dev	APC	3.1 ± 1.4	3.0 ± 0.9	0.87
	Enterobacteriaceae	1.4 ± 1.4	1.2 ± 1.2	0.57
	Coliforms	1.2 ± 1.1	0.9 ± 1.1	0.47
	E. coli	0.1 ± 0.0	0.2 ± 0.4	0.44
Rapid tests	ATP (Log RLU/100cm²) Mean ± Std Dev	2.6 ± 0.7	2.2 ± 0.9	0.22
	Glucose/lactose residue swab	Pass: 66.7% Fail: 33.3%	Pass: 40.2% Fail: 59.8%	ND [B]

[A] Number of samples
[B] Not determined

Discussion

One of the objectives of this study was to evaluate the populations of APC, *Enterobacteriaceae,* coliforms, and *E. coli* at the different unit operations within an apple packinghouse after cleaning and sanitation procedures. For APC populations, means varied from 2.7 to 3.3 log CFU/100 cm^2. Unit operations in both wet and dry areas showed significantly higher counts of these indicator organisms. In previous studies, where food contact surfaces were evaluated after cleaning and sanitization procedures, similar values of APC mean populations were found. APC mean counts of 3.4 to 3.5 log CFU/100 cm^2 were obtained on food contact surfaces in a facility that processed fresh-cut carrots and lettuce (Lehto et al., 2011), and 2.1 to 4.6 log CFU/100 cm^2 on raw vegetable and meat preparation surfaces in a university canteen (Osimani et al., 2014).

The lower mean values obtained after the washing/sanitizing/rinsing step for *Enterobacteriaceae* populations, except for the tunnel drying unit operation, could be explained by the fact that bacteria belonging to the *Enterobacteriaceae* family, which are part of the regular microbiota on apples (Wassermann et al., 2019), are easily inactivated by chemicals used for sanitation purposes (Kornacki et al., 2015). Because coliforms and *E. coli* populations represent sub-populations of the larger *Enterobacteriaceae* family, the total *Enterobacteriaceae* population is expected to be higher than either of the sub-populations (Baylis et al, 2011). Therefore, it was reasonably foreseeable that this relationship was also observed in this study. Other evaluations of *Enterobacteriaceae* populations on food contact surfaces in food manufacturing environments showed higher counts with 3 to 3.3 log CFU/100 cm^2 reported in Finnish vegetable processors (Lehto et al., 2011), and 2.1 to 2.5 log CFU/100 cm^2 observed in US meat processors (Gómez et al., 2012). However, these results could be explained by the nature of the vegetable and meat product growing/handling environment, in that these commodities are commonly associated with soil and/or fecal contamination, in contrast to the tree fruit packing environment.

Lower coliform populations during sorting and packing (0.6 and 0.8 log CFU/100 cm^2, respectively) may be attributed to the removal of potential sources of coliforms that come with the fruit from the orchards within the wet area. Thus, lower carry-over after a sanitation event. In contrast to our findings, Williamson et al., (2018), evaluated automated sorting systems surfaces during peach packing and reported a higher coliform population mean of 2.9 log CFU/100 cm^2 after sanitation procedures. According to the authors, this value was expected since it represented natural microbiota present on peach fruits, which was also evaluated. Also, the difference of values could be explained

by commodity-specific factors, specifically that unlike peaches, the apple surface is smoother, has a natural wax layer, and is less prone to punctures. Hence, apples may carry a smaller microbial load than peaches. In another study in bell pepper packinghouses, a similar mean value of coliforms of 0.6 ± 0.2 log CFU/100 cm^2 was found on food contact surfaces of equipment such as unloading ramp, roller, conveyor belt and packing bin (Soto-Beltran et al., 2015). Regarding *E. coli,* population means were low throughout all unit operations (0.2 to 0.3 log CFU/100 cm^2). *E. coli* is highly related to and used as an indicator for fecal contamination and is regularly employed for water quality standards. In spite of all the tested packinghouses using recirculated water in the dump tank, no higher population was found at this unit operation (the washing step). Indeed, the use of sanitizers, such as chlorine and PAA, in the dump tank could explain this result (Pietrysiak et al., 2019). Similarly, no detectable *E. coli* contamination of the water used for wash produce, was observed by Ailes et al., (2008), who evaluated microbial concentrations on different types of produce during post-harvest processing. Besides, tree fruit traditionally has low populations of *E. coli*. Since fruit is grown on trees above ground, apples are rarely in contact with soil. Therefore, a lower introduction of this microorganism should be seen during tree fruit packing. Duffy et al., (2005), evaluated *E. coli* populations in orange, parsley, and cantaloupe in the field, finding that the only commodity where *E. coli* was not detected was oranges (also a tree fruit).

Moore (2003) did a review from different authors and countries of recommended microbiological limits for acceptable general microbial counts (not a specific type of microorganism) on food contact surfaces. Results for an "appropriate" hygienic surface ranged from < 2.3 to 5 Log CFU/100 cm^2 for different types of industries. No specifications for the fresh produce industry were included in this analysis. Additionally, no US regulatory agency currently provides specific standards to define acceptable levels of microbial loads on food contact surfaces. Any such standards should also address differences that may arise given the sampling method employed, surface area sampled, type of product that has been processed, and the processing step at which the samples have been taken. Therefore, it is suggested to use populations of indicator organisms for trend analysis to compare samples that are routinely taken under the same conditions. It is recommended that each facility construct its own thresholds for accepting or rejecting the cleanliness of a surface based upon target standards obtained after a validated sanitation procedure that has been duly and fully performed (Blackburn, 2003; Forsythe, 2000).

The second objective of this research project was to evaluate the association between rapid tests with populations of indicator organisms and the detection of *Listeria* spp. Even though the coefficients of determination (r^2) between ATP assay with APC and *E. coli* populations were statistically significant ($p<0.05$), ATP values explained less than 1% of the variance in APC and *E. coli* counts, suggesting that, while a weak positive correlation was found, ATP values alone do not provide significant predictive power for APC and *E. coli* populations. Additionally, no statistically significant correlation was found between the ATP assay and either *Enterobacteriaceae* ($p=0.17$) or coliform ($p=0.38$) populations.

The lack of association observed between the quantification of indicator organisms via the ATP test and the actual populations could be attributed to different factors. ATP is very sensitive to low levels of residual matter on a surface; however, it is not capable of distinguishing if the contamination on the surface originates from microbial or non-microbial sources (Moore, 2003). The amount of ATP varies based upon the type of microorganisms present on the surface. Various studies have shown different amounts of ATP in bacteria, yeast, and fungal spores (Shama and Malik, 2013). Furthermore, ATP tests do not detect whether cells present on the surface are dead or alive (Alfa et al., 2015). Factors such as nutrient level, environmental stress level, and the stage of growth are also known to influence the amount of ATP present (Betts and Blackburn, 2009; Shama and Malik, 2013). Additionally, ATP quantity differs depending on the type of product. Raw fruits and vegetables typically contain a higher amount of ATP compared to dry products (Griffith, 2005). Other factors affecting ATP readings include the use of sanitizers and cleansers (Green et al., 1999), the state of the surface (wet or dry) (Davidson et al., 1999), presence of salts and metal ions that affect the stability of

the enzyme luciferase within the reagent of the ATP test (Moore, 2003). In order to establish acceptance limit levels for ATP values, similar factors, as discussed for populations of indicator organisms need to be considered.

Many studies have shown no or low associations between APC populations and ATP quantities, including in retail delis (r^2=0.10) (Hammons et al., 2015), milking equipment such as bulk tank (r^2=0.12) (Vilar et al., 2008), stainless steel milk contact surfaces (Costa et al., 2006), hospital environments (r^2=0.09) (Raia et al., 2018), (r^2=0.29) (Amodio et al., 2014), and on hands and surfaces in the home (r^2=-0.001, and 0.002 respectively) (Larson et al., 2003).

In contrast, studies have reported strong linear positive correlations between APC populations with ATP, including those on plastic cutting boards (r^2=0.97) (Leon and Albrecht, 2007), whole unwashed cantaloupe surfaces (r^2=0.995) (Ukuku et al., 2001), and in retail delis (Hammons et al., 2015). However, the detectable sensitivity threshold ranged only from 3.6 to 5.6 log CFU/100 cm^2 for the first study, and a minimum detectable level of 6 log CFU/100 cm^2 and 3 log CFU/sponge for the second and third study, respectively. These APC populations were significantly higher values than the ones obtained in this study. Also, Ukuku et al., (2001), utilized ATP extractants such as Tris-EDTA rather than commercial ATP swabs. Another study conducted to evaluate the correlation between *E. coli* populations and ATP reported that a minimum concentration of 4 log CFU/100 cm^2 of *E. coli* was needed in either wet or dry surfaces to be detectable by an ATP test (Davidson et al., 1999). In addition, one of the limitations of the previous studies (Davidson et al., 1999; Leon and Albrecht, 2007), is that they were performed under laboratory conditions. In real life, situations involving microbial populations at these concentrations are unlikely to occur since microorganisms are not present as pure culture in the environment (Davidson et al., 1999; Turner et al., 2010).

The association between the glucose/residue test swab and APC populations could be explained by the fact that glucose is an energy source and the major nutrient required for microorganism metabolism (Galant et al., 2015). While significantly different APC values (i.e. higher APC counts for a 'failed' hygienic surface), these values, from a practical standpoint, may not represent a numerical difference when establishing thresholds for acceptance or rejection. A study to evaluate cleanliness in cattle barns was conducted using glucose/lactose residues swabs. No difference between outcomes for a 'clean' or 'dirty' surface was found (Kymäläinen and Kuisma, 2016). The authors analyzed different cattle barn soils, which contained different nutrients including sugars such as carrot juice and milk, nevertheless the color of the soil could have interfered with the color detection of the test. Additionally, when assessing this type of rapid test, it is important to note that an absence of detectable sugar residues on a surface does not necessarily mean a clean surface, but rather that the residual contaminants were not present in levels high enough then the detection limit of the test (Schmitt and Moerman, 2016) or the contaminant did not contain sugar residues.

The packing unit operation showed one of the highest readings in both rapid tests: ATP (2.5 log RLU/100 cm^2) and glucose/lactose swab (72.7% of "failed" hygienic surface). These values may be due to physical contaminants, such as stickers and labels, that are not easily removed from belts and packing tables, making cleaning procedures harder to perform. Furthermore, the dry area was not cleaned and sanitized as often as the wet area in order to avoid water residues on the dry side of the plant. However, the dry area did not present higher microbial counts of APC, *Enterobacteriaceae*, coliforms, and *E. coli* than the wet side. Thus, it has been hypothesized that since the fruit has already been sanitized within the wet area, less carryover of bacteria was taken to the dry area.

Lastly, the lack of association between both rapid tests and populations of indicator organisms with the positive detection of *Listeria* spp. is supported by previous data. No associations between the detection of *Listeria monocytogenes* with APC (D'Amico et al., 2008; Jackson et al., 2012; Van Kessel et al., 2004), *Enterobacteriaceae* (Jackson et al., 2012), coliforms (Jackson et al., 2012; Martin et al., 2016) and *E. coli* (Jackson et al., 2012) populations have been reported in the dairy industry. APC is not considered an indicator of food safety because it does not specify the presence of any pathogen (Ryser and Schuman, 2015). It has been suggested that the presence of organisms from the *Enterobacteriaceae* family including coliforms and generic *E. coli*, are not suitable to assess the

presence of *Listeria* spp. since these species are more resistant to environmental factors than enteric pathogens such as salmonellae*, Shigella dysenteriae,* or pathogenic *E. coli* (Baylis et al., 2011; Tortorello, 2003). However, studies have also observed positive correlations between the growth of *L. monocytogenes* and APC in other environments such as minimally processed fresh endive (Carlin et al., 1995), and retail delis (Hammons et al., 2015), likely due to similar favorable growing conditions for mesophilic bacteria and *L. monocytogenes* (Carlin et al., 1995).

The results of this study suggest that apple packinghouses should use both rapid tests and traditional microbiological methods for indicator organism populations when assessing cleaning and sanitation practices. Rapid tests are valuable for monitoring residual matter on a surface, thus validating the efficacy of cleaning procedures prior to sanitation. However, to validate sanitation practices, traditional microbiological methods are still needed. These findings can help guide packinghouses when establishing microbiological thresholds of indicator organisms (e.g. APC, *Enterobacteriaceae,* coliforms and *E. coli)*. Also, to assess a trend analysis of microbial populations or rapid test readings over a packing season. Future studies should seek to improve dry cleaning and sanitation methods for the dry area. Moreover, it is important to emphasize that a risk of *L. monocytogenes* harborage in apple packinghouses may not be detected when utilizing indicator organisms other than *Listeria* spp. as demonstrated through these findings.

Acknowledgments

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Citations

Ailes, E.C., Leon, J.S., Jaykus, L.A., Johnston, L.M., Clayton, H.A., Blanding, S., Kleinbaum, D.G., Backer, L.C., Moe, C.L., 2008. Microbial concentrations on fresh produce are affected by postharvest processing, importation, and season. J. Food Prot. 71, 2389–2397. https://doi.org/10.4315/0362-028X-71.12.2389

Alfa, M.J., Olson, N., Murray, B.L., 2015. Adenosine tri-phosphate (ATP)-based cleaning monitoring in health care: how rapidly does environmental ATP deteriorate? J. Hosp. Infect. 90, 59–65. https://doi.org/10.1016/j.jhin.2015.01.020

Amodio, E., Cannova, L., Villafrate, M.R., Merendino, A.M., Aprea, L., Calamusa, G., 2014. Comparison of ATP bioluminescence and aerobic bacterial count for evaluating surface cleanliness in an Italian hospital. J. Occup. Environ. Hyg. 11, D23–D27. https://doi.org/10.1080/15459624.2013.852281

Angelo, K., Conrad, A.R., Saupe, A., Dragoo, H., West, N., Sorenson, A., Barnes, A., Doyle, M., Beal, J., Jackson, K.A., Stroika, S., Tarr, C., Kucerova, Z., Lance, S., Gould, L.H., Wise, M., Jackson, B.R., 2017. Multistate outbreak of *Listeria monocytogenes* infections linked to whole apples used in commercially produced, prepackaged caramel apples: United States, 2014-2015. Epidemiol. Infect. 145, 848–856. https://doi.org/10.1017/S0950268816003083

Azizkhan, Z., 2014. Comparison between ATP bioluminescence technique and traditional microbiological method to detect contamination within food facilities in Saudi Arabia (Jiddah). Public Heal. Front. 3, 11–18. https://doi.org/10.5963/phf0301003

Baylis, C., Uyttendaele, M., Joosten, H., Davies, A., 2011. The *Enterobacteriaceae* and their significance to the food industry, International Life Sciences Institute. Brussels, Belgium.

Betts, R., Blackburn, C., 2009. Detecting pathogens in food, in: Blackburn, C., McClure, P. (Eds.), Foodborne Pathogens: Hazards, Risk Analysis and Control. Woodhead Publishing Limited, Boca Raton, pp. 17–65.

Blackburn, C., 2003. Microbiological analysis and food safety management: GMP and HACCP systems. Detect. Pathog. food 3–19.
Bott, A., 1998. Electrochemical methods for the determination of glucose. Curr. Sep. 17, 25–31.
Caputo, P., Ferri, E.N., Girotti, S., Gozzi, S., Saracino, P., 2011. Application of luminescent ATP rapid checks at ready-to-eat foods producing plant. Czech J. Food Sci. 29, 382–390. https://doi.org/10.17221/197/2010-CJFS
Carlin, F., Nguyen-the, C., da Silva, A.A., 1995. Factors affecting the growth of *Listeria monocytogenes* on minimally processed fresh endive. J. Appl. Bacteriol. 78, 636–646. https://doi.org/10.1111/j.1365-2672.1995.tb03110.x
Carrascosa, C., Saavedra, P., Millán, R., Jaber, J.R., Pérez, E., Grau, R., Raposo, A., Mauricio, C., Sanjuán, E., 2012. Monitoring of cleanliness and disinfection in dairies: Comparison of traditional microbiological and ATP bioluminescence methods. Food Control 28, 368–373. https://doi.org/10.1016/j.foodcont.2012.05.001
Centers for Disease Control and Prevention (CDC), 2015. Multistate outbreak of listeriosis linked to commercially produced, prepackaged caramel apples made from Bidart Bros. apples (Final update). URL https://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/index.html (accessed 10.10.19).
Costa, P.D., Andrade, N.J., Brandão, S.C.C., Passos, F.J.V., Soares, N.D.F.F., 2006. ATP-bioluminescence assay as an alternative for hygiene-monitoring procedures of stainless steel milk contact surfaces. Brazilian J. Microbiol. 37, 345–349. https://doi.org/10.1590/S1517-83822006000300026
D'Amico, D.J., Groves, E., Donnelly, C.W., 2008. Low incidence of foodborne pathogens of concern in raw milk utilized for farmstead cheese production. J. Food Prot. 71, 1580–1589. https://doi.org/10.4315/0362-028X-71.8.1580
Davidson, C.A., Griffith, C.J., Peters, A.C., Fielding, L.M., 1999. Evaluation of two methods for monitoring surface cleanliness - ATP bioluminescence and traditional hygiene swabbing. Luminescence 14, 33–38.
Dostálek, P., Brányik, T., 2005. Prospects for rapid bioluminescent detection methods in the food industry - A review. Czech J. Food Sci. 23, 85–92.
Duffy, E.A., Lucia, L.M., Kells, J.M., Castillo, A., Pillai, S.D., Acuff, G.R., 2005. Concentrations of *Escherichia coli* and genetic diversity and antibiotic resistance profiling of *Salmonella* isolated from irrigation water, packing shed equipment, and fresh produce in Texas. J. Food Prot. 68, 70–79. https://doi.org/10.4315/0362-028X-68.1.70
Forsythe S.J, 2000. The microbiology of safe food, First. ed. Blackwell Science Ltd, London.
Galant, A.L., Kaufman, R.C., Wilson, J.D., 2015. Glucose: detection and analysis. Food Chem. 188, 149–160. https://doi.org/10.1016/j.foodchem.2015.04.071
Gómez, D., Ariño, A., Carramiñana, J.J., Rota, C., Yangüela, J., 2012. Sponge versus mini-roller for the surface microbiological control of *Listeria monocytogenes*, total aerobic mesophiles and *Enterobacteriaceae* in the meat industry. Food Control 27, 242–247. https://doi.org/10.1016/j.foodcont.2012.03.031
Green, T.A., Russell, S.M., Fletcher, D.L., 1999. Effect of chemical cleaning agents and commercial sanitizers on ATP bioluminescence measurements. J. Food Prot. 62, 86–90. https://doi.org/10.4315/0362-028X-62.1.86
Griffith, C., 2005. Improving surface sampling and detection of contamination, in: Lelieveld, H.L.., Mostert, M., Holah, J. (Eds.), Handbook of Hygiene Control in the Food Industry. Woodhead Publishing Limited, pp. 588–618. https://doi.org/10.1533/9781845690533.3.588
Griffiths, M., 1997. Rapid microbiological methods with Hazard Analysis Critical Control Point. J. AOAC Int. 80, 1143–1150.
Hammons, S.R., Stasiewicz, M.J., Roof, S., Oliver, H.F., 2015. Aerobic plate counts and ATP levels correlate with *Listeria monocytogenes* detection in retail delis. J. Food Prot. https://doi.org/10.4315/0362-028X.JFP-14-500

Hitchins, A.D., Jinneman, K., Chen, Y., 2017. Detection of *Listeria monocytogenes* in foods and environmental samples, and enumeration of *Listeria monocytogenes* in foods, in: U.S. Food and Drug Administration (Ed.), Bacteriological Analytical Manual.

Jackson, E.E., Erten, E.S., Maddi, N., Graham, T.E., Larkin, J.W., Blodgett, R.J., Schlesser, J.E., Reddy, R.M., 2012. Detection and enumeration of four foodborne pathogens in raw commingled silo milk in the United States. J. Food Prot. 75, 1382–1393. https://doi.org/10.4315/0362-028X.JFP-11-548

Kornacki, J.L., Gurtler, J.B., Stawick, B.A., 2015. *Enterobacteriaceae*, coliforms, and *Escherichia coli* as quality and safety indicators, in: Salfinger, Y., Tortorello, M.L. (Eds.), Compendium of Methods for the Microbiological Examination of Foods. American Public Health Association, Washington,DC, pp. 103–120.

Kymäläinen, H.R., Kuisma, R., 2016. Detection methods for cleanness in cattle barns. Agric. Eng. Int. CIGR J. 18, 11–24.

Larson, E.L., Aiello, A.E., Gomez-Duarte, C., Lin, S.X., Lee, L., Della-Latta, P., Lindhardt, C., 2003. Bioluminescence ATP monitoring as a surrogate marker for microbial load on hands and surfaces in the home. Food Microbiol. 20, 735–739. https://doi.org/10.1016/S0740-0020(03)00041-8

Lehto, M., Kuisma, R., Määttä, J., Kymäläinen, H.R., Mäki, M., 2011. Hygienic level and surface contamination in fresh-cut vegetable production plants. Food Control 22, 469–475. https://doi.org/10.1016/j.foodcont.2010.09.029

Leon, M.B., Albrecht, J.A., 2007. Comparison of adenosine triphosphate (ATP) bioluminescence and aerobic plate counts (APC) on plastic cutting boards*. J. Foodserv. 18, 145–152. https://doi.org/10.1111/j.1745-4506.2007.00060.x

Martin, N.H., Trmcic, A., Hsieh, T.H., Boor, K.J., Wiedmann, M., 2016. The evolving role of coliforms as indicators of unhygienic processing conditions in dairy foods. Front. Microbiol. 7, 1–8. https://doi.org/10.3389/fmicb.2016.01549

Moore, G., 2003. Rapid methods for assessing surface cleanliness within the food industry: their evaluation, design and comparison to traditional techniques. University of Wales.

Moore, G., Griffith, C., 2002. A comparison of traditional and recently developed methods for monitoring surface hygiene within the food industry: An industry trial. Int. J. Environ. Health Res. https://doi.org/10.1080/0960312021000056429

Moore, G., Griffith, C., 2001. A comparison of traditional and recently developed methods for monitoring surface hygiene within the food industry: A laboratory study. Dairy, Food Environ. Sanit. 21, 478–488. https://doi.org/10.1080/0960312021000056429

Omidbakhsh, N., Ahmadpour, F., Kenny, N., 2014. How reliable are ATP bioluminescence meters in assessing decontamination of environmental surfaces in healthcare settings? PLoS One 9, 15–19. https://doi.org/10.1371/journal.pone.0099951

Osimani, A., Garofalo, C., Clementi, F., Tavoletti, S., Aquilanti, L., 2014. Bioluminescence ATP monitoring for the routine assessment of food contact surface cleanliness in a university canteen. Int. J. Environ. Res. Public Health 11, 10824–10837. https://doi.org/10.3390/ijerph111010824

Pietrysiak, E., Smith, S., Ganjyal, G.M., 2019. Food safety interventions to control *Listeria monocytogenes* in the fresh apple packing industry: A review. Compr. Rev. Food Sci. Food Saf. 0, 1–22. https://doi.org/10.1111/1541-4337.12496

Raia, D.D., Cannova, L., Provenzano, S., Santangelo, O.E., Piazza, D., Alagna, E., Bonanno, V., Aprea, L., Firenze, A., 2018. Comparison between adenosine triphosphate bioluminescence and aerobic colony count to assess surface sanitation in the hospital environment. Epidemiol. Biostat. Public Heal. 15, e12710-1-e12710-4. https://doi.org/10.2427/12710

Ryser, E., Schuman, J., 2015. Mesophilic aerobic plate count, in: Salfinger, Y., Tortorello, M.L. (Eds.), Compendium of Methods for the Microbiological Examination of Foods. American Public Health Association, Washington,DC, pp. 95–101.

Schmitt, R., Moerman, F., 2016. Validating cleaning systems, in: Handbook of Hygiene Control in

the Food Industry. Elsevier Inc., pp. 587–601. https://doi.org/10.1016/B978-0-08-100155-4.00038-8
Shama, G., Malik, D.J., 2013. The uses and abuses of rapid bioluminescence-based ATP assays. Int. J. Hyg. Environ. Health 216, 115–125. https://doi.org/10.1016/j.ijheh.2012.03.009
Soto-Beltran, M., Castro-del Campo, N., Campos-Sauceda, J., Avena-Bustillos, R., Chaidez, C., 2015. Prevalence of *Salmonella*, *Escherichia coli* and coliforms on bell peppers from the field to the packing house process. African J. Agric. Res. 9, 718–724. https://doi.org/10.5897/A
Tortorello, M.L., 2003. Indicator organisms for safety and quality - Uses and methods for detection: Minireview. J. AOAC Int. 86, 1208–1217.
Turner, D.E., Daugherity, E.K., Altier, C., Maurer, K.J., 2010. Efficacy and limitations of an ATP-based monitoring system. J. Am. Assoc. Lab. Anim. Sci. 49, 190–195.
U.S Food and Drug Administration, 2017. Control of *Listeria monocytogenes* in Ready-To-Eat foods : Guidance for industry. URL https://www.fda.gov/media/102633/download#page=39%0A (accessed 9.18.19).
Ukuku, D.O., Pilizota, V., Sapers, G.M., 2001. Bioluminescence ATP assay for estimating total plate counts of surface microflora of whole cantaloupe and determining efficacy of washing treatments. J. Food Prot. 64, 813–819. https://doi.org/10.4315/0362-028X-64.6.813
Van Kessel, J.S., Karns, J.S., Gorski, L., McCluskey, B.J., Perdue, M.L., 2004. Prevalence of salmonellae, *Listeria monocytogenes*, and fecal coliforms in bulk tank milk on US dairies. J. Dairy Sci. 87, 2822–2830. https://doi.org/10.3168/jds.S0022-0302(04)73410-4
Vilar, M.J., Rodríguez-Otero, J.L., Diéguez, F.J., Sanjuán, M.L., Yus, E., 2008. Application of ATP bioluminescence for evaluation of surface cleanliness of milking equipment. Int. J. Food Microbiol. 125, 357–361. https://doi.org/10.1016/j.ijfoodmicro.2008.04.024
Wassermann, B., Müller, H., Berg, G., 2019. An apple a day: Which bacteria do we eat with organic and conventional apples? Front. Microbiol. 10, 1–13. https://doi.org/10.3389/fmicb.2019.01629
Whiteley, G.S., Nolan, M., Fahey, P.P., 2018. Improving the reliability of adenosine triphosphate (ATP) testing in surveillance of food premises: A pilot study. J. Environ. Health 81, E1–E8.
Williamson, K., Pao, S., Dormedy, E., Phillips, T., Nikolich, G., Li, L., 2018. Microbial evaluation of automated sorting systems in stone fruit packinghouses during peach packing. Int. J. Food Microbiol. 285, 98–102. https://doi.org/10.1016/j.ijfoodmicro.2018.07.024

EXECUTIVE SUMMARY

Project Title: Utility of rapid tools to assess cleanliness in apple packinghouses

Key words: ATP, glucose/lactose residue, cleaning, sanitation, apple packing

Abstract

The 2014 listeriosis outbreak caused by caramel-coated apples was linked to apples cross-contaminated within an apple packing facility. This outbreak has increased the focus on effective cleaning and sanitation methods that must be validated and monitored during apple packing. Thus, rapid and reliable testing methods are necessary for assessing cleanliness in the apple packing industry. The objectives of this study were to assess the prevalence of common indicator organisms [Aerobic plate count (APC), *Enterobacteriaceae,* coliforms*, Escherichia coli*, and *Listeria* spp.] on food contact surfaces (zone 1) in apple packinghouses and to evaluate the utility and accuracy of currently used rapid tests (ATP and glucose/lactose residue swabs). Food contact surfaces were sampled over a 100 cm^2 area in five commercial apple packinghouses to evaluate populations of indicator organisms APC, *Enterobacteriaceae,* coliforms*, E. coli* (n=741), and rapid test readings (n=659). Petrifilm plates were used for the quantification of APC, *Enterobacteriaceae*, and coliform/*E. coli*. Rapid tests [ATP swabs (UltraSnap) and glucose/lactose residue swabs (SpotCheck Plus)] were processed on-site. A larger area (0.93 m^2) was sampled for the detection of *Listeria* spp. (n=747), following a modified protocol of the FDA's Bacteriological Analytical Manual method, and confirmed with PCR and gel electrophoresis via the *iap* gene. No significant association was found between either rapid test and populations of APC, *Enterobacteriaceae*, coliforms, *E. coli*, and *Listeria* spp. detection. However, recovery of APC (log CFU/100cm^2) was higher with a failed glucose/lactose residue swab surface hygiene result (3.1) than a passed result (2.9) ($p=0.03$).

Populations of APC, *Enterobacteriaceae,* and coliforms were significantly different at each unit operation during the packing process ($p \leq 0.05$). This study concluded that ATP and glucose/lactose residue rapid tests were poorly suited for determining microbial load since they were not related to populations of any common indicator organisms or the detection of *Listeria* spp. These findings emphasize the need to utilize a rapid test, which can be a good indicator of residual matter on a surface, along with traditional microbiological methods to assess cleaning and sanitation practices in apple packinghouses.

FINAL PROJECT REPORT **YEAR**: 2 of 2

PROJECT TITLE: Complying with the FMSA Preventive Controls for Human Food Rule

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COOPERATORS: Claudia Coles (WSDA), Ines Hanrahan (WTFRC) and Various Packing Houses (Stemilt Growers, Double Diamond Fruit, Borton Fruit, Crane and Crane, Allan Brothers, Kershaw, Washington Fruits, Cowiche, Blue Bird, McDougall & Sons Inc, Columbia Reach)

Budget: **Year 1:** $48,711 **Year 2**: $50,260

Other funding sources: The PI has some funds from the WSDA-SCBG program to support the one training on the PSFA-PCHF one day training. The event coordinator's (Cathy Blood) time will be covered through the WSDA grant.

Budget 1
Organization Name: WSU **Contract Administrator:** Katy Roberts
Telephone: 509-335-2885 **Email address:** arcgrants@wsu.edu

Item	2017	2018
Salaries	28,418	29,555
Benefits	2,580	2,683
Wages	7,261	7,552
Benefits	452	470
Training Materials	5,000	5,000
Supplies	2,000	2,000
Travel	3,000	3,000
Miscellaneous	$0	$0
Plot Fees	$0	$0
Total	48,711	50,260

Footnotes:
The majority of the funding requested is to cover graduate student wages. Funds are also requested for wages to support an undergraduate student to help with the project. Funds are requested to cover travel costs related to the project work, such as trips to the packing facilities in Wenatchee and Yakima for the work related to the first objective and for the trainings. Funds are also requested to cover supplies and training material costs that will be provided to the training attendees.

ORIGINAL OBJECTIVES

In this project, we proposed to conduct a thorough assessment of a range of apple packing lines and identify the common practices that can be improved. These assessments were further to be utilized to develop a model food safety plan for the apple packing process.

The specific objectives of the proposal are as detailed below:

1) Develop a thorough assessment of the current apple packing process and packing house environments.
2) Develop model food safety plans for apple packing processes to help comply with the FSMA-PCHF rule.
3) Summarize the peer-reviewed literature available on the different technology interventions that are currently used in different produce industries.
4) Offer one, 2.5-day training on the FSMA-PCHF rule with an emphasis on the apple packing process, and two additional, 1-day trainings, focused on the implementation of the FSMA-PCHF rule.

SIGNIFICANT FINDINGS

Assessment of current packing facility practices:

Most apple packing houses fall under the FSMA Produce Safety Rule; however, the customers often expect them to comply with Preventive Control for Human Food Rule, which requires the development and implementation of the food safety plan.

- The cleaning and sanitation of the packing line are of crucial importance. However, it is often difficult because of the insufficient amount of time and problems with the rotation of employees.
- The biggest challenges identified by food safety managers were;
    - Design of facility and equipment.
    - Limited time for cleaning and sanitizing due to the high production rate.
    - The availability of water and the amount of water that would be used to conduct a proper sanitation of the flume piping and pump systems.
    - Restricted capacities of wastewater allowed for the municipal sewage system.
    - Budget limitation.
    - Personnel unawareness and high turnover.

Food safety model plan development:

Model food safety plan for the apple packing house was developed.

- The observations of the current practices from the first objective were incorporated into this plan.
- A model food safety plan was shared, explained, and discussed with attendees of 1-day special FSMA-PCHF class (November 2nd 2018, and May 6th 2019).

Summary of literature on different interventions:

- *L. monocytogenes* is a persistent, highly pathogenic microorganism that can pose a high risk in fresh produce operations. Abundant amounts of water used during the apple packing process, presence of wet surfaces, and difficult to clean equipment provide ideal conditions for *Listeria* growth and numerous paths for produce contamination.
- Removal of bacteria from the surface of the apple is difficult due to its morphology. The irregular shape of apples and the presence of microstructures on the apple peel surface

facilitate bacterial attachment. Bacteria harbored in the microstructures may be protected from cleaning interventions.

- Attacking bacteria by several different mechanisms through hurdle technology may help to improve the apple decontamination efficiency.
- Significant research is still needed for the development of effective strategies for reducing microbial loads on fresh apples. Critical aspects that should be considered include morphological characteristics of apples, conditions, and scale of the packing process, and influence of the interventions on apple quality.

RESULTS & DISCUSSION

Objective #1: Develop a thorough assessment of the current apple packing process and packing house environments.

The food safety practices vary significantly across the industry. Apple packing houses across the WA State are very motivated to improve food safety in their facilities. Substantial investment in food safety system was observed in recent years, and it continues to be one of the priorities in the management and development of apple packing facilities.

The cleaning and sanitation of the packing line are of crucial importance. However, it is often difficult because of the difficulty of cleaning equipment, an insufficient amount of time allowed for cleaning and sanitation, and problems with rotation of employees. Other challenges reported by food safety managers are a limited amount of water used to conduct proper sanitation of the flume piping and pump systems. Recycled systems are not designed to perform adequate sanitation. Restricted capacities of wastewater allowed for the municipal sewage system.

Currently used and potential solutions for improving food safety during apple packing process, based on the assessment of the current apple packing process and literature review are summarized in Table 1.

Table 1. Currently used and potential solutions for improving food safety during apple packing process.

	Potential solution for reducing microbial load
Post-harvest fungicide treatment (drenching/fogging)	● Use of fogging method rather than drenching to avoid reuse of fungicide solution and minimize the possibility of cross-contamination of apples. Alternatively, the use of a fungicide solution does not support pathogen growth (Gomba et al., 2017; Guan et al., 2001; Ng, Fleet, and Heard, 2005).
Dump tank and flumes water treatments	● Use of double dump tank. The role of the first tank is to remove most of the debris and organic matter from the surface of the bins. It will greatly decrease the amount of organic matter, which causes a significant decline in sanitizer concentration. Thus, the concentration of sanitizer in the second tank and flumes will be more stable and easier to control (Luo et al., 2011).

	● Aeration in dump tank to help with total apple saturation while in the dump tank ● Separation of dump tank from flumes. ● Maintaining the quality of the water by use of sanitizing agents such as: chlorine, PAA, or EOW combined with surfactant. ● Proper monitoring system (Suslow, 2004).
Brush bed: Fruit may be treated with soaps and/or sanitizers	● Application of surfactant combined with sanitizing solution. Brushing and rotating apples can help evenly cover apples with cleaning solution, increase detachment of microorganisms. Use of sanitizer ensure bacteria deactivation and prevent contamination of brushes and cross-contamination of subsequently washed apples. ● Steam cleaning of wax brushes.
Wax coating: Fruit coated with food grade wax	● Application of wax with antimicrobial treatment (Jo et al. 2014)
Drying	● Automated dryer cleaning system to allow for more frequent dryer cleaning.
Personnel	● Frequent personnel training on understanding basics of food safety and personal hygiene.
Cleaning and sanitation	● Allowing enough time for sanitation crew to perform adequate cleaning, especially in Zone 1 and 2. ● Reward system for sanitation crew, recognition of importance of their work.
Packing plant environment	● Automatic door foamers. ● Forklifts designed only to the specific areas (i.e., forklifts used in cold room do not enter the packing area, forklifts in wet area are not used in the dry area). ● Drain system accessible for cleaning
Other	Traceability system - geolocation system, room identification, specific lot and grower tagging. Efficient environmental monitoring program - seek and destroy approach. Support from chemicals and sanitation systems suppliers (often they provide trainings, ensure calibration and maintenance of the equipment, and provide information about new food safety interventions)

Objective #2: Develop a model food safety plan for apple packing processes to help comply with the FSMA Preventive Controls for Human Food Rule.

Most of apple packing houses fall under the FSMA **Product Safety Rule,** which does not require the implementation of a food safety plan; however, the customers often expect them to comply with **Preventive Control for Human Food Rule,** which in turn requires a food safety plan.

Required or not, a food safety plan can help facilities in managing the food safety system and ensuring the safety of the final product.
In this objective, we aimed to develop a model food safety plan that can be used by industry as an example, guide in developing their proper food safety plans. The food safety plan is based on hazard analysis for each step of the apple packing process. It is crucial to recognize all potential risks that can lead to contamination of the final product and identify the appropriate preventive controls for managing these hazards. In the case of the apple packing process majority of the hazards can be addressed by good manufacturing practices (GMPs) and sanitation preventive controls.
The drafted model food safety plan was reviewed by industry and by regulators (FSPCA), and based on obtained comments, the final version of the model food safety plan was developed.

Objective #3: Summarize the peer-reviewed literature available on the different technology interventions that are currently used in different produce industries.

Current FSMA-PCHF regulations require interventions in food safety to be based on scientific data. It is essential for the apple packing industry to find appropriate peer-reviewed literature to support the use of these technologies. This review provides the fresh apple packing industry with peer-reviewed literature on the effectiveness of these technologies. Based on the presented information, apple packers can make decisions on the use of different interventions. It can also aid in developing food safety plans.
The review includes supplementary information such as the possible routes of produce contamination, bacteria attachment, bacteria resistance mechanisms, and the mode of action of the common decontamination agents. This information can help to better understand the food safety risks, how cleaning treatments work, and why bacteria removal is so important.
Current methods of produce decontamination can be divided into chemical, physical, and biological methods that can be used individually or in combination. Scientific investigations on the efficacy of various decontamination methods have been conducted by numerous research groups. However, there is still a need for studies that will evaluate the suitability of a given method for application in the packing process of apples or other types of produce.
Lack of standard methodology for evaluating the efficacy of antimicrobial agents on fresh produce, laboratory-scale experiments, as well as differences between fresh produce morphologies makes it difficult to compare the results between studies and hard to predict their effectiveness in the industrial settings. A standardized methodology for the validation of the antimicrobial potential of sanitizing agents would facilitate a more objective and standardized evaluation.
Manuscript titled, "*Food Safety Interventions to Control Listeria Monocytogenes in Fresh Apple Packing Industry: A Review"* has been accepted for publication in the Comprehensive Reviews in Food Science and Food Safety Journal (Pietrysiak et al., 2019).

Objective #4: Offer one, 2.5-day training, on the FSMA-PCHF rule with an emphasis on the apple and pear packing process, and two additional, 1-day trainings, focused on the implementation of the FSMA-PCHF rule.

We offered 2.5-day training as a part of the WSDA-SCBG in 2017 and two 1-day trainings on implementation of the FSMA-PCHF rule with an emphasis on the apple packing process (November 2nd in Yakima and May 17th in Wenatchee). The 1-day training has been designed specifically for the attendees who have gone through the FSPCA standard Preventive Controls for Human Food (PCHF) Course. During the training, we shared with participants the food safety plan model, draft of literature review, and presentation slides. The training was well received, with full attendance and great feedback from attendees. During the training, we were able to

assist some of the packers with their food safety plans and answer questions related to a different aspect of food safety.

REFERENCES:

Gomba, A., Chidamba, L., and Korsten, L. (2017). Viable microbial loads on citrus carpoplane during packhouse processing and survival of foodborne pathogens in reconstituted postharvest fungicides. Journal of Food Safety.

Guan, T. Y., Blank, G., Ismond, A., and Van Acker, R. (2001). Fate of foodborne bacterial pathogens in pesticide products. Journal of the Science of Food and Agriculture. 81: 503-512.

Jo, W.-S., Song, H.-Y., Song, N.-B., Lee, J.-H., Min, S. C., and Song, K. B. (2014). Quality and microbial safety of 'Fuji'apples coated with carnauba-shellac wax containing lemongrass oil. LWT-Food Science and Technology. 55: 490-497.

Luo, Y., Nou, X., Yang, Y., Alegre, I., Turner, E., Feng, H., Abadias, M., and Conway, W. (2011). Determination of free chlorine concentrations needed to prevent Escherichia coli O157: H7 cross-contamination during fresh-cut produce wash. Journal of Food Protection. 74: 352-358.

Pietrysiak, E., Smith, S., & Ganjyal, G. M. (2019). Food safety interventions to control Listeria monocytogenes in the fresh apple packing industry: a review. Comprehensive Reviews in Food Science and Food Safety, 18(6), 1705-1726.

Ng, P. J., Fleet, G. H., and Heard, G. M. (2005). Pesticides as a source of microbial contamination of salad vegetables. International Journal of Food Microbiology. 101: 237-250.

Suslow, T. V. (2004). Oxidation-reduction potential (ORP) for water disinfection monitoring, control, and documentation. University of California. Division of Agriculture and Natural Resources (2004) Publication 8149.

EXECUTIVE SUMMARY

Complying with the FMSA Preventive Controls for Human Food Rule

Keywords: Food Safety, FSMA, Food Safety Plan, Food Safety Training

The overall goal of this project was to increase the effectiveness of the food safety systems and help apple packing houses in complying with new FSMA-PCHF regulations. Based on (i) visits to various facilities; (ii) survey outcomes; (iii) scientific literature review; (iv) comments from industry and FSPCA, the **food safety plan model** was finalized.

The biggest challenges identified by food safety specialists are difficult (or impossible) to clean equipment, very limited time for cleaning and sanitizing, use and treatment of water, budget limitation, and personnel unawareness, and high turnover due to high production rate. Currently used and potential solutions for improving food safety during the apple packing process, based on the assessment of the current apple packing process and literature review were summarized.

Current methods of produce decontamination were reviewed and presented in a manuscript titled, *"Food Safety Interventions to Control Listeria Monocytogenes in Fresh Apple Packing Industry: A Review"* (is available online @ https://doi.org/10.1111/1541-4337.12496). Additionally, this review contains supplementary information such as the possible routes of produce contamination, bacteria attachment, bacteria resistance mechanisms, and the mode of action of the common decontamination agents. This information can help to better understand the food safety risks, how cleaning treatments work, and why bacteria removal is so important.

Scientific investigations on the efficacy of various decontamination methods have been conducted by numerous research groups. However, there is still a need for studies that will evaluate the suitability of a given method for application in the packing process of apples or other types of produce.

Project outcomes:

- Developed a model food safety plan.
- Provided one, 2.5-day training on the FSMA-PCHF rule with an emphasis on apple and pear packing process (2017), and two additional, 1-day trainings, focused on the implementation of the FSMA-PCHF rule (2018, and 2019)
- Summary of the peer-reviewed literature available on the different technology interventions that are currently used in different produce industries presented in a published manuscript titled, "*Food Safety Interventions to Control Listeria Monocytogenes in Fresh Apple Packing Industry: A Review" (*https://doi.org/10.1111/1541-4337.12496)

FINAL PROJECT REPORT

Project Title: Optimizing harvest time for WA38

Project Award: AP-19-105B

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Cooperators: Ryan Sheick, Ines Hanrahan, Stemilt Growers (Quincy)

Other funding sources:
Stemilt Growers (Quincy) orchard

Total Project Funding: $95,419

WTFRC Budget:
Organization Name: WTFRC **Contract Administrator:** Kathy Coffey
Telephone: (509) 665-8271, ext. 2 **Email:** kathy@treefruitresearch.com

WFTRC	2019
Salaries[1] (include benefits)	$ 8,140
Wages[2]	$ 14,188
Benefits[3]	$ 7,565
Supplies	$ 1,500
Travel	$ 1,000
total	$ 32,393

[1]Salary and benefits for Manoella Mendoza and Tory Schmidt.
[2]Wages and [3]benefits for hourly employees.

Budget

Organization name: WSU **Contract Administrator:** Katy Roberts/Shelli Tompkins
Telephone: 509-293-8803 **Email address**: arcgrants@wsu.edu/ shelli.tompkins@wsu.edu

Musacchi-Serra-Ross	**2019**
Salaries[1]	**$ 28,800**
Benefit[2]	**$ 11,226**
Plot fee[3]	**$ 3,000**
Sensory evaluation[4]	**$ 9,000**
Supplies[5]	**$ 8,000**
Travel[6]	**$ 3,000**
total	**$ 63,026**

Footnotes:
[1] Salary for 60% Research assistant ($4000/month) (Musacchi-Serra)
[2] Benefit on salary at 38.98%
[3] Plot fee for plots
[4] Sensory evaluation Ross lab.
[5] Labware/consumable, fruit sample reimbursement (Musacchi)
[6] 5,556 miles/year for domestic travel (0.54$/mile) to go to the orchard.

RECAP OBJECTIVES:

1. Determine optimum timing for WA38 harvest based on fruit production, pack out, and quality.
2. Validate the new WA38 starch scale as a tool to predict harvest time.
3. Assess consumers' acceptance of WA38 fruit harvested at a different time (6 consecutively weekly picks[¥]).

[¥] *From the original project submitted and funded, the number of picks to study increased from 4 to 6 picks for 6 weeks in a row. No budget modification was requested.*

SIGNIFICANT FINDINGS:

- The time of harvest impacted the pack-out: cull fruit/tree increased significantly from Pick3 to Pick6, and consequently, the amount of "good" fruit/tree decreased.
- With the latest pick, a significant increase of average fruit dropped per tree was noticed: about 1.7 Mton/Acre (4.4 bins/A) production could be lost if the harvest date is delayed to the third week of October (Pick6), while at Pick1 the lost yield was only 0.5 Mton/A (1.3 bins/A).
- The delay in harvesting is influencing the grading quality of fruit: early harvest date (Pick1) had the lowest percentage of cull fruit (8.2%) versus 34% six weeks later (Pick6).
- More delayed was the harvest date, higher was the incidence of defects like bird peck and split (Pick6: 27% of cull for bird peck and 40% cull for split).
- An increase of greasiness has been observed in the late picks. Pick3 showed 3.1% of the fruit affected, while Pick4 reach 10.9%. At Pick5, the percentage of fruit affected increase to 18.8%.
- Apple flavor at harvest showed a higher incidence of starchy/unripe flavor in Pick1 apples than in all the other 5 picks, while from Pick2, 80% of the tasted apples showed a ripe/good flavor.
- Parameters correlating the most with starch index were firmness and I_{AD} both at T0 and T1 quality assessment. I_{AD} drop from 0.73 (September 17th) of the first pick to 0.40 of the fourth pick (October 8th). In many varieties, an I_{AD} below 0.4 can be considered a threshold for harvest.
- Non-destructive estimation of dry matter and soluble solids did not increase with later pick dates, suggesting no benefit to internal fruit properties with longer on-tree time.
- However, later picks did improve coloring, which is a known factor in determining consumer purchasing behavior, though also at the risk of increasing proportions of culled fruit.
- A threshold of consumer liking was identified between pick 1 and 2, indicating starch levels of 2.2 or greater are necessary to achieve the most positive consumer outcomes.

METHODS

Objective 1) Determine optimum timing for WA38 harvest based on fruit production, pack out, and quality.

Within WA38 P3 block in Quincy (trees planted in 2008 and grafted on M9-337, 12 ft x 3 ft, 1210 trees/Acre, and 1360 ft of elevation), in August 2019, we selected 48 trees for this trial. Trees had similar TCSA (average 43.5 cm^2) with a number of fruits per tree ranging from 93 to 175 apples per tree. Eight trees per each pick have been utilized to represent the crop load variability in the field. For each of the 6 harvests (picks), we randomly choose 8 trees available as repetitions. WA38 apple's internal quality varies depending on the date of harvest. Little is known about the optimum picking date and how to monitor the fruit once received at the storage facility. We harvested weekly for 6 weeks. For each pick, WA38 apples were sampled to understand the variation of internal fruit quality based on the harvest dates. Fruits were collected for quality analysis at harvest and one month after harvest (precisely 30 days in cold storage RA after each of the picks) as well as at the beginning of December 2019 (when fruit started to be sold in the retail stores for the first time in WA38's history).

The first harvest was planned at an average of starch index ≈ 1.4. That date set the beginning of the entire experiment. Here the dates of harvest in blk P3 and the corresponding average of the starch index based on the WA38 T0 quality run immediately after harvest.

Pick	Harvest dates	Starch index (1-6)
Pick1	09/17/2019	1.4
Pick2	09/24/2019	2.2
Pick3	10/01/2019	3.3
Pick4	10/08/2019	3.3
Pick5	10/15/2019	3.4
Pick6	10/22/2019	4.9

The following parameters were collected for each pick:

- Yield (kg/tree as net weight)
- Number of total fruits harvested from the tree
- Number of dropped fruit and their weight
- Size of all fruit/tree in mm (sizer 65 to 110 mm)
- Good (extra fancy and fancy) vs. cull fruit count (pack out by pick) and cull reasons.

Objective 2) Validate the new WA38 starch scale as a tool to predict harvest time.

A set of 16 apples/pick/month was assigned to a monthly "starch degradation" assessment for 5 months until March 2019 to understand the evolution of starch index in storage.

Objective 3) Assess consumers' acceptance of WA38 fruit harvested at a different time (6 consecutive weekly picks).

Fruit from the samples previously described were provided to Dr. Ross's lab at the WSU Sensory Evaluation Facility end of November 2019. Fruits from regular cold storage were brought up to room temperature 24 hours before analysis. Apples were evaluated by consumers (80-120) using a ballot where preferences about different apple attributes were scored with a 1 to 9 hedonic scale. Consumers were asked to express their preference for apple firmness, crunchiness, juiciness, sweetness, flavor, overall liking. The sensorial analysis was performed in two parts: on December 3rd, 2019, for shelf-life 1 day and December 10th, 2019, for shelf-life 7 days (apples were kept at room temperature for 7 days).

RESULTS & DISCUSSION:

Objective 1) Determine optimum timing for WA38 harvest based on fruit production, pack out, and quality.

As expected, yield data in 2019 did not reveal differences in kg/tree between picking times (trees have the same crop load level), but significant differences in average fruit weights of apples harvested at Pick6 and Pick1; with 47 grams more per fruit on average with the latest pick (Table 1).

*Table 1: WA38 harvest data for 2019 in block P3 Quincy by picking dates from September 17th to October 22nd. Significance: *, p<0.05, **p<0.01, ***, p<0.001, NS= not significant difference.*

PICK	date	N=	August 2019 TCSA (cm^2)	Crop Load (n frt/TCSA)	tot num frt/tree	yield 2019 (kg/tree)	avr apple weight (g)		% cull from n frt		% good from n frt		I_{AD} (10 apples/tree)		bins/A (1 bin=880lb)	Mton/A
1	9.17.19	8	45.1	3.2	139	28.6	206	b	8.2	c	91.8	a	0.73	a	86.8	34.7
2	9.24.19	8	44.2	2.9	125	27.0	219	ab	12.5	c	87.5	a	0.62	ab	81.9	32.7
3	10.1.19	8	43.7	3.1	134	30.3	226	ab	11.7	c	88.3	a	0.48	bc	91.7	36.6
4	10.8.19	8	43.2	3.4	145	32.6	225	ab	16.5	bc	83.5	ab	0.40	cd	98.8	39.5
5	10.15.19	8	42.6	3.1	129	30.9	239	a	23.9	ab	76.1	bc	0.31	d	93.8	37.4
6	10.22.19	8	42.3	2.9	120	28.5	243	a	33.4	a	66.6	c	0.28	d	86.4	34.5
Significance			NS	NS	NS	NS	**		***		***		***		NS	NS

The time of harvest impacted the pack out of the fruit: kg of cull fruit increased significantly from Pick3 to Pick6, and consequently, the amount of "good" fruit/tree decreased (presented as % in

Table 1). I_{AD} index measured at harvest showed a consistent decrease with the delay of harvest and a significant drop from Pick3 to Pick6.

The size of WA38 fruit improved significantly more delayed was the pick (Figure 1); indeed, Pick6 had 21.8% more fruit belonging to the size 64 apples/box (=85 mm) than Pick1 (with only 4.7%). Pick3 and 4 showed a similar fruit size distribution; starting from Pick3, there was only 16% of apples in the sizes below or equal to 70 mm (=113 apples/box) with 80-72 apples/box (80 mm) being the most representative sizes (Figure 1).

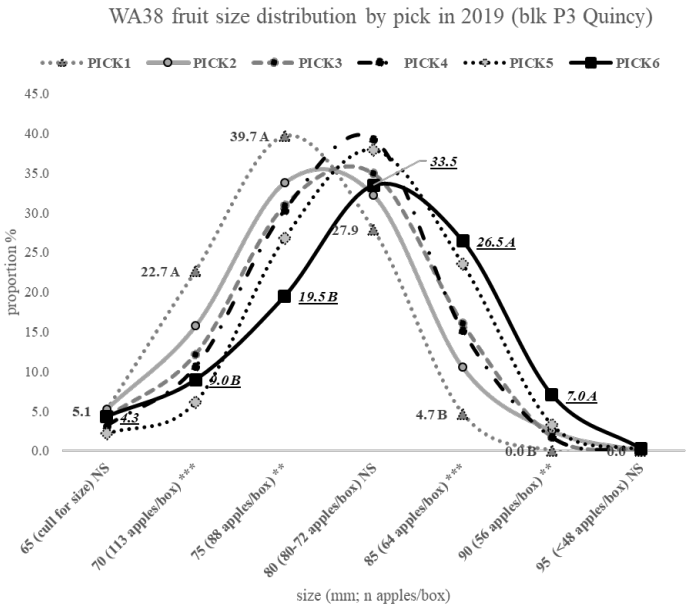

Figure 4: WA38 fruit size distribution by pick in 2019. Significance: *, p<0.05, **p<0.01, ***, p<0.001, NS= not significant difference.

The fruit grading carried out at each harvest showed significant differences in pack-out (Figure 2). Pick1 had the lowest percentage of cull fruit (8.2%) versus a 34% six weeks later (Pick6); from Pick4, the number of cull apples increased to 16.5%, statistically similar to Pick5 with 24%. The proportion of extra fancy (XF) apples was the highest at Pick1 with 73.8%, while at Pick6, they were representing only 39.3% of the harvested fruit. The delay in harvesting is influencing the grading quality of fruit (Figure 2). Among all the possible reasons to cull the fruit, we observed that later was the harvest date higher was the incidence of defects like bird peck and split (data not shown). The split was reason to cull apples for the 4% at Pick1 while, 6 weeks later, the proportion got tenfold (40% culled for the split, mainly stem split); after Pick4, the split incidence reached worrisome levels (data not shown).

The color was always at the highest level (50 to 100% red colored surface) since Pick1 to Pick6, ranging from 94% to 100% (data not shown). Green spot did not significantly affect this production, reaching a maximum of only 4.4% at Pick2, while all the other harvest dates were affected at lower incidence (data not shown).

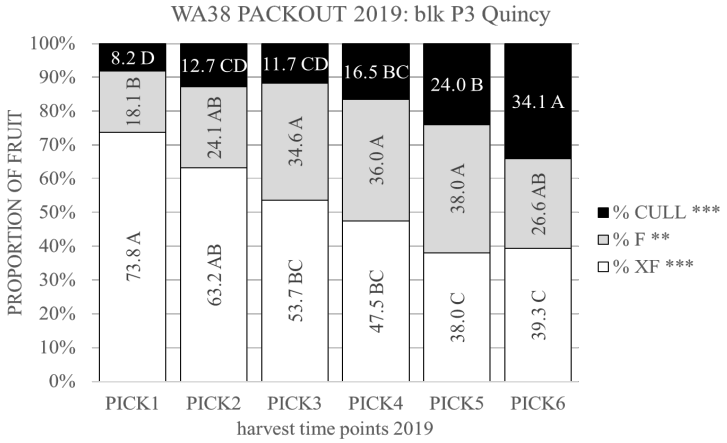

Figure 5: WA38 fruit grading and pack-out2019 by pick: XF= extra fancy apples, F=fancy, CULL=cull.
*Significance: *, p<0.05, **p<0.01, ***, p<0.001, NS= not significant difference.*

Instrumental fruit quality assessment at each time of picking (T0=24h after harvest) revealed differences in apple physiology/quality related to delay in the harvest (Table 2). The starch index increased significantly from Pick1 to Pick6 (1.4 to 4.9 respectively on a 1-6 scale), showing starch degradation of 0.8-0.9/week for the first 2 picks. From Pick 3 to Pick5, the index did not drop, probably due to the critical decrease in temperature registered in October 2019 in the Wenatchee/Quincy area. Pick3 registered an average starch index around 3.3 (across 80 apples), a value already higher than the recommended 2.5, while Pick2 was 2.2, so closer to the recommended values to star WA38 harvest. A significant drop of the I_{AD} (index of

absorbance difference measured by the DA meter) was noticed between Pick2 and Pick3, reflecting decreased firmness (from 19.4 lb to 17.9 lb, Table 2). Soluble solids instead increased significantly only at Pick4, reaching 12.2 ° Brix. At the same time, titratable acidity (TA) showed similar values from Pick2 to Pick5 (0.56 to 0.55%), with a spike at Pick6 challenging to explain (Table 2).

Same instrumental quality analyses were done precisely 30 days after each of the 6 harvest dates to see how the quality changed after one month of regular air storage at 34°F. For Pick1, firmness

Table 2: WA38 quality at harvest 2019 (T0) by picking dates from September 17th to October 22nd.
*Significance: *, p<0.05, **p<0.01, ***, p<0.001, NS= not significant difference.*

pick	time	rep =trees (10apples/ tree)	Starch index (1-6)	Avr frt weight (grams)	Red color (1-4)	Backgr. Color (0.5-6.0)	Red intensity (1-5)	I_{AD} index	Firmness (lb.)	Soluble solids (SSC, %brix)	TA (% malic ac.)
PICK1	T0	8 (10)	1.4 D	210 B	3.9	5.8	4.6	0.73 A	20.0 A	11.1 C	0.80 A
PICK2	T0	8 (10)	2.2 C	215 AB	3.8	5.5	4.2	0.65 A	19.4 A	11.2 C	0.56 C
PICK3	T0	8 (10)	3.3 B	228 AB	4.0	5.7	4.5	0.48 B	17.9 B	11.7 BC	0.52 C
PICK4	T0	8 (10)	3.3 B	224 AB	4.0	5.8	4.7	0.40 BC	16.8 C	11.6 BC	0.58 C
PICK5	T0	8 (10)	3.4 B	245 A	4.0	5.8	4.8	0.31 C	16.9 C	12.2 A	0.55 C
PICK6	T0	8 (10)	4.9 A	240 AB	4.0	5.7	4.6	0.28 C	16.7 C	12.1 AB	0.72 B
Significance			***	*	NS	NS	NS	***	***	***	***

decreased significantly by about 0.88 lb from T0 to T1, but no other significant differences were seen in the comparisons between T0 and T1 within each picking dates (data not shown). At T1 (+30d), the starch index was already mostly degraded and on average above 5 from Pick4 to Pick6. SSC showed an increase between T0 and T1 only at Pick1 and Pick4, while titratable acidity reported higher values at T1 (+30d) than at T0 for Pick 1, 3, 4, and 5 (data not shown).

In general, non-significant correlations were reported between the starch index and titratable acidity at T0 and T1. Non-destructive dry matter (DM %) predicted by Felix F750 at harvest showed that the DM did not significantly change, keeping the fruit on trees for 6 weeks longer. Values were ranging on average from 13.9% to 14.4% across the 6 picks (NS). No significant differences after 30d of storage emerged in the dry matter across the 6 picks (data not shown).

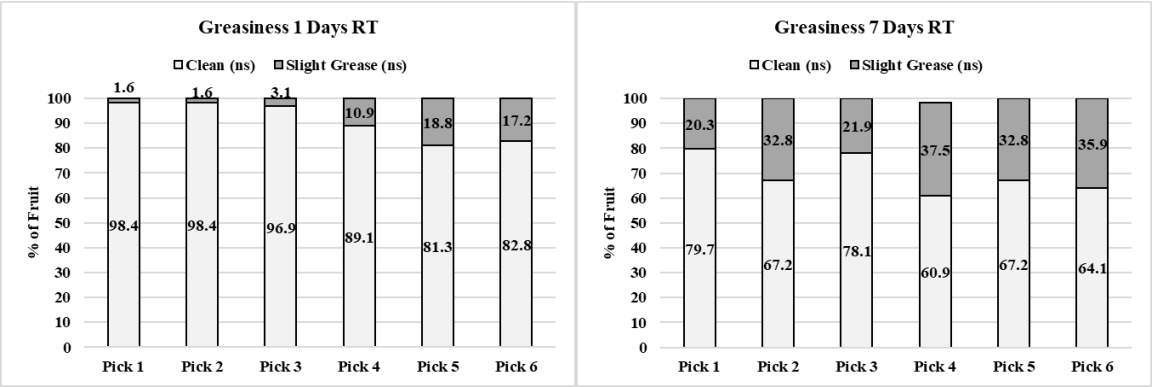

Figure 3: WA38 greasiness assessment at the industry selling time (T2) plus 1 (left) or 7 (right) days of room temperature ripening by picking dates from September 17th to October 22nd.

After storage (T2) and with 1 day of room temperature ripening, the proportion of fruit considered clean and grease-free appeared on average to be greatest in early pick dates (Pick1 through 3, > 95% of fruit considered "clean"). However, overall, differences in picks were not significant. Similarly, the average proportion of apples with some degree of greasiness (slight grease) was higher in later picks (Pick5 and Pick6, > 15% of fruit considered to have "sight grease"), though again, these trends were not statistically significant (Figure 3). After 7 days of room temperature ripening, fruit from

all picks appeared relatively similar in terms of greasiness, though Pick1 displayed the greatest proportion of clean fruits (Figure 3).

Within each given pick date, the average proportion of fruit displaying slight greasiness increased significantly from 1 to 7 days of room temperature ripening except for pick 5 where the increase from 18.8 to 32.8% of fruit displaying slight grease was not determined to be significantly different (Figure 3).

Non-destructive assessment of dry matter and soluble solids revealed no significant differences among pick dates at both 1 and 7 days of room temperature ripening at the industry selling time (T2). While some differences were apparent at harvest, it appears that starch conversion during storage may have had a homogenizing effect on fruit, leading to largely similar values of predicted dry matter and SSC at T2 (Figure 4).

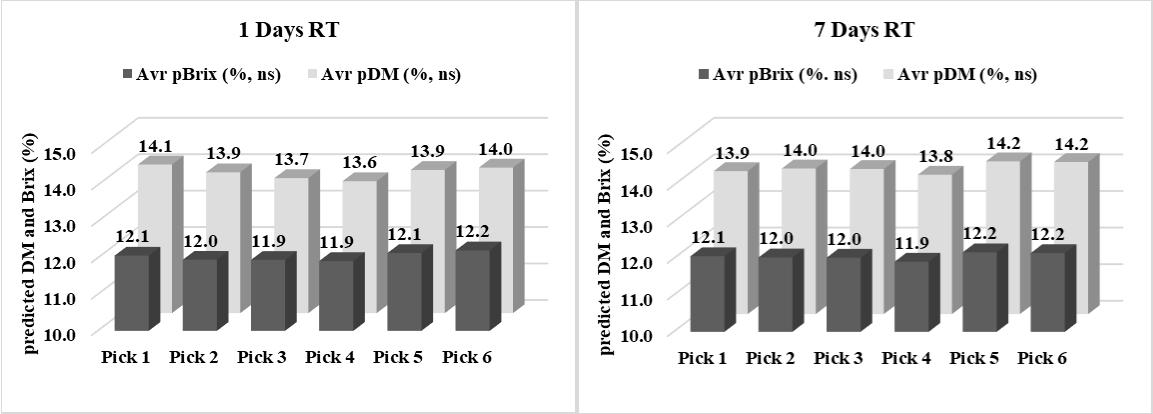

Figure 4: WA38 non-destructive dry matter and soluble solids assessment at the industry selling time (T2) plus 1 or 7 Days of room temperature ripening by picking dates from September 17th to October 22nd.

On average, predicted dry matter was slightly higher in fruit at T2-7 days than T2-1 day of room temperature ripening, likely due to loss of water content during this time. However, this difference was not significant for any pick date. Meanwhile, predicted sugar content remained similar on day 7 compared to day 1 (Figure 4).

At industry selling times (T2, December 2019), starch differences among pick dates were subtle but significant and reflected the patterns as seen at-harvest (i.e., Pick1 and Pick2 had the lowest levels of the starch index, Pick5 and Pick6 had the highest), indicating more conversion to sugar in later picking dates (Table 3). However, this did not translate to meaningful patterns in soluble solids content, though significant differences were present. After 7 days of room temperature ripening, no significant differences in soluble solids were detectable. However, fruit color was often significantly greater in later pick dates relative to early pick dates in terms of the amount and intensity of red color and the amount of background color. After 7 days of ripening, firmness was significantly higher in early pick dates relative to later picking dates (Table 3).

*Table 3: WA38 quality at the industry selling time (T2) among picking dates from September 17th to October 22nd plus 1 or 7 Days of room temperature ripening. Significance: *, p<0.05, **p<0.01, ***, p<0.001, NS= not significant difference.*

Pick	Ripening Period	rep=trees(8 apples/tree)	Starch Index (1-6)	Avr Fruit Weight (g)	Red Color (1-4)	Backgr. Color (0.5-6.0)	Red Intensity (1-5)	I_{AD} (DA Index)	Firmness (lb)	Soluble Solids (SSC %Brix)	TA (% malic acid)
Pick 1	1 days RT	8 (8)	5.4 b	207	3.9	5.6	4.2	0.36	17.8	12.0	0.68 a
	7 days RT	8 (8)	5.9 a	207	4.0	5.7	4.1	0.26	17.5	12.3	0.50 b
		Significance	***	ns	ns	ns	ns	ns	ns	ns	***
Pick 2	1 days RT	8 (8)	5.4 b	212	4.0	5.7	4.7	0.39	17.1	11.4 b	0.68 a
	7 days RT	8 (8)	5.8 a	209	4.0	5.8	4.5	0.30	17.0	12.7 a	0.52 b
		Significance	**	ns	ns	ns	ns	ns	ns	**	**
Pick 3	1 days RT	8 (8)	5.5 b	228	4.0	5.8	4.8	0.36 a	17.1 a	11.2 b	0.67 a
	7 days RT	8 (8)	5.9 a	217	4.0	5.9	4.8	0.29 b	16.6 b	12.5 a	0.50 b
		Significance	***	ns	ns	ns	ns	***	*	***	***
Pick 4	1 days RT	8 (8)	5.7 b	223	4.0	5.9	4.9	0.32	16.9 a	11.3 b	0.65 a
	7 days RT	8 (8)	5.9 a	221	4.0	6.0	4.9	0.28	16.2 b	12.6 a	0.49 b
		Significance	**	ns	ns	ns	ns	ns	**	***	***
Pick 5	1 days RT	8 (8)	6.0	233	4.0	5.9	4.8	0.26	17.3	11.8 b	0.71 a
	7 days RT	8 (8)	6.0	223	4.0	5.9	4.7	0.22	16.9	12.9 a	0.53 b
		Significance	ns	ns	ns	ns	ns	ns	ns	*	***
Pick 6	1 days RT	8 (8)	5.9	249	4.0	5.9	4.7	0.26	16.9	12.0 b	0.70 a
	7 days RT	8 (8)	6.0	237	4.0	5.9	4.8	0.24	16.9	13.0 a	0.49 b
		Significance	ns	ns	ns	ns	ns	ns	ns	**	***

Comparing quality within each pick date, we see significant increases in starch index between 1 and 7 days of room-temperature ripening in Picks1-4, indicating further conversion of starch to sugars as the fruit ripens at room temperature (Table 3). No further change in the starch index was detected in Picks5 and 6, suggesting no further evolution of these fruits was possible as measured via starch index. However, significant increases in sugars were present for most picks and even in Picks5 and 6 despite no starch index changes. This would suggest that more starch was present in these fruits and available for sugar conversion than was detectable at the starch index level. As is typical, firmness decreased as fruits remained at room temperature though this decrease was only significant in Pick3 and Pick4. In terms of titratable acidity, the percent of malic acid in fruits decreases significantly from 1 to 7 days for all picks. No significant changes were found in fruit appearance or the I_{AD} index for most picks except Pick3, which showed a slight, though significant, drop in I_{AD} (Table 3).

Objective 2) Validate the new WA38 starch scale as a tool to predict harvest time.

Starch levels in the first two months after harvest reflected differences at-harvest (i.e., Pick1 had lowest, Pick6 had highest). After two months of storage, all apples, regardless of pick, had roughly the same starch, indicating a homogenization of fruit during storage (Figure 5).

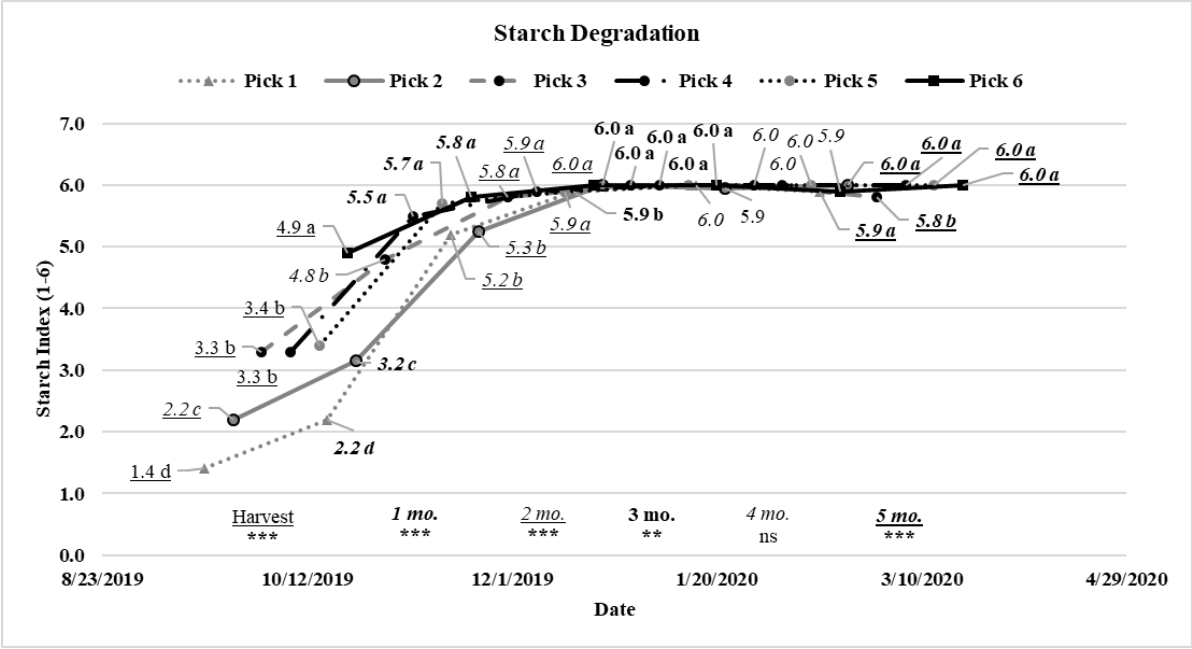

*Figure 5: WA38 starch degradation by picking dates from September 17th to October 22nd. Significance: *, p<0.05, **p<0.01, ***, p<0.001, NS= not significant difference.*

Objective 3) Assess consumers' acceptance of WA38 fruit harvested at a different time (4 consecutive weekly picks).

Apple flavor was assessed from T0 to T2. Results revealed a higher incidence of starchy/unripe flavor in Pick1 apples, significantly higher than in all the other 5 picks. Starting from Pick2, 80% of the tasted apples showed a ripe/good flavor. Only at Pick6, there was a small percentage of apples tasting bland/no flavor (2.5%, data not shown). Flavor after 30 days (T1) showed that apples from Pick1 decreased the proportion of starchy/unripe apples from 92.5% to only 32.5%, while some bland/no flavor apples appeared in Pick4 and Pick5 (but not statistically different across the 6 picks) and from Pick4 to Pick6, at least the 88% of tasted apples were in the good/ripe flavor range (Figure 6).
After 1 day at room temperature, no significant differences emerged for flavor. However, the highest proportion of apples with good flavor were those in Pick3, while later picks revealed some bland/off-flavor (Figure 6). After 7 days at room temperature, the best-flavored apples belonged to Pick 1 to 4.
As judged by panels of untrained consumers consisting of over 200 unique participants in 2 days, picking date did not lead to many noticeable differences in perceived taste prior to many days of ripening except for consumer liking of apple flavor where Pick4 displayed significantly lower consumer liking relative to pick 5, with other picks falling in-between. However, after 7 days of room-temperature (RT) ripening, consumer liking of apple flavor was significantly higher in Picks 2, 3, and 6 relatives to the lowest liking of Pick1, resulting in the significantly better overall liking of Pick 2 and the least overall liking of pick 1. No significant differences in consumers' preference regarding firmness, crunchiness, juiciness, or sweetness were found among picks at either 1 or 7 days of room temperature ripening. These results would indicate that the effect of the pick date was not perceivable for individual liking attributes. However, as fruits ripened at room temperature, consumers could distinguish a better liking for later apple picks, with Pick1 standing out as the significantly least-liked apple for flavor and overall liking. In this sense, we can identify a threshold in consumer preference between Pick 1 and Pick 2 in terms of apple flavor and overall liking (Table 5).

Table 4: Flavor assessment on WA38 from P3 orchard Quincy by pick and comparison between T0 and T1 (at harvest vs after 30d of storage) in 2019.

*Significance: *, p<0.05, **p<0.01, ***, p<0.001, NS= not significant difference.*

pick	time	rep =trees (10apples/ tree)	Flavor 1 (unripe-sour/starchy) %	Flavor 2 (ripe/apple flavor) %	Flavor 3 (bland/no flavor) %
PICK1	T0	8 (5)	93 A	8 B	0
PICK1	T1-30d	8 (5)	33 B	68 A	0
Significance T0-T1			***	***	
PICK2	T0	8 (5)	20	80	0
PICK2	T1-30d	8 (5)	20	78	3
Significance T0-T1			**NS**	**NS**	**NS**
PICK3	T0	8 (5)	20 B	80 A	0
PICK3	T1-30d	8 (5)	58 A	43 B	0
Significance T0-T1			**	**	
PICK4	T0	8 (5)	38 A	63 B	0
PICK4	T1-30d	8 (5)	3 B	93 A	5
Significance T0-T1			**	*	**NS**
PICK5	T0	8 (5)	13	88	0
PICK5	T1-30d	8 (5)	5	88	8
Significance T0-T1			**NS**	**NS**	**NS**
PICK6	T0	8 (5)	10	88	3
PICK6	T1-30d	8 (5)	0	100	0
Significance T0-T1			**NS**	**NS**	**NS**

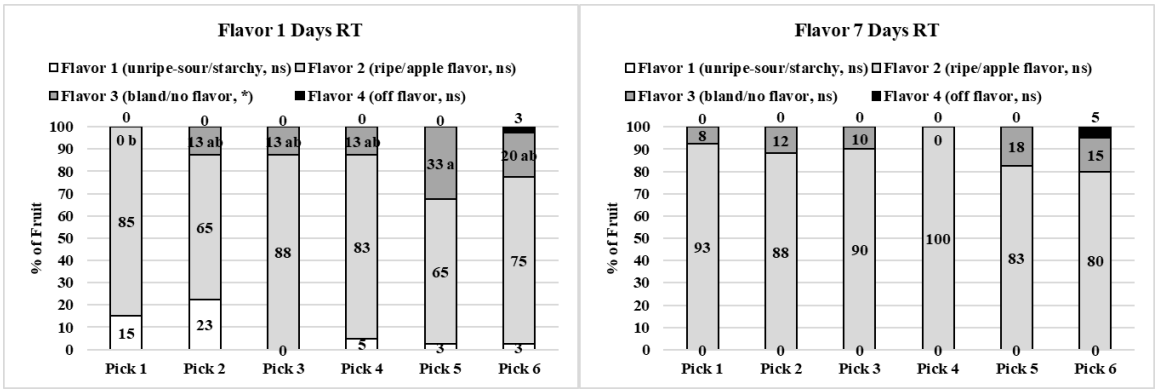

Figure 6: WA38 flavor assessment at the industry selling time (T2) plus 1 or 7 Days of room temperature (RT) ripening by picking sates from September 17th to October 22nd.

*Table 5: WA38 consumer preference ratings at the industry selling time (T2) plus 1 or 7 Days of room temperature (RT) ripening by Picking Dates from September 17th to October 22nd (by Prof. Carolyn Ross). Significance: *, p<0.05, **p<0.01, ***, p<0.001, NS= not significant difference.*

Pick	Evaluation	Firmness	Crunchiness	Juiciness	Sweetness	Apple Flavor	Overall Liking
Pick 1	1 Days RT	7.43	7.65	7.44	6.98	7.04 ab	7.03
Pick 2		7.62	7.61	7.63	7.02	7.05 ab	7.04
Pick 3		7.6	7.7	7.64	7.03	7.26 ab	7.26
Pick 4		7.48	7.63	7.55	6.88	6.95 b	7.13
Pick 5		7.74	7.79	7.64	7.03	7.38 a	7.35
Pick 6		7.65	7.69	7.68	7.17	7.29 ab	7.34
	Significance	ns	ns	ns	ns	*	ns

Pick	Evaluation	Firmness	Crunchiness	Juiciness	Sweetness	Apple Flavor	Overall Liking
Pick 1	7 Days RT	7.27	7.39	7.25	6.89	6.68 b	6.73 b
Pick 2		7.37	7.32	7.39	7.02	7.17 a	7.13 a
Pick 3		7.38	7.4	7.44	7.13	7.12 a	7.12 ab
Pick 4		7.5	7.46	7.51	7.01	7.07 ab	6.99 ab
Pick 5		7.42	7.41	7.4	7.03	6.99 ab	7.06 ab
Pick 6		7.39	7.32	7.44	7.02	7.15 a	7.10 ab
	Significance	ns	ns	ns	ns	*	*

PROJECT OUTCOMES

Presentations:
Musacchi S., 2020. WA38 Pre-harvest Q/A. Date Time: September 23rd, 2020.
Musacchi S., 2020. WA38 Live Field Day Webinar. Date Time: September 16th, 2020.
Musacchi S., Serra S., Ross C., Mendoza M., Schmidt T. 2020. Optimizing harvest time for WA38 Sub-quality committee meeting. March 24th, 2020.
Musacchi S., Serra S., Ross C., Mendoza M., Schmidt T. 2020. Optimizing harvest time for WA38. Continuing report 7M out of 12M Apple Review Yakima January 29th, 2020.

Publications:
WA 38 Defects Guide. WA 38 Common Defects and Unique Characteristics Near Harvest and During Storage. Written by Ines Hanrahan and Carolina Torres. Collaborators: Stefano Musacchi, Sara Serra, Kate Evans, Karen Lewis, David Rudell, Manoella Mendoza, Mackenzie Perrault, Jill Burberry.

FUTURE DIRECTIONS

Our preliminary research (one-year) highlights the harvest time's role in fruit quality and defects appearance and incidence—one trait of WA38 has been determined as a potential problem, the greasiness. More studies on greasiness, especially in post-harvest, can lead to the optimization of the WA38 storage.

EXECUTIVE SUMMARY

Project Title: Optimizing harvest time for WA38
Keywords: WA38, fruit quality, consumer preference, greasiness, starch

The project wants to investigate the effects of harvest time on fruit quality, starch degradation, and consumer acceptance of WA38 apples. WA38 internal quality of the fruit at harvest and during storage varies depending on the date of harvest. The most utilized method to determine harvest time in the apple is to estimate internal starch content by an iodine-staining index. Growers, packers, and researchers widely utilize the iodine test because it is a feasible and fast tool to adopt from the field to the lab. WTFRC has recently developed a specific WA38 starch scale (starch index 1-6) because the cultivar presents two patterns of starch degradation. The project wants to determine the starch degradation trends on different harvest days.

Within a WA38 P3 block in Quincy (trees planted in 2008 and grafted on M9337, 12 ft x 3 ft, 1210 trees/Acre, and 1360ft of elevation), in August 2019, we selected 48 trees for this trial. For each of the 6 harvests (picks), we randomly choose 8 trees available as reps. The first pick starts on September 17th, 2019, and the last ends on October 22nd, 2019.

The size of WA38 fruit improved significantly more delayed was the pick; indeed, Pick6 had 21.8% more fruit belonging to the size 64 apples/box (=85 mm) than Pick1 (with only 4.7%).

The color was always at the highest level (50 to 100% red colored surface) since Pick1 to Pick6, ranging from 94% to 100%. Green spot did not significantly affect this production, reaching a maximum of only 4.4% at Pick2, while all the other harvest dates were affected at a lower incidence.

The delay in harvesting is affecting the grading quality of fruit. Among all the possible reasons to cull the fruit, we observed that later was the harvest date higher was the incidence of defects like bird peck and split. The split was reason to cull apples for the 4% at Pick1, while 6 weeks later, the proportion got tenfold (40% culled for the split, mainly stem split).

After storage, the proportion of fruit considered clean and "grease-free" appeared on average to be greatest in early pick dates (Pick1 through 3, > 95% of fruit considered "clean"). Similarly, the average proportion of apples with some greasiness ("slight grease") was higher in later picks (Pick 5 and 6). After 7 days of room temperature ripening, apples from all picks showed an increase of greasiness.

Non-destructive assessment of dry matter and soluble solids revealed no significant differences among pick dates at both 1 and 7 days of room temperature ripening at the industry selling time, confirming a previous project's findings.

At industry selling times (December 2019), starch differences among pick dates were subtle but significant and reflected the patterns seen at-harvest. Comparing each pick date, we saw significant increases in starch index between 1 and 7 days of room-temperature ripening in Pick1-4, indicating further conversion of starch to sugars as the fruit ripens at room temperature. No further change in the starch index was detected in Pick5 and Pick6, suggesting no further evolution of these fruits were possible as measured via starch index. Starch levels in the first two months after harvest reflected differences at-harvest (i.e., Pick1 had lowest, Pick6 had highest). After two and a half months of storage, all apples had roughly the same starch, regardless of the picking date, indicating a homogenization of fruit during storage.

Flavor after 30 days (T1) showed that apples from Pick1 decreased the proportion of starchy/unripe apples from 92.5% (at harvest, T0) to only 32.5%, while some bland/no flavor apples appeared in Pick4 and Pick5 and from Pick 4 to Pick6, at least 88% of tasted apples were in the good/ripe flavor range.

As judged by panels of untrained consumers, consisting of over 200 unique participants in two days, picking date did not lead to many noticeable differences in perceived consumer preference. After 7 days at room temperature, Pick2 and Pick3 showed a numerically higher overall liking value than the other harvest dates.

FINAL PROJECT REPORT

Project Title: Calcium fertilization efficacy

PI: Bernardita Sallato
Organization: Washington State University
Telephone: 509 4398542
Email: b.sallato@wsu.edu
Address: 24106 N Bunn Rd,
City/State/Zip: Prosser, WA, 99350

Cooperators: Denny Hayden (Hayden's Orchards), Paul Carter (Stemilt), Aaron Avila and Dan Griffith (GS Long), Andy Dolph (Redox), Paolo Sanguankeo (Wilbur Ellis).

Total Project Request: **Year 1:** 13,000 **Year 2:** **Year 3:**

Other funding sources: **Awarded**
Root Growth Management to Reduce Ca Deficiency Disorders in Apples and Cherries. Washington State USDA- Specialty Crop Block Grant. $152,938. P.I. B. Sallato. Co-P.I.s; L. Kalcsits, M. Whiting.

Budget 1
Organization Name: Washington State University **Contract Administrator:** Katy Roberts
Telephone: (509) 335-2885 **Email address:** arcgrants@wsu.edu

Item	2020
Salaries	
Benefits	
Wages	4800
Benefits	480
Equipment	
Supplies	7312
Travel	653
Miscellaneous	
Plot Fees	
Total	13,100

Footnotes: Supply include Soil, Tissue and Fruit nutrient analyses; 328 x 18 USD for tissue (leaf, fruit, buds, flowers and fruitlets) and 64 x 22 USD for complete soil test (includes WSU discount) = 7312. Travel for two sites (10 times x 94 miles average x 0.54 = 508), Wages (320 hours at 15 USD/hour plus 10% benefits) for temporary support during sampling.

OBJECTIVES

Calcium (Ca^{+2}) nutrient has been recognized as one key element due to its many roles plant physiological processes and fruit development (Marschner, 2002). Several fruit disorders have been associated to Ca^{+2} deficiencies, being bitter pit (BP) the most important Ca deficiency disorder in apples in Washington (Kalcsits 2017). The application of Ca to reduce Ca related disorders have been widely used in apple production (Ferguson et al., 1979). In Washington, growers have reported an intensive Ca spray program with inconsistent results or no improvement at all. The objective of this project was to evaluate different calcium treatments in two different conditions, developing a thoughtful diagnostic process to determine:

1) Calcium fertilizer efficacy on nutrient uptake.

2) Calcium fertilizer efficacy on fruit quality in 'Honeycrisp' apples.

SIGNIFICANT FINDINGS

- The two sites had significant differences in BP incidence at harvest and after storage, with high incidence at Site 1 with average of 54% compared to 24% on Site 2.
- Among treatments, there were no statistical differences in controlling BP incidence. However, when compared with the control receiving no Ca, F_CaCO3 and F_ Ca+N where effective in reducing BP on Site 1 and Site 2, respectively.
- At Site 2, where sulfate (S) levels in the soil were deficient, S_CaSO4 improved fruit firmness when compared with the control, but there were no differences between the other Ca treatments.
- On both sites, Ca treatments had no effect on Ca uptake by the fruitlets, leaves of fruit flesh and peel, and none of these measurements correlated with BP incidence, thus should not be utilized as BP predictors.
- Possible causes for the higher BP incidence exhibited at Site 1 can be associated to: reduced root growth, excessive levels of soil K (above 250 mg/kg) and oversized fruit.
- Results from the treatments receiving 12 lbs of Ca/acre in this trial, did not differ from the results observed from the grower-managed areas, where total applied Ca was more than 200 lbs/acre and 400 lbs/acre on Site 1 and Site 2, respectively.
- From this one year trial, it appears that prophylactic applications of calcium are ineffective for reducing BP.

RESULTS AND DISCUSSION

This research was conducted in two 'Honeycrisp' orchards located near Pasco, WA. The application method, date and formulations for each orchard are indicated in table 1. The total amount of actual Ca applied was equal for all treatments at 12 lbs/acre, equivalent to 36 lbs of CaCl/acre. Dry products were dissolved in water and soluble products were diluted according to the label recommendations. Treatments were applied on 6 or 4 dates for foliar and soil, respectively, every 14 days starting at petal fall. Gyspum ($CaSO_4$) was applied in one application with the second irrigation. Foliar sprays were applied with a Flow-Zone FZSAAJ-2 4-Gallon Cyclone Multi-Use 18V Lithium-Ion Backpack Sprayer, with automatic PSI controller. The experimental unit consisted of 10 trees, replicated 4 times in a complete randomize design with one border rows on each side.

Table 1. Treatments: commercial name, method and total cost for 12 lbs of actual Ca/acre.

Treatment	Commercial name	Method	USD/acre[b]
Control	Control (No Calcium)	-	-
F_CaCl	Mora-Leaf® Calcium (Wilbur-Ellis Company LLC)	Foliar	$16
F_$CaCO_3$	Mainstay ™ Calcium (Redox Chemical LLC)	Foliar	$120
F_Ca+1%-N	ProNatural ® Calcium Plus (Wilbur-Ellis Company LLC)	Foliar	$444
S_CaCl	Mor-Calcium (Genesis Agri Products, Inc)	Soil	$16
S_$CaCO_3$	Mainstay ™ Calcium (Redox Chemical LLC)	Soil	$120
S_$CaSO_4$	Pro-Pell-it (Marion Ag Service Inc.)	Soil	$13

Note: 1. Soil CaCl was not applied to the soil in orchard 2 (Sagemore) as it is not labelled for organic production. b. Cost estimate for the 12 lbs/acre of actual Ca applied. Includes only the fertilizer at a standard rate for bulk purchase and does not include application cost.

While not included in the experimental design, additional sampling unit was included as the grower's managed area from three representative trees to compare with the standard grower's practice. The Ca program for each site is described below:

- Site 1: Two applications of 500 lbs/acre of CaSO4 (Gypsum) plus 30 lbs/acre of CaCl spread in 5 sprays and 8 gallons/acre of Ca+1%N spread in 20 dates during the growing season. The total Ca unit applied summed 210 lbs/acre by ground and 14 lbs/acre by foliar spray. Fertilizer cost was approximately 378 USD/acre.
- Site 2: One application of 2000 lbs/acre of CaSO4 (Gypsum) during spring plus 9 lbs/acre of CaCl spread in 5 sprays, one spray of 0.2 lbs/acre with Ca+1%N and 12.2 lbs/acre of $CaCO_3$ alone or in combination with Silicon (Si). The total Ca unit applied summed 430 lbs/acre via ground and 11.5 lbs/acre via spray. Fertilizer cost was approximately 650 USD/acre.

Complete details about the methods can be found in the proposal Sallato_Ca_New_2019.

Initial conditions

Both sites were mature 'Honeycrisp' orchard located in Franklin county, WA with mean annual precipitation between 6 to 12 inches.

Site 1: 'Honeycrisp' grafted onto Malling 9 (M9- Nic 29) rootstock on a spindle system at 12 ft x 1.5 ft spacing. The block is located near Pasco 46°20'35.8"N 119°08'57.8"W. The soil series at this site is associated to Quincy loamy fine sand, an Entisoil formed in sands on dunes and terraces. This soil series cover 714,600 acres of eastern Washington, representing a large portion of apple orchards located in Quincy, Mattawa, Basin City and Pasco area (Figure 1, left.) The soil profile had a surface layer ranging from 4 to 60 inches of loamy find sand and a second strata of fine sand, with excessive drainage in some areas.

Site 2: 'Honeycrisp' grafted onto EMLA (M26) rootstock on a V trellis system at 12 ft x 6 ft spacing. Each rootstock supports 4 leaders tied horizontally to the first wire plus 6 upright leaders (3 per side). The orchard is in Sagemoor 46°24'14.1"N 119°14'05.5"W. The soil type at this site can be associated to Warden silt loam series, and Aridisoil, silt loam soil, alkaline, well drained. Warden series cover 486,111 acres, representing a large proportion of orchards in the Yakima valley and the Basin (Figure 1, right).

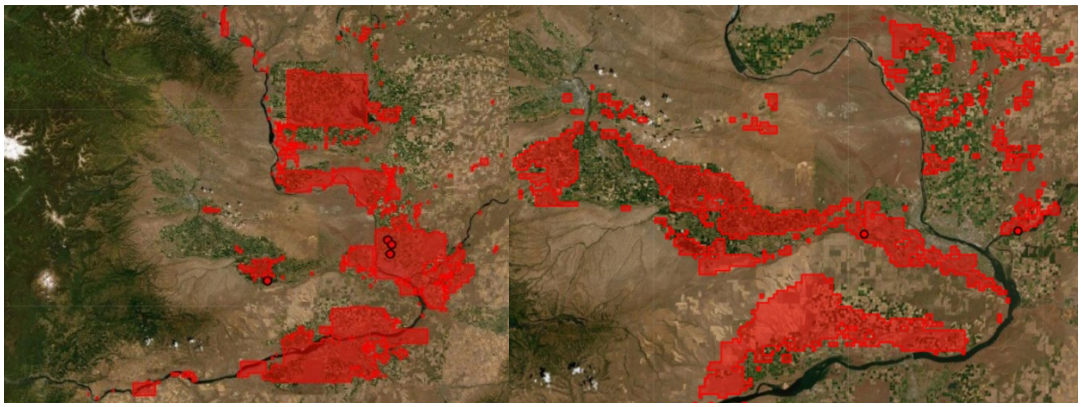

Figure 1. Map of soil series associated to Quincy (left) and Warden (right). (USDA-NRCS Web Soil Survey).

For Site 1, the first strata had nine inches of loamy sand (75% sand) followed by fine sand of at least 20 inches deep. Most fine roots were observed in the first strata with few roots observed in the second strata (Figure 2, left). Site 2 had 2 feet of effective soil depth with varying soil conditions, but predominantly silt loam soil with large volume of roots growing throughout the soil profile (Figure 2, right).

Figure 2. Soil profile for the top 3 foot. left: Site 1 (Quincy series) near Pasco, right: Site 2 (Warden series) in Sagemoor.

For each site, soil samples were collected from the strata where most fine roots were observed. Soil chemistry for Site 1 was representative of a Quincy series with neutral pH, cation exchange capacity (CEC) of 8 meq/100g and within the low range of Ca and Mg. However, it had elevated levels of P and K (42 and 325 mg/kg respectively), uncommon on sandy soils. High levels of P and K in sandy textured soil are indicative of drainage impediments in the soil profile, which can be attributed to the texture differences within the soil profile. Site 2 had alkaline pH and higher CEC (10 meq/100g), representative of Warden silt loam series. Cation levels were adequate, while P, S and B levels were low.

To evaluate inherent nutrient variability of each orchard, dormant buds and blooms were collected from spurs on 2 year old wood, from 20 random trees, prior to the application of treatments. Both sites had consistent nutrient levels within the orchard, reflecting low initial variability between trees (data not shown). Between orchards, nutrient levels were equivalent, except for Ca and B were Site 1 had 30% more Ca and almost half the amount of B.

Table 2. Soil nutrient and physical initial conditions for Site 1 (Quincy soil series) and Site 2 (Warden soil series). Average of four randomly collected samples.

Soil test	Unit	Optimal	Site 1 (Pasco)	Site 2 (Sagemoor)
pH	-	5.0 – 7.0	6.9	**7.9**
O.M	%	> 1	1.1	1.3
E.C paste	mmhos/cm	< 2.5	0.4	0.4
Nitrate _(NO_3_N)	mg/kg	-	3.4	2.3
Ammonium _(NH^4_N)	mg/kg	-	2.6	2.5
Phosphorus (P)	mg/kg	15 - 40	**42**	**8**
Potassium (K)	mg/kg	150 - 250	**325**	217
Potassium (K)	meq/100g	0.4 - 0.6	0.8	0.6
Calcium (Ca)	meq/100g	4.1 - 20	5.8	7.4
Magnesium (Mg)	meq/100g	0.5 – 2.5	2.8	3.9
Sodium (Na)	meq/100g	< 0.5	0.2	0.2
Total Base	meq/100g	-	9.6	12.0
CEC	meq/100g	11 - 40	9.6	12.0
Boron (B)	mg/kg	1.0 – 1.5	**0.2**	**0.3**
Sulfur (S)[b]	mg/kg	9 - 20	12.3	**5.0**
Zinc (Zn)	mg/kg	> 1.0	2.6	4.0
Copper (Cu)	mg/kg	> 1.0	4.6	3.2
Manganese (Mn)	mg/kg	1 - 4	3.9	1.3
Iron (Fe)[c]	mg/kg	-	26.3	8.8
Sand	%	-	75.0	40.0
Clay	%	-	1.0	5.0
Silt	%	-	24.0	55.0

Methods: Methods: Plant, Soil and Water Reference Methods for the Western Region. 2005. R. G. Gavlak, D. A. Horneck, and R. O. Miller. http://www.naptprogram.org

1. Treatment effect on nutrient uptake

Treatment effect on nutrient concentration was determined for each replicated unit on fruitlets at 10 mm size (of golf ball), leaves during late July (when middle shoot leaves were mature) and in fruit peel and flesh during harvest (more details about the methodology can be found in the proposal).

Fruitlet nutrient analyses

Fruitlet nutrient concentration was not affected by treatments on either site except for B levels on Site 1 (Table 3 and 4). Despite the higher amount of initial Ca in blooms on Site 1, fruitlet Ca levels were equivalent in both sites (0.12 – 0.14 %) with no difference among treatments. There were significant differences between nutrient levels when comparing between orchards ($p < 0.001$) except for Ca fruitlet levels that were equivalent (0.12 %). Site 1 had higher levels of N, P and K and lower levels of B. Based on the fruitlet concentration recommendation from the pomological fruit center of Universidad de Talca in Chile (Centro de Pomaceas, 2011), Site 1 was above adequate concentration on N, P, K and Mg, while Site 2 was within range except of P and Mg slightly above the recommended levels.

Table 3. Effect of calcium treatments on 'Honeycrisp' fruitlet mineral concentration (dry weight) in Pasco orchard (Site 1).

Treatments	%					mg/kg
	N	P	K	Ca	Mg	B
Control	0.95	0.17	1.46	0.12	0.1	23 a
F_CaCl	0.92	0.17	1.39	0.12	0.1	16 bc
F_CaCO	0.95	0.17	1.44	0.13	0.11	15 c
F_Ca+N	0.97	0.17	1.45	0.14	0.1	22 ab
S_CaCl	0.87	0.15	1.3	0.12	0.1	20 abc
S_CaCO	0.94	0.16	1.34	0.12	0.1	19 abc
S_CaSO	0.94	0.16	1.38	0.12	0.1	16 bc
Pr > F(Model)	ns	ns	ns	ns	ns	0.006

Different letters within column indicate significant difference determined by Tukey mean separation test (a = 0.05).

Table 4. Effect of calcium treatments on 'Honeycrisp' fruitlet mineral concentration in Sagemoor orchard (Site 2).

Treatments	%					mg/kg
	N	P	K	Ca	Mg	B
Control	0.65	0.13	1.17	0.13	0.09	22.2
F_CaCl	0.59	0.13	1.1	0.12	0.08	21.3
F_CaCO	0.64	0.12	1.05	0.13	0.09	21.5
F_Ca+N	0.58	0.13	1.09	0.13	0.09	21.1
S_CaCO	0.57	0.12	1.04	0.12	0.09	22.3
S_CaSO	0.62	0.12	1.07	0.12	0.09	22.4
Pr > F(Model)	ns	ns	ns	ns	ns	ns

ns: no significance determined by ANOVA test ($p < 0.05$).

Leaf nutrient analyses

Leaf nutrient levels have been utilized for more than 50 years as an indicator for nutrient uptake by the plant and yield. For leaves tissue analyses, there are validated standards that can be utilized as reference to determine overall nutrient status, health of the trees, deficiencies, or toxicities.

Treatment effect on nutrient concentration varied between orchards. At Site 1, soil treatment S_CaCO had the highest amount of N in leaves followed by S_CaSO and F_Ca+N with no significant differences (Table 5). The lowest concentration was observed in the control and all foliar sprays. However, all treatments were within adequate range for N concentration (Shear and Faust. 1980, Riguetti et al 1990). The improved N uptake with the soil Ca treatments might be a consequence of the removal of weeds around the trunk done on the soil treatments. Thus, improving the root zone environment. Leaf treatments had higher K levels, however, only F_Ca+N was higher than the control (Table 5). The F_Ca+N impact on K uptake was not determined. Despite the differences between treatments in N and K, all nutrient levels were within the adequate ranges (Shear and Faust. 1980, Riguetti et al 1990).

Table 5. Effect of calcium treatments on 'Honeycrisp' leaves mineral concentration in Pasco orchard (Site 1).

Treatments	%					mg/kg
	N	P	K	Ca	Mg	B
Control	2.2 c	0.21	1.4 bcd	1.68	0.40	33
F_CaCl	2.3 bc	0.23	1.6 ab	1.64	0.42	39
F_CaCO	2.3 bc	0.23	1.5 abc	1.90	0.42	37
F_Ca+N	2.2 c	0.23	1.6 a	1.72	0.40	35
S_CaCl	2.4 abc	0.22	1.3 d	1.71	0.42	30
S_CaCO	2.5 a	0.22	1.3 cd	1.65	0.41	40
S_CaSO	2.4 ab	0.23	1.4 bcd	1.78	0.44	35
Pr > F(Model)	0.037	ns	0.018	ns	ns	ns
Grower*	2.1	0.18	1.1	2.2	0.49	25

ns: no significance determined by ANOVA test ($p < 0.05$). Different letters within column indicate significant difference determined by Tukey mean separation test ($a = 0.05$). *grower managed area not included in the statistical analyses.

Although not included in the statistical analyses, samples from the grower managed area, outside the trial site but within the same block, were also collected to utilize as a reference added at the bottom of each table. Here, nutrient levels were slightly lower on N, P and K, but higher on Ca and Mg.

Leaf tissue analyses of P and K did not correlate with soil elevated nutrient levels of these two elements, which suggest that there is a limiting factor at the uptake level: poor root growth, physical impediment or bad drainage. In this condition, while the demand of the trees remains the same, the efficiency is reduced, and the supply should be increased until cause of the limited uptake is resolved.

At Site 2, the treatments did not affect nutrient uptake (Table 6). Only B levels were different between treatments, being slightly lower on F_CaCl, and in both soil treatments (S_CaSO and S_CaCO) when compared with the control. However, in this site all samples were within adequate levels (Shear and Faust. 1980, Riguetti et al 1990). The grower managed sample were also within adequate range and equivalent to those obtained in all treatments.

Table 6. Effect of calcium treatments on 'Honeycrisp' leaves mineral concentration in Sagemoor orchard (Site 2).

Treatments	%					mg/kg
	N	P	K	Ca	Mg	B
Control	1.8	0.40	1.9	2.1	0.51	36 bc
F_CaCl	2.0	0.44	2.0	2.4	0.56	43 ab
F_CaCO	2.1	0.38	1.5	2.4	0.57	32 c
F_Ca+N	2.2	0.32	1.4	2.2	0.53	46 a
S_CaCO	2.1	0.40	1.6	2.1	0.57	33 c
S_CaSO	2.0	0.35	1.5	2.0	0.54	32 c
Pr > F(Model)	ns	ns	ns	ns	ns	0.006
Grower*	2.0	0.29	1.3	2.3	0.59	37

ns: no significance determined by ANOVA test ($p < 0.05$). Different letters within column indicate significant difference determined by Tukey mean separation test ($a = 0.05$). *grower managed area not included in the statistical analyses.

When comparing between the two orchards, all nutrients were significantly different except for B. Site 1 had higher levels of N, while, while Site 2 had higher levels of P (despite the low P-Olsen observed in the soils), K, Ca and Mg. The greater amount of P and K on Site 2, despite the reduced level of soil supply can be associated to the greater volume of roots observed in the soil pit, which is particularly important for P uptake. Higher levels of Ca and Mg in Site 2 can also be attributed to the increased levels of these nutrients in the soil.

Fruit nutrient analyses

Fruit nutrient analyses were determined in a sub sample of 20 fruit per replicated unit. Each fruit was weighed and a ring of 1 inch from the center of the fruit was then obtained and separated into peel and flesh for elemental analyses. Each tissue component was weighed and dried at 60 °C (140 °F). Once there was no more weight loss, samples were removed from the oven, ground to powder, and sent to Soiltest Lab for chemical analyses.

At Site 1, the treatments had no effect on nutrient concentration in the fruit peel nor the flesh (Table 7). Similarly, in Site 2, only N and Mg concentration of the peel was affected by the spray treatments.

In the peel, N concentration was higher on all soil treatments and with F_Ca+N (between 0.25 and 0.27 %), compared to the control (0.23%). Levels of Mg in the peel were also higher in both soil treatments, but with no different from the control (Table 8). Regarding fruit nutrient concentration, values obtained in this study were equivalent to those obtained by Cheng and Raba (2009), however N levels were below the reported values for 'Honeycrisp', while Ca were higher.

Overall, the treatments did not affect Ca concentration on fruitlets, leaf tissue or in the fruit (peel and flesh). The response to nutrient uptake was different for each orchard, which might be due to their specific limiting factors. Initial diagnostics at Site 1 reflected root growth limitations: shallower root growth and reduced effective soil depth, with high accumulation of P and K in the upper layer. And while Ca uptake was not impacted by any of the treatments, soil treatments CaCO and CaSO improved N uptake. This effect could be attributed to modifying the environment around the root zone (weed removal, temperature) which relate to the site limiting condition. On Site 2, where there were no evident limiting factors, nutrient application of Ca treatment had no significant effect on nutrient uptake.

Correlations between nutrient levels from blooms, fruitlets, leaves and fruit peel and flesh were weak, though some were statistically significant ($p < 0.001$). The strongest relations were within tissue tissue (with correlation above 60%) (data not shown). The lack of correlation between nutrient levels and tissue suggest that nutrient analyses of fruitlets and leaves are not a good predictor for fruit nutrient concentration, which has been indicated previously by numerous authors (Manganaris et.al, 2005, Torres et al, 2015). The lack of prediction within nutrient levels is that trees are able to regulate their demand and needs when nutrient are sufficiently supplied.

2. Effect of treatments on fruit quality

At harvest, overall vigor assessment was determined on each site by measuring 20 shoot, trunk diameter and total fruit count from 3 representative trees on each site. Crop load was calculated as the number of fruit per square centimeter of trunk cross sectional area (fruit/cm^2 of TCSA). Site 1 had an average of 5 inches of shoot growth, 91.33 fruit per tree and 11.6 cm^2 TCSA, leading to a crop load of 8 fruit/cm^2 TCSA. Site 2, with grafted trees had an average of 3 inches of shoot growth, 216 fruit per

Table 7. Effect of calcium treatments on 'Honeycrisp' flesh and peel mineral concentration in Pasco orchard (Site 1).

Treatments	Flesh (% dry weight)					Peel (% dry weight)				
	N	P	K	Ca	Mg	N	P	K	Ca	Mg
Control	0.20	0.06	0.70	0.04	0.04	0.38	0.13	0.92	0.12	0.12
F_CaCl	0.19	0.06	0.71	0.05	0.04	0.36	0.13	1.00	0.12	0.11
F_CaCO	0.20	0.06	0.66	0.04	0.04	0.38	0.12	0.92	0.11	0.11
F_Ca+N	0.22	0.07	0.73	0.05	0.04	0.42	0.15	1.05	0.12	0.12
S_CaCl	0.19	0.05	0.64	0.04	0.04	0.38	0.12	0.92	0.10	0.11
S_CaCO	0.20	0.05	0.69	0.04	0.04	0.36	0.14	1.05	0.10	0.13
S_CaSO	0.21	0.06	0.71	0.05	0.04	0.39	0.13	0.94	0.12	0.12
Pr > F(Model)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

ns: no significance determined by ANOVA test ($p < 0.05$). Different letters within column indicate significant difference determined by Tukey mean separation test ($a = 0.05$).

Table 8. Effect of calcium treatments on 'Honeycrisp' flesh and peel mineral concentration in Sagemoor orchard (Site 2).

Treatments	Flesh (% dry weight)					Peel (% dry weight)				
	N	P	K	Ca	Mg	N	P	K	Ca	Mg
Control	0.12	0.05	0.67	0.04	0.03	0.23 c	0.10	0.80	0.07	0.07 ab
F_CaCl	0.13	0.05	0.66	0.04	0.04	0.25 bc	0.10	0.71	0.07	0.07 ab
F_CaCO	0.12	0.05	0.63	0.04	0.03	0.25 b	0.08	0.68	0.07	0.07 ab
F_Ca+N	0.13	0.05	0.63	0.04	0.03	0.25 ab	0.09	0.68	0.07	0.07 b
S_CaCO	0.13	0.05	0.63	0.04	0.04	0.26 ab	0.10	0.72	0.07	0.08 a
S_CaSO	0.14	0.05	0.62	0.04	0.04	0.27 a	0.09	0.75	0.07	0.08 a
Pr > F(Model)	ns	ns	ns	ns	ns	0.009	ns	ns	ns	0.041

ns: no significance determined by ANOVA test ($p < 0.05$). Different letters within column indicate significant difference determined by Tukey mean separation test ($a = 0.05$).

tree (with 4 leaders per tree) and 19.9 cm^2 of TCSA, leading to a crop load of 10 fruit/ cm^2 TCSA. A crop load above 8 had no negative impact on nutrient concentration (Serra et al. 2016) or bitter pit (BP) incidence (Robinson, et al., 2009), so this factor was not a limiting condition on either location.

The treatments had no impact on fruit firmness, weight, or diameter on Site 1. On Site 2, fruit was firmer with S_CaSO4 (Gypsum) compared to the control that had the softest fruit (Table 9). However, there was no statistical difference among treatments. On both sites, the grower managed condition had similar values to the ones obtained in this trial. When comparing values between orchards (data not shown), fruit firmness in Site 1 had significantly lower (17 lbs) and fruit was significantly bigger. Fruit weight in Site 1 ranged from 248 to 345 g, compared to 211 to 323 g on Site 2.

Table 9. Treatment effect on fruit quality indicators at harvest on 'Honeycrisp' apples on Site 1 and Site 2.

Treatments	Site 1- Pasco			Site 2- Sagemoor		
	Firmness (lbs)	Weight (g)	Diameter (mm)	Firmness (lbs)	Weight (g)	Diameter (mm)
Control	17.1	321	97	18.9 a	290	88
F_CaCl	16.8	299	89	21.2 ab	278	87
F_CaCO	15.6	289	88	20.9 ab	245	84
F_Ca+N	17.7	308	90	19.9 ab	262	85
S_CaCl	16.3	303	90	-	-	-
S_CaCO	16.6	298	89	19.3 ab	256	84
S_CaSO	17.7	292	89	22.0 b	262	86
Pr > F(Model)	0.134	0.738	0.293	**0.027**	0.339	0.399
Grower*	17	298	88	19	271	86

*Grower site was not included in the statistical analyses. Different letters within column indicate significant difference determined by Tukey mean separation test (a = 0.05).

Treatment effect on bitter pit (BP) development

To evaluate harvest BP, 40 fruit per replicated unit were randomly harvested and taken to the laboratory for fruit quality assessment and bitter pit incidence. For storage BP, half the fruit was stored at 39 F (the other half was utilized for the nutrient analyses). After 4 weeks of storage, fruit were removed from the cold room and kept at room temperature for 12 hours prior to BP evaluation.

Bitter pit incidence varied significantly between the orchard sites. Fruit from Site 1 exhibited very high levels of BP at harvest, ranging from 53% to 74% (data not shown), however there were no differences among treatments. After four weeks of storage, significant differences were observed between the control, with 54% BP and the F_$CaCO_3$ treatment with 5% BP. There were no statistical differences among the other treatments (Figure 3). The grower managed sample developed 60% BP after storage.

At Site 2, BP incidence at harvest and after storage was lower than at Site 1. At harvest, BP incidence ranged from 0 to 14% with no statistical differences ($p<0.05$). The grower managed sample exhibited only 4% BP incidence (data not shown). After storage, BP increased, though with no statistical differences at 95% probability. However, when considering 86% probability ($p = 0.136$), the F_Ca+N

treatment had lower BP incidence (3%) compared to the control (24%), with no differences among the other treatments (Figure 4).

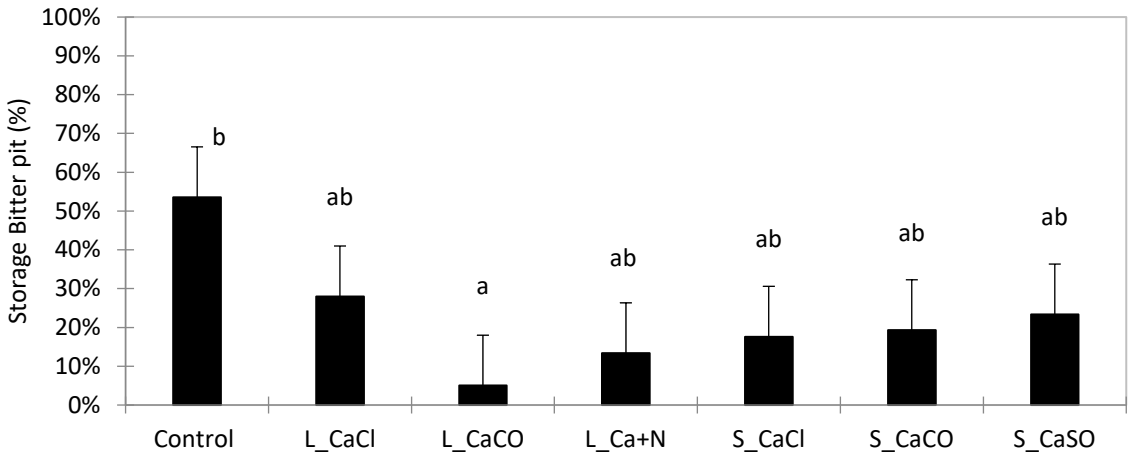

Figure 3. Effect of Ca treatments on Bitter pit development after 4 weeks of storage (bottom) on Site 1. Different letters indicate significant difference determined by Tukey mean separation test (a = 0.05).

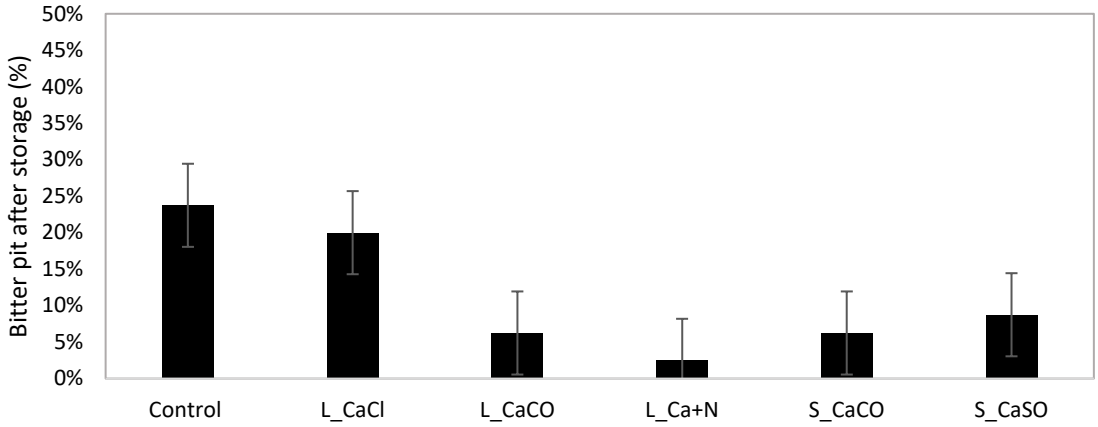

Figure 4. Effect of Ca treatments on Bitter pit development after 4 weeks of storage on Site 2. Treatment were not significantly different ($p > 0.05$)

The relation between parameters and bitter pit incidence vary between sites. Site 1, had a strong relation between fruit weight and BP incidence. The linear regression linear regression $y = 0.0069x - 1.4643$ had a correlation value *r* of 0.8, and coefficient of determination R2 of 0.64 (Figure 5). If the *r* value is close to 1 or -1, the relation is very strong, while the R2 value reflects how much of the variation on BP incidence can be explained by the fruit weight, in this case 64% of BP at harvest can be explained by the fruit weight. However, Site 2 had a weak relation between BP and fruit weight ($r = 0.33$) and the relation only explained 10% of the variation (Figure 5)

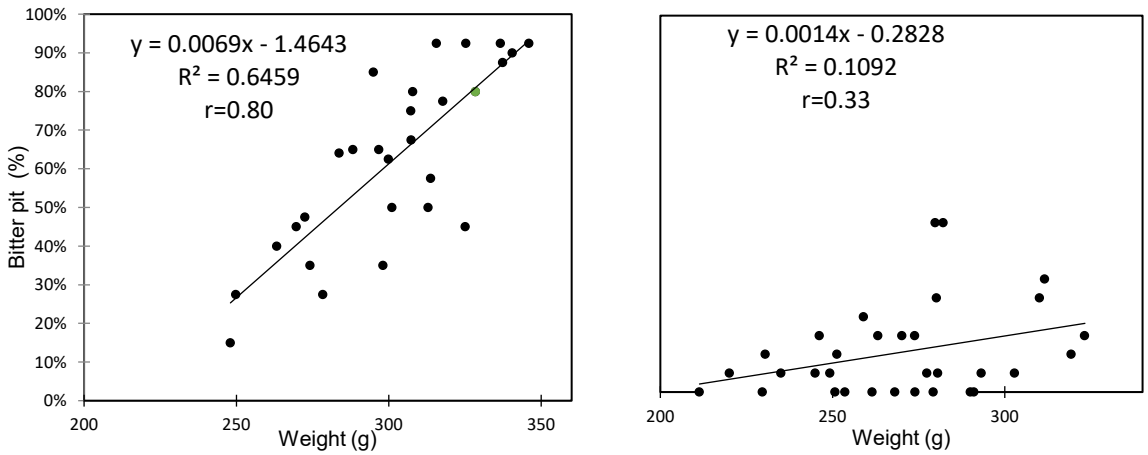

Figure 5. Relation between Bitter pit and fruit weight on Site 1 (left) and Site 2 (right) ($p < 0.001$).

Overall, the different treatments of Ca applications at a fixed rate of 12 lbs of actual Ca/acre had no statistical differences in controlling BP incidence after storage on 'Honeycrisp' apples. The two sites had significant differences in BP incidence at harvest and after storage, with high incidence at Site 1 with average of 54% compared to 24% on Site 2. Among treatments, there were no statistical differences in controlling BP incidence. However, when compared with the control receiving no Ca, F_$CaCO_3$ and F_ Ca+N where effective in reducing BP on Site 1 and Site 2, respectively. At Site 2, where sulfate (S) levels in the soil were deficient, S_$CaSO_4$ improved fruit firmness when compared with the control, but there were no differences between the other Ca treatments. On both sites, Ca treatments had no effect on Ca uptake by the fruitlets, leaves of fruit flesh and peel, and none of these measurements correlated with BP incidence, thus should not be utilized as BP predictors. Only at Site 1, where fruit was oversized (above 300 g), fruit weight had a strong relation with BP development. However, under adequate fruit weight and size (below 300 g), there were no strong predictors of BP development.

Possible causes for the higher BP incidence exhibited at Site 1 can be associated to: reduced root growth due to soil stratification, excessive levels of soil K (above 250 mg/kg) and oversized fruit (above 300g). Interestingly, results from the treatments receiving 12 lbs of Ca/acre in this trial, did not differ from the results observed from the grower-managed areas, where total applied Ca was more than 200 lbs/acre and 400 lbs/acre on Site 1 and Site 2, respectively. Therefore, from this one year trial, it appears that prophylactic applications of calcium are ineffective for reducing BP.

LITERATURE

Atkinson, D and G.C. White. 1980. Some effects of orchard soil management on the mineral nutrition of apple trees. p. 241-254 In: D. Atkinson, J.E. Jackson, R.O. Sharples and W.M. Waller (eds.), Mineral nutrition of fruit trees. Butterworths, London and Boston.

Ferguson, I. B.; Reid, M. S.; Prasad, M. 1979. Calcium analysis and the prediction of bitter pit in apple fruit. New Zealand Journal of Agricultural Research Vol.22 No.3 pp.485-490.

Gavlak, R. G., D. A. Horneck, and R. O. Miller. 2005. Plant, Soil and Water Reference Methods for the Western Region. 2005. http://www.naptprogram.org

Kalcsits, L., G. van der Heijden, M. Reid and K. Mullin. 2017. Calcium Absorption during Fruit Development in 'Honeycrisp' Apple Measured Using 44Ca as a Stable Isotope Tracer. HORTSCIENCE 52(12):1804–1809. 2017. doi: 10.21273/HORTSCI12408-17

Manganaris, G.A., Vasilakakis, M., Diamantidis, G., Mignani, I., 2005. Effect ofpost-harvest calcium treatments on the physicochemical properties of cellwall pectin in nectarine fruit during ripening after harvest or cold storage. J.Hortic. Sci. Biotechnol. 80, 611–617.

Marschner H. 2002. *Mineral Nutrition of Higher Plants*. 3rd edition. *Academic Press*, *London*, U.K
Shear, C.B. and M. Faust. 1980. Nutritional ranges in deciduous tree fruits and nuts. Horticultural Reviews 2, 142-163

Robinson, T. L, Lopez, S., Iungerman, K. and Reginato, G. 2009. Crop load management for consistent production of Honeycrisp apples. NY Fruit Q. 17(1): 24–28.

Sera, S., R. Leisso, L. Giordani, L. Kalcsits, S. Musacchi. 2013. Crop load Influences Fruit Quality, Nutritional Balance, and Return bloom in 'Honeycrisp' apple. HortScience 51 (3): 236 – 244. https://doi.org/10.21273/HORTSCI.51.3.236

Torres, E., Recasens, I., Peris, J.M., Alegre, S., 2015. Induction of symptomspre-harvest using the 'passive method': an easy way to predict bitter pit.Postharvest Biol. Tehnol. 101, 66–72.

EXECUTIVE SUMMARY

Project title: Calcium fertilization efficacy

Key words: Calcium, Bitter pit, Honeycrisp.

Abstract: Calcium (Ca^{2+}) has been recognized as one key element due to its many roles in plant physiological processes and fruit development. Several fruit disorders have been associated to Ca^{2+} deficiencies, including bitter pit (BP), the most important for apple growers in Washington. The application of Ca to reduce Ca-related disorders has been widely used in apple production with inconsistent results or no improvement at all. The objective of this research was to evaluate Ca fertilizer efficacy on nutrient uptake and fruit quality, including BP development at harvest and after storage in two orchards. The treatments included a control with no Ca, three foliar sprays (F): F_CaCl, F_$CaCO_3$ and F_Ca + N (Calcium plus 1% N), and three soil applications (S): S_CaCl (only in the conventional site), S_$CaCO_3$ and $CaSO_4$ (Gypsum). The total amount of actual Ca applied was equal for all treatments at 12 lbs/acre. Treatments were applied on 6 or 4 dates for foliar and soil, respectively, every 14 days starting at petal fall. Gyspum ($CaSO_4$) was applied in one application with the second irrigation. The two sites had significant differences in BP incidence at harvest and after storage, with high incidence at Site 1 with average of 54% compared to 24% on Site 2. Among treatments, there were no statistical differences in controlling BP incidence. However, when compared with the control receiving no Ca, F_$CaCO_3$ and F_ Ca+N where effective in reducing BP on Site 1 and Site 2, respectively. At Site 2, where sulfate (S) levels in the soil were deficient, S_$CaSO_4$ improved fruit firmness when compared with the control, but there were no differences between the other Ca treatments. On both sites, Ca treatments had no effect on Ca uptake by the fruitlets, leaves of fruit flesh and peel, and none of these measurements correlated with BP incidence, thus should not be utilized as BP predictors. Possible causes for the higher BP incidence exhibited at Site 1 can be associated to: reduced root growth, excessive levels of soil K (above 250 mg/kg) and oversized fruit. Interestingly, results from the treatments receiving 12 lbs of Ca/acre in this trial, did not differ from the results observed from the grower-managed areas, where total applied Ca was more than 200 lbs/acre and 400 lbs/acre on Site 1 and Site 2, respectively. Therefore, from this one year trial, it appears that prophylactic applications of calcium are ineffective for reducing BP.

FINAL PROJECT REPORT Year: 2 of 2

Project Title: Pollination, flower biology and fruit development in 'WA38' apples

Project Award: AP-19-102

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Total Project Request: Year 1: $67,156 Year 2: $69,262

In-kind support:

- TASC (2014-2019): "Identifying and Managing Sources of Quarantine Significant Post-Harvest Diseases in Pacific Northwest Apple and Pear Orchards" total funds $ 1,913,832 provided several of the pollinizers we will use and contributed to develop expertise in flower biology.
- Project #AP14-103A: "WA38 rootstocks and training systems" (2014-2016+1yr NCE) total funds $ 242,519 provided the support to maintain the orchard for this project.

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Serra-Musacchi		
Costs	Year 1 (2019)	Year 2 (2020)
Salaries [1]	$ 36,000	$ 37,440
Benefit [2]	$ 14,033	$ 14,594
Wages [3]	$ 2,400	$ 2,496
Benefit [4]	$ 223	$ 232
Supplies [5]	$ 10,000	$ 10,000
Plot fee [6]	$ 1,500	$ 1,500
Travel [7]	$ 3,000	$ 3,000
Total	$ 67,156	$ 69,262

Footnotes:

[1] Salary for a 75% Research assistant/Research intern ($4,000/month)

[2] Benefit on salary at 38.98%

[3] One non-student temporary for 4 wks: 40hrs/wk at $15/hr

[4] Benefits on temporary wage

[5] Labware/consumable, includes $ 1,200 membership for Franceschi Microscopy & Imaging Center (Pullman, WA) and $ 600 for electronic fruit sizer.

[6] Plot fee for the orchard

[7] 5,556 miles/year for domestic travel (0.54$/mile).Travel to orchard and to Pullman for microscopy work.

Recap objectives:

1. Assess the effective pollination period for 'WA38' and identify limiting factors (2019).
2. Evaluate pollen tube growth of different crabapples in 'WA38' flowers (2019-2020).
3. Analyze seed set, fruit drop, and fruit growth potential based on pollen source (2020).

Significant findings:

1. Assess the effective pollination period for 'WA38' and identify limiting factors (2019).
    - Effective pollination period (EPP) for 'WA38' apples was at least 2 days in 2019:
        - Stigmatic receptivity: 9 days
        - Pollen tube growth ('Granny Smith') from stigma to ovule: 7 days
        - Ovule longevity: 9 days.

2. Evaluate pollen tube growth of different crabapples in 'WA38' flowers (2019-2020).
    - Pollen tubes of 'Snowdrift' tend to grow faster inside 'WA38' styles than other pollinizers tested in 2019.
    - In 2020, no significant difference was found across the pollen tube growth of the 5 pollen sources three days after pollination.
    - Between Day 4 and Day 5, after pollination, all pollen sources passed the style base (between 2 years).
    - No significant differences in the fruit set (%) between the 5 pollen sources in May, June nor September (2020).
    - 'WA38' fruit weight and diameter and number of healthy seeds did not significantly differ comparing the 5 pollen sources.

3. Analyze seed set, fruit drop, and fruit growth potential based on pollen source (2020).
    - When only one stigma (out of 5) in the flower gets pollinated, there is a higher possibility that apples are misshapen and have incomplete seed set.
    - By the end of May, 82% of flowers/fruitlets naturally dropped, and no significant shedding occurred in the following weeks.
    - At harvest (150 DAFB), 51% of tracked clusters ended up with single fruit, 12% with double fruit, 35% empty (no fruit), and 2% broken (out of trial).
    - Imposing king and lateral occupancy treatments within a cluster at the end of April did not result in significant differences in the proportion of clusters that retained fruit at harvest.

Results and Discussion

Objective 1: Effective pollination period

Stigmatic receptivity

The effective pollination period (EPP) is defined as the time period in which a pollination event with compatible pollen can result in ovule fertilization (Sanzol and Herrero, 2001, *Scientia Horticulturae* 90(1):1-17). This period can be limited by the stigmatic receptivity, pollen tube growth, and ovule longevity. We observed a significant decrease in pollen germination on the stigmatic surface area on Day 4; this is probably related to the quality of 'Granny Smith' pollen used on this specific day, since the ability of the stigmas to support pollen adhesion was not affected (80% but not statistically different from Day 1 to Day 3; data not shown). On Day 10, only 8% of the stigma samples analyzed contained adhered pollen, and none of the stigmas pollinated on Day 10 carried germinated pollen. Thus, in our 2019 study, stigmas of WA38 were considered receptive for nine days (data not shown).

Pollen tube growth

'Granny Smith' pollen tubes reached the ovules seven days after pollination (data not shown). There was no variation between sample replicates; however, pollen tubes from different pollinizers could potentially reach the ovules earlier or later (see Objective 2: Pollen tube growth of five different pollinizers for details). Pollen tube growth rates in WA38 pistils could differ depending on the location because environmental factors also influence pollen tube growth. In general, higher temperatures increase pollen tube growth rate. Pollen tube growth from the style to the ovule in 2020 was reported in the paragraph "Objective 2A: Pollen tube growth" below.

Ovule viability

Ovules were classified by microscopy assessment either as viable or senescent based on the fluorescence signal's absence/presence (data not shown). In our 2019 sampling, the first fluorescent 'WA38' ovules were observed on Day 9 (out of 10 days of samples available). Not all ovules from Day 9 and 10 showed fluorescence, which suggests there is some variability between flowers in the timing of ovule senescence. Day 9 and Day 10 samples showed an "ovule senescence index" with an estimated mean of probability of 0.3 and 0.7, respectively, indicating that ovules started becoming senescent (index=1 means ovules are senescent).

Overall, based on the present data, the effective pollination period for 'WA38' in 2019 was two days (Figure 1). This window of time was calculated by subtracting the time required for the pollen tube to reach the ovules (7 days) from the longevity of the ovules (9 days), knowing that the stigmatic receptivity was not a liming factor until Day 10. All three components of the pollination period are temperature-dependent.

Usually, a higher temperature increases pollen tube growth but decreases ovule longevity. In this experiment, we decided to use 'Granny Smith' as a fully compatible pollen source based on bloom phenology, full compatibility, and availability; however, pollen tube kinetics may vary depending on the pollen source (see results Objective 2).

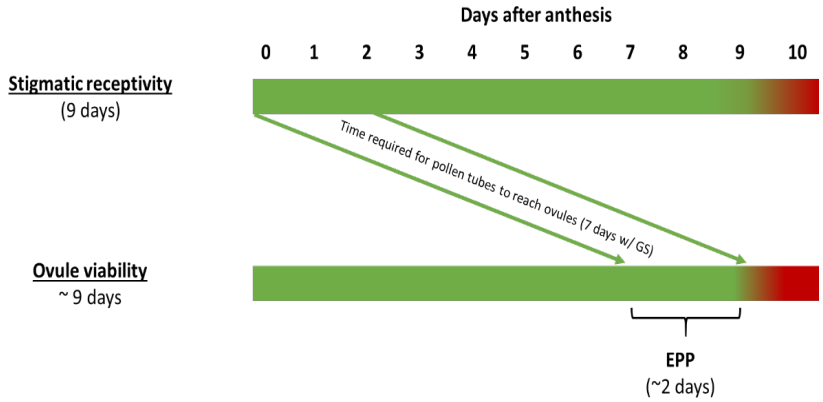

Figure 1: *Effective pollination period (EPP) in WA38 flowers. EPP is based on duration of stigmatic receptivity, pollen tube kinetics, and ovule longevity. The bracket indicates the EPP approximately 2 days with the present experimental conditions in 2019.*

Objective 2: Evaluate pollen tube growth of different crabapples in 'WA38' flowers

Objective 2A: Pollen tube growth

In 2019, WA38 flowers were cross-pollinated with 5 different pollen sources: 'Evereste', 'Indian Summer', 'Granny Smith', 'Snowdrift', and 'Frettingham' where the last pollen source was substituted for 'Mt Blanc' due to an off-blooming year in our 'Mt. Blanc' collection. Blossoms were harvested from pollinizers planted in Sunrise Research Orchard in 2016 and trained to a spindle (spacing 5 ft × 12 ft). In 2020, the 5 pollen sources used for this objective were 'Evereste', 'Indian Summer', 'Granny Smith', 'Snowdrift', and 'Mt Blanc' as originally planned. Anthers were manually separated from

blossoms and dried at 25 ± 1°C (RH: 24.4 ± 5.3 %) for 2-3 days (until anther dehiscence) prior to pollination. Fifty-four flowers on spurs (best lateral available) at pink balloon stage (4/15/2020) were tagged, emasculated, and cross-pollinated with each of five pollen sources across 27 WA38/NIC29 biaxis trees, for a total of 270 flowers. After hand-pollination, flowers were isolated with Kleenguard™ A20 protective sleeves. Six flowers pollinated from each of the five pollen sources (2 flowers/tree rep) were harvested in 24 h intervals for 9 days. Flowers were fixed and prepared following the protocol described in report Year 1.

In 2019, pollen tubes from 'Snowdrift' reached the base of the 'WA38' style four days after pollination **(**data not shown**),** while the other four genotypes required one additional day (Day 5, data are shown in previous report Year 1). 'Frettingham' and 'Indian Summer' were slower than 'Evereste' and 'Granny Smith' at Day 3 (=72 h after pollination (AP)). The same trial repeated in 2020 showed some different results. Firstly, by Day 4 all the pollen had already reached the base of the style, one day earlier than in 2019. The difference in pollen tube lengths between pollen sources in 2020 (as proxy for pollen tube growth rate) was significant only at 48 hours AP (Day 2), where Snowdrift was confirmed to be the fastest pollen tube grown among the 5 pollen sources compared, as reported in 2019 for Day 3. In order of descending pollen tube length at Day 2 we found 'Indian Summer', 'Granny Smith' and 'Mt Blanc' (statistically similar), and the slowest was 'Evereste' in 2020. After 72 hours AP, however, there were no significant differences in pollen tube length between the 5 pollen sources, and by the following Day (4) all of them had passed the base of the style (Table 1). A high pollen tube growth rate is recommended for a good pollinizer to ensure flowers get fertilized in the shortest period of time: the more time it takes for pollen tubes to reach the ovule, the shorter the EPP becomes. Upon feedback from the first-year project report, the sampling of pollinated flowers by 5 different pollen sources was extended to Day 9 in 2020 (up to Day 6 in 2019) to dig further into the pollen tube journey after surpassing the base of the style. In general, from the microscopy observation of pollinated flowers on Day 5 we have not found any pollen tubes reaching the ovules, while on Day 6 and Day 7, about the 64% and 95% of the discerned pollen tubes, respectively (excluding flowers where pollen tubes were not able to be visualized) were in close proximity of the ovules (data not shown). On Day 8, 100% of discernable pollen tubes reached the ovules. Pollen tube growth is highly dependent on temperature, so a comparison between 2019 and 2020 is necessary to

Table 1: *'WA38' pollen tube length measurements in 2020 for obj. 2A: comparison between 5 pollen sources for 3 days and across the 3 days within each pollen source. Significance reported in the legend: *= p<0.05, **=p<0.001, ***p<0.001.Means are separated with post-hoc SNK test within each parameter, where different letters indicate significant difference between those means.*

Pollen sources 2020	**N pistils (avr 5 styles)**	**Day 1 (=24h AP)**		**Day 2 (=48 h AP)**		**Day 3 (=72h AP)**		***Signif. across days***
Evereste	6	0.96	C	2.66	c B	8.33	A	***
Mt Blanc	6	0.83	C	3.81	b B	7.66	A	***
Granny Smith	6	0.91	C	3.67	b B	7.58	A	***
Indian Summer	6	0.91	C	4.09	ab B	8.57	A	***
Snowdrift	6	0.92	C	4.47	a B	7.96	A	***
Significance across pollens		***NS***		***		***NS***		

AP= after pollination. Capital letters discriminate means horizontally for each pollen source (significance across days)

Small letter discriminate means vertically for each day (significance across pollen sources). WA38 styles = 10.2 mm on average in 2020. The number of styles measured range from 25 to 30 for each time point and pollen source.

draw some conclusions. To quantify and display the potential year-to-year variation in weather, we plotted the growing degree hours (GDH) with a baseline of 41°F from Sunrise AWN weather station from Day 1 to Day 9 (AP) in 2019 and 2020 (Figure 2). The 24 hours of difference between 2019 and 2020 for the average (avg) pollen tube to reach the base of the style can be due to: 1) higher GDH in 2020 (warmer AP week) from Day 3 to Day 9 with respect to 2019 and 2) the average style length of 'WA38' in 2020 was 1 mm shorter than in 2019 (10.2 mm and 11.2 mm respectively). An approximate amount of GDH ranging from 1440 to 1700 (GDH °F) was needed for the pollen tube to successfully reach the base of the style based on the two years of the experiment (Figure 2).

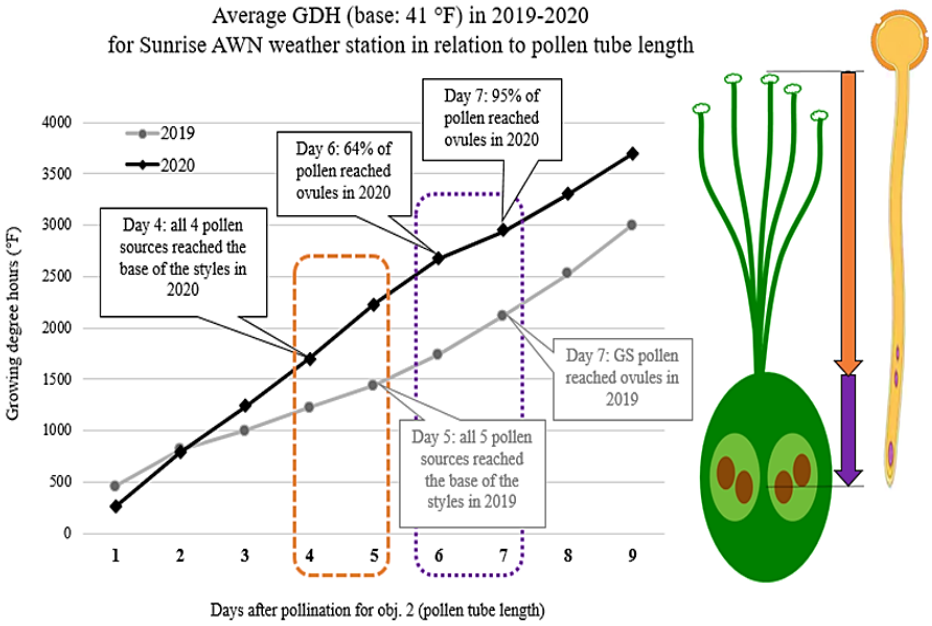

Figure 2: *Comparison between 2019 and 2020 in growing degree hours (GDH) with a base temperature of 41°F from Sunrise AWN weather station from Day 1 to Day 9 (days after pollination) for objective 2A. Dashed-line box marks the days AP when the pollen tubes reached the base of the 'WA38' styles, while the dotted-line box indicates when pollen tubes were in general proximity of ovules. Arrows on the right indicate the two meaningful position (based of the style and ovule proximity) in the "pollen tube journey".*

Objective 2B: Fruit set

The pollen performance was also assessed in terms of 'WA38' fruit set after hand pollination with the 5 pollen sources in trial. Fifteen mature trees of 'WA38'/NIC29 trained to V (planted in 2013 at 2,997 trees/Acre) were selected for this objective. Pollination was performed on 4/16/20 at balloon stage, and the best lateral flower on spur was selected and thinned to a single (10 flowers x 3 trees x 5 pollen sources). Starting one month after pollination (5/15/20), fruit set ranged between 70% and 87%, with no significant differences across the 5 pollen sources (data not shown). No significant differences, neither in June nor in September (at harvest), were found related to the fruit set. Despite the lack of statistical significance between the fruit set for the different pollen sources, a clear reduction in fruit set, regardless of the pollen source, was evident between May and June. For instance, 'Granny Smith' reported a decrease in fruit set from 87 % in May to 43 % in June, but after the "shedding wave," all the apples left on the tree were retained until harvest (data not shown). Seed analysis was conducted on all apples harvested from this objective, and no significant differences were found comparing the 5 pollen sources regarding average 'WA38' fruit weight, fruit diameter, number of good/healthy seeds, number of underdeveloped seeds. The average number of "healthy" (as determined by morphology) seeds/fruit ranged from 8.7 in 'Mt. Blanc' to 9.6 for 'Snowdrift', and the average number of seeds/fruit was 9.2 across all the pollen used.

Objective 3: Analyze seed set, fruit drop, and fruit growth potential based on pollen source

This set of experiments designed to investigate Objective 3 was carried out in 2020, according to the original project proposal. The present objective is divided in 3 sub-objectives: A) pollination intensity and seed set analysis, B) tracking natural shedding inside clusters from pre-bloom to harvest, and C) fruit development with or without king flower in the cluster.

Objective 3A: pollination intensity and seed set analysis

In this experiment, we simulated different pollination situations by removing stigmas and saturating the remaining stigmas with compatible pollen to investigate if varying degrees of "pollination intensity" can affect the fruit shape and size. Eighteen mature trees of WA38/NIC29 trained to bi-axis (planted in 2013 at a density of 1,499 trees/acre and headed back in 2014) were selected for this objective. For each of the six treatments, thirty king flowers on spurs (10 flowers × 3 trees) were singularized (no laterals) at late balloon stage, emasculated and pollinated with compatible pollen of 'Granny Smith' on 4/14/20. The six treatments were established to test the number of stigmas and therefore, the level of pollination intensity needed to achieve a full seed set. Treatments were applied by removing 0, 1, 2, 3, 4, or 5 stigmas and then hand pollinating the remaining stigma(s) in each flower. 'Granny Smith' pollen utilized for this pollination was previously collected, sieved, and dried at room temperature for 48 hours. Pollen was germinated on 1% agar plates in the field on the day of pollination to confirm its viability. No pollination bags were installed for this task. Starting from 1 month after pollination day, the fruit set was assessed for each of the 180 king flowers as presence or absence. The assessment was repeated after June drop and then at harvest on 9/15/20 (150 DAFB). Apples were picked and stored at 34 °F until further analysis. Six weeks after harvest, apple dimensions were measured, including equatorial diameter, maximum and minimum diameter, maximum and minimum height, and individual fruit mass. Additional parameters were calculated based on fruit dimensions: Symmetry Index A = Min diameter/Max diameter, Symmetry Index B = Min height/Max height, H/D ratio = average height/average diameter. Moreover, the seed analysis included the following parameters: number of carpels/fruit, distribution of seeds among the carpels (following the template from Sheffield 2014, *Journal of Pollination Ecology*, 12(13):120-128), number of seeds per carpel, seed types (healthy and underdeveloped) and healthy seed weight.

Starting at one month after pollination (5/15/20), the significant difference between the six treatments highlighted a lower fruit set when all 5 stigmas were cut before hand pollination in comparison to 0 stigmas cut, and a slight decrease when only 1 stigma remained (Figure 3). In June, a similar fruit set confirmed the significant difference between the fruit set of 93% in flowers with 5 stigmas remaining (no cut) and the flowers from which all 5 stigmas were removed (17%). In September, the final % fruit left per treatment did not result in statistically significant differences (Figure 3). The uneven pollen deposit between the five stigmas during bee visitation can result in variable seed distribution that directly affects fruit size and shape. Regarding apple dimensions and weight, the treatment that reported the lowest symmetry indices was "4 stigmas cut, 1 left" confirming our hypothesis (data not shown). The average differences between maximum and minimum diameter and maximum and minimum height were confirmed to be larger in the "4 stigmas cut, 1 left" (followed by "5 stigmas cut" and "3 stigmas cut"), suggesting this condition can lead to more asymmetric and misshapen fruit. Treatments equal to and fewer than "3 stigmas cut" produced apples with the least variation in dimensions. No significant differences emerged in relation to Height/Diameter (H/D) ratio nor average fruit weight across the 6 treatments (avg H/D=0.91 and avg apple weight 290 g). The seed occupancy in the carpels showed that leaving at least 3 stigmas in the flower resulted in 100% of fruit with a full seed set (100% apples had "healthy" seeds in the 5 carpels). When the number of stigmas remaining in the flowers decreased (2 or fewer stigmas), we observed carpels without "healthy" seeds (data not shown). The treatments with the highest number of stigmas remaining (3 to 5

remaining/flower) showed the best seed set where on average, 9.7-9.8 of "healthy" seeds were found in each apple **(**data not shown**)**. WA38 often exceeded the standard 10 seeds/fruit and sometimes carried as many as 14 seeds.

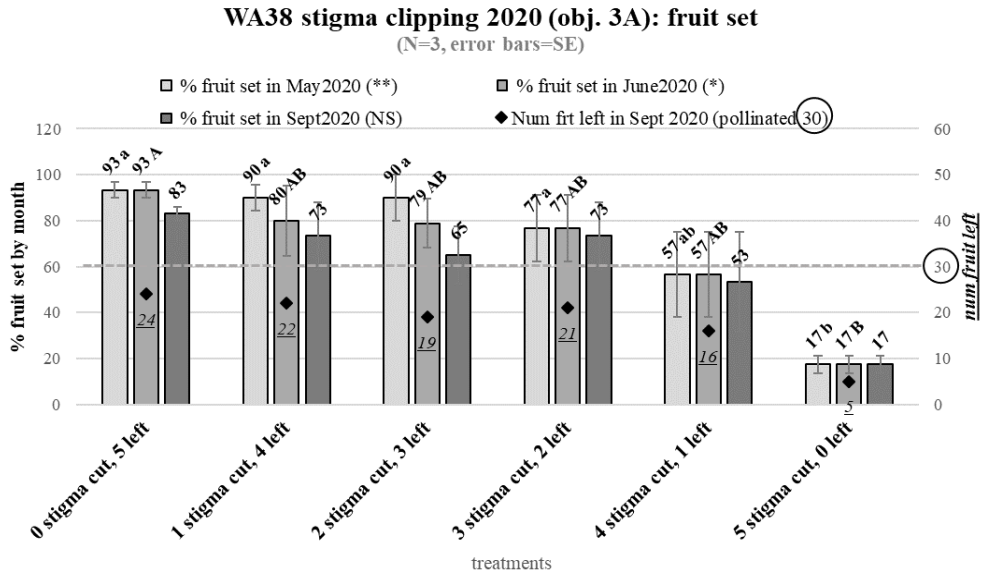

Figure 3: *'WA38' fruit set (%) in May, June and September 2020 based on the numbers of stigma left on the flower (0, 1, 2, 3, 4 and 5) and then hand pollinated with 'Granny Smith' pollen. Three trees with 10 flowers each were used as replication (N=3), error bars represent the standard error of the mean. For each of the 6 treatments 30 flowers were selected and in the secondary Y axis the number of fruit retained until harvest/treatment is reported. Significance reported in the legend: *= p<0.05, **=p<0.001, NS= not significant. Means are separated with post-hoc SNK test within each month, where different letters indicate significant difference between those means.*

Objective 3B: tracking natural shedding inside clusters from pre-bloom to harvest

One hundred WA38 clusters were selected across 2 days on 4/15/20 and 4/16/2020 across 14 WA38/NIC29 bi-axis trees planted in the same rows described for objective 3A. Each cluster was numbered, and each flower within a cluster was marked by acrylic paint to identify it by cardinal position from that moment on as follows: King (no color), blue (west lateral), yellow (northwest lateral), red (northeast), white (east), black (south). The assessment of phenology and presence/absence of each flower in the clusters were carried out daily from 4/16/20 to 4/29/20, then every 2-3 days from 4/29/20 to 6/6/20, then weekly until harvest on 9/15/2020 (150 DAFB). The WA38 natural shedding of flowers/fruitlets was the primary focus of this objective, as this variety is characterized by a self-thinning tendency that reduces the need for chemical thinning. This 22-week long cluster assessment allowed us to track the natural shedding and define the duration of the different phenological stages from bloom to fruit set. In contrast to objectives 2A-B and 3A, the data collected in objective 3B represents fruit set under open-pollination conditions instead of hand-pollination. Starting at 32 DAFB, maximum apple diameters of attached fruitlets were measured with digital calipers, and a fruit growth curve was plotted for fruit that were retained on the tree throughout the season; the frequency of the measurements were first every 2-4 days for a month, then weekly until harvest.

Tracking 100 clusters from balloon stage until harvest allowed us to observe several peculiar traits of WA38. The time window from first bloom to all open flowers in our experimental condition

was from 4/15/20 to 4/20/20; in the 5 days, 549 flowers (across 100 clusters) opened. At the time of cluster selection flowers were at the balloon stage. However, by 4/16/20, 94% of the king blooms had opened, followed by lateral flowers facing southwest and southeast (34% and 25%, respectively), while just 7% of the north-facing lateral flowers had opened. Within 48 hours from first bloom, the percentages of open flowers ranged from 77% (lateral north-facing flowers) to 97% king flowers (data not shown). April 18th was recorded as the "full bloom date" for king blossoms and served as the reference date for counting days after full bloom (DAFB). Petal fall also started early for the king flowers (starting in the first 24-48h), and within 48 hours from the beginning of bloom, 38% of the king flowers were in petal fall while the lateral flowers had just begun shedding petals after 72 hours (=4/19/20). The petal fall phase lasted 9 days and ended on 4/24/20 (data not shown). The difference in phenology between king and lateral flowers was around 48-72 hours, with the king flowers blooming earlier as reported in the literature and regularly observed.
The flower/fruitlet drop started on 4/21/20 and ended 49 days later. Within the first 3 weeks post-bloom, the drop was around 23% regardless of the flower's specific position in the cluster, and in the following week (5/11/20= week 4) almost doubled up to 45% (Figure 4).

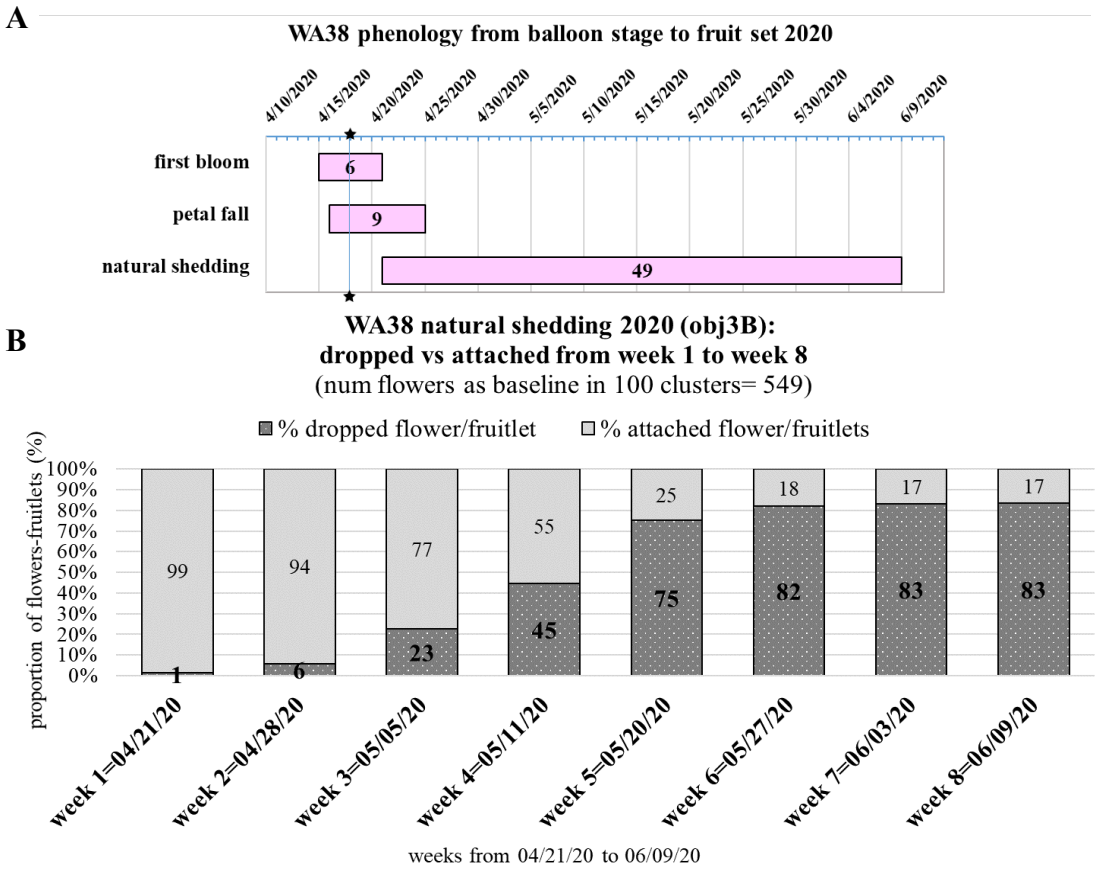

Figure 4: *A) 'WA38' phenology from balloon to fruit set in 2020, length of bars represents duration of each stage in days. B) 'WA38' natural shedding from 4/21/20 to 6/09/20 (=8 weeks) considering 100 clusters in trial.*

During the second half of May, the natural shedding of flowers/fruitlets reached 82% and did not change much for the following two weeks (83%). We consider the "shedding wave" of the variety ending on 6/9/20 (=52 DAFB=8 weeks of shedding); the remaining fruitlet drop until harvest was minimal and likely due to random causes unrelated to genetic factors (broken branch, bird damage, etc.). A survey was conducted to understand the destiny of the clusters up to harvest. Of the one hundred clusters

selected before bloom, 52% consisted of 6 flowers (1 king and 5 laterals), 45% of 5 flowers, and 3% were made of only 4 flowers. At 150 DAFB, 35% of the clusters had completely dropped (no fruit retention), and 2% were broken during evaluation and excluded from the trial. Clusters supported two apples until harvest in 12% of the inflorescences monitored, whereas 51% of the clusters yielded only one fruit at harvest (Figure 5). Of the clusters that supported a single apple until harvest, the king was set in 78% of "single" clusters, whereas 22% were set on laterals (most frequently the blue, west-facing lateral, Figure 5). King fruitlets reported a higher diameter in comparison to lateral fruitlets for 8 weeks, between 5/20/20 and 7/8/20 (data not shown**)**, but after 7/8/20 the diameter of the two types of fruit did not statistically differ (average size 86 mm at harvest). A difference in fruit size emerged when comparing apples that were growing in double in the same cluster with respect to apples grown in single-fruit clusters **(**data not shown). The "double" apples were penalized from 35 to 42 DAFB, reporting a significantly lower diameter on average in comparison to single fruit/cluster, but this aspect did not impact the final fruit size.

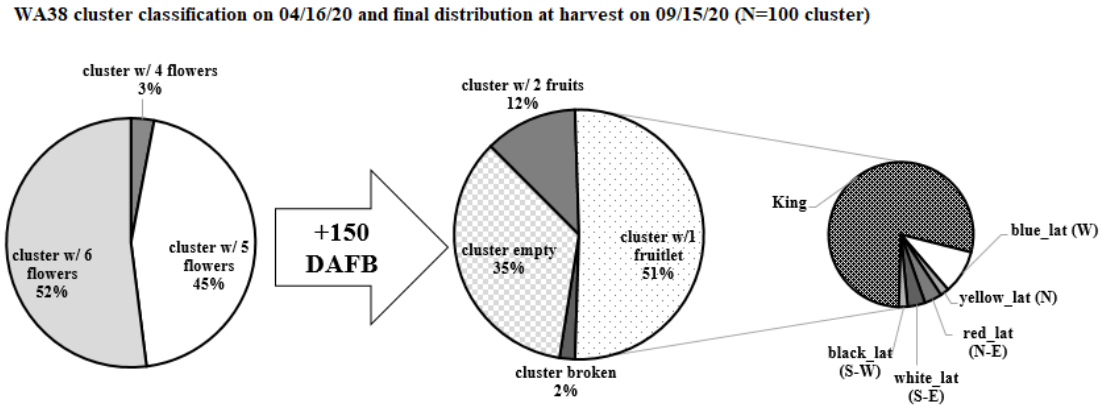

Figure 5*: 'WA38' clusters classification from bloom (4/16/20) to harvest (9/15/20) for obj. 3B.*

Analyzing the characteristics of the apples after harvest, we identified some major differences comparing firstly, apples originating from king flowers versus apples borne from lateral flowers (regardless of their position inside the cluster). Apples produced by king flowers and those coming from lateral flowers did not differ significantly in average diameter, nor for any of the symmetry indices (Table 2). The main parameters that highlighted differences between these two types of fruit were the average apple height, and the H/D ratio, for which fruit originating from king flowers showed high values. Those apples indeed tended to be more spherical than the apples from lateral flowers, which were shorter in height and had lower average H/D ratios. Despite the absence of statistical differences in diameter (avg 85 mm) between those types of fruit, the "king apple" registered a higher average fruit weight than the "lateral apple" with a difference of 33 g between average values (Table 2). Seed counts and weights did not reveal significant differences between the two types of fruit (data not shown), with an average number of healthy seeds/apple of 9.9 and 9.5 respectively for "king apples" and "lateral apples" (67 mg/seed for both, NS). This comparison did not consider the number of apples per cluster.

Looking at the comparison between fruit that were retained until harvest as single apples in the cluster (named "single", N=52) versus apples that were retained as doubles (named "double", N=24) allowed us to shed more light into the cluster dynamics. "Double" apples were less round and shorter in height than "single" apples, which were more spherical with a significantly higher H/D ratio. The latter also tended to have a higher average fruit weight than the former with 292 g for the "single" and 267 g for the "double" (but this difference was not statistically significant, data not shown). Interestingly,

"single" apples presented a more complete seed set with respect to the "double"; single fruit in clusters presented an average of 9.9 healthy seeds/apple versus 9.3 for the "double." Moreover, the number of underdeveloped seeds were significantly higher in the "double" fruit with respect to the "single" (0.8/apple vs 0.4/apple).

***Table 2**: WA38 apple dimensions and weights for obj. 3B: comparison between flower of origin (king versus lateral) regardless to the number of fruits/cluster. Significance reported in the legend: *= p<0.05, **=p<0.001, ***p<0.001, NS= not significant. Means are separated with post-hoc SNK test within each parameter, where different letters indicate significant difference between those means.*

FLOWER TYPE	N=	Avr. apple diameter (mm)	Avr. apple height (mm)		Avr. Symmetry Index A	Avr. Symmetry Index B	Avr. H/D		Avr. apple weight (g)	
KING	46	85.50	77.55	A	0.96	0.94	0.91	A	297	A
LATERAL	30	84.08	71.18	B	0.96	0.93	0.85	B	264	B
Significance		*NS*	***		*NS*	*NS*	***		**	

Objective 3C: fruit development with or without king flower in the cluster

This part of the study was focused on attaining a deeper understanding of the fate of the flower clusters with and without the presence of the king flowers. We started with 30 clusters for each possible pattern of king flower with 0, 1, 2, 3, 4, or 5 laterals (6 combinations with king; K+0L to 5L) and a duplicate set of clusters without the king flower (5 combinations without king; 1L to 5L) for a total of 11 combinations and 330 flower clusters selected. On 4/17/20, eighty WA38/NIC29 V-system trees were chosen, and king flowers were removed the same day for the 5 combinations without king (noK). On 4/29/20, clusters were labeled by combination and excess of laterals manually clipped to meet the target number flowers/cluster. From 4/29/20 to 5/20/20, the clusters were tracked weekly to assess the drop and from 5/20/20 to harvest on 9/18/20, fruit(lets) were also measured for their diameter at their widest point. At harvest (9/18/20), all the apples remaining from the original 330 clusters were picked and brought back to the lab. Overall, at harvest, 31% of clusters were empty (fruit dropped prematurely), 55% supported a single fruit and only 14% retained two apples ("double" clusters). Comparing the 11 combinations the highest percentage of clusters with single fruit was found in K+0L (84%) and the lowest in noK+4L (30%, Figure 6). The proportions of empty cluster and "single" cluster across the combinations did not show

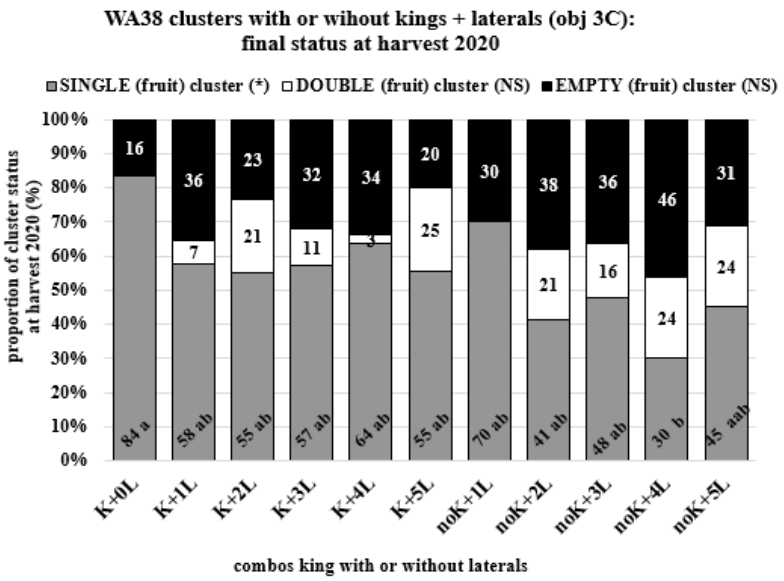

***Figure 6:** WA38 proportion (%) of clusters at harvest classified as empty (no fruit), single (one apple/cluster) and double (two apples/cluster). The comparison is presented between the 11 combinations. Significance: *= p<0.05, no asterisks means=NS. Means separation by SNK.*

specific statistical trends (Figure 6). Manipulating king and lateral occupancy treatments within a cluster at the end of April did not result in significant differences in the proportion of clusters that retained fruit at harvest.

To understand the fruit growth along the season based on the presence or absence of the king inside the cluster, we considered only the measurable fruit that were retained until harvest (N=264). Then, we classified those apples based on all possible patterns recorded at harvest as follows: K_K_single, K_king_w_lat, K_lat_single, K_lat_w_king, K_two_lats, noK_lat_single, noK_two_lats, where "K" or "noK" indicates the original treatment at the beginning of the trial, while K_single or king_lat indicate apples that originated from king flowers alone in the cluster (without laterals) until harvest, and apples that originated from king flowers, but from clusters that also carried lateral fruit, respectively. The highest proportions of fruit that arrived to harvest belonged to apples originating from king flowers alone in the cluster (32% K_K_single), followed by apples produced by a lateral ending alone in the cluster (27% noK_lat_single, data not shown). The fruit diameters measured for 18 weeks showed only a few differences across the combinations in the early part of the season from 5/20/20 to 6/3/20, where K_K_single fruit were larger since the beginning of measurements and significantly different than fruitlets grown in shared clusters, such as K_lat_w_king, K_two_lats, noK_two_lats. On the other hand, both treatments in which a single lateral ended up alone in a cluster at harvest despite the original "K/noK" treatment (such as K_lat_single and noK_lat_single) resulted in statistically similar diameters to the "king" apples alone on 5/27 and 6/3/20 (data not shown). No further significant differences in diameters emerged from June to harvest 2020 with a final diameter of 87 mm as average across the 7 combinations at harvest.

Another way to look at the fruit growth for this sub-trial is comparing apples that ended up as "double" in the same cluster with respect to "single" apples retained in the cluster at harvest within each of the original scenarios: clusters with king and clusters without king (data not shown). In both scenarios, we observed significantly higher diameters for fruit that ended up being "single" at harvest versus apples that were picked as "double" from 5/20/20 to 6/3/20. Looking at the average daily fruit growth (mm/day) calculated from the weekly measurements and comparing the four "simplified" scenarios at harvest such as K_single, K_double, noK_single, noK_double, we can report some differences between 6/24/20 and 7/15/20 (approx. 2 and 3 months after full bloom, data not shown). Both combinations with double fruit in the cluster with or without king at bloom showed higher growth rates after "June" drop in the weeks 5, 6, and 8 of measurements (6/24/20, 7/1/20, and 7/15/20) than "single" apples in cluster with or without kings (data not shown).

'WA38' apples picked from "double" clusters at harvest exhibited a faster average growth rate throughout the growing season (18 weeks) than fruit detached from "single" clusters at harvest in the scenario where king was removed.

EXECUTIVE SUMMARY

Project Title: Pollination, flower biology and fruit development in 'WA38' apples

Keywords: *pollinizers, effective pollination period (EPP), fruit set, natural shedding*

The project originally sought to address WA growers' concerns regarding the selection of the most suitable pollinizers for the new 'WA38' variety as well as to gain knowledge about the timing of fruitlet drop and potential fruit growth based on flower of origin. This investigation firstly focused on understanding the *effective pollination period* (EPP) of 'WA38' which is defined as the time window during which a pollination event turns into fertilization and successful fruit set. EPP is linked to the duration of three other factors occurring in the flowers during bloom. The first is the *stigmatic receptivity* expressed as the number of days the stigmas support pollen adhesion, second is the *pollen tube growth* (rate) which is the time the pollen tube takes to reach the ovules and last is the *ovule longevity,* namely, the duration of the ovules' viability. We learned that in 2019 'WA38' stigmatic receptivity lasted 9 days and was not a limiting factor in the successful pollination event. The EPP was calculated to be approximately 2 days by subtracting the duration of time required for the pollen tube to reach the ovules (7 days for 'Granny Smith' pollen) from the duration of ovule viability (9 days; 9 days - 7 days = 2 days). Therefore, the first 2 days after anthesis are the most important to have a successful pollination event that leads to fruit set (assuming adequate flower visitation). *Pollen tube growth* of 5 potential compatible pollinizers commonly planted in the apple orchards and selected with a similar bloom window with 'WA38' were tested for two years. In 2019, 'Snowdrift' pollen tubes were fastest to reach the base of the style, while 'Indian Summer' pollen tubes were the slowest; however, all 5 pollen sources reached the base of the style by the fifth day after pollination. In 2020 the same distance was covered by the pollen tube in 24 hours less than the previous year, and by 4 days post-pollination all the pollen had passed the base of the style with no significant differences across pollen sources. Also, *fruit set from hand pollination* experiments confirmed the 5 pollen sources were equally efficient in fertilizing 'WA38' flowers with no relevant differences in fruit set throughout the growing season. Moreover, 'WA38' fruit mass and diameter and number of healthy *seeds* did not significantly differ based on the pollen source. Fruitlet drop typical in the early stages after bloom (approx. 8 weeks) was particularly evident in 'WA38'; this self-thinning variety, indeed, tends to retain one or two fruit per cluster until harvest. In this study we aimed to investigate the dynamics of the natural shedding and quantify the drop of 'WA38' fruitlets throughout the season. By the end of May, 82% of flowers/*fruitlets naturally dropped* and no significant shedding occurred in the following weeks. About 30% of the clusters ended up being unfruitful at harvest 2020. This investigation provided information about 'WA38' fertility and fruitlet abscission traits that could be valuable to WA38 growers when making management decisions in the orchard.

PROJECT OUTCOMES

Presentations:

- Serra S., Musacchi S., Roeder S., Sheick R..: "Pollination, flower biology, and fruit development in 'WA38' apples" (oral presentation by Serra S. continuing report). January 29th 2020, Yakima.
- Serra S., Roeder S., Sheick R., Musacchi S. "Assessing 'WA38' Pollination and Fruit Development" (invited oral presentation by Serra S.) during the Pomology Professional Interest Group workshop: "Reproductive Development and Environmental Stress: Tree Fruit Crops". ASHS Annual Conference. August 10th-13th, 2020.

FUTURE DIRECTIONS

The natural evolution of this study would be using the acquired knowledge about the 'WA38' natural fruitlets shedding and aim to mitigate it.

FINAL PROJECT REPORT **YEAR**: 3 of 3

Project Title: Crop Load and Canopy Management of WA Tree Fruit

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Requested WTFRC Funds for Project:

Item	2018	2019	2020
Salaries	5950	6130	na
Benefits	2440	2510	na
Wages	25,000	27,500	30,250
Benefits	13,250	14,580	16,040
RCA Room Rental			
Shipping			
Supplies	1500	1500	1500
Travel	1000	1000	1000
Plot Fees	5040	4400	4600
Miscellaneous	500	500	500
Total gross costs	54,680	58,120	53,890
Anticipated Income (contracts and gift grants)	67,560	60,300	60,000?
Total net costs	(12,880)	(2180)	(6110?)

Footnotes:
Salaries: salary costs reflect time for Mendoza only in 2018 & 2019; no salary costs reflected in internal projects starting in 2020
Increase in wages & benefits include increase in WA minimum wage through 2020
Supplies include tractor/sprayer fuel & maintenance, spray suits, occasional chemical purchase, etc.
Plot fees assume use of 2 blocks at WSU Sunrise Research Orchard

NOTE: Budget for informational purposes only; research is funded through WTFRC internal program

OBJECTIVES:

1. Determine best use practices for metamitron including appropriate rates, timings, use of adjuvants, and weather considerations.
2. Explore other novel bloom and postbloom chemical thinning programs utilizing new chemistries and/or new use patterns for existing products, especially those approved for organic use.
3. Explore new uses of plant growth regulators to help manage apple crop load and orchard canopy systems.

SIGNIFICANT FINDINGS 2018-2020:

No treatments reduced fruit set in a chemical bloom thinning trial in 2018 and 2019 chemical bloom thinning trials, but fruit finish was improved by Regalia (Table 1)

The most efficacious options for chemical bloom thinning of apple continue to be spray oil + lime sulfur programs (Table 2)

Metamitron products continue to reduce fruit set, improve harvest fruit size, and increase return bloom more consistently than current industry standard thinning programs (Tables 3-5)

Metamitron efficacy can be promoted by tank mixing with non-ionic surfactants, increasing rate, or use of multiple applications (Tables 3, 4)

2019-EXP-01 significantly boosts the performance of 6-BA as a chemical thinner (Table 3) and of GA_7 as an inhibitor of return bloom (Table 6)

Applications of GA_7 effectively reduce return bloom in biennial apple blocks (Tables 6, 7); this new product has been registered as "Arrange" and is approved for use in organic and conventional blocks; grower should be able to purchase this product for the 2021 season

Collaborative research efforts continue to help develop new models, information, and technologies to improve crop load management of WA apples

Work restrictions due to COVID-19 precautions limited the scope of field trials in 2020

BACKGROUND:

After years of robust efforts to evaluate various aspects of bloom and postbloom chemical thinning programs, our current focus is to screen new chemistries and provide collaborative support for external research programs working on crop load and canopy management. Most of our current trials are funded in part or wholly by third party companies that contract our services to independently evaluate their products alongside industry standard programs. We continue to evaluate the relative success of thinning programs through three measurable targets which are directly tied to a grower's economic bottom line:

1. Reduction of green fruitlet hand-thinning
2. Improved fruit size and quality
3. Increased return bloom/annual bearing

The degrees to which our chemical thinning programs achieve each of these goals are reflected in our data labeled fruitlets/100 floral clusters, harvest fruit size, and percent return bloom, respectively.

BLOOM THINNING:

For years, chemical bloom thinning programs in Washington have predominantly featured lime sulfur or combinations of lime sulfur and horticultural spray oils. While these programs have been largely efficacious for most growers, there have been few alternative chemistries that have demonstrated potential as cost-effective chemical thinners, especially for organic growers. After hearing anecdotal reports of reduced fruit set in some commercial organic apple blocks and in pathology research trials by Regalia, a biofungicide derived from extracts of knotweed, we began testing the material as a chemical bloom thinner in 2018. Results from that initial Gala trial did not demonstrate any significant treatment effects from Regalia on fruit set, fruit finish, or return bloom, but we did observe an increase in fruit size in one Regalia treatment, as well as the industry standard oil + lime sulfur program.

In 2019, we tried thinning with Regalia again, this time in a Jonagold block (Table 1). As with the 2018 Gala trial, no treatment significantly affected fruit set, but there was a clear improvement in fruit finish across most treatments, both from Regalia and oil + lime sulfur. While we were unable to document statistically significant improvements in fruit size in 2019, some Regalia treatments once again suggested a trend toward that effect.

Table 1. Crop load and fruit quality effects of bloom chemical thinning programs. WTFRC 2019.

Treatment	Fruitlets/100 floral clusters	Blanked spurs	Singled spurs	Harvest fruit weight	Relative box size	Russet free fruit
Jonagold / M.26 - Rock Island		*%*	*%*	*g*		*%*
2% Regalia	45 abc	63 ab	30 ab	247 ns	74	43 ab
4% Regalia	49 ab	57 b	37 a	221	82	60 a
1.5% CFO + 1% Regalia	40 abc	65 ab	31 ab	232	78	21 b
1% WES + 1% Regalia	52 a	57 b	35 ab	241	75	59 a
1% WES + 2% LS	36 c	69 a	27 b	231	79	51 ab
Control	37 bc	67 a	29 ab	215	84	16 b

While the lack of clear thinning or improvements in return bloom in our two Regalia trials was disappointing, it is worth noting how infrequently our replicated field trials have documented significant treatment effects in other bloom thinning trials (Table 2). Regardless, improvements in fruit finish and size were intriguing and may be worth considering for organic growers seeking to improve their packouts.

Table 2 reflects the cumulative success rates of our most frequently tested chemical bloom thinners over time at achieving our three main criteria for effective thinning and demonstrates the overall superiority of programs featuring lime sulfur.

Table 2. Incidence and percentage of results significantly superior to untreated control. Apple chemical bloom thinning trials. WTFRC 1999-2020.

Treatment	Fruitlets/100 blossom clusters	Harvested fruit size	Return bloom[1,2]
ATS	15 / 60 (25%)	10 / 63 (16%)	4 / 55 (7%)
NC99	15 / 32 (47%)	7 / 34 (21%)	2 / 28 (7%)
Lime sulfur	26 / 58 (45%)	12 / 52 (23%)	9 / 52 (17%)
CFO + LS	62 / 115 (54%)	27 / 106 (25%)	22 / 105 (21%)
JMS + LS	14 / 24 (58%)	8 / 23 (35%)	4 / 22 (18%)
WES + LS	15 / 32 (47%)	5 / 31 (16%)	4 / 31 (13%)
ThinRite	7 / 22 (32%)	0 / 23 (0%)	0 / 12 (0%)

[1]Does not include data from 2020 trials.

[2] (no. blossom clusters year 2/sample area) / (no. blossom clusters year 1/sample area)

POSTBLOOM THINNING:

Our primary focus for postbloom chemical thinning research continues to be to identify and develop alternatives to carbaryl, which faces regulatory scrutiny as well as mounting pressure from elements of the consumer market seeking to reduce overall use of broad-spectrum pesticides. Even though WTFRC pesticide residue studies have been unable to detect any trace of carbaryl at harvest when used as a chemical thinner on Gala apples, some retail grocers have established policies prohibiting the sales of produce which has been treated with specific pesticides, including carbaryl.

Most of our recent postbloom thinning work has featured metamitron, a sugar beet herbicide that has been recently registered by Adama under the trade name “Brevis” as a postbloom thinning agent in several countries including Italy, France, Spain, and South Africa. We began working with small quantities of metamitron in 2011 and have scaled up the number and size of our trials in recent years as more product has become available. Our results have consistently found it to be a promising chemistry when used aggressively in our relatively low plant stress environment.

Like many other research programs, we were forced to scale back the scope of our planned field trials in 2020 due to rapidly evolving restrictions and guidelines for safe workplaces due to the COVID-19 pandemic. It was unclear if and when we could return to work during the early spring, but we were given approval to follow modified work protocols just in time to get two postbloom thinning trials in the field before bloom.

Unfortunately, we were unable to collect fruit set data at our Frenchman Hills trial site before the grower accidentally hand-thinned most of our trial plots in June, despite our regular communication with the orchard manager. As such, any data based on counts of fruit set for that trial is fatally compromised and not appropriate for analysis or reporting (Table 4). This was particularly unfortunate because this was the only 2020 site where we applied an experimental surfactant which had clearly amplified the efficacy of BA products in 2019 (Table 3). Nonetheless, we were still able to collect relevant data regarding harvest fruit size and quality, including fruit weight results which hint at some sort of thinning effect in fruit treated with metamitron due to their slightly larger fruit size at harvest. We will collect return bloom data from these plots in Spring 2021 to complete the assessment of the effects of chemical thinning programs included in the trial.

Our 2020 Golden Delicious postbloom thinning trial was executed without a hitch and produced yet another strong set of results showing clear reductions in fruit set for all treatments including several variations of programs utilizing metamitron, as well as some standard industry treatments featuring carbaryl, BA, and NAA (Table 4). Interestingly, each chemical thinning treatment also significantly

improved harvest fruit size, while a June green fruit hand-thinning treatment did not. As we have seen in previous studies, the thinning effects of metamitron tend to be amplified with the use of a non-ionic surfactant such as Regulaid; this option may prove to be valuable to WA growers interested in more aggressive thinning tactics once metamitron is registered for use here.

Table 3. Crop load and fruit quality effects of postbloom thinning programs. WTFRC 2019.

Treatment	Fruitlets/100 floral clusters	Blanked spurs	Singled spurs	Harvest fruit weight	Relative box size	Russet free fruit
		%	%	g		%
Fuji / M.9 - Wapato						
FAL 551 25.6 fl oz PF & 10-12mm	121 b	34 cd	26 bc	200 c	91	78 ns
FAL 551 25.6 fl oz + 2019-EXP-01 16oz PF & 10-12mm	103 bcd	35 cd	36 ab	227 abc	80	86
FAL 551 25.6 fl oz + 2019-EXP-01 32oz PF & 10-12mm	84 cde	43 cd	36 ab	228 abc	80	90
FAL 551 25.6 fl oz + 2019-EXP-01 64oz PF & 10-12mm	32 f	74 a	22 c	261 a	70	86
ADA 46343 40 oz PF&10-12mm	108 bc	38 cd	30 abc	205 bc	89	93
ADA 46343 40 oz + Regulaid 32 oz PF & 10-12mm	49 ef	60 ab	31 abc	248 ab	73	85
Carbaryl 4L 36 oz + Fruitone L 2 oz PF & 10-12mm	67 def	49 bc	39 a	232 abc	78	91
Control	158 a	28 d	21 c	188 c	97	80
Gala / M.9 – Frenchman Hills (George)						
FAL 551 25.6 fl oz PF & 10-12mm	140 ab	28 c	28 ab	158 c	115	16 ns
FAL 551 25.6 fl oz + 2019-EXP-01 16oz PF & 10-12mm	168 a	19 c	28 ab	166 bc	109	16
FAL 551 25.6 fl oz + 2019-EXP-01 32oz PF & 10-12mm	144 ab	27 c	28 ab	165 bc	110	11
FAL 551 25.6 fl oz + 2019-EXP-01 64oz PF & 10-12mm	136 ab	31 bc	26 ab	164 bc	111	18
ADA 46343 40 oz PF&10-12mm	88 cd	46 ab	32 ab	180 ab	101	No data
ADA 46343 40 oz + Regulaid 32 oz PF & 10-12mm	58 d	56 a	32 ab	191 a	95	24
Carbaryl 4L 36 oz + Fruitone L 2 oz PF & 10-12mm	122 bc	30 bc	33 a	171 abc	106	6
Control	152 ab	29 bc	23 b	152 c	119	25
Gala / M.26 - Orondo						

ADA 46343 40 oz PF	66 d	56 c	27 b	155 cd	117	38 ns
ADA 46343 40 oz 10-12mm	71 d	52 c	32 ab	159 bc	114	43
ADA 46343 40 oz PF & 10-12mm	19 e	86 b	11 c	186 ab	98	44
ADA 46343 32 oz + Regulaid 32 oz	8 e	93 a	7 c	187 a	97	35
Carbaryl 4L 36 oz + Fruitone L 2 oz	20 e	82 b	16 c	188 a	97	36
CFO 1 gal + LS 1 gal @ 400 GPA 10-12mm	99 c	41 cd	33 ab	129 d	141	38
CFO 1 gal + LS 1 gal @ 400 GPA PF & 10-12mm	129 b	24 de	37 a	143 cd	127	33
Control	163 a	16 e	36 a	135 cd	135	40
Golden Delicious / Bud.9 – Rock Island						
ADA 46701 1.3 pt 12-14mm	25 b	77 e	21 ab	213 bc	85	45 b
ADA 46701 2 pt 12-14mm	20 bc	83 cde	15 bcd	239 abc	76	49 ab
ADA 46701 2.7 pt 12-14mm	12 cd	88 bc	11 de	259 ab	70	60 ab
ADA 46701 3.3 pt 12-14mm	8 de	92 ab	8 ef	259 ab	70	54 ab
ADA 46701 3.3 pt + Regulaid 32 oz 12-14mm	3 e	97 a	3 f	286 a	63	39 b
Carbaryl 4L 36 oz + Fruitone L 2.5 oz12-14mm	14 cd	87 bcd	13 cde	246 abc	74	51 ab
Exilis 9.5SC 25.6 oz + Fruitone L 2.5 oz 12-14mm	21 bc	81 de	18 bc	198cd	92	78 a
Control	41 a	66 f	28 a	154 d	118	58 ab
Granny Smith / M.9 – Rock Island						
ADA 46701 3.0 pt PF	59 a	48 b	45 a	214 ab	85	94 ns
ADA 46701 3.0 pt 8-11mm	32 b	72 a	25 b	220 ab	83	84
ADA 46701 3.0 pt 12-15mm	28 b	73 a	26 b	232 a	78	94
ADA 46701 3.0 pt 16-20mm	29 b	71 a	29 b	220 ab	83	94
Carbaryl 4L 36 oz + Fruitone L 2 oz	34 b	68 a	31 b	217 ab	84	89
Control	67 a	42 b	49 a	177 b	103	86

Table 4. Crop load and fruit quality effects of postbloom thinning programs. WTFRC 2020.

Treatment	Fruitlets/100 floral clusters	Blanked spurs	Singled spurs	Harvest fruit weight	Relative box size	Russet free fruit
		%	%	g		%
Gala / M.9 – Frenchman Hills (George)						

ADA 46701 2 pt PF & 10-12mm	na	na	na	206 a	88	60 ns
ADA 46701 3 pt PF & 10-12 mm	na	na	na	201 ab	90	45
ADA 46701 2pt + Regulaid 1pt PF & 10-12 mm	na	na	na	200 ab	91	66
FAL 551 25.6 fl oz PF & 10-12 mm	na	na	na	194 ab	94	55
FAL 551 25.6 fl oz + 2019-EXP-01 32 fl oz PF & 10-12 mm	na	na	na	189 ab	96	53
CFO 1 gal 1 + LS 1 gal @ 400 GPA PF & 10-12mm	na	na	na	186 b	98	63
Carbaryl 4L 36 oz + Fruitone L 2 oz PF & 10-12 mm	na	na	na	190 ab	96	53
Control	na	na	na	191 ab	95	51
Golden Delicious / Bud.9 – Rock Island						
ADA 46701 2.0 pt PF & 12-16 mm	42 b	65 b	31 bc	189 abc	96	46 ab
ADA 46701 2.0 pt + Regulaid 1 pt PF & 12-16 mm	28 bc	75 ab	23 cd	196 ab	93	33 b
ADA 46701 2.5 pt PF & 12-16 mm	31 bc	71 ab	28 bcd	182 bc	100	43 ab
ADA 46701 2.5 pt + Regulaid 1 pt PF & 12-16 mm	26 bc	74 ab	25 cd	218 ab	83	38 ab
ADA 46701 3.0 pt PF & 12-16 mm	36 b	67 b	29 bcd	188 abc	97	53 ab
ADA 46701 3.0 pt + Regulaid 1 pt PF & 12-16 mm	19 c	82 a	16 d	223 a	81	53 ab
Carbaryl 4L 36 oz + Fruitone L 3 oz PF & 12-16 mm	31 bc	70 ab	28 bcd	189 abc	96	55 ab
Exilis 9.5SC 25.6 oz + Fruitone L 3 oz PF & 12-16 mm	32 bc	69 ab	29 bcd	182 bc	100	63 a
Hand thinned mid June	72 a	41 c	48 a	152 cd	119	53 ab
Control	62 a	49 c	41 ab	138 d	132	46 ab

Table 5 demonstrates the strong performance of BA + NAA programs and metamitron products as compared to other postbloom thinning options featuring carbaryl over the course of all our studies across varieties and locations. While we used to think of metamitron only as an acceptable alternative to carbaryl, we continue to see more consistent performance of those programs relative to current industry standards, suggesting that metamitron may ultimately prove to be a superior option to carbaryl, BA, and/or NAA products.

Table 5. Incidence and percentage of results significantly superior to untreated control. Apple chemical postbloom thinning trials. WTFRC 2002-2020.

Treatment	Fruitlets/100 blossom clusters	Harvested fruit size	Return bloom[1,2]
BA	7 / 29 (24%)	0 / 30 (0%)	0 / 28 (0%)
Carb + BA	33 / 91 (36%)	10 / 89 (11%)	13 / 86 (15%)
Carb + NAA	30 / 79 (38%)	20 / 78 (26%)	16 / 76 (21%)
BA + NAA	20 / 42 (48%)	9 / 41 (22%)	8 / 37 (22%)
Metamitron	19 / 30 (63%)	14 / 30 (47%)	9 / 27 (33%)

[1]Does not include data from 2020 trials.

[2] (no. blossom clusters year 2/sample area) / (no. blossom clusters year 1/sample area)

GIBBERELLIC ACID FOR BLOOM INHIBITION:

Over many years of trials, we have established that multiple applications of modest concentrations of GA_3 can be effective at reducing return bloom across multiple apple varieties as a tool for mitigation of biennial bearing. In the absence of GA products registered for this use pattern, we focused most of our work on GA_3 products because of their relatively low price point. Despite ample data demonstrating their efficacy, the registrants of these products have been reluctant to add this use pattern to their labels, primarily due to the abundance of competitive generic products in the market and relatively poor prospects for making a return on investment for such a label amendment.

In recent years, however, we have been testing a new formulation of GA_7 from Fine Americas alongside our standard GA_3 programs. GA_7 is known to be a more potent isomer than GA_3 in terms of inhibiting floral initiation and can produce analogous results at lower concentrations. Our 2018 trial on biennial Golden Delicious (Table 6) with this GA_7 product (FAL 900) demonstrated dramatic reductions in 2019 return bloom when combined with a proprietary surfactant or partnered with a series of applications of GA_4 (Novagib). All GA_3 (Falgro 2XLV) and GA_7 (FAL 900) treatments in our 2019 trial on Honeycrisp (Table 7) generally reduced 2020 return bloom, although not all results were statistically significant. As we have seen in previous studies, application of GA_4 (Novagib) did not clearly affect return bloom.

After many delays in the regulatory process, we are pleased to report that this new GA_7 product known as "Arrange" has received full registration and should be available for use in the 2021 growing season. This product has also been approved by OMRI and may become an important crop load management tool for organic apple growers who have relatively few plant growth regulators in their toolboxes.

The use recommendations for Arrange largely reflect the treatments we found to be efficacious in our studies of FAL 900. The product label recommends up to 4 applications of materials totaling no more than 100 ppm per season, or a single application of 100 ppm if multiple sprays are not an option. Our results have indicated that multiple small doses of any GA product are generally more effective than single large doses. The product label also provides recommendations for annual maintenance sprays of Arrange, including use in the "on" year of a biennial bearing cycles. We did not test these programs and are unsure of their potential risks or benefits.

Table 6. Effects on tree vigor, fruit size, and return bloom of GA applications. WTFRC 2018.

Treatment	2018 harvest fruit weight	2018 relative box size	2018 shoot growth	2019 return bloom	2019 return bloom per CSA
	g		*cm*	*%*	*clusters/cm²*
Golden Delicious / M.9 – Rock Island					
4 x FAL 900 25ppm	245 ns	74	22.6 ns	2583 bc	1.2 a
FAL 900 100ppm @ petal fall	215	84	24.3	2398 bc	1.9 a
FAL 900 100ppm @ PF+14	216	84	24.2	1390 cd	1.2 a
FAL 900 100ppm + 2019-EXP-01 @ PF	216	84	24.9	154 d	0.2 b

FAL 900 50ppm; 4 x 20 oz Novagib	234	78	25.2	828 d	0.3 b
FAL 900 100ppm; 4 x 20 oz Novagib	246	74	16.8	650 d	0.2 b
4 x Falgro 4L 100ppm	211	86	22.5	3023 ab	1.6 a
Control	192	95	21.2	4399 a	1.9 a

Table 7. Effects on tree vigor, fruit size, and return bloom of GA applications. WTFRC 2019.

Treatment	2019 harvest fruit weight	2019 relative box size	2019 shoot growth	2020 return bloom	2020 return bloom per CSA
	g		*cm*	*%*	*clusters/cm²*
Honeycrisp / B.118 – Brewster					
FAL 900 (25 ppm) 32 oz @ PF, PF + 7, PF + 14, PF + 21	287 abc	63	30.7 ab	309 b	1.3 c
FAL 900 (100 ppm) 128 oz @ PF + 7	308 a	59	33.8 ab	659 ab	1.7 bc
FAL 900 (150 ppm) 192 oz @ PF + 7	299 ab	61	31.3 ab	604 ab	2.2 abc
FAL 900 (200 ppm) 256 oz @ PF + 7	299 ab	61	34.3 ab	563 ab	1.7 bc
FAL 900 128 oz @ PF+7; Novagib 20oz @ PF, PF+7, PF+14, PF+21	258 c	70	29.6 b	741 ab	1.6 bc
Novagib 20 oz @ PF, PF + 7, PF + 14, PF + 21	282 abc	64	32.7 ab	1011 a	2.6 ab
Falgro 2XLV 473 ml @ PF, PF + 7, PF + 14, PF + 21	311 a	58	34.8 a	629 ab	2.1 abc
Control	271 bc	67	31.8 ab	957 ab	2.8 a

COLLABORATIVE CROP LOAD MANAGEMENT RESEARCH:

"Optimizing light and water for orchards covered with netting" (AP-18-102; PI: Kalcsits) – support for labor intensive data collection, harvest sampling, and postharvest fruit quality analysis; also support for project leadership team including sharing of relevant WTFRC projects and protocols, as well as editing of project manuscripts

"Development and validation of a precision pollination model" (TR-16-102; PI: DeGrandi-Hoffman) – coordination of local data collection for bee foraging, bloom phenology, and fruit sampling activity at sites near Yakima and Chelan; active member of project leadership team (project funded through WTFRC technology committee)

"Developing and validating models for tree fruit" (TR-17-102; PI: Jones) – coordination of data collection for fruit growth at 39 blocks throughout Central Washington (primarily Golden Delicious, Fuji, Honeycrisp, and WA 38); help with outreach activities for new horticultural models (project funded through WTFRC technology committee)

“Precision Crop Load Management for Apples” (USDA-NIFA Specialty Crop Research Initiative (SCRI) - PD: Terence Robinson, Cornell) – project will begin in 2021 and include work in WA, NY, VA, MI, MA, and NC; objectives focus on development of predictive models and horticultural strategies to develop/optimize crop load, as well as development of vision systems, robots, & other automated tools to assess and adjust crop load as various phenological stages

Proposed to WTFRC Apple Horticulture Committee: “Maximize pollination window to improve fruit set in WA38” (PI: Serra) – help coordinate field activities including trial layout, data collection, spray application, reflective material deployment, sample collection, and harvest analysis; intent is to improve fruit set in WA38 to promote consistently high annual yields

EXECUTIVE SUMMARY

Project title: Crop load and canopy management of WA tree fruit
Key words: chemical thinning, PGR, metamitron, return bloom, GA

Abstract: Effective crop load management is fundamental to the financial success of commercial apple production. This project sought to identify and develop cost-effective management strategies primarily through the use of chemical thinners and plant growth regulators to help Washington apple growers produce consistent annual crops featuring large yields of high-quality fruit.

Our initial tests of Regalia as a chemical blossom thinner did not elicit significant reductions in fruit set but showed some encouraging trends toward improving fruit finish. The best available option for chemical bloom thinning continues to be combinations of horticultural oils and lime sulfur as were developed in prior WTFRC studies.

We continue to refine best management practices for metamitron, a new postbloom thinning chemistry that is nearing registration for the US market. Our studies clearly demonstrate that this product generally competes with or outperforms current standard postbloom chemical thinning programs featuring carbaryl, NAA, and/or BA products. Metamitron efficacy can be boosted with the use of a non-ionic surfactant such as Regulaid. We are confident that metamitron will represent a step forward for apple crop load management in WA and we look forward to its commercial release.

Our studies also validate the relatively strong performance of tank mixes of BA and NAA, programs which may be of increasing interest as regulatory and marketplace pressures on carbaryl continue to mount. We have also been impressed with the performance of a proprietary developmental adjuvant which significantly boosted the thinning and fruit sizing performance of BA products in thinning trials and the efficacy of GA products in inhibiting return bloom in apple.

We have worked for years to develop PGR programs to help mitigate alternate bearing in apple, primarily through the application of bloom-inhibiting gibberellins during the "off" year of the biennial cycle. We have had considerable success with multiple applications of GA_3 products, but commercial registrants have been reluctant to adapt those product labels to accommodate that use pattern. In more recent years, we have achieved similar positive results with a formulation of GA_7, which has now been registered as the commercial product "Arrange" and approved for use in organic orchards as soon as this upcoming growing season.