2023 Apple Horticulture and Postharvest Research Review

Apple Breeding Program Phase 3 harvest in Quincy, WA.

Photo Source: Manoella Mendoza

January 25, 2023

Hybrid Format
Wenatchee, WA

Project/Proposal Title: Retraction of netting near harvest: risks vs. rewards

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Cooperators: Noah Willsea (WSU), Harold Schell (Chelan Fruit); Tom Gaussman (Agrimacs); Garrett Grubbs (Agrimacs); Felipe Castillo (Extenday); Jonathan Toye (Extenday)

Report Type: Continuing Project Report

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $ 37,761
Total Project Request for Year 2 Funding: $ 39,107

Other related/associated funding sources: Requested
Funding Duration: 2023 - 2028
Amount: $6.2 million
Agency Name: USDA SCRI
Notes: This was a our third time submitting this proposal that is focused on mitigating the impacts of temperature extremes on pome fruit. While not funded, it was, again, ranked highly and will be resubmitted in 2023.

WTFRC Collaborative Costs: none

Budget 1
Primary PI: Lee Kalcsits
Organization Name: Washington State University
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Item	2021	2022
Salaries	17,514[1]	18,215[1]
Benefits	6,548[1]	6,811[1]
Wages	7,800[2]	8,112[2]
Benefits	1,749[2]	1,819[2]
Equipment		
Supplies	3,000[3]	3,000[3]
Travel	1,150[4]	1,150[4]
Miscellaneous		
Plot Fees		
Total	37,761	39,107

Footnotes:
[1]Funding is requested for a scientific assistant at 35% during August to November of each year of the project. Benefits rates for the scientific assistant are equal to 37.4%
[2]Funding is requested for a summer staff member to work on netting set up at Sunrise research orchard, fruit thinning and horticultural management, and experimental set up in August. Benefits for this position are equal to 22.4%
[3]Supplies are for netting set up and consumables for field and lab experiments that may include new data loggers, solar panel hardware, as well as lab supplies for fruit quality analysis.
[4]Funding for travel is requested for weekly trips to Sunrise research orchard as well as twice-weekly trips to Quincy in August and September for conducting retraction experiments.

OBJECTIVES

This project has two objectives aimed at quantifying risks and rewards of using retractable netting systems for high-value apple cultivars.

1. Test the timing of retraction of netting across two growing seasons to determine how much netting retraction enhances red color development and how close to harvest deployment should occur.
2. Determine whether fruit under netting is at a greater risk of developing sunburn when netting is retracted.

SIGNIFICANT FINDINGS

After two seasons, retraction 7 days before harvest had equal red color as when netting was retracted 14 days before harvest. While netting retraction had a significant benefit in 2021, it did not in 2022 for the commercial trial. However, color development was extremely poor in 2022 for Honeycrisp across the state and as such, differences between treatments were not as great.

When comparing additional losses from sunburn to gains in red color in 2021, these changes translated to an additional 1.5 packed boxes per bin when retraction was used compared to leaving netting up. These differences were mostly consistent between the commercial and research orchard locations. In 2022, the commercial orchard only had an additional 0.25 packed boxes per bin.

When these differences are calculated for a 60 bin/acre crop and a box price of $56/box for 'Honeycrisp', it translates to an additional $5040/acre in revenue in 2021 and only $840/acre in 2022 for the commercial orchard site.

Netting had the greatest benefit to reducing sunburn and EC reduced severe sunburn when used in conjunction with retractable netting systems. Evaporative cooling alone was not sufficient to limit sunburn development on fruit in 2021.

There was no evidence of the development of photo oxidative sunburn from removing netting prior to harvest even when netting was retracted at higher temperatures (above 100 °F in 2022).

METHODS

Experiment 1: Removal timing for netting retraction

This experiment was performed in a Honeycrisp orchard that was planted in 2018. It consists of Honeycrisp on G890 rootstocks planted to a tall spindle training system. Netting was installed and covered the orchard in 2020. It consists of a panel and cable system that extends over the entire orchard. The experimental design will be a randomized complete block design. Each panel is 55'

wide and covers 4 rows. In August, 14 days before harvest, in 2021 and 2022, netting was removed from a 55' section within the block. Then, 7 days before harvest another 55' wide section will be retracted. These two treatments were compared against a control that was left covered until after harvest. These treatments helped determine the impact of duration of retraction before harvest on color development for previously netted trees. This will lead to stronger recommendations for netting retraction near harvest.

Measurements (Summarized in Table 1):

On the day prior to netting retraction 7 days before harvest, thermocouples that measure fruit surface temperatures were installed on four fruit on each of two trees per replicate. There were a total of 18 dataloggers installed for the entire experiment. Fruit surface temperatures were continuously measured for 8 days to determine if there were differences in fruit surface temperatures of fruit between treatments. To assess fruit quality for each treatment, 100 fruit were harvested from the upper canopy area of each replicate to look at sunburn incidence and fruit color development. After harvest, fruit was run on an AWETA sorting line that can measure fruit diameter, weight, red color coverage and intensity as well as background color. Sunburn incidence and severity was graded on all fruit using a six-point scale adapted from Schraeder et al. (2003). These two factors are part of a trade-off in risk that growers must navigate in response to highly variable weather conditions that are normally experienced this time of year.

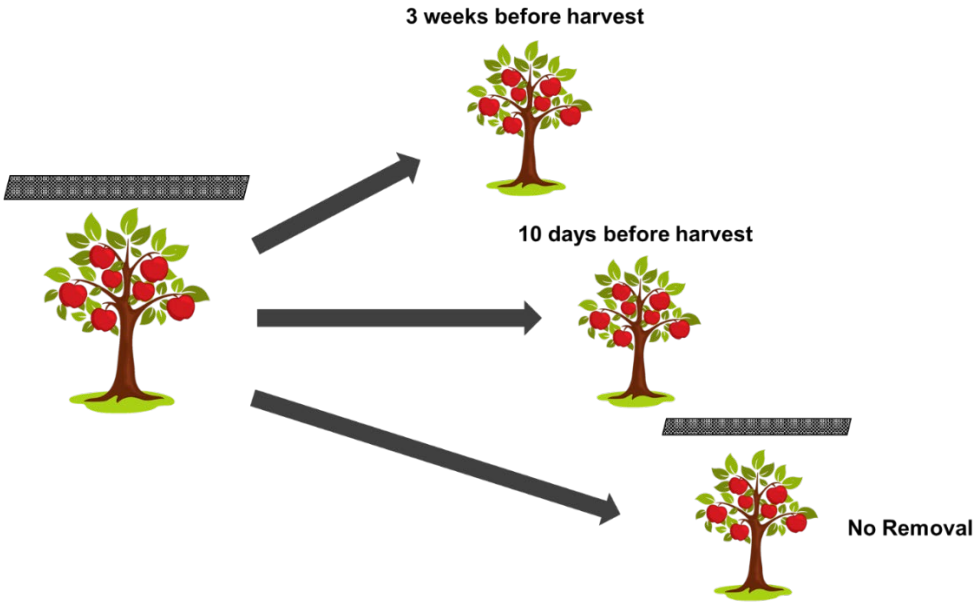

Figure 1. Experimental design of commercial netting retractions study taking place in Quincy, WA. Each treatment will have three replicates. Note: Actual timing was 14 days and 7 days before harvest instead of 21 and 10 days before harvest as described above.

Table 1. Measurements made on fruit in the orchard and at harvest for experiment 1 which is focused on identifying optimum timing of net retractions near harvest for Honeycrisp apple.

Measurement	What	When	Where	Why
Fruit surface temperature	Thermocouples	Entire duration of the experiment in 2021 and 2022	Two trees per replication and four fruit per tree	Assessing sunburn risk and differences in acclimation between treatments
Fruit surface temperature	Infrared camera	One day after retraction	5 fruit per replication	Image development for use in Extension material
Fruit sizing	AWETA Sorting Line	Within one week of harvest	WSU TFREC	Grading for size, color area, and color intensity
Sunburn incidence and severity	Graduate student and technician	One week after harvest	WSU TFREC	Assessing the impact of netting retraction on sunburn risk
Postharvest disorders	Graduate student and technician	January 2022	WSU TFREC	Assessment of postharvest sunburn development along with other external and internal disorders that might emerge from retracting netting near harvest

Experiment 2: Combining netting retraction with evaporative cooling

This second experiment was conducted at the Sunrise Research Orchard in Wenatchee, WA in a top-worked Firestorm® Honeycrisp orchard that was regrafted in 2016. The experimental design had six treatments arranged in a split plot design with evaporative cooling treatments as a main plot and then retraction as a secondary plot. There were three replications for each treatment. Netting was deployed in early June using a modified retracted netting setup from Extenday (See Figure 2). Evaporative cooling was available from June 15 to harvest with automated sprinklers that were triggered when air temperatures reached 85 °F. Cycling was set to be 15 minutes on and 45 minutes off during those times. Netting was retracted two weeks prior to harvest for replications with either evaporative cooling or no cooling and there was a completely uncovered control to compare all sunburn mitigation treatments against to look at effect on red color and sunburn.

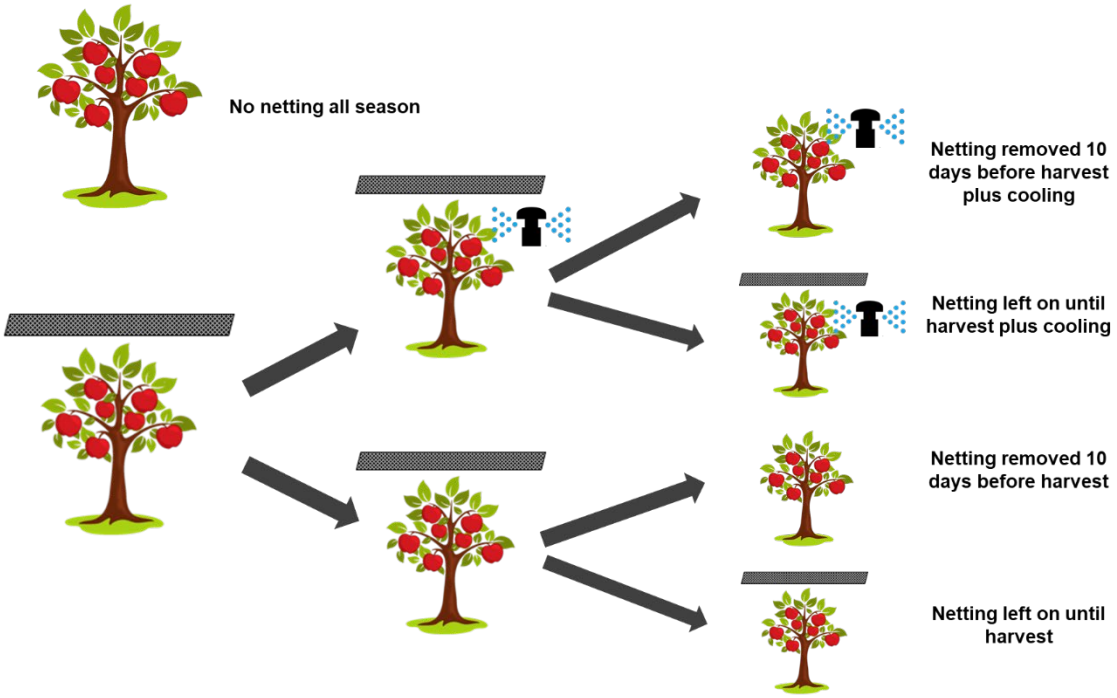

Figure 2. Experimental design of netting retractions study taking place at the Sunrise Research Orchard near Wenatchee, WA. There are five treatments and an untreated control. The five treatments will include either evaporative cooling or not and then netting removed 14 days (This was originally described as 10 days above) before harvest or not. There was also a treatment added with just evaporative cooling and no netting. Each treatment had three replicates.

Experiment 2 Measurements (Summarized in Table 1):

Thermocouples that measure fruit surface temperatures were installed on the day of retraction on four fruit on each of one tree per replicate. There was a total of 15 dataloggers used for the entire experiment in 2021 and 2022. Fruit surface temperatures were monitored for the whole 10 days to determine differences in fruit surface temperatures among treatments. Environmental conditions were pulled from the WSU AgWeatherNetwork (Sunrise Weather Station). Like experiment 1, fruit quality was assessed for each treatment. Approximately 100 fruit were harvested from the upper canopy area of each replicate to look at sunburn incidence and fruit color development. After harvest, fruit was run on an AWETA sorting line that can measure fruit sizing, weight, red color coverage and intensity as well as background color. Sunburn incidence and severity was graded using a six-point scale adapted from Schraeder et al. (2003). In 2022, fruit was also stored at 33 °F in regular atmosphere for three months to assess fruit quality after storage. Here, we tested whether there is added value in evaporative cooling under netting and whether netting retraction is beneficial when used with evaporative cooling.

RESULTS AND DISCUSSION

Experiment 1: Quincy Experiment

For both years in the commercial trial, retraction produced higher proportions of fruit with premium red color coverage (>33%) but were not statistically significant ($\alpha = 0.05$). Whether retraction was done 14 days or 7 days before harvest had no difference in red color coverage or the proportion of fruit with premium red color (>33% coverage).

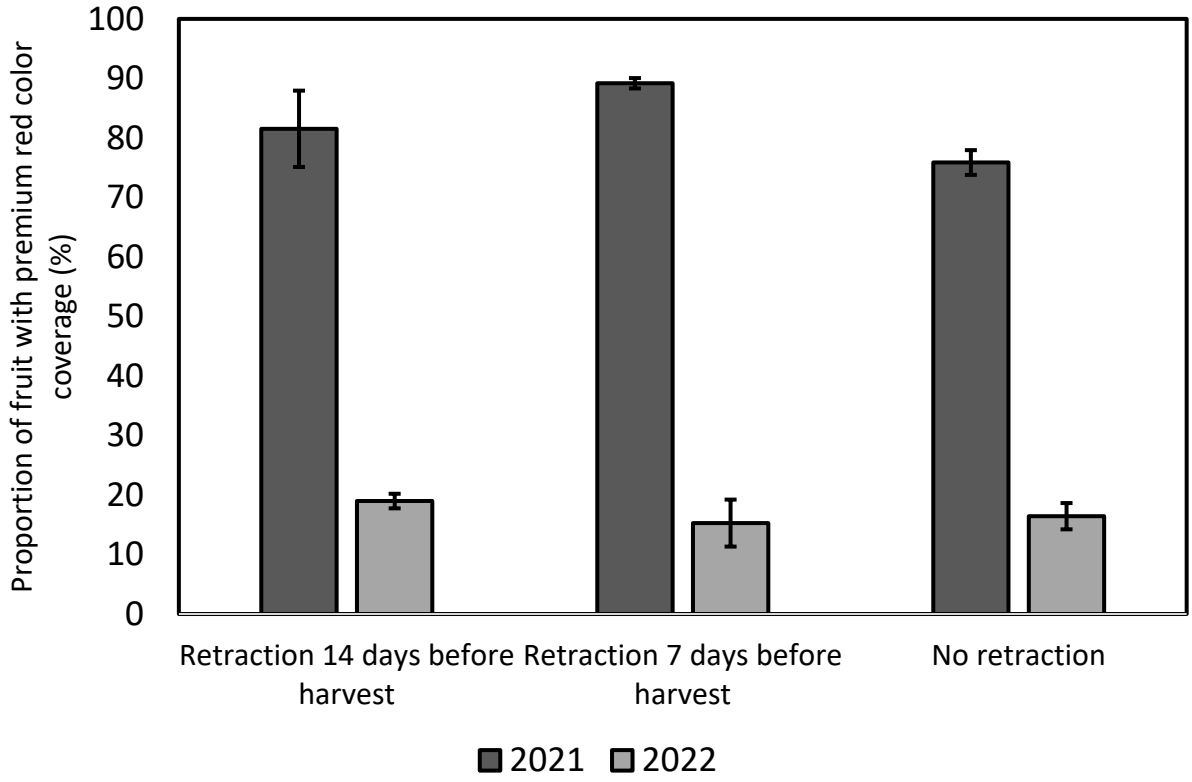

Figure 3. The proportion of fruit with premium red color coverage (%) in 2021 and 2022 when netting was retracted either 14 days or 7 days before harvest compared to a control where netting remained in place until after harvest.

Retraction increased the proportion of fruit culled from sunburn, even in 2021 when sunburn pressure was lower during retraction (daily maximum temperatures were approximately 85 °F during this period) (Figure 4). In 2022, retraction was delayed until after September 5 to limit the risk of fruit sunburn in the commercial orchards as daytime maximum temperatures neared 100 °F. In 2021, 7% of fruit had severe sunburn whether it was retracted 7 days or 14 days before harvest. However, less than 4% of fruit had severe sunburn when netting was left in place until after harvest. Trends were similar in 2022 between treatments but sunburn incidence was lower. Between 4 and 5% of fruit was culled from sunburn for both retraction treatments compared to only 2% when netting was left in place until after harvest.

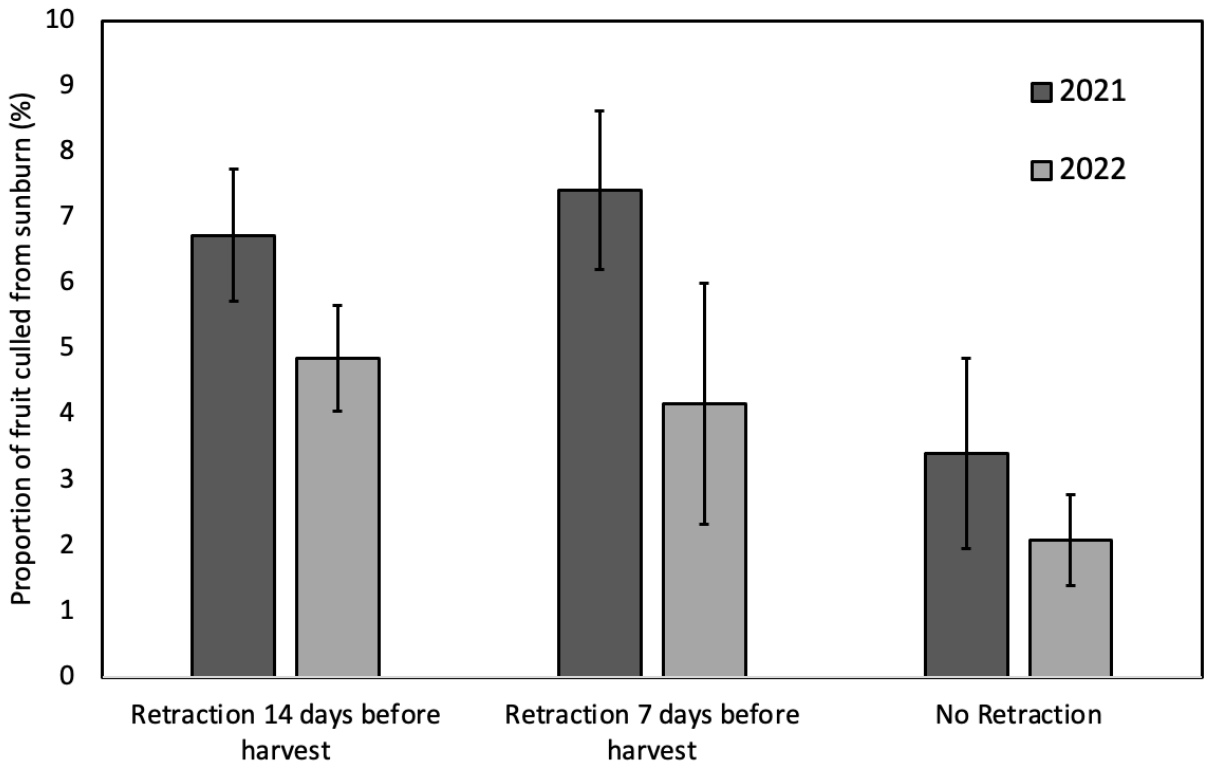

Figure 4. The percentage of fruit culled from sunburn (belonging to either 3 or 4 on the 6-point sunburn scale) for Honeycrisp apple for the Quincy experiment in 2021 and 2022. Error bars indicate standard error of the mean (N=3).

Experiment 2: Sunrise Experiment

Table 2. Comparisons of the average red color coverage, retraction period, and average maximum temperature for 2021 and 2022.

	2021	2022
Average red color coverage (%)	58.3	17.7
Retraction period	August 18-August 30	August 29-September 8
Average daily maximum temperature during retraction (°F)	83.8	91.5

Since maturity was delayed in 2022 compared to 2021, the retraction period occurred 11 days later in 2022 (Table 2). However, the daily maximum temperature was approximately 8 °F greater in 2022 than 2021 during the retraction period. Fruit color development was poor, even in red Honeycrisp strains like Firestorm. Although color development was so poor, the mean starch rating was 3.5-4 for all fruit harvested at Sunrise and the background color was breaking from green to yellow indicating maturity of fruit on the tree. Delaying harvest longer would have resulted in excessive fruit drop and poor storability.

Unsurprisingly, uncovered fruit had the highest proportion of fruit with severe sunburn compared to netted fruit (Figure 5). Evaporative cooling only reduced the proportion of fruit with severe sunburn when it was used for uncovered or retracted trees. In 2021, when trees were left covered until after harvest, evaporative cooling did not significantly reduce the proportion of fruit with severe sunburn. We did not observe this same pattern in 2021. Looking at the main effects, evaporative cooling decreased losses from severe sunburn and netting, whether retracted or not, was effective at reducing severe sunburn. Interestingly, red color coverage (%) was improved when evaporative cooling was used in 2021 but while also higher in 2022, there was low statistical confidence in those differences. Overall, there were 10% more fruit with >33% red color coverage when EC was used in 2021 and 2.5% more fruit with >33% red color coverage when EC was used in 2022.

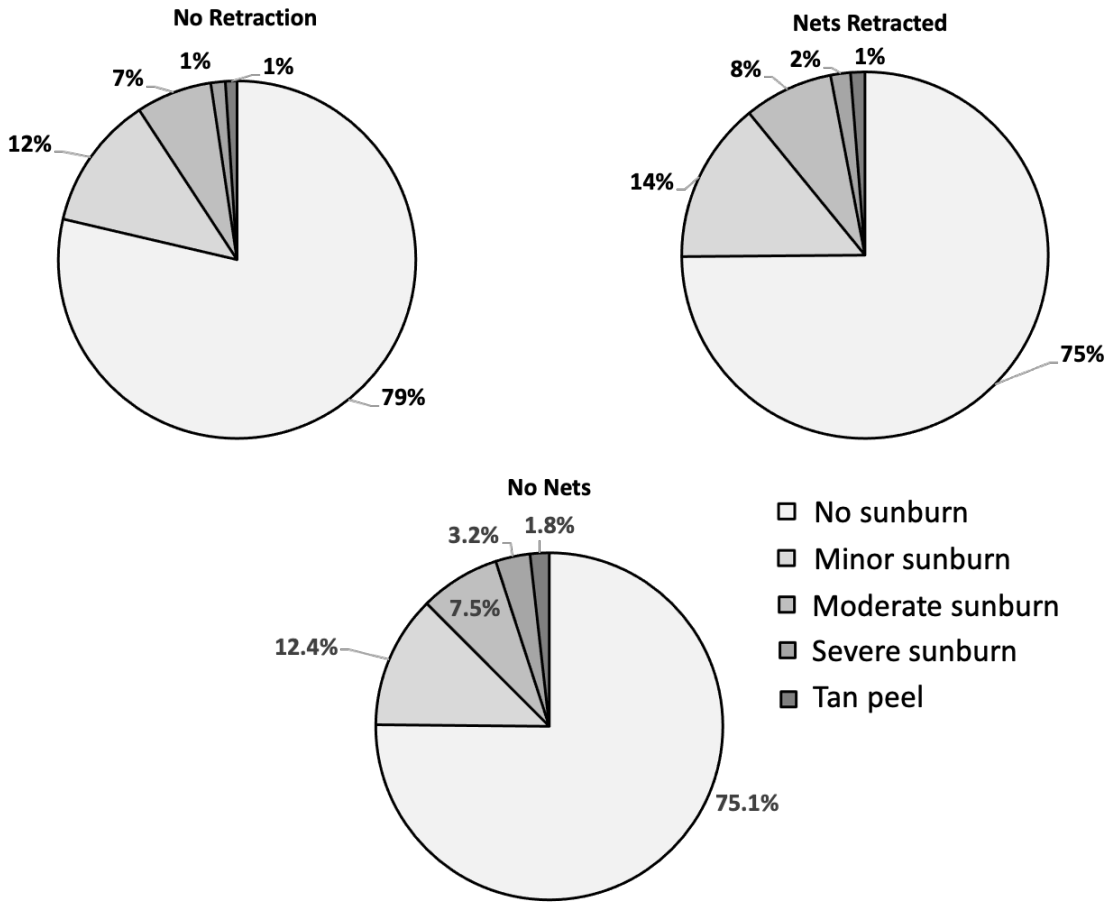

Figure 5. Sunburn incidence for trees with nets remaining all season, nets retracted 10 days before harvest and then trees with no nets all season. Sunburn incidence follows the Schrader/McPherson scale (0-4) where 0 = no sunburn, 1 = minor sunburn, 2 = moderate sunburn, 3 = severe sunburn, and 4 = tan sunburn on the peel.

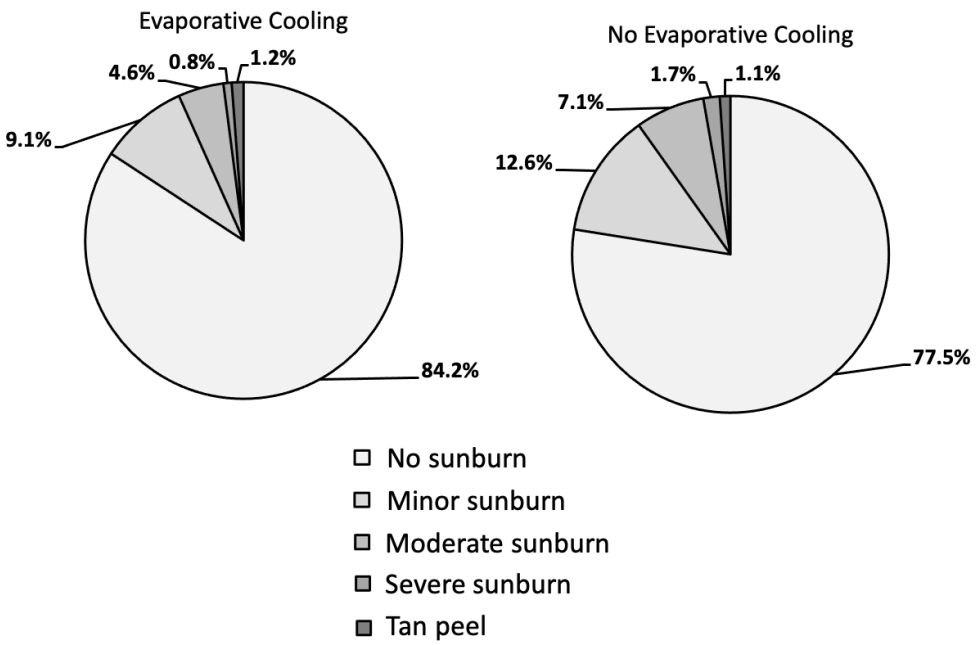

Figure 6. Sunburn incidence for trees with either evaporative cooling or no cooling when air temperatures exceeded 85 °F. Sunburn incidence follows the Schrader/McPherson scale (0-4) where 0 = no sunburn, 1 = minor sunburn, 2 = moderate sunburn, 3 = severe sunburn, and 4 = tan sunburn on the peel.

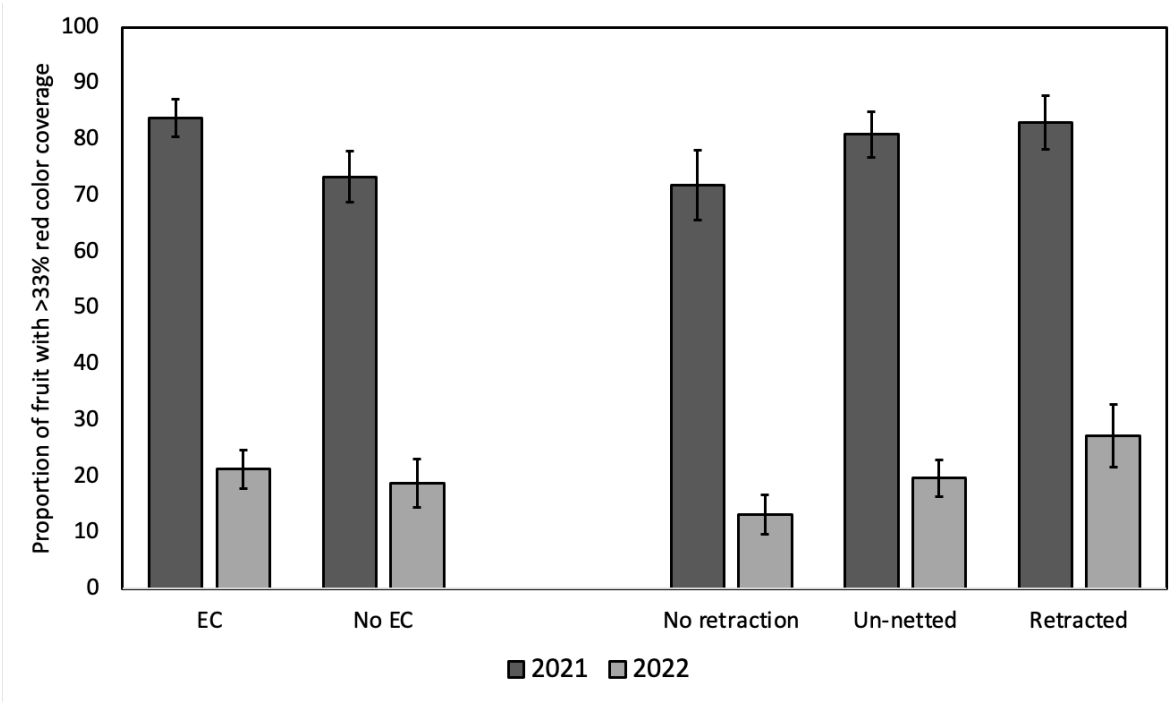

Figure 7. The proportion of fruit with red color coverage exceeding 33% for trees treated with evaporative cooling (EC) or not (No EC) and then with netting left on until harvest (No retraction), un-netted, and then netting retracted 10 days before harvest. Error bars indicate standard error of the mean (N=3).

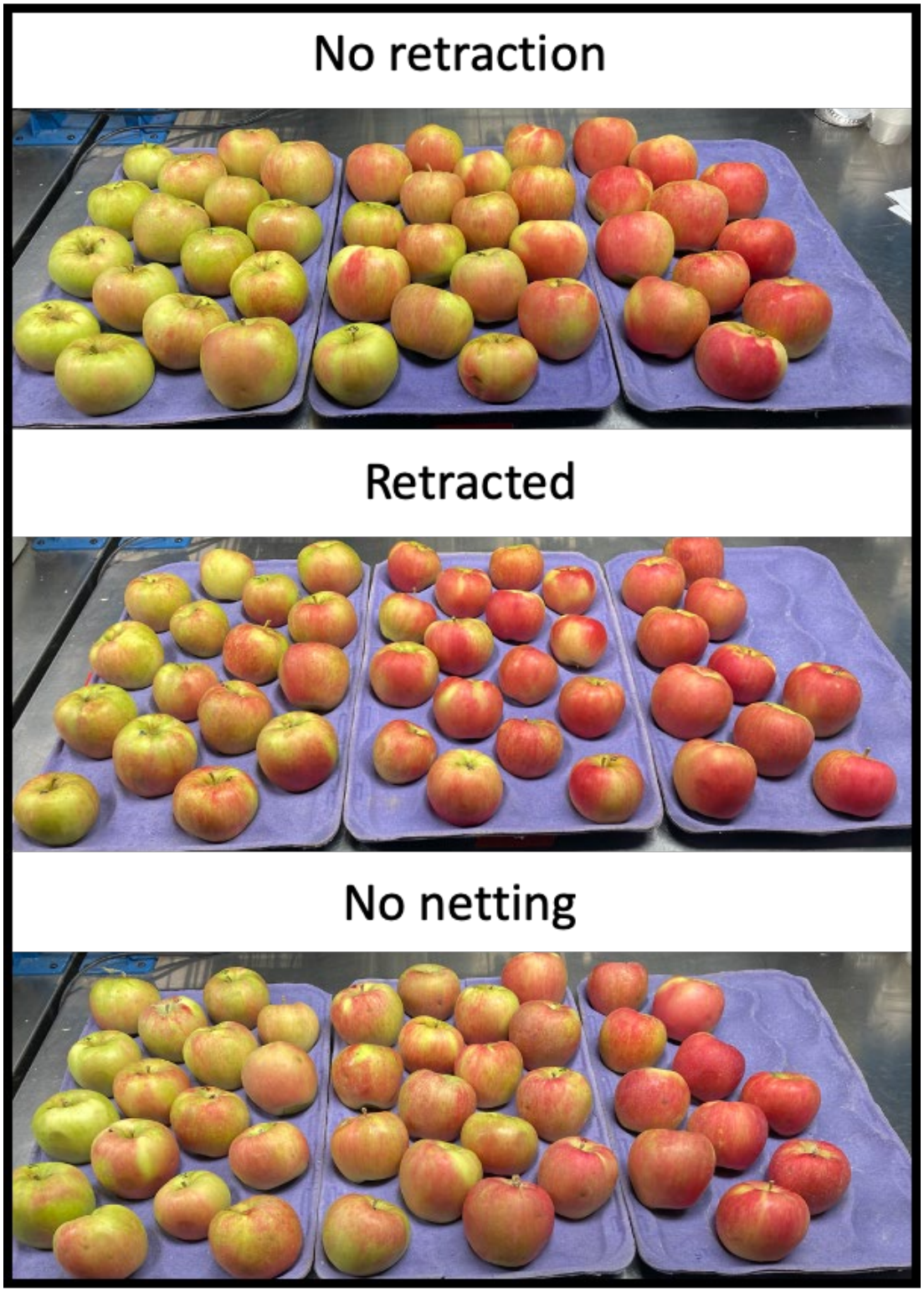

Figure 8. Representative Honeycrisp fruit samples from each netting treatment from the Sunrise research orchard in Rock Island, WA.

OUTPUTS IN 2022

- Lee Kalcsits and Noah Willsea. Netting retraction focused discussion at monthly meeting for the Apple Horticulture and Protection metting. Yakima, WA. May 14, 2022.
- Noah Willsea and Victor Blanco. Heat impacts and management. Columbia Growers Club meeting. Pasco, WA. June 30, 2022.
- Noah Willsea and Lee Kalcsits. Netting retraction as a tool to improve red color in apple. American Society for Horticultural Sciences Annual Meeting. Chicago, Illinois. August 1, 2022.
- Noah Willsea and Lee Kalcsits. Netting Retraction to Improve Red Color in Apple. WSTFA Annual Meeting, Wenatchee, WA. December 7, 2022.

CONTINUING PLANS

We will evaluate the incidence of postharvest disorders as recommended by the WTFRC Apple Hort Committee in January 2023.

We need to complete the economic analysis based on improvements in the proportion of fruit reaching minimum premium color standards, the cost of implementation of sunburn management practices, and labor considerations (retracting during harvest compared to immediately following harvest). This will be part of an Extension publication that will be published by WSU Extension.

Noah Willsea will also continue to develop Extension material and publish videos that we have recorded in 2022.

We anticipate publishing a peer-reviewed publication from this research in addition to presenting this research at winter meetings in 2023.

Project Title: Efficient heat stress management for improved apple fruit quality

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Cooperators:

1. **Hancock Farmland Services. In-kind support valued at: $30,000** (+ yearly upkeep cost)
   Roy C "Dewey" Holliday, *SVP Pacific Northwest Operations*
   Casey Hubbs, *Quality Assurance Specialist*
   Nature of cooperation: Orchard access (Honeycrisp); Establish and maintain overhead netting, and convention sprinkler as well as fogging systems (16 acers) for heat stress management

2. **Jain Irrigation Inc. USA**. **In-kind support valued at: $6,000**
   Brad Holliday, *Territory Sales Manager– Pacific Northwest*
   Nature of cooperation: Provide hardware (foggers, supply tubing and fittings) to establish overhead evaporative fogging systems for heat stress management in Honeycrisp and WA-38.

Report Type: Continuing Project Report

Project Duration: 3 -Years

Total Project Request for Year 1 Funding: $68,717
Total Project Request for Year 2 Funding: $66,232
Total Project Request for Year 1 Funding: $68,035

Other funding sources: **AwardedAwarded**
Amount: $450,000
Agency Name: USDA NIFA/ NSF Cyber Physical System
Notes: Funded in 2018 to develop localized orchard climate and crop physiology sensing system for apple fruit surface temperature and heat stress monitoring.

Budget 1
Primary PI: Lav Khot
Organization Name: WSU-IAREC **Contract Administrator: Anastasia Mondy**
Telephone: 509-335-2885 **Email address: arcgrants@wsu.edu**
Station Manager: Naidu Rayapati **Email address: naidu@wsu.edu**

Item	2021	2022	2023
Salaries	40,500	42,120	43,804
Benefits	14,875	15,470	16,089
Wages			
Benefits			
Equipment			
Supplies	10,210	5,510	5,010
Travel	3,132	3,132	3,132
Miscellaneous			
Plot Fees			
Total	68,717	66,232	68,035

Footnotes: Year 1 -- Salaries of $20,000 will support 5-months at 100% FTE of postdoc jointly supervised by Khot & Peters; $14,000 to support 7-months research associate at 50% FTE supervised by PI-Torres and $6,500 to support lab technician for 4-months at 50% FTE supervised by PI-Sallato. Pertinent HR benefits for these three personnel will be $14,875.Supplies include procurement of material to integrate crop physiology sensing nodes (8 nodes, $700/unit), telemetry (wifi router, cellular subscription, $620), pressure transducers w/ data logging capability ($250 × 4 units), misc. hardware, harness & related costs ($150) and orchard diagnostics/testing supplies for Soil test, Tissue samples, Fruitlets and Fruit ($1,350). Travel include 60 trips (× 90 miles/round × 0.58/mile) for members of team to travel to field sites for research and extension activities. **Year-2 and -3 –** Salaries are inflated by 4% respectively and pertinent benefits. Supplies include $2,670 to upkeep the sensing nodes and $1,350 for orchard diagnostics/testing supplies. Travel costs will remain unchanged from year-1.

Objectives

1. Evaluate the impact of three different heat stress management techniques on fruit quality at harvest and after storage.
2. Assess the effectiveness of sensing technology for automated stress monitoring and management.
3. Estimate the economic cost-benefits of each technology.
4. Deliver new knowledge to the apple industry through extension and outreach.

For this continuation report, we have focused reporting on objectives 1 through 4.

Significant findings

Honeycrisp:

- Lower sunburn damage (0.8 to 11%) compared to 2021 season (6 to 30%) with untreated control being the highest.
- Fogging and netting combined (i.e., fognet) had lowest sunburn damage. Damage in fogging and conventional evaporative cooling are comparable.
- Browning contributed more towards total sunburn damage followed by photooxidative type sunburn. Top canopy section had higher damage than other sections, expect fogging where mid-canopy section was higher than other two sections.
- Crop load in fognet treatment was comparable to conventional EC and fogging in terms of fruit weight per tree. However, fognet had larger size fruit (62.4 mm) compared to conventional evaporative cooling (59.9 mm). Like last year, netting continued to show a greater number of fruits per tree but of a smaller size (55.3 mm).
- Water based cooling methods seem to propagate exponential increase in fruit growth (size in mm) compared to netting alone.
- Localized thermal-RGB imagery and dew point temperature were able to quantify treatments reliably compared to other weather variables.
- Imagery data based fruit surface temperature snapshot for entire day suggests that fognet seem to maintain fruit temperature more uniformly throughout the day compared to other treatments and cyclic conventional EC tends to underperform at peak heat stress hours.
- Postharvest fruit quality analysis over 6-month storage period for 2022 is being performed. For 2021 season, conventional EC had had higher soft scald incidence than other treatments. All treatments except control had comparable fruit firmness over the storage.

WA-38:

- 2022 season had lower and less uniform crop load in all heat mitigation treatments and control.
- Like 2021 season, WA-38 had less sunburn damage with drape type netting being efficient in mitigating sunburn compared to fogging. With comparable fruit count per tree, fogging (253 g) had fruits with higher weight compared to netting (220 g) and control (202 g).
- Dew point temperature and fruit surface temperature data is relatable to the treatment effects.
- Postharvest fruit quality analysis over 6-month storage period for 2022 is being performed. For 2021 season, all three treatments had comparable fruit firmness and soft scald over the storage.

Methods

Objective 1. **Evaluate the impact of three different heat stress management techniques on fruit quality at harvest and after storage.** The project is being conducted in two independent sites: 1. Honeycrisp block (of Farmland Services commercial orchard near Prosser, WA); and 2. WA-38 research block (WSU Roza farm, Prosser, WA). Honeycrisp trees are on M9-339 rootstock planted in 2016 on vertical system with three leaders per trees planted at 10’×4’ tree spacing. WA-38 trees are

on M9-Nic 29 rootstock planted in 2013 on a vertical system with a bi-axis training system with 10'× 3' spacing.

Table 1 has treatment details for 2022 field season. At 'Honeycrisp' site (fig. 1), we have established four replicates of netting, fogging, conventional evaporative cooling (EC), fogging and netting combined (termed as 'fognet' hereafter) treatments and untreated control (UTC). The overhead netting (with approximately 12% shade, Orchard & Vineyard Supply Inc., WA) was established on 4-acre block by Farmland Services as **in-kind support.** As a treatment, nets were on from July 15 to September 7 in 2021 and between June 23 to September 23 in 2022. The overhead fogging system was installed in about 1-acre block that utilizes 2-way fogging type emitters (1.8 GPM/side, Jain Irrigation Inc., USA) spaced every 10' in each row. The foggers were provided by Jain-USA as **in-kind support**. The fogging system was turned on at about 80°F air temp. The conventional evaporative cooling treatment block uses overhead sprinklers (P2 9Red, Nelson Irrigation Corp., USA; flow rate 0.51 GPM @50 psi) and were spaced every 20x20'. They were turned on when temp reached 90°F and in 25-min ON/OFF cycles. The 'fognet' treatment consisted of netting and fogging combined. Four blocks of 10 trees each were included as UTC. All other management practices including irrigation, pest and disease control and sunburn sprays were recorded and equivalent to all the treated area.

Table 1. Heat stress management treatment details for 2022 field season.

Cultivar	Treatment type*	Treatment details
Honeycrisp M9-339 rootstock planted in 2016 Training system: tall spindle	Untreated control	-
	Fogging [26.2 GPM/A]	Emitter: two way@ 1.8 GPM/side, spacing 10x10' Operation: Continuous (On at 80 °F)
	Conventional evaporative cooling [55.5GPM/A]	Emitter: 0.51 GPM @ 50 psi, spacing 20x20' Operation: Cyclic (~25 min ON/Off), On at 90°F
	Netting (Shade net)	12% shade
	Fognet	Fogging at 26.2 GPM/A and netting combined
WA-38 M9-nic 29 rootstock planted in 2013 Training system: bi-axis	Untreated control	-
	Fogging [26.2 GPM/A]	Emitter: two way@ 1.8 GPM/side, spacing 10x10' Operation: Continuous (On at 80°F)
	Netting (DrapeNet)	15% shade

*Honeycrisp: 10 trees/replicate × 4 replicates/treatment; WA-38: 10 trees/replicate × 3 replicates/treatment.

Figure 1. Heat stress mitigation treatments (Netting [left], Fogging [middle], Fogging + Netting [right]) in Honeycrisp block.

The WA-38 trial consists of three replicates of 33 ft with 10 trees each. The treatments included shadenet (approximately 15% shade, DrapeNet as **in-kind support**), fogging (as described above) and an untreated control. The drape net covered trees between July 8 to October 7 in 2021 and June 25 to October 21 in 2022.

Monitoring

In both locations, several monitoring systems were installed within the canopy to record environmental, soil and plant conditions during the growing season; (Table 2). A localized orchard climate/crop physiology sensing system (CPSS) nodes have been developed by our team through the NSF/USDA-NIFA funded project. Each of the CPSS node utilizes thermal-RGB imager (FLIR Inc., OR) and an all-in-one weather station (ATMOS 41, Meters Group, Pullman, WA) to estimate real-time apple fruit surface temperature (FST). The sensing nodes (fig. 2) are programmed to autonomously acquire thermal-RGB images (fig. 2 insert) and microclimate data at 5 min intervals, process those data on the Raspberry Pi computer (edge computing) for real-time FST estimation and wirelessly share the data with user host computing devices.

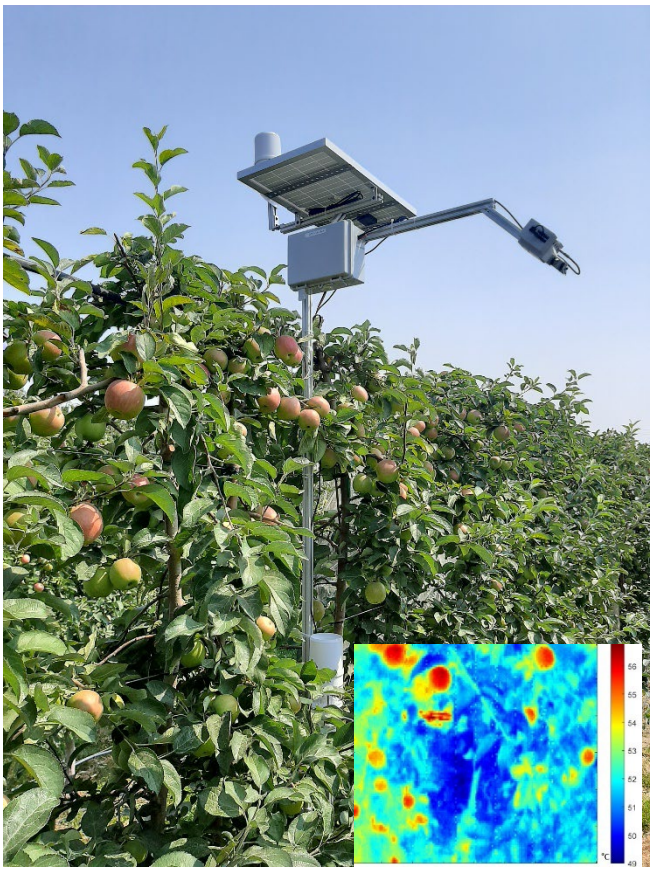


Figure 2. Crop physiology sensing node installed in cosmic crisp heat stress trial block.

Table 2. Quantification of canopy and fruit parameters in 2022 season to evaluate heat stress management treatments.

Monitoring type	Parameter [Frequency]	
	Cultivar: Honeycrisp	WA-38
Localized weather	Above canopy temperature (T, °C), Relative humidity (RH, %), Wind speed (WS, m/s) and direction (WD, degrees	-same-

	from N), Solar radiation (SR, w/m^2) [1 min] Open field T, RH, WS, WD, SR [1 min]	
Crop physiology	In-canopy weather and FST_w in °C [1 min] Thermal RGB imagery and FST_i in °C [5 min]	-same-
	Mid-day stem water potential [6 distribuend days in July 2022]	-NA-
	PAR (moles per m^2 per second) [15-days in June and July each] *Only in netting and UTC*	-NA-
	Drone imagery [thermal and multispectral, 11 AM and 5 PM each of the 3 days in July, 1 time after netting removal]	-NA-
Fruit size	Diameter (mm) and length (mm) [Bi-weekly: May through August]	-same- Monthly [in July and August]
Soil moisture	Volumetric water content (m^3/m^3, [1 min]	-NA-
Ground truthing	FST_a in °C using Thermopen and as an image using thermal-RGB imager [6-days in July 2022]	FST_a in °C using Thermopen and as an image using thermal-RGB imager [3 times once in July and twice in August 2022]
	Pre-harvest fruit quality [3 weeks prior]	NA- due to low crop load
	Post-harvest fruit quality [0, 3, 6 months]	-same-

A contact type thermal probe with ±0.4 °C accuracy (model: Thermapen, ThermoWorks Inc., USA) was utilized to validate the apple FST (FST_a) measurements. This ground truth data was collected on three fruits per tree and fives tress in each treatment. For Honeycrisp, ground truth data were collected in July 2022 for 6 days. Similar data was collected in WA-38 three times in July and twice in August 2022. We also quantified actual fruit surface temperature as ground truth data using a handheld thermal-RGB imager (FLIROne, Teledyne Inc., USA).

Thermal-RGB imagery data help derive the mean measured FST (FST_i), maximum FST ($FST_{i\text{-}max}$), and mean FST of the 10%, 15% and 20% hottest part of the fruit surface (i.e., FST_{10}, FST_{15}, and FST_{20}, respectively). The weather data helps derive weather-model-predicted FST (FST_w). Detailed methods are in Ranjan et al. (2020). The FST_w has been found to be highly sensitive to fruit size, color, and shading. In 2021 we had quantified fruit albedo (Model: SP-710-SS, Apogee Instruments, Logan, UT) changes with respect to size (vernier caliper) of the fruit. In 2022 season, we quantified fruit size by measuring 50 fruits per treatments with two measurements on each fruit as ground truth data.

Evaluations

At commercial harvest five to ten representative trees with equivalent trunk cross sectional area and crop load within replicated unit were selected for individual tree analysis. In 2021, trees were strip harvested and sunburn damage was assessed in the field, classifying sunburn in a scale from 1 to 3: 1. no external symptoms of sunburn, 2. browning, and 3. necrosis (fig. 3). In 2022, due to significant color and maturity differences between treatments in Honeycrisp block, we evaluated a set of three trees per replicated unit, in two harvest timing; first: when 60% of the fruit in the most

advanced treatment reached commercial harvest guidelines (over 50% of red color) and second: when the least advanced treatment reached commercial harvest guidelines. In 2022, these dates were September 23 and October 7, 2022, for the first and second harvest, respectively. In 2022 trees were strip harvested by section top, middle and bottom and taken to IAREC fruit laboratory for at harvest sunburn, bitter pit and other defect assessment, and fruit color and size distribution. In addition, 110 representative fruits per replicated unit were collected and transported to WSU-TFREC Wenatchee (PI-Torres lab) for quality evaluation. General fruit quality per treatment was assessed using a commercial sorting line (Aweta Inc., The Netherlands). Additionally, 10 fruits per replicate/treatment were used to determine maturity indexes (flesh firmness (lb), soluble solids (ºBrix), titratable acidity (% malic acid), starch index (1-6)). Postharvest evaluations for 2022 harvest is ongoing.

For WA-38, fruits were strip harvested on October 21, 2022. Crop load and % sunburn assessments were done after harvest and all the fruits, due to low crop load, were send to WSU-TFREC Wenatchee (PI-Torres lab) for quality evaluation.

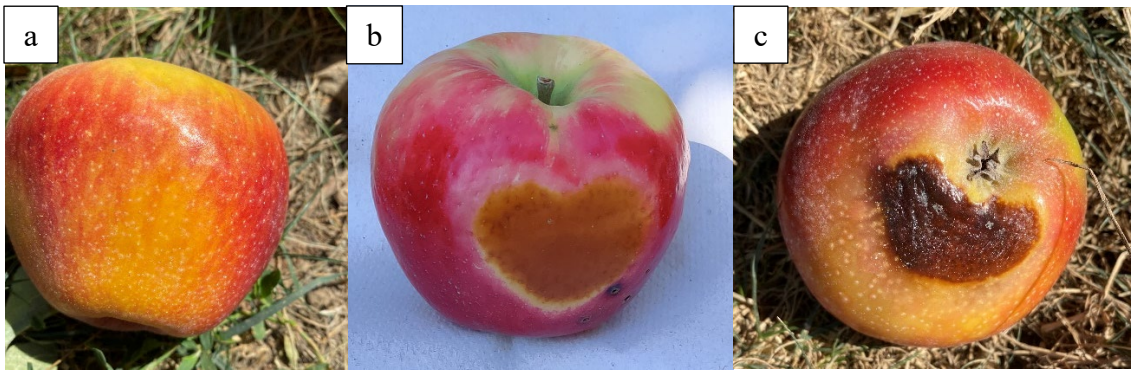


Figure 3. Sunburn in Honeycrisp. a: browning, b: oxidative damage and c: necrosis.

Objective 2. Assess the effectiveness of sensing technology for automated stress monitoring and management. *Although this objective will come in effect in year-3 of the project, we have piloted automated fogging system in WA-338 block. The system actuation is based on the localized air temperature data with threshold set to 80 ºF.*

In year 3, with two seasons data-based learning, a localized orchard climate/crop physiology sensing system developed by our team will be used for automated stress monitoring and management. We will manage about 1-acre replicate blocks using such system. The sensing nodes will monitor the in-field apple fruit surface temperature and actuate evaporative cooling system if FST is above set threshold. Pertinent to WA-38, our field trials season have shown that ***fruit surface temperate*** of 116.6 ºF (47 ºC) and 123.8 ºF (51 ºC) or higher results in sunburn browning and necrosis, respectively (unpublished data). Having such scientific data and other published work (Rasco & Schrader, 2012), we will set 112.1 ºF (44.5 ºC) as an evaporative cooling system actuation threshold. Similar thresholds will be applied for Honeycrisp` cultivar in consultation with grower cooperator. Similar to objective 1, we will conduct at harvest and after storage fruit quality analysis. The in-season data on water usage will also be collected and contrasted with trials that didn't actuate cooling using sensing data.

Objective 3. Estimate the economic cost – benefits of each technology. In terms of economic analysis, we are keeping record of initial installation costs (including hardware and labor) for all three heat stress management systems. We also have estimated water and energy usage for fogging and conventional evaporative cooling for 2021 and 2022 season. Next year, we will use a water pressure

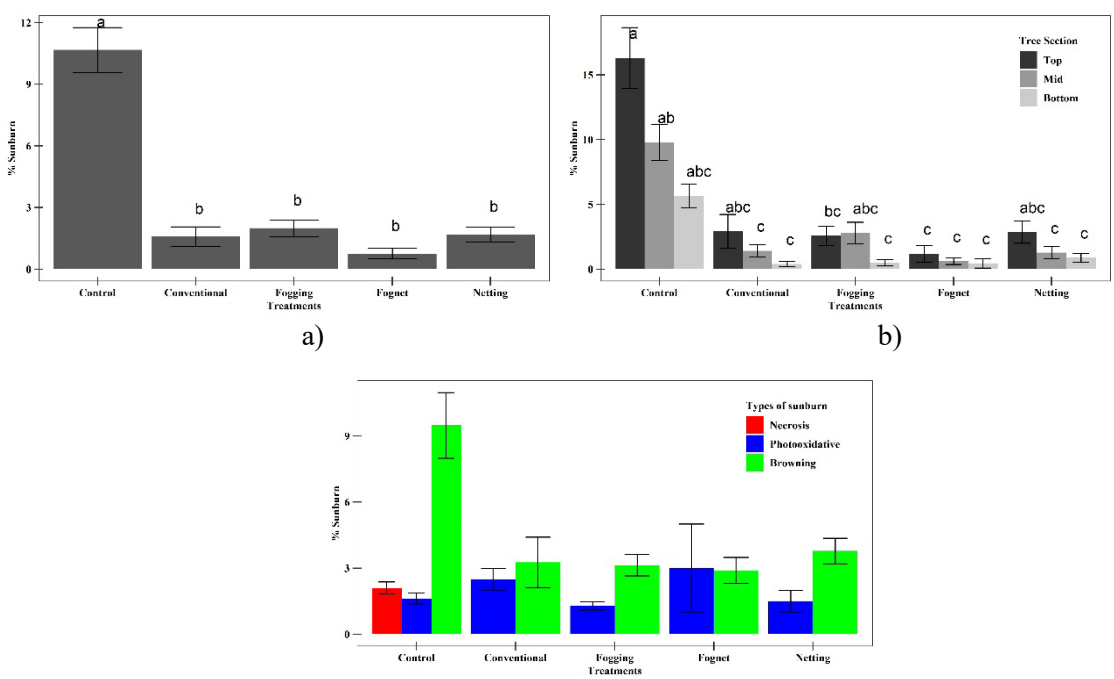
datalogger to measure on and off times and durations. We will calibrate this with an ultrasonic flow meter to know how much water is applied and when throughout the growing season. We will compare the amount of water applied with an estimate of the amount of water required for evapotranspiration as estimated by the Irrigation Scheduler Mobile app. At the end of the project, we will monitor water and energy usage for the different treatments and pertinent costs analysis will be contrasted with fruit yield, quality and pack-outs to develop cost benefit analysis.

Objective 4. Deliver new knowledge to apple industry through extension and outreach. *In 2022, we conducted two field days in 'WA 38'; organized by J. Bolivar and Co PI- B. Sallato, where the technology and pertinent knowledge was shared with industry members. Information and preliminary results were shared in the Columbia Basin Tree Fruit grower meeting (25 attendees) and research flash talks at 118th NW Hort Expo, 2022 (300+ attendees).*

Results and Discussion

Objective 1. **Evaluate the impact of three different heat stress management techniques on fruit quality at harvest and after storage.**

Honeycrisp trial. Sunburn damage: The precent sunburn damage of the harvest fruits varied between 0.8 and 10.7% (Fig. 4a). Fognet had lowest sunburn damage (0.8±0.3% [mean ± standard error]), followed by netting (1.7±0.4%), conventional EC (1.6±0.5%) and fogging (2.0±0.4%), respectively. Except control, none of the treatments were significantly different. Overall, sunburn damage was lower compared to 2021 season which had 30% damage in control treatments. In 2022 season, browning contributed more towards total damage followed by photooxidative type sunburn. Necrosis contributed to higher damage in control treatments only. In all treatments, except fogging, top section of the canopy had higher sunburn damage compared to other sections.



a) b) c)

Figure 4. Percent sunburn damage on Honeycrisp with plots a) overall damage, b) section wise damage and c) contribution from type of sunburn. Bars represent mean values; error bars represent

standard error. Different letters indicate statistical differences between means (Welch ANOVA p = 0.001).

Crop load: as total weight of fruits per tree was significantly higher for conventional EC compared to control treatment. Although higher in conventional EC, crop load was not significantly different compared to other treatments except netting (Fig. 5a). In terms of average number fruits per tree, control and netting had higher number of fruits per tree compared to other treatments, with fognet being the lowest. However, fognet had bigger size (& fruit weight: 227 g) fruits, followed by fogging (fruit weight: 196 g) and conventional EC (fruit weight: 217 g) with no significant difference between latter two treatments (Fig. 5c). The control and netting had smaller size fruits compared to other treatments. The ground truth data on fruit size (Fig. 5d) quantified throughout the production season (2022) suggest that water-based cooling impacts fruit development positively compared to netting treatments.

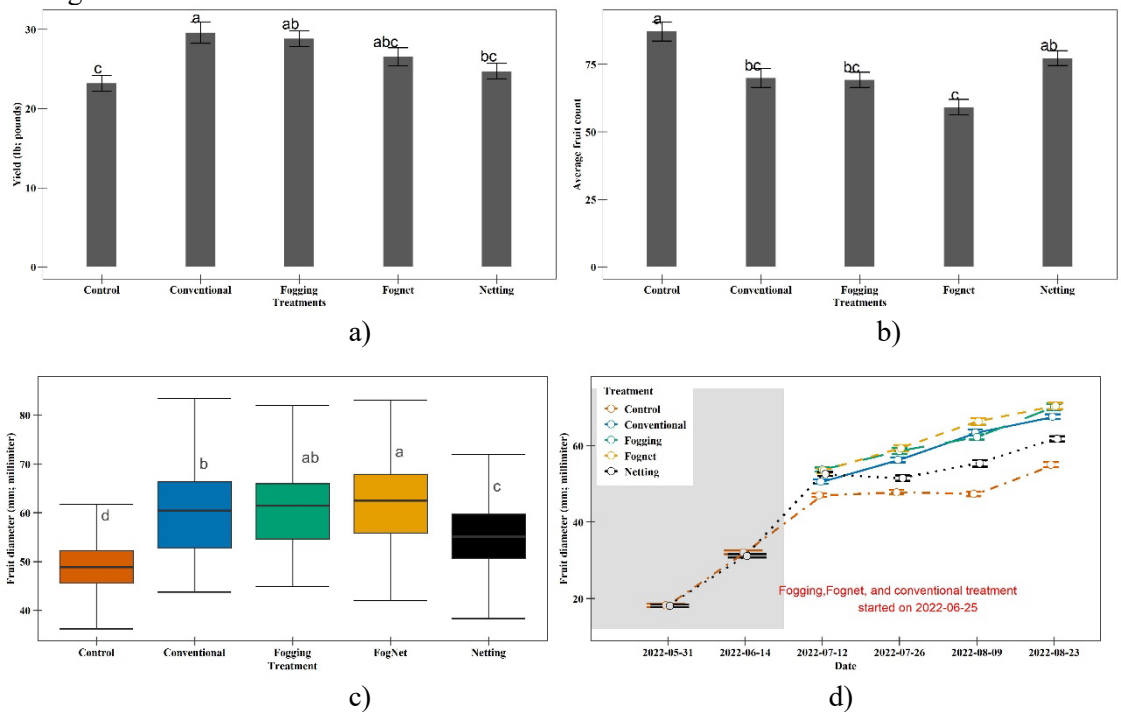


Figure 5. Honeycrisp a) yield (lb/tress), b) average fruit count, c) box-wisher of fruit size (mm) at harvest, and c) fruit size changes during the treatment period.

Heat stress mitigation: Like last year, 2022 data suggest that imagery based FST is a reliable measure of heat stress compared to air temperature and pertinent use of existing weather-based energy balance model based FST estimation. Localized thermal and RGB imagery does benefit in that it captures the fruit shading and non-shading aspects and related fruit surface temperature gradients (see fig. 6a and b). Regarding localized weather, dew point temperature can help understand the heat stress mitigation treatment effects more reliably than air temperature alone. Dew point temperature is derived from air T and RH as a measure to indicate water vapor saturation in air (Sonntag, 1990). Overall, conventional EC treatments had highest amount of moisture in the air followed by fogging, fognet control and netting (fig. 6c). This is somewhat reflected by the actual ground-truth FST.

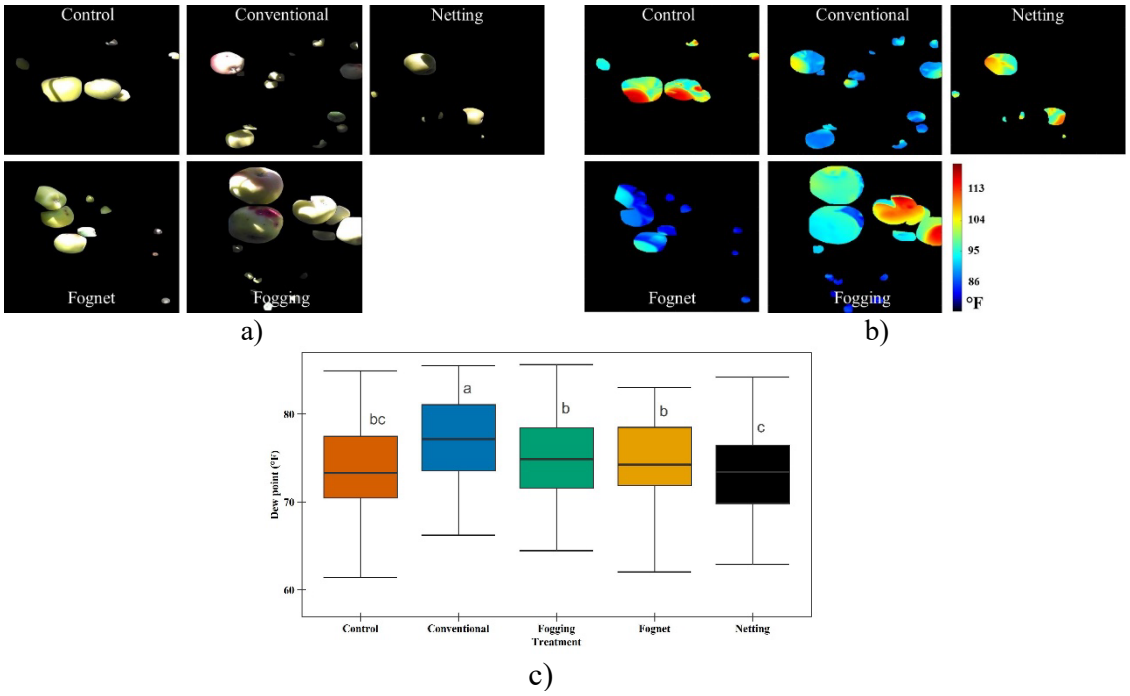


Figure 6. Localized a) RGB, b) thermal imagery of fruits showing shading effects on FST, and c) dew point temperature quantified in each of the heat stress management treatments.

To provide day's snapshot on efficacy of mitigation techniques to lower/maintain fruit surface temperature below sunburn threshold, figure 7 was done using 5-min interval thermal-RGB imagery captured by CPSS on one of the hottest (& ground truth data collection) day. The data is of July 25, 2022, from noon through 9:00 PM. As seen, control and netting treatments had FST >113 °F (threshold) during afternoon hours. Overall, fognet seem to maintain fruit surface temperature uniformly throughout the day and that cyclic conventional EC tend to underperform at peak heat stress hours. Also, industry needs to rethink a fixed frequency cycle based EC approach and consider varying the cycle frequency based on the instantaneous heat stress levels.

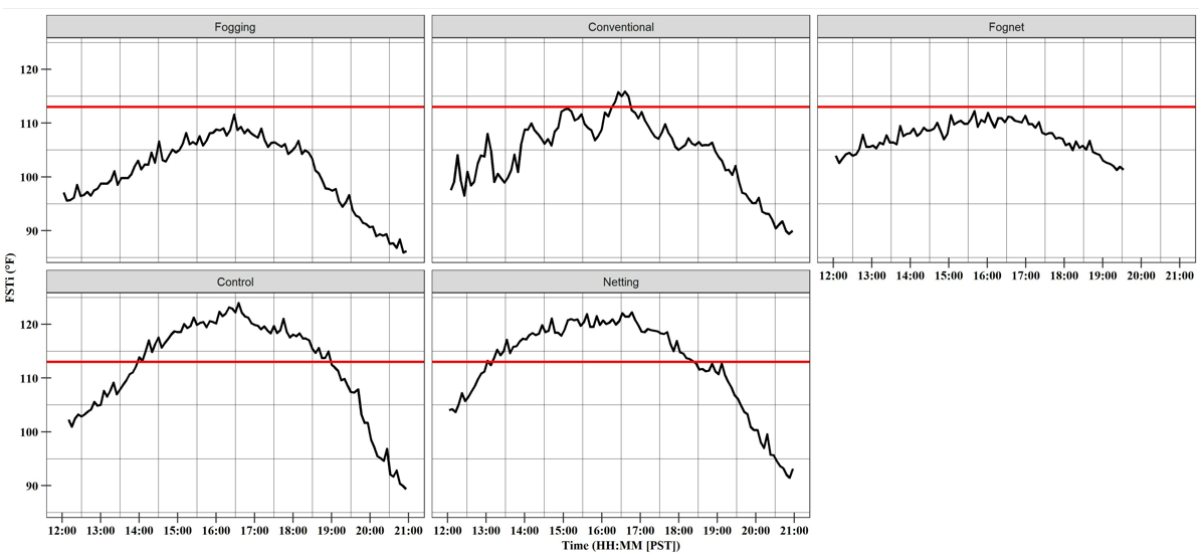


Figure 7. Localized thermal-RGB imagery based FST showing fruits temperature variation for all treatments for the day of July 25, 2022.

Postharvest fruit quality: analysis of 2021 season fruits stored up to 6 months after harvest and analyzed at 3 months (1 and 7th day), 6 months (1 and 7th day) suggest that conventional EC had higher soft scald incidence (Table 3) than other treatments. Fruit firmness differences between treatments and the control group were observed at the end of storage.

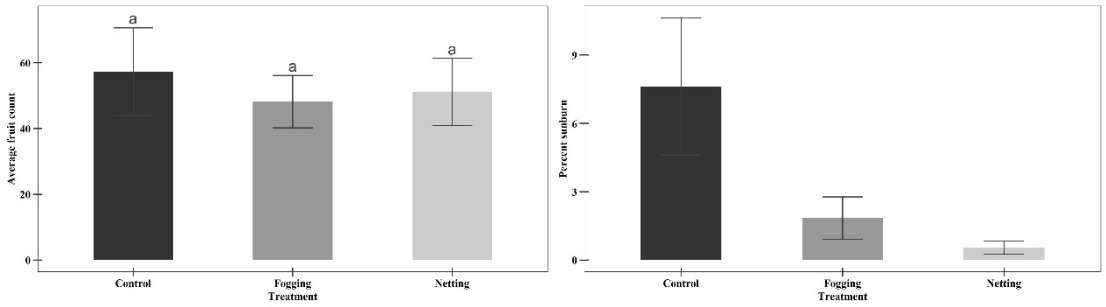
Table 3. Mean Firmness of fruits over storage up to 6 months.

Treatment	Initial	3mo+1d	3mo+7d	6mo+1d	6mo+7d
Control	14.2 ab	14.4	14.7	14.3 a	13.4 a
Conventional EC	14.6 ab	14.5	14.5	14.5 a	14.3 ac
Fogging	13.6 a	14.2	14.5	13.9 a	14.0 ab
Fognet	14.1 ab	14.3	14.3	14.4 a	14.5 bc
Netting	14.7 b	14.9	14.9	15.5 b	15.0 c
Significance	*	ns	ns	**	**

ANOVA (*: P<0.05; ** P<0.01; ns: non-statistically significant).
Different letters within columns and time points
indicate differences between treatments (Tukey, HSD (P<0.05)).

On-going data analysis: we continue to ingest and analyze the season long thermal-RGB imagery, weather data in each of the treatments. Postharvest fruit quality analysis is also underway. Before 2023 season field trials, we will have all two seasons' data analyzed and results inferred to develop peer-reviewed publications as well as extension articles in WSU Extension Fruit Matters magazine.

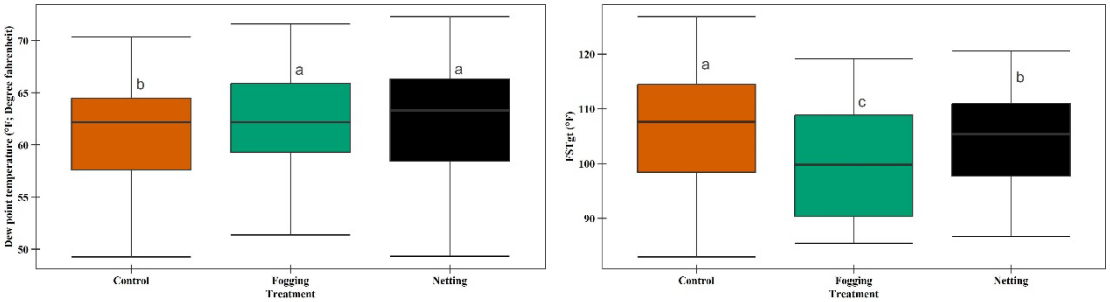
WA 38 trial. Crop Load: was low and not uniform this season in the research block. Hence, at harvest fruit quality evaluation was limited to fruit counts and sunburn damage assessments only. As shown in fig.7a, average fruits per tree and size in netting and fogging were comparable to control. Average fruit weight was higher in fogging (253 g), compared to netting (220 g) and control (202 g). In terms of sunburn, damage was considerably lower in both netting (0.6%) and fogging (1.8%) compared to control (7.6%). Overall, browning was prominent compared to other types of sunburn.



a) b)

Figure 7. WA-38 a) average fruits per tree, and b) associated sunburn damage for the implemented treatments.

Heat stress mitigation: Dew point temperature in fogging and netting was comparable and significantly higher than control treatment (fig. 8a). This relates to the sunburn damage data shown in fig. 7b. Ground truth FST quantification showed relatable trend with control treatment having significantly higher FST, followed by netting and fogging (fig. 8b).



a) b)

Figure 8. a) dew point temperature, and b) ground truth FST in the implemented treatments in WA-38 block.

Postharvest fruit quality: analysis of 2021 season fruits stored up to 6 months after harvest and analyzed at 3 months (1 and 7th day), 6 months (1 and 7th day) suggest that fruit firmness and soft scald were not different over storge for all three treatments.

On-going data analysis: We continue to ingest and analyze the season long thermal-RGB imagery, weather data in each of the treatments. Postharvest fruit quality analysis is also underway. Before 2023 season field trials, we will have all two seasons' data analyzed and results inferred to develop peer-reviewed publications as well as extension articles in WSU Extension Fruit Matters magazine.

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

Project Title: Apple Crop Load Management

PI: Tory Schmidt
Organization: WA Tree Fruit Research Commission
Telephone: (509) 669-3903
Email: tory@treefruitresearch.com
Address: 1719 Springwater Ave.
City/State/Zip: Wenatchee, WA 98801

Cooperators: Stefano Musacchi (WSU), Sara Serra (WSU), Karen Lewis (WSU), Gerardo Garcia, Manoella Mendoza, private chemical companies

Total Project Request: **Year 1:** $0 **Year 2:** $0 **Year 3:** $0

Other funding sources: **Awarded**
Amount: $127,283 (4 year total)
Agency Name: NIFA – SCRI: Precision Crop Load Management for Apples (PD: Terence Robinson, Cornell University)
Notes: funding primarily supports 2 research assistants for 3 months/year to be shared with co-PIs Musacchi and Lewis; selected trial sites will be jointly utilized for WTFRC and SCRI projects

Other funding sources: **Requested**
Amount: Unknown
Agency Name: Contract work with private chemical companies (i.e. Adama, Fine Americas, Valent)
Notes: amount requested & awarded typically offsets all costs (excluding PI salary) associated with execution of trial protocols; annual total contributions from registrants (typically $30-50K) vary depending on trial number and complexity of protocols

WTFRC Budget

Item	**2021**	**2022**	**2023**
Salaries	na	na	na
Benefits	na	na	na
Wages	28,000	28,000	28,000
Benefits	15,000	15,000	15,000
Travel	1000	1000	1000
Plot Fees	4600	4600	4600
Miscellaneous	400	400	400
SCRI funding	*(20,000)*	*(20,000)*	*(20,000)*
Contract funding	*(29,000)*	*(29,000)*	*(29,000)*
Total net cost	**$0**	**$0**	**$0**

Footnotes:
All budget figures are rough estimates and will change depending on the number of trial sites and complexity of individual trial protocols in any given year; regardless of costs incurred, external funding should likewise adjust to offset cost totals
NOTE: Budget for informational purposes only; research is funded through WTFRC internal program

OBJECTIVES:

1. Ongoing screening of novel thinning chemistries (i.e. metamitron) for bloom and post-bloom thinning of apple including development of best practices regarding rates, timings, and use of adjuvants.

2. Ongoing screening of plant growth regulators (i.e. gibberellins) with potential to influence shoot growth, flowering, fruit set, fruit growth, fruit quality, etc. to the benefit of commercial apple production.

3. Collaborate with state and national research team on SCRI grant "Precision Crop Load Management for Apples."

SIGNIFICANT PROGRESS & FINDINGS 2022:

No thinning treatment produced significant reductions in fruit set or increases in harvest fruit size vs. untreated controls across three trial sites and cultivars

Historically cool, wet conditions during spring of 2022 rendered many postbloom chemical thinning applications ineffective in research trials and commercial orchards

The most efficacious options for chemical bloom thinning of apple continue to be spray oil + lime sulfur programs (Table 1)

Despite lackluster performance in 2022, metamitron continues to consistently reduce fruit set, improve harvest fruit size, and increase return bloom (Tables 2 & 3)

One ACC treatment reduced harvest fruit size in Gala (Table 2), but none significantly reduced fruit set; previous research trials with ACC from other regions have shown promise when thinning conditions are favorable

ABA failed to significantly reduce fruit set or improve fruit size; previous research trials with ABA from other regions have shown promise when thinning conditions are favorable

A 2021 trial featuring GA_7 (Arrange) failed to significantly affect return bloom in an East Wenatchee Fuji block (Table 4), but multiple previous studies have demonstrated the product's potential to mitigate biennial bearing for conventional and organic apple growers

Collaborative research efforts improve our understanding of cropping physiology and help develop new models, strategies, and technologies to improve crop load management of WA apples

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. Reduced fruit set and need for 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:

Much of our early work in chemical thinning (1998-2010) focused on screening of dozens of potential bloom thinners including various formulations of salts, sulfur compounds, oils, weak acids, and bioregulators. Very few of those products proved to be sufficiently efficacious, whether alone or in combination with other products, to offer viable options for commercial use. Over time, programs featuring the use of lime sulfur, whether applied by itself at higher concentrations (6-8%) or partnered with various spray oils at lower concentrations (2-3%) emerged as relatively consistent performers effective at achieving the three primary goals for chemical thinning described above.

While we have not conducted any chemical bloom thinning studies in apple in recent years, we continue to seek out new chemistries and novel thinning programs to evaluate. We remain confident in the efficacy of lime sulfur thinning programs based on the robust set of trial results we have built through the years across locations and varieties. Table 1 summarizes the results of more than 200 chemical bloom thinning trials conducted by the WTFRC since 1999, indicating how frequently various thinning chemistries produced results in fruit set, harvest fruit size, and return bloom that were statistically superior to untreated control treatments in those field trials.

Table 1. Incidence and percentage of results significantly superior to untreated control. Apple chemical bloom thinning trials. WTFRC 1999-2022.

Treatment	Fruitlets/100 blossom clusters	Harvested fruit size	Return bloom[1]
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] (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, some retail grocers have already established policies prohibiting the sales of produce which has been treated with specific pesticides, including carbaryl.

Fortunately for apple growers, there are multiple alternatives that are now or will soon be available for postbloom chemical thinning. Our ongoing trials seek to evaluate several of those products:

Metamitron – this chemistry was initially developed as an herbicide for use in sugar beets and is currently being developed by Adama. It is already registered as a postbloom thinner of apple in several countries including Italy, France, Spain, South Africa, Chile, and New Zealand under the trade name "Brevis." Metamitron has been shown to induce temporary reductions in carbon fixation by inhibiting Photosystem II; this effect tends to be more pronounced during weather conditions associated with increased carbohydrate stress in apple trees, namely when days are hot and cloudy and nighttime temperatures are warm.

We have been fortunate to work with metamitron since 2011 and have found it to be very effective under Washington field conditions. Our early metamitron studies explored various chemical formulations, application rates and timings, use of adjuvants, and combinations with other thinning chemistries. Results from these trials have been key in helping develop best use patterns for metamitron and will help guide the development of a product label when the commercial product is finally registered, hopefully in 2024.

Much of our early work with metamitron utilized high product rates (64+ ounces/acre) and aggressive timings to establish its efficacy and to determine a red line of what would be "too much" for our conditions in WA. After several instances of over-thinning when the product was applied during hot conditions (85+ F), we concluded that more modest rates of 24-28 ounces/acre would be more appropriate for most chemical thinning scenarios, especially when the product would be tank-mixed with a non-ionic surfactant such as Regulaid, which consistently has improved thinning efficacy. Use of these lower rates in recent years has reduced the incidence of phytotoxicity as wells as the degree of thinning.

The spring of 2022 was the coldest and wettest on record throughout Central WA, creating very poor conditions for chemical thinning across the region. As such, none of our experimental treatments with metamitron (ADA 46701) or any other thinning chemistry produced significant reductions in fruit set across all our trial sites (Table 2). Interestingly, some WA apple growers reported strong "thinning" in some of their blocks, but it is unclear in most cases if that was due to the action of their chemical thinners or simply due to poor pollination and fruit set.

ABA (abscisic acid) – ABA has been sold by Valent under the trade name "ProTone" for a few years. It was initially registered to enhance color in table grapes but now also has a label for postbloom thinning of apples and pears. ABA is known to boost ethylene biosynthesis, causing increased abortion of developing fruit. It is generally considered to be a mild thinner of apples, but has been approved by OMRI, making it a welcome option for organic growers.

As with all other products, ProTone failed to provide significant thinning in our 2022 trials (Table 2). Our first-hand experience with ABA is still quite limited and we look forward the opportunity to use it in more favorable conditions across multiple cultivars, locations, and growing seasons.

ACC (1-aminocyclopropanecarboxylic acid) – ACC is a metabolic precursor of ethylene, which promotes fruitlet abscission in apples. Unlike ethephon which produces a sudden burst of ambient ethylene gas, ACC is taken up by the plant and subsequently metabolized, resulting in a more steady, controlled production of ethylene in the plant tissue. Research trials in the Eastern US have proven it to be an effective chemical thinner of apples, especially when applied late in the spring (15-20mm fruitlet size). Due to its efficacy at the tail end of chemical thinning season, ACC may offer some potential as a "rescue" thinner in circumstances when apple growers may feel they need additional

thinning after assessing early fruit set. ACC was available for commercial use under the trade name "Accede" for the first time in the 2022 thinning season.

We conducted a trial on Gala in 2022 trying to learn more about the affects of spraying concentrate (50 gal water/acre) vs. dilute (100 gal water/acre) of similar concentrations of ACC (VBC-30452), as well as targeting sprays to the entire tree canopy vs. only spraying the tops of trees. Unfortunately, none of those treatments proved effective compared to an untreated control (Table 2), leaving our questions regarding carrier volume and targeted spraying unanswered. Nonetheless, we hope to gain more experience with ACC in the coming seasons and help determine a role for this new product in our WA chemical thinning programs.

BA (6-benzyladenine) – BA is a type of cytokinin which can induce some fruitlet abortion and increase fruit size by promoting cell division. Previous WTFRC trials with BA have shown it to be a relatively weak thinner of apples in our conditions and typically requires tank mixing with other chemistries like NAA or carbaryl to provide adequate reductions in fruit set. Many BA products including MaxCel and Exilis have been available to industry for several years, but in 2022 we had the opportunity to screen several new formulations (FAL 547, FAL 567, FAL 570) in a Monitor Cripps Pink orchard. Once again, our treatments did not produce any significant thinning effects (Table 2), but those results were certainly affected by the cold, wet conditions that dominated our spring.

Table 2. Crop load and fruit quality effects of postbloom thinning programs. WTFRC 2022.

Treatment	**Fruitlets/100 floral clusters**	**Blanked spurs**	**Singled spurs**	**Harvest fruit weight**	**Relative box size**	**Russet free fruit**
		%	*%*	*g*		*%*
Gala / M.9 - Frenchman Hills						
400 ppm VBC-30452 + Reg 50 gpa-tree tops	109 ab	55	8	143 ab	127	61
400 ppm VBC-30452 + Reg 50 gpa-whole trees	121 ab	48	11	135 ab	135	58
400 ppm VBC-30452 + Reg 100 gpa	150 b	39	10	138 ab	132	59
535 ppm VBC-30452 + Reg 75 gpa	106 ab	51	10	129 a	141	64
800 ppm VBC-30452 + Reg 50 gpa-tree tops	133 ab	47	5	133 ab	137	59
800 ppm VBC-30452 + Reg 50 gpa-whole trees	103 ab	59	7	134 ab	136	59
48 fl oz Carbaryl 4L + 10 ppm PoMaxa	92 a	58	10	141 ab	129	64
Control	139 ab	44	8	147 b	124	61
Significance (p value)	*0.024*	*0.081*	*0.181*	*0.012*		*0.978*
Cripps Pink / M.26 - Monitor						
200 ppm Exilis 9.5SC + 12 fl oz Reg	93	62	7 ac	188	97	90 ab
200 ppm FAL 547 + 12 fl oz Reg	85	64	5 ab	183	99	98 b
200 ppm FAL 567	88	59	10 c	183	99	88 ab
200 ppm FAL 570	84	63	8 bc	184	99	89 ab

24 oz ADA 46701 + 12 fl oz Reg	85	63	8 bc	190	96	98 b
28 oz ADA 46701 + 12 fl oz Reg	86	64	5 ab	178	102	99 b
32 oz ADA 46701 + 12 fl oz Reg	93	61	6 ac	188	97	93 ab
28 oz ADA + 32 fl oz MaxCel	81	63	9 bc	180	101	93 ab
48 fl oz Carbaryl 4L + 10 ppm PoMaxa	81	65	8 bc	191	95	95 ab
Control	92	67	2 a	184	99	81 a
Significance (p value)	*0.498*	*0.291*	*0.001*	*0.465*		*0.005*
Fuji / Mounded scion rooted – East Wenatchee						
300 ppm Accede	72 ab	61	17	214	85	59 ab
500 ppm ProTone + 12 oz Reg	82 b	57	16	213	85	70 b
40 oz ADA 46701 + 12 oz Reg	57 a	66	17	214	85	60 ab
44 oz ADA 46701 + 12 oz Reg	66 ab	62	18	223	81	56 ab
48 oz ADA 46701 + 12 oz Reg	65 ab	63	17	220	83	58 ab
44 oz ADA + 32 fl oz MaxCel	59 a	65	18	226	80	44 a
48 fl oz Carbaryl 4L + 10 ppm PoMaxa	69 ab	61	16	221	82	60 ab
Control	78 ab	59	15	213	85	48 ab
Significance (p value)	*0.003*	*0.465*	*0.997*	*0.952*		*0.076*

Given the variability in results from one chemical thinning trial to the next, it is important to look at the "big picture" of research data. Similar to an earlier table which demonstrated chemical bloom thinning results, Table 3 summarizes the results of every chemical postbloom thinning trial conducted by the WTFRC over the last 20 years. These data confirm that apple growers can use thinning programs based on BA and NAA (naphthaleneacetic acid) and reasonably expect results comparable to those produced with thinning programs based on carbaryl. Further, Table 3 reveals the increasingly impressive performance of metamitron, suggesting that when that chemistry is finally registered for commercial use, it may offer a more consistently efficacious option for postbloom thinning than any other program that is currently available.

Table 3. Incidence and percentage of results significantly superior to untreated control. Apple chemical postbloom thinning trials. WTFRC 2002-2022.

Treatment	Fruitlets/100 blossom clusters	Harvested fruit size	Return bloom[1,2]
BA	7 / 31 (23%)	0 / 32 (0%)	0 / 30 (0%)
Carb + BA	33 / 91 (36%)	10 / 89 (11%)	13 / 86 (15%)
Carb + NAA	30 / 86 (35%)	23 / 86 (27%)	18 / 81 (22%)
BA + NAA	20 / 42 (48%)	9 / 41 (22%)	9 / 38 (24%)
Metamitron	20 / 34 (59%)	16 / 33 (48%)	10 / 30 (33%)

[1]Does not include data from 2022 trials.
[2] (no. blossom clusters year 2/sample area) / (no. blossom clusters year 1/sample area)

GIBBERELLIC ACID FOR BLOOM INHIBITION:

Our interest in using gibberellins to help promote annual cropping in apple grew out of several years of unsuccessful trials trying to promote return bloom with flowering promotors like auxins (i.e., NAA) and ethylene (i.e., ethephon). Despite enthusiastic testimonials from several prominent industry figures, we were simply unable to demonstrate any increase in flowering from summer applications of NAA or ethephon. We decided to instead, explore a strategy of attacking biennial bearing from the opposite direction by applying a flowering inhibitor like gibberellic acid (GA) in the "off" year of a biennial cycle in hopes of reducing the return bloom in the "on" year and ultimately producing more flowers in the subsequent "off" year approximately 23 months after the GA application.

This strategy has proven much more successful, and over 15+ years of testing, we have demonstrated the efficacy of several GA products at reducing return bloom and ultimately mitigating the amplitude of year-to-year swings in apple flowering. Most of our early work focused on GA_3 products like Falgro and ProGibb which are primarily used to delay harvest and promote fruit firmness in cherry. While these programs were effective and relatively inexpensive, the registrants of these products were reluctant to pursue expanded labels for chemistries whose patents had already expired. More recently, Fine Americas developed a new formulation of GA_7 that has proven to be effective at lower concentrations than GA_3 products; that product is now sold as "Arrange" and is approved for use by OMRI, providing a valuable tool to organic growers who have limited chemical options for managing crop load.

As with GA_3 products, our work has shown Arrange to be most effective around 10mm fruitlet size timing. Generally speaking, most bioregulator spray programs benefit from multiple applications of lower doses but in prior trials, Arrange has been reasonably effective in a single dose, especially when partnered with an effective adjuvant. While we saw no significant reductions in return bloom from our 2021 trial on a commercial East Wenatchee Fuji block (Table 4), the performance of Arrange did seem to improve with the addition of experimental adjuvants (EXP 1-21 and EXP 2-21).

Based on our work with Arrange and other GA formulations, we feel that the best use pattern for Arrange would be to make 2-4 weekly applications of reduced rates of the product starting around petal fall in a block with uniformly lightly cropped (but not blank) apple trees. Obviously, application of a GA product to the occasional heavily cropped tree would only further inhibit return bloom and increase the severity of its alternation. As such, growers with blocks that are mixed with heavily and lightly bloomed trees should consider the option of spraying individual light trees with a handgun to bring the entire block into more synchronous and consistent cropping.

Table 4. Effects on tree vigor, fruit size, and return bloom of GA applications. WTFRC 2021.

Treatment	**2021 harvest fruit weight**	**2021 relative box size**	**2021 shoot growth**	**2022 return bloom**
	g		*cm*	*%*
Gale Fuji / Mounded scion rooted - East Wenatchee				
Arrange 32oz + Reg 12oz PF, PF+7, PF+14, PF+21	246 ns	74	24.7 ns	114 ab
Arrange 128oz + Reg 12oz Petal fall	227	80	26.4	184 b
Arrange 128oz + EXP 1-21 16oz Petal fall	238	76	27.6	146 ab
Arrange 128oz + EXP 2-21 16oz Petal fall	239	76	27.8	96 a
Arrange 128oz + Reg 12oz PF + 7 days	238	76	24.7	138 ab

Arrange 128oz + EXP 1-21 16oz PF + 7 days	249	73	25.4	100 a
Arrange 128 oz + EXP 2-21 16oz PF +7 days	238	76	24.1	92 a
Control	239	76	24.9	120 ab
Significance (p value)				*0.01*

COLLABORATIVE CROP LOAD MANAGEMENT RESEARCH:

"Precision Crop Load Management for Apples" (USDA-NIFA Specialty Crop Research Initiative (SCRI) - PD: Terence Robinson, Cornell) – field work for project initiated in 2021 and includes trials 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; 2022 WTFRC efforts focused on support for Musacchi trial work in the field and lab investigating effects of pruning severity and floral density on cropping in Gala and WA38, as well as facilitating evaluation of mobile phone-based technology to count and measure fruit on the tree throughout the growing season (Farm Vision)

"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; see Serra final report for more detail

Proposed to WTFRC Apple Horticulture Committee: "Real-time fruit growth measurement and tracking" (PI: Karkee) – provide historical fruit growth data for 6 apple cultivars across multiple seasons in WA; will also advise project team regarding development of new fruit growth models

Evaluation of smart sprayer technology for chemical thinning – work with representatives from BB Leap (Netherlands) to test performance of commercial sprayers modified with vision system and nozzle control technology designed to target spray application of chemical bloom thinners to densely flowered trees while reducing spray to lightly bloom trees; project planning is still in progress

Project/Proposal Title: Measuring the impact of leaf removal on spur and tree health

Report Type: Continuing Project Report

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Cooperators: Thiago Campbell, Orlando Howe, McDougall and Sons, Gebbers Farms,

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 60,344
Total Project Request for Year 2 Funding: $ 66,377
Total Project Request for Year 3 Funding: $ 52,580
Other related/associated funding sources: None

Budget 1
Primary PI: Lee Kalcsits
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Station Manager/Supervisor: Chad Kruger
Station manager/supervisor email address: cekruger@wsu.edu

Item	2022	2023	2024
Salaries[1]	$40,777	$43,826	$31,460
Benefits[2]	$6,637	$7,393	$10,895
Wages[3]	$5,187	$5,394	$0
Benefits[4]	$518	$539	$0
Equipment	$0	$0	$0
Supplies[5]	$3,000	$5,000	$5,500
Travel[6]	$4,225	$4,225	$4,225
Miscellaneous[7]	$0	$0	$500
Plot Fees	$0	$0	$0
Total	$60,344	$66,377	$52,580

Footnotes:

[1] Salary is requested for a 25% post-doc in years 1 and 2 and then 50% in year 3 as well as a graduate assistant in year 1 and 2 to complete the applied physiology experiments.

[2] Benefits are calculated at 34.6% for the post-doc and 12.6% for the graduate assistant.

[3] Wages are for covering summer salary for the graduate assistant

[4] Benefits are calculated at 10% for summer graduate students

[5] Supplies are for field and lab consumables to conduct applied experiments for objective 1 and 2 and then Extension material for objective 3.

[6] Travel funds are requested for frequent travel to the Sunrise research orchard for PIs and personnel and to commercial orchards to conduct deleafing trials.

[7] Funding is requested for a small personal service contract for a videographer to capture some of the applied experiments being conducted for this project.

Objectives

1. Quantify improvements in leaf color and changes to sunburn incidence from leaf removal for an early and late-season bicolor apple cultivar.

2. Determine whether differences in leaf removal severity and timing before harvest impacts energy and nutrient storage and subsequent spur health the following season or an early and late-season bicolor apple cultivar.

3. Develop practical operating guidelines and economic cost-benefit thresholds for leaf removal based on commercial trials in WA.

Significant Findings

- In 2022, color development was poor for earlier cultivars
- Leaf removal significantly enhanced color development but also increased sunburn damage for Honeycrisp. Benefits were observed as low as 25% leaf removal. Unsurprisingly, above 75% leaf removal increased sunburn damage in unprotected fruit.
- Leaf removal had limited benefit for a high coloring cultivar like WA 38, but also had limited sunburn risk.
- Leaf removal timing had little impact on red color development in 2022.
- Leaf senescence was abnormal in 2022 with leaves staying on the tree much later than normal and not abscising as expected. We are unsure whether this will have an impact on results.
- Carbohydrate and nutrient analysis on buds has not been completed this winter and will be presented in the next report.

Methods

1. Leaf removal timing

An experiment was started in 2022 to answer when the optimum timing is for defoliation to maximize fruit red color development and decrease risks of sunburn of previously shaded fruit. Treatments included early defoliation (14 days before harvest) and defoliation closer to harvest (7 days before harvest). Weather conditions during this period are presented in Figure 1 below for both experiments with Honeycrisp and WA 38. 50% of the leaves will be removed for both defoliation treatments. We will also have an undefoliated control to compare fruit quality with no interventions. Five trees will be selected for each treatment selecting for uniformity of fruit distribution in the canopy and vigor for both Honeycrisp and Fuji. This experiment will be continued in 2023 by Orlando Howe (MS student). Whole tree fruit samples were single picked at commercial harvest timing to assess fruit color coverage. 48 fruit per tree were used for each tree to capture a full assessment of fruit quality. Fruit was run on a commercial sorting line at WSU TFREC (AWETA) to measure fruit weight and diameter, red color coverage, intensity, and background color. Sunburn incidence was also evaluated in harvest fruit using the Schraeder and McFerson (2003) sunburn scale.

1. Leaf removal severity - part I

This experiment was also conducted by Orlando Howe (MS Student). There are five treatments with five single-tree replications for each treatment. The five treatments were: 0% removal, 25% removal, 50% removal, 75% removal or 100% removal of foliage. Both Honeycrisp and WA 38 were used as the two cultivars for these experiments. We will add Fuji in 2023 as a third cultivar. These

experiments were conducted in single-axis tall spindle plantings at a density of 3’ x 12’ that are entering their sixth leaf. Defoliation was conducted 14 days before harvest for all severity treatments.

For each treatment, leaf number was counted per tree (5 trees per treatment). Defoliation was evenly applied to the entire tree. Whole tree fruit samples was taken from all five trees for each treatment. Fruit was hand-graded for sunburn and then run on a commercial sorting line to measure fruit weight and diameter, red color coverage, intensity, background color. Then, we will sample spur and non-spur reproductive buds on January 1, and March 1 to analyze nutrient and non-structural carbohydrate concentrations. Nutrient concentrations will be analyzed for all macro and micronutrients at a commercial analysis lab. To measure sugar concentrations (adapted from Chow & Landhausser, 2004), 10 mg of previously freeze-dried and ground tissue will be weighed and then extracted with 80% hot ethanol followed by colorimetric analysis with phenolsulfuric acid. The resulting bulk sugar extract will be read at 490 nm on a microplate reader (Epoch Microplate Spectrophotometer; Bio-Tek Instruments, Winooski, VT, USA) or a spectrophotometer (Thermo Fisher Scientific GENESYS 10S UV-Vis, Waltham, MA, USA). Sugar concentrations (expressed as mg sugar per g dry wood) will be calculated from a 1:1 :1 glucosefructosegalactose (Sigma Chemicals, St Louis, MO, USA) standard curve. To determine starch concentrations, the remaining tissue will be solubilized in NaOH and then digested with an a-amylase/amyloglucosidase digestive enzyme solution. Glucose hydrolysate will be determined using a PGO-colour reagent solution (Sigma Chemicals) and read at 525 nm. Starch concentrations (expressed as mg starch per g dry material) will be calculated based on a glucose (Sigma Chemicals) standard curve.
The same trees will also be monitored for return bloom using two approaches: 1. Dissecting five spur buds per tree in late March, and 2. Counting flower clusters per tree at king-bloom stage.

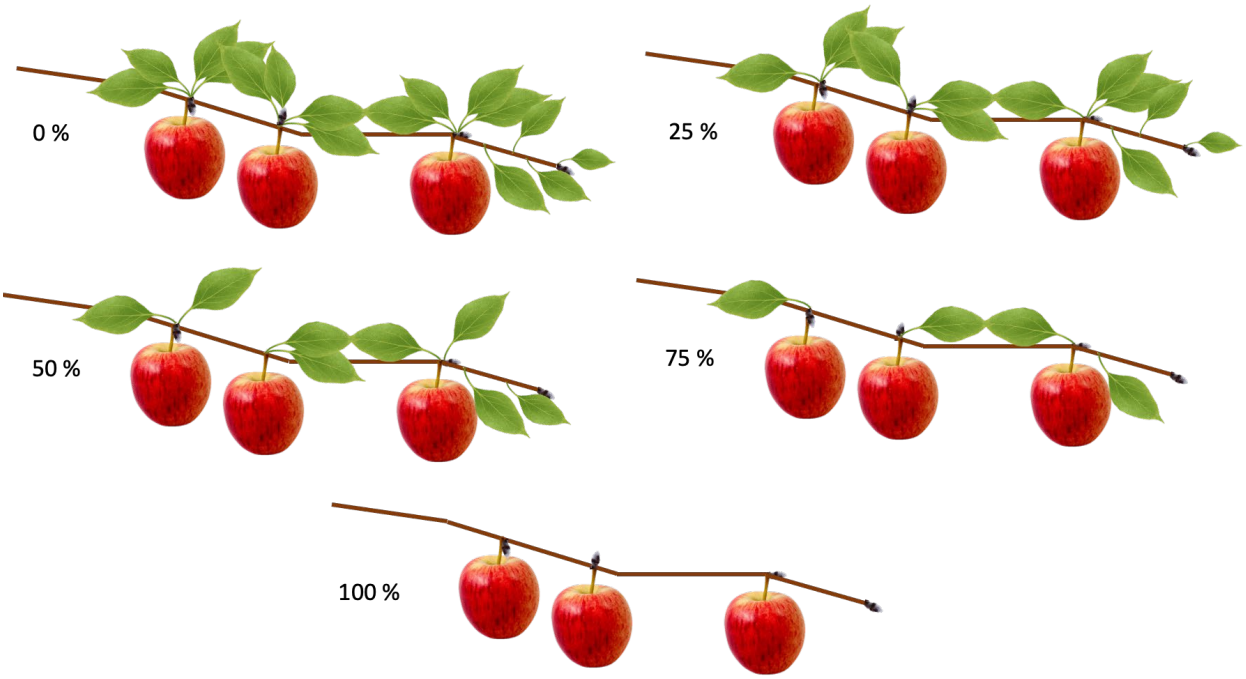

Figure 3. Leaf removal severity treatments of 0% leaf removal, 25% leaf removal, 50% leaf removal, 75 % leaf removal and 100% leaf removal.

Results and Discussion

The focus of this section will be on fruit quality from the different treatments since the nutrient, carbohydrate analysis, and return bloom assessments will be completed at the beginning of 2023. Overall, we completed sampling from two commercial orchards ('Gala' and 'Honeycrisp') and will complete nutrient and carbohydrate sampling and return bloom assessments on another two commercial orchards in 2023. Although we can assess gain in red color as well as changes in sunburn for individual orchards, we need to have more fruit samples from commercial orchards in 2023 before we can start the economic analysis.

Although 50% deleafing increased red color, it didn't matter whether it was done 7 or 14 days before harvest for WA 38 (Figure 1). However, for Honeycrisp, fruit color coverage was higher when deleafing was done 14 days before harvest compared to 7 days before harvest (Figure 2). Sunburn incidence was less consistent. 7 days before harvest, temperatures exceeded 100 °F and there was overall little color development for Honeycrisp for any treatment. Treatments did not impact sunburn development for WA 38.

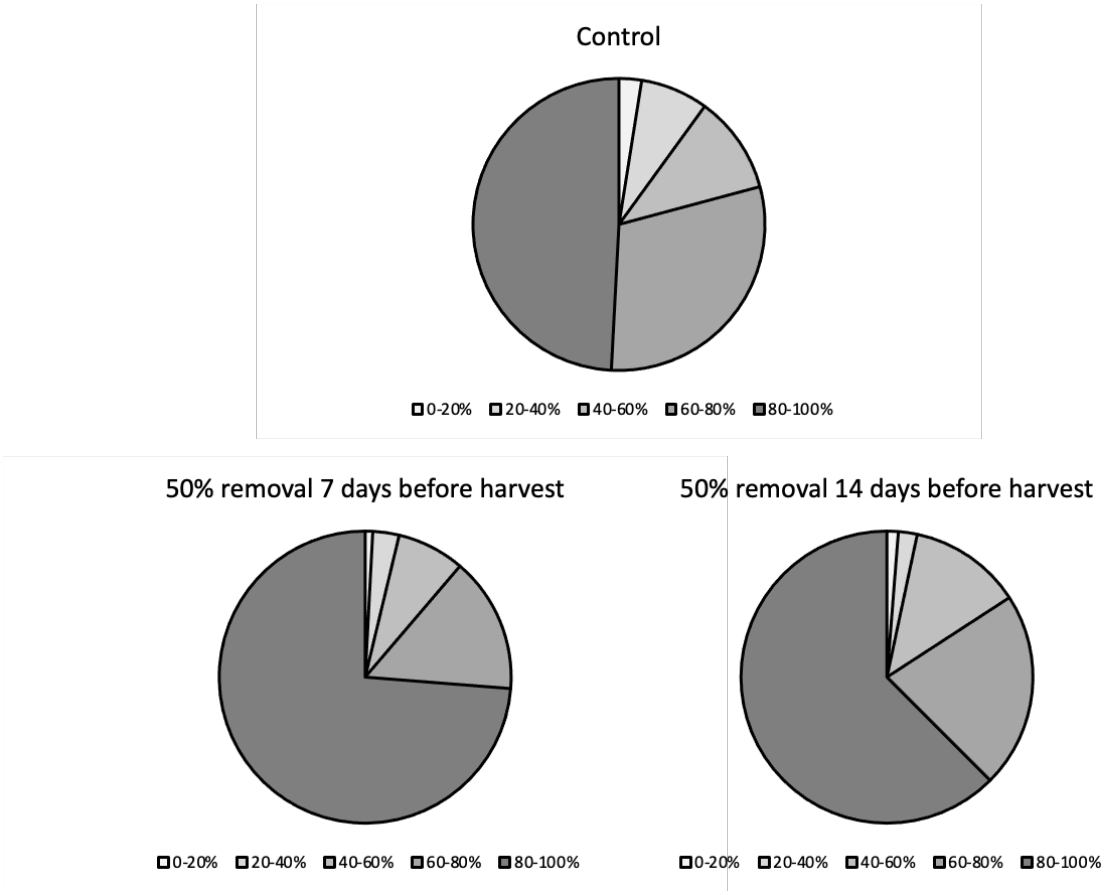

Figure 1. The proportion of fruit (%) with 0-20%, 20-40%, 40-60%, 60-80%, or 80-100% red color for 'WA 38' trees where 50% of leaves were removed either 7 or 14 days before harvest compared to an untreated control.

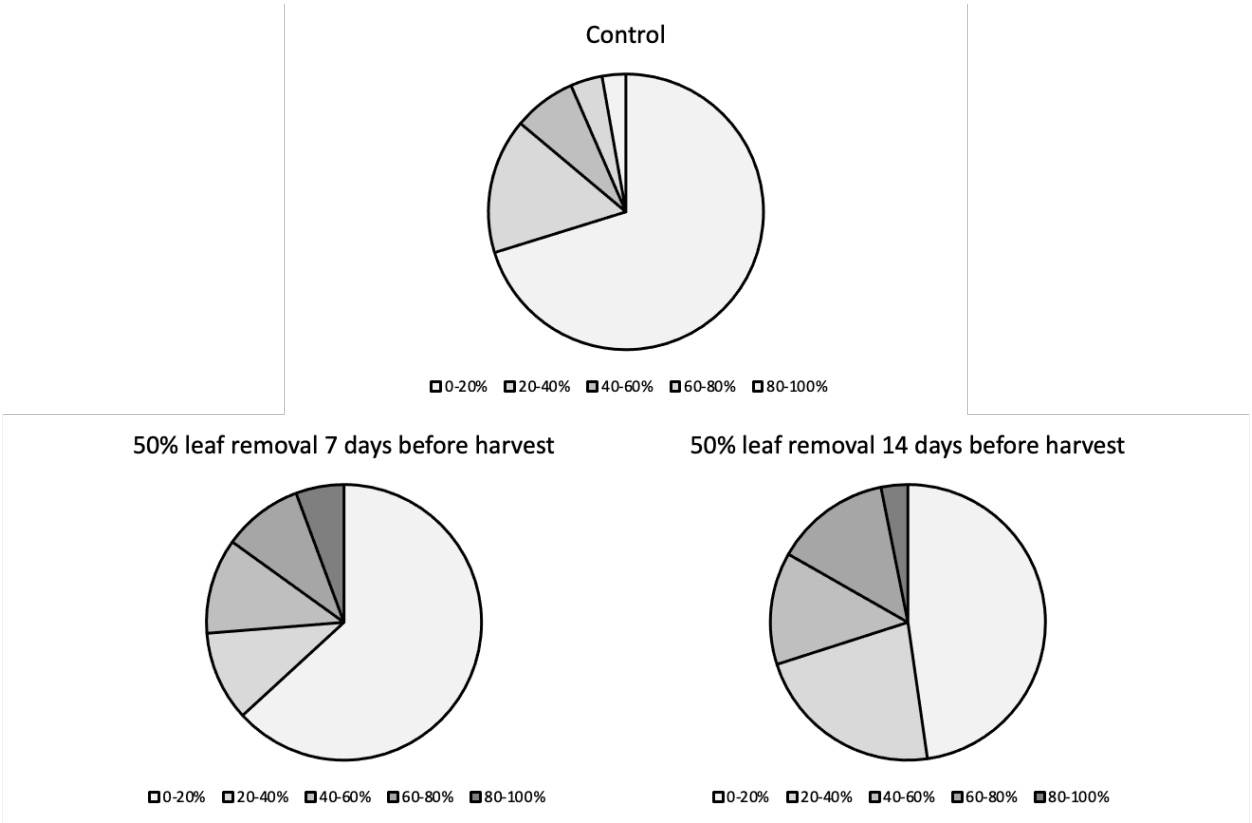

Figure 2. The proportion of fruit (%) with 0-20%, 20-40%, 40-60%, 60-80%, or 80-100% red color for 'Honeycrisp' trees where 50% of leaves were removed either 7 or 14 days before harvest compared to an untreated control.

Overall, the fruit quality results from the deleafing severity were unsurprising and consistent with the benefits observed in previous commercial deleafing trials and experiments. The impacts of deleafing on the incidence of sunburn and red color coverage were greater for the earlier cultivar, 'Honeycrisp', than the later cultivar, 'WA 38', which naturally developed color easier than 'Honeycrisp'. Still though, there were more fruit meeting minimum color standards (>50% red color coverage) for when more than 50% of leaves were removed for WA 38 compared to the control (Figure 6).

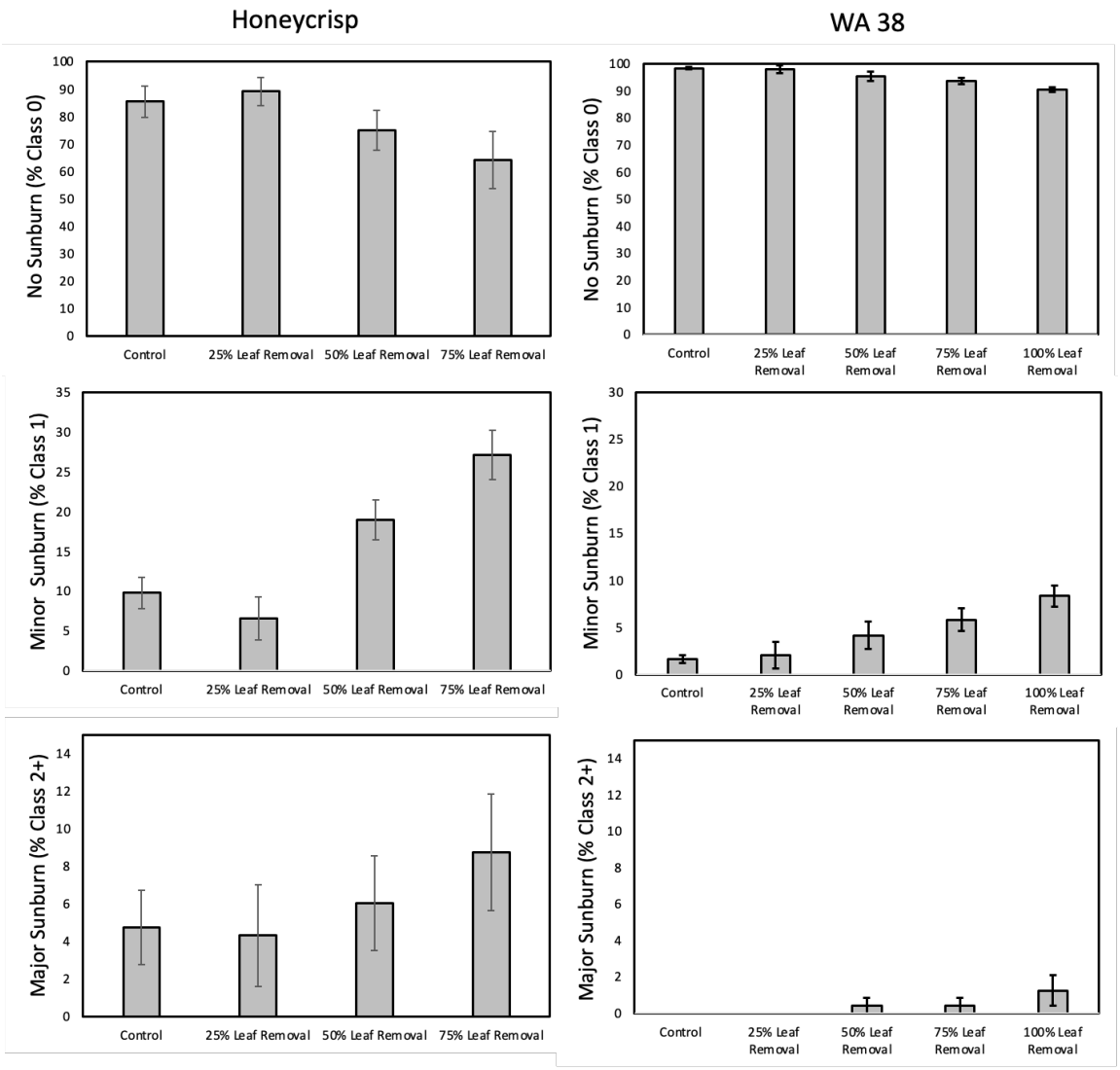

Figure 3. Sunburn incidence for 'Honeycrisp' (left) and 'WA 38' (right) fruit from trees with 25%, 50%, 75%, or 100%* leaf removal 14 days before harvest compared to an untreated control. Error bars denote standard error (N=5). *Fruit assessment of sunburn will be completed for this treatment after storage along with evaluation of other treatments.

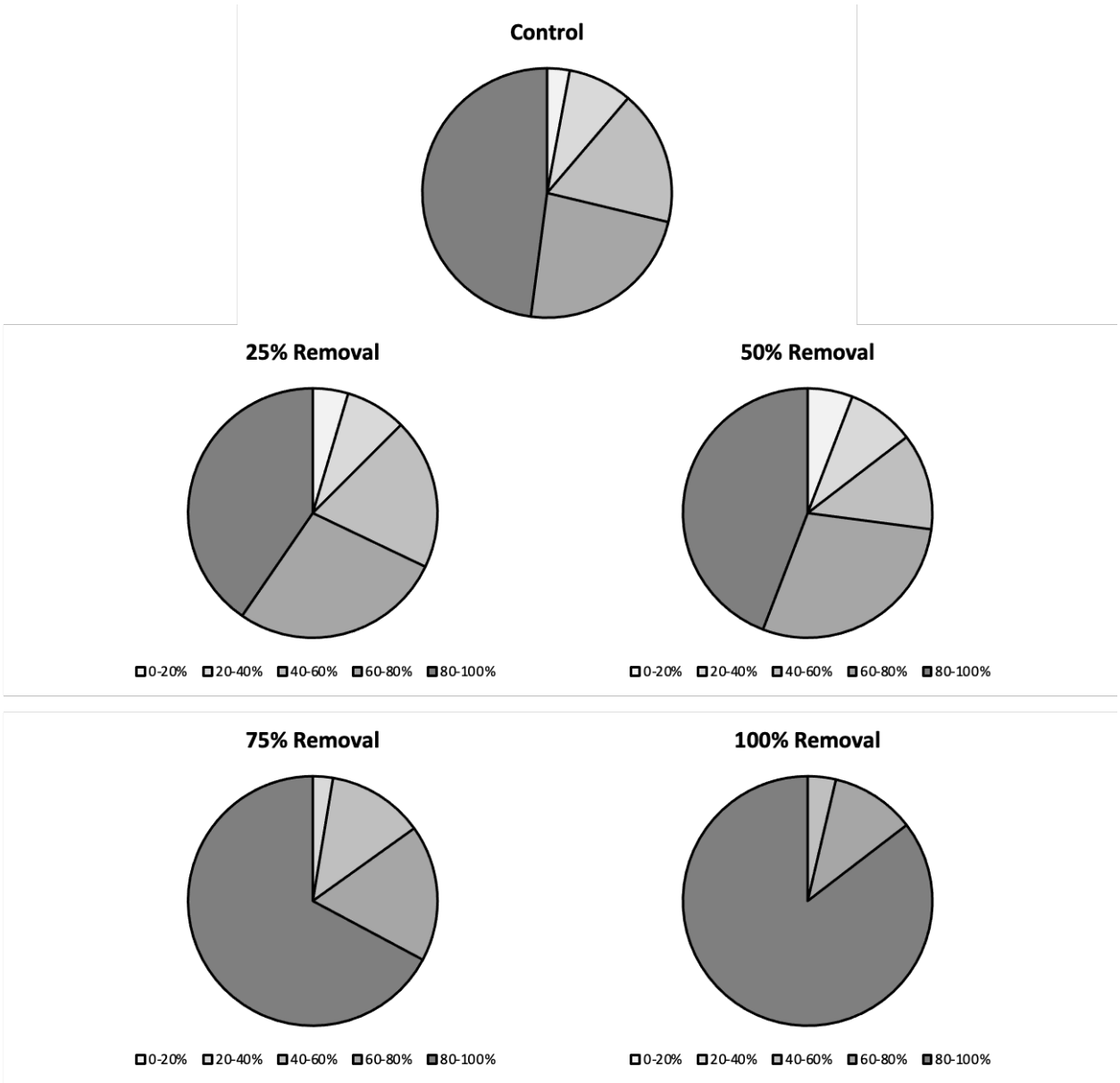

Figure 4. Percentage of fruit with 0-20%, 20-40%, 40-60%, 60-80%, 0r 80-100% red color coverage for 'WA 38' fruit treated with five defoliation severities (control, 25% removal, 50% removal, 75% removal, or 100% leaf removal).

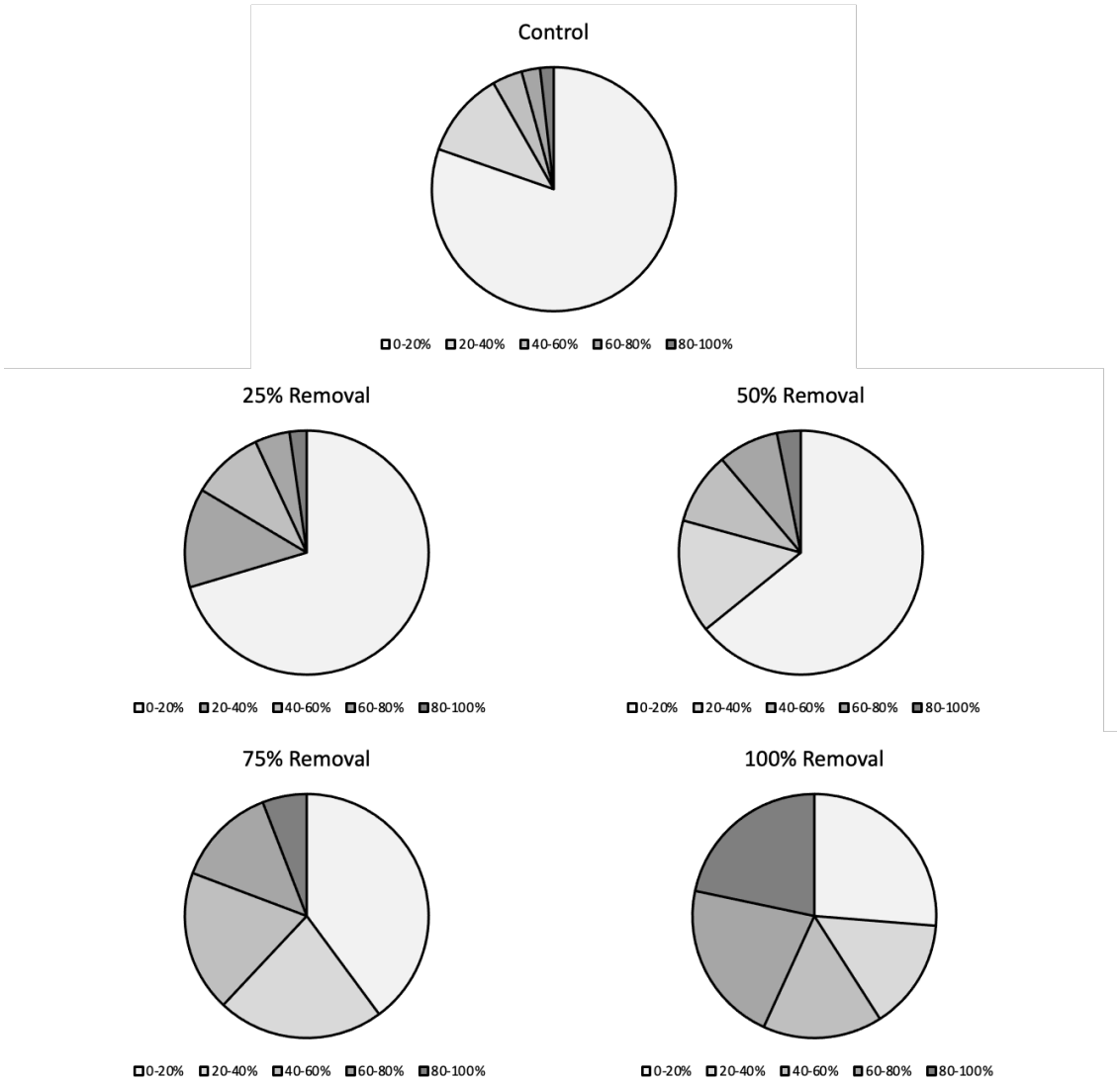

Figure 5. Percentage of fruit with 0-20%, 20-40%, 40-60%, 60-80%, 0r 80-100% red color coverage for 'Honeycrisp' fruit treated with five defoliation severities (control, 25% removal, 50% removal, 75% removal, or 100% leaf removal).

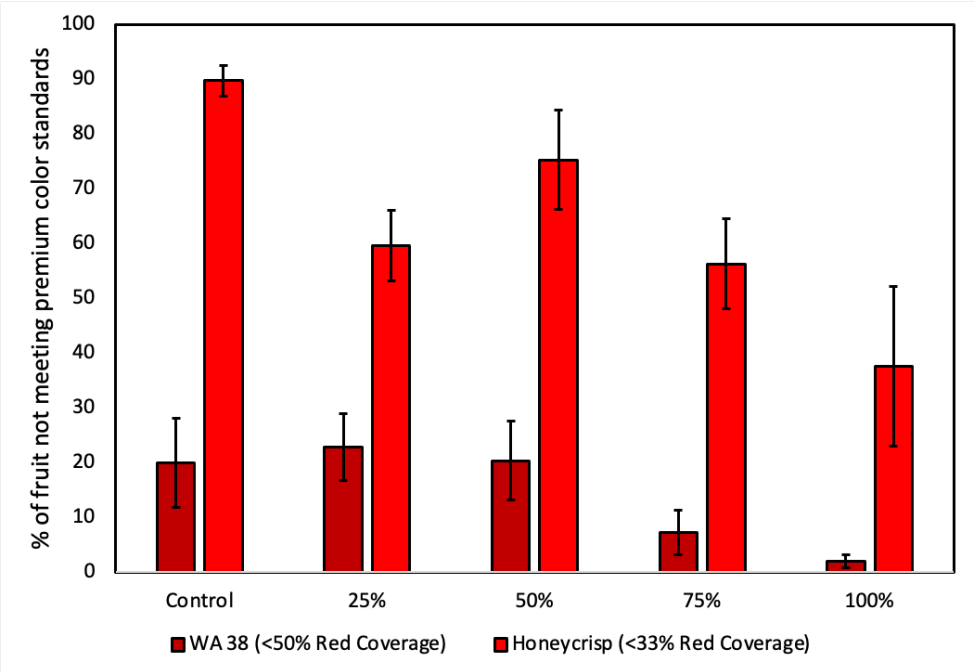

Figure 6. The proportion of fruit not meeting minimum premium color standards for five defoliation treatments (control, 25%, 50%, 75%, or 100% leaf removal). Error bars denote standard error (N=5)

Plans for 2023

Table 1. Project timeline for the completion of objectives 1-3

	2022				2023				2024			
	Spring	Summer	Fall	Winter	Spring	Summer	Fall	Winter	Spring	Summer	Fall	Winter
Objective 1												
Commercial defoliation trials on four cultivars and fruit quality evaluation		■	■			■	■			■	■	
Evaluation of changes in nutrient and carbohydrate reserves				■				■				■
Assessment of changes to return bloom and shoot vigor					■	■			■	■		
Objective 2												
Defoliation timing experiment												
Treatments			■				■					
Fruit quality evaluation			■	■			■	■			■	■
Defoliation severity experiment part I												
Treatments			■				■					
Evaluation of changes in nutrient and carbohydrate reserves				■				■				
Assessment of changes to return bloom and shoot vigor					■	■			■	■		
Defoliation severity experiment part II												
Treatments and monitoring of leaf health and longevity			■				■					
Evaluation of changes in nutrient and carbohydrate reserves				■				■				
Assessment of changes to return bloom and shoot vigor					■	■			■	■		
Objective 3												
Field Days							■					
Fruit Matter Publications								■	■	■	■	■
Share updates at winter meetings				■				■				■
Extension publications								■	■	■	■	■
Economic Analysis				■				■			■	■

Project Title: Phase 3 Evaluations of Apple Breeding Program Selections

Report Type: Final Project Report

Primary PI: **Manoella Mendoza**
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Cooperators: Agrofresh Inc., Legacy Fruit. Growers: Stemilt Inc. and Allan Brothers. Apple Breeding Program Advisory Committee: Aylin Moreno (Washington Fruit and Produce Co.), Brent Milne (McDougall), Paul Cathcart (Columbia Reach), Dale Goldy (Gold Crown), Dave Gleason (Kershaw), Dena Ybarra (WTFRC commissioner), Jeff Cleveringa (Starr Ranch), Jeff LaPorte (Chelan Fruit), Jim Mattheis (USDA-ARS), Lauren Gonzalez (GS Long), Mike Robinson (Double Diamond), Sarah Franco (Allan Bros.), Suzanne Bishop (Allan Bros.), Tim Welsh (Columbia Fruit), Rob Blakey (Stemilt), Hannah Walters (Stemilt), Anne Morrell (Columbia Fruit), Erick Smith (Taggares Fruit Company), Craig Anderson (Gilbert Orchards), Matt Miles (WTFRC commissioner), Technical consultants: Stefano Musacchi, Carolina Torres, Bernardita Sallato, Lee Kalcsits

Project Duration: 3 Year

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

Other related/associated funding sources:
in kind contributions: $30,000.
Notes: Stemilt and Allan Brothers provide farm crew assistance for pruning, thinning, and field maintenance, Agrofresh donates Smartfresh, and Stemilt assists with SmartFresh and donates and apply postharvest fungicides.

Primary PI: Manoella Mendoza
Organization: WA Tree Fruit Research Commission
Contract Administrator: Paige Beuhler
Telephone: (509)665-8271
Contract administrator email address: paigeb@treefruitresearch.com

Item	2020	2021	2022
Salaries			
Benefits			
Wages	$24,938.00	$25,401.00	$25,831.00
Benefits	$11,375.00	$11,396.00	$11,407.00
RCA Room Rental	$13,500.00	$13,905.00	$14,321.00
Shipping			
Supplies	$500.00	$500.00	$500.00
Travel	$500.00	$500.00	$500.00
Plot Fees			
Miscellaneous			
Total	$50,813.00	$51,702.00	$52,559.00

Footnotes:
Wages/Benefits: Calculated based on expected staff wage adjustments.
RCA room rental: 1.5 rooms @ $6500/room plus $2500/room warehouse fees, adjusted yearly
Supplies: consumables for fruit quality lab (KOH, distilled water, iodine solution, etc.)
Travel: In-state travel

New and improved apple varieties are essential to enhance a successful Washington apple industry. The WSU apple breeding program (WABP) aims to produce a portfolio of new, improved, unique varieties, specially selected for the environment of central Washington and available to Washington's growers. Phase 3 (P3), the pre-commercialization phase of the WABP, includes larger plot plantings of the elite selections to determine potential commercial suitability. Having the WTFRC manage P3 provides an independent and industry-oriented evaluation that, with the input of industry representatives in the apple breeding program advisory committee (BPAC), ensures that the data collected, and information provided align with stakeholders' interests.

Objectives

1. Evaluate and determine the 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).

1. Selections P and L have a good shelf-life potential granted by the low incidence of disorders and diseases in the field and during storage.
2. Selection L was preferred by consumers in two pairwise tastings in spring 2022 for overall liking, texture, and flavor when compared with Honeycrisp or Cripps Pink. Preference for texture was significantly higher, outperforming both selections.
3. Selections Q, R, and S were grafted in Quincy (2020), and Sagemoor (2021), and grew to the top wire in their first year. Quality analysis of fruit from Quincy is ongoing.

Methods

General Procedures

<u>Bud and Bloom observation</u> Field observations started as the trees began to bloom, occurring at least twice a week, considering the weather pattern and its influence on blooming. The full bloom date was determined for each P3 selection and the standard varieties near the P3 plots. Starting at this stage, every field visit includes general observations on disease incidence, tree growth habits, and health. Standard management practices (rodent activity monitoring, powdery mildew sprays, row mowing, etc.) were conducted and discussed with field managers. Pest and disease incidence and monitoring were documented during the entire season.

<u>Fruitlet development and pre-harvest:</u> Field activities for this stage start after June drop. Orchard visits occurred at least every other week until a month prior to predicted harvest. Observations on fruit set and self-thinning were documented. The orchard crew performed hand-thinning and summer pruning when appropriate, as if the selections were being produced commercially. In this phase, no plant growth regulator

or thinning products were applied, because we are interested in observing the natural growth and cropping of each selection.

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. From 2020 to 2022, all apple selections were strip-picked. Apples were harvested using picking bags and placed in blue crates (30 lb.).

The apples with cracks, insect damage, chemical damage, splits, severe sunburn damage, bitter pit, and birds peck are classified as culls in the field. These apples were collected during harvest and weighed separately; the reason for cullage was assessed on individual fruit, and data was used to calculate the percentage of fruit loss in the field.

The storage samples were weighed in the field and separated into two storage conditions: Refrigerated air (RA, 33°F), and controlled atmosphere (CA, 34°F 1% CO_2, 2% O_2), with and without 1-MCP treatment. This fruit was drenched with a postharvest fungicide 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 48 hours of harvest using 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. DA index was recorded in 2022.

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. Quality analysis was conducted after 7 days at room temperature to determine the potential quality for consumers after shipping, handling and purchase. Box size distribution data was generated from individual fruit weights. Fruit flavor and eating quality were evaluated in the laboratory, by the Apple Breeding Program Advisory Committee (BPAC), and through informal consumer tasting.

Advanced Phase 3

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

- commercial packing line handling: glossiness and bruising will be evaluated on the same day, after 3 and 7 days in RA storage, and 3 and 7 days at room temperature.
- formal consumer taste panels: coordinated with Kate Evans (co-PI and WSU apple breeder) and performed in locations or events with diverse consumer demographics (i.e., Spokane mall, Apple Blossom Festival). The protocol utilized was generated by Carolyn Ross (Professor and Director of the Sensory Evaluation Facility, WSU Pullman).

Selection specific evaluations

Selection P (Honeycrisp × Southern Snap):

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

Selection L (Honeycrisp × Cripps Pink):

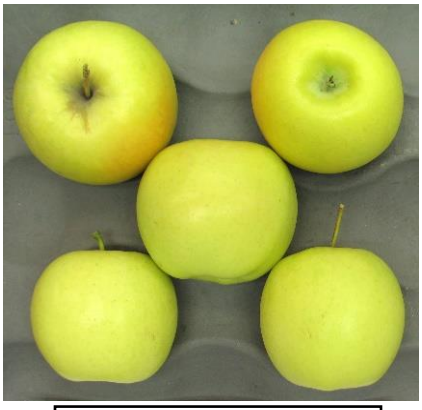
- establish optimum harvest window based on maturity parameters, field cullage, and storage disorders incidence
- evaluate packing line handling (waxing and bruising)
- assess consumer acceptance

Results and Discussion

Selection Q, R, and S

Q: Cripps Pink × Honeycrisp

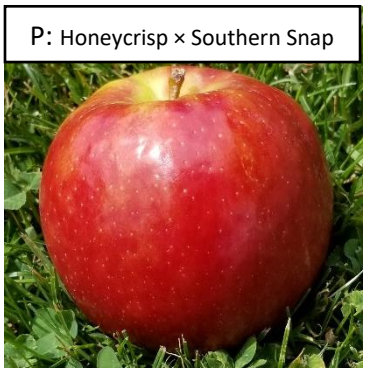
R: Cripps Pink × WSU 3



S: Honeycrisp × WA 2

These three selections were top worked in Quincy and Sagemoor in 2020 and 2021, respectively. In Quincy, the selections are performing well, and the overall mortality rate was low; 4 trees out of 128 total trees. At the Sagemoor orchard site, all grafts of Q and R have survived, but 11 trees (out of 28) of selection S have died. Most of the trees reached the top wire within one year. Both locations were defruited in the first year, and trees from Quincy were harvested for the first time in 2022. Storage evaluation is ongoing.

Selection P



Selection P is a bicolored apple that develops good red color coverage on the fruiting wall (Prosser) and the spindle system (Quincy). The apples have low sunburn incidence, and pre-harvest drop has not been observed. This selection was grafted in Quincy and Prosser in 2017 and 2018, respectively. The trees reached the top wire in the first year. Harvest occurs typically in mid to late September (Honeycrisp timing).

Its unique trait is the flavor profile. The apples are crispy and juicy and have a unique tart-sweet flavor resulting from high acidity and mid to high-soluble solids. The fruit texture is typically praised by potential consumers.

Selection specific evaluations

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

One unique characteristic of this selection is the high titratable acidity (T.A., % malic acid) values at harvest (between 0.9 to 1.2) that remain high throughout storage, especially on early picks. Later harvested fruit can be prone to greasiness. Three consecutive weekly picks were performed in Quincy in 2020 and 2021 to observe the effects of advanced maturity at harvest on titratable acidity and firmness degradation, greasiness prevalence during storage, and incidence of stem bowl splitting.

Maturity parameters:

In 2021, at harvest, the T.A. and firmness decreased by 0.1 (% m.a.) and 1.0 lb., respectively, between the first and the third pick (Table 1). SSC increased by 2.3 units (% Brix) in the same timeframe. All quality parameters remained stable during long-term RA and CA storage, 6 and 9 months, respectively. The flavor classification was similar between harvest and storage for the three picks, and no off-flavor was found. Comparable results were found in 2020, indicating that quality parameters and fruit flavor is not negatively affected by advanced maturity at harvest.

Table 1. Summary of titratable acidity (T.A.), soluble solids concentration (SSC), firmness, and flavor classification (good, bland, and off flavor) of selection P at harvest and after RA or CA storage in 2021 from Quincy. The storage results are the last timepoint for RA (1) = 6 months and CA (2) = 9 months.

	1st pick (9.21.21)			2nd pick (9.27.21)			3rd pick (10.4.21)		
Parameters	**Har**	**RA (1)**	**CA (2)**	**Har**	**RA (1)**	**CA (2)**	**Har**	**RA (1)**	**CA (2)**
T.A.(%m.a.)	1.167	0.932	1.008	1.088	0.887	0.933	1.058	0.765	0.892
SSC (%Brix)	12.8	12.8	13.9	12.5	13.5	14.2	14.5	14.3	14.9
Firmness (lb.)	18.7	18.3	17.5	17.4	17.1	18.8	17.7	17.6	18.1
Flavor Classification									
Good (%)	100	95	100	100	95	95	100	100	95
Bland (%)	0	5	0	0	5	5	0	0	5
Off (%)	0	0	0	0	0	0	0	0	0

Field cullage and storage disorder incidence:

Field cullage was assessed by the methods described under general procedures. Overall incidence was low, at 2.2% and 2.7% of total yield, in 2020 and 2021, respectively (data not shown). Bird peck was the main reason for cullage in both years, ranging from 0.6% to 1.9% (Figure 1). Bird peck occurrence increased over time in 2020, but not in 2021. Cracking, insect damage, sunburn, and limb rub were equal to or less than 0.5%.

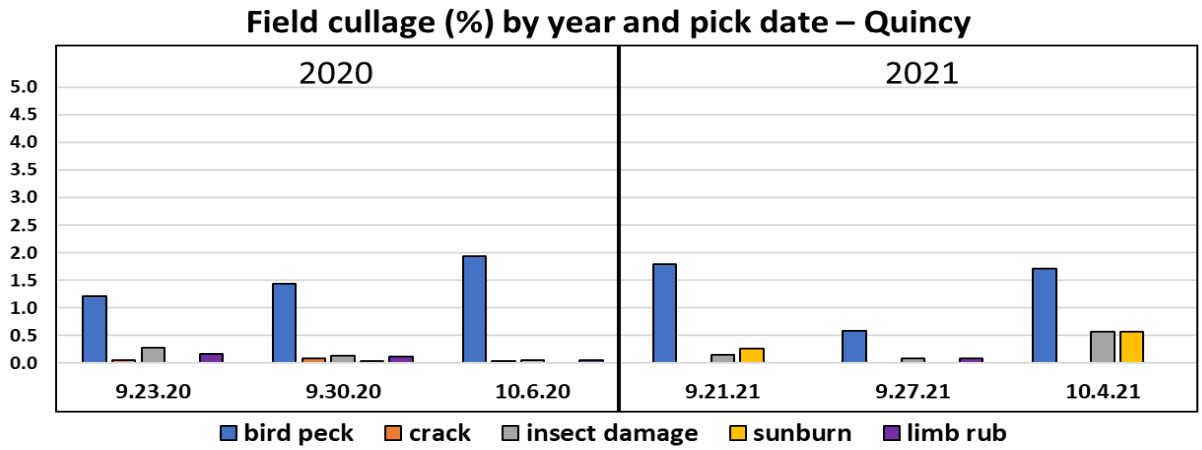


Figure 1. Percentage of field cullage caused by bird peck, cracking, insect damage, sunburn, and limb rub at harvest for selection P harvested at Quincy in 2020 and 2021 by harvest date.

Maximum stem bowl split incidence was 3% overall, combining harvest and storage. It did not increase in the latest pick of 2020 but increased by 1% in 2021 (Table 2). Greasiness was not found at harvest, but during storage, it increased slightly from the 1st to the last pick in both years, and from 2020 to 2021. The elevated incidence of greasiness in 2021 is explained by higher greasiness prevalence on fruit stored in RA without 1-MCP treatment (data not shown). Overall, greasiness is mostly slight, rather than severe. 1-MCP treatment can inhibit greasiness but is not always consistent.

Table 2. Incidence in percentage of stem bowl split (at harvest and during storage combined) and greasiness (during storage only as no greasiness was found at harvest) of selection P harvested in 2020 and 2021 by harvest sequence.

	Split (%)		Greasiness (%)	
Harvest Sequence	2020	2021	2020	2021
1st	3	1	9	20
2nd	0	1	7	21
3rd	3	2	11	24

Superficial scald, cavity, lenticel breakdown, and shrivel were not found during four years of evaluation. Watercore was found sporadically during harvest. Internal browning occurred during long-term storage on fruit from young trees (1st and 2nd leaf) at very low rates (0.2% and 0.6%). Soft scald was found

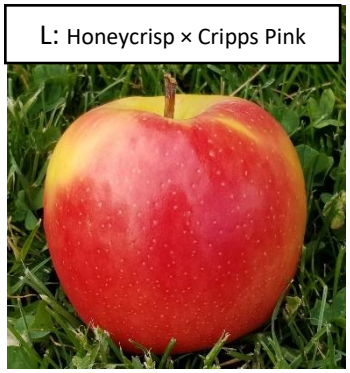
in 2020 at 0.3%. Bitter pit, stem puncture, sunburn, and russet were higher on fruit harvested from one-year-old trees. The prevalence of these disorders is less than 7%.

Based on the 2020 and 2021 results, harvesting selection P in early October did not affect fruit quality parameters, the onset of greasiness, or the incidence of other storage disorders.

- *Identify potential market*

Due to the Covid-19 pandemic, we were not able to perform industry tastings or a formal consumer taste panel. Some batches of fruit were evaluated by the BPAC and potential consumers. Generally, people that like tart apples enjoy selection P, and most tasters like its texture, regardless of flavor profile preferences. Assessing consumer preference is a goal for the 2023 season.

Selection L:



Selection L is a bicolored apple that colors well when exposed to sunlight. It is slow to brown, easy to pick, and pre-harvest drop has not been observed. Some concerns are sensitivity to sunburn and powdery mildew (on the leaves). This selection was grafted in 2015 on both Prosser and Quincy locations. It is typically harvested from late September to early October (Golden Delicious timing).

Its unique trait is high firmness retention during storage, which, combined with the low incidence of disorders and diseases in the field and during storage, grants this selection a long shelf-life potential. Because of its desirable characteristics, selection L was moved to an advanced P3 in 2019.

Selection specific evaluations

- *establish optimum harvest window based on maturity parameters, field cullage, and storage disorder incidence*

Maturity parameters:

Prior to 2019, color (1- 4), background color (0.5-6.0), and starch (Cornell, 1-8) were the parameters used to establish harvest timing. Consecutive picks were initiated when the average for color was at 3 (= 51 to 75% of red color coverage), background color at 4.0 (light yellow), and starch above 2.5 (Cornell, 1-8).

Selection L can maintain high levels of fruit firmness (above 20 lb.) throughout storage, sometimes resulting in apples that are perceived as too hard to eat. Starting in 2020, in addition to the above-mentioned parameters, firmness was monitored pre-harvest, and harvest began when firmness averaged 20 lb. (Figure 2). Although fruit was harvested with advanced starch (above Cornell 5) from 2020 to 2022, fruit firmness was stable during storage, decreasing only 1 to 2 lb. after 6 or 9 months of storage (data not shown).

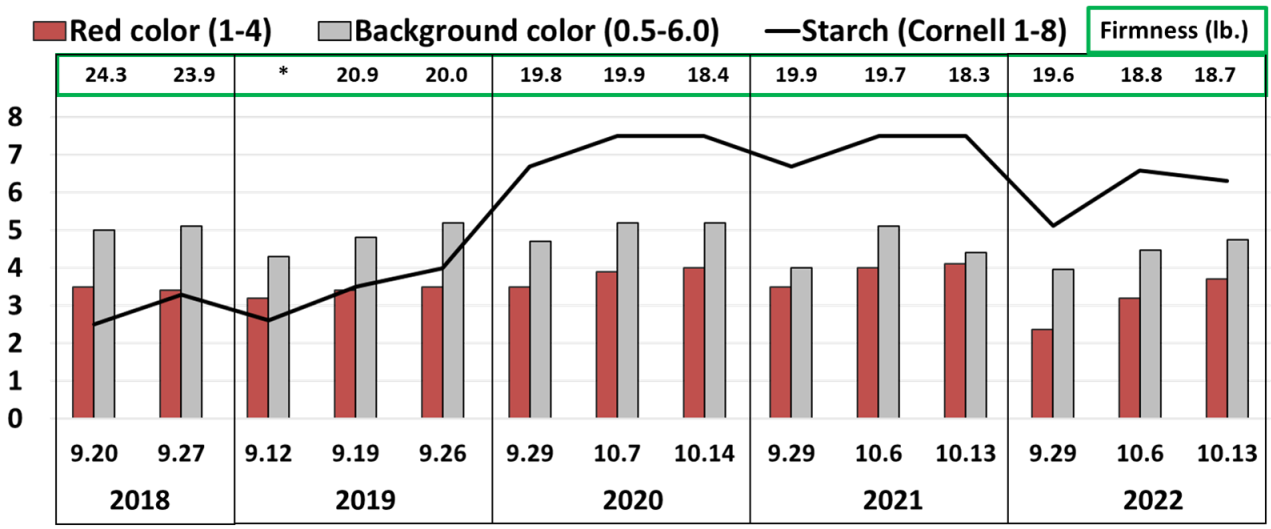


Figure 2. Color (1 = 0 to 25%, 26 to 50%, 3 = 51 to 75%, 76 to 100%) background color (0.5 to 3.5 = shades of green, 4.0 = light yellow/break, 4.5 to 5.0 = shades of yellow, 5.5 = orange, 6.0 pink/red) starch (Cornell starch scale, 1 to 8), and firmness (lb.) at harvest from 2018 to 2022 for selection L from Prosser.

Field cullage and storage disorders:

Field cullage was assessed by the methods described under general procedures. In five years of evaluation, field cullage ranged from 0.2% to 4%, and 1% to 6% in Quincy and Prosser, respectively. In Prosser, sunburn was the main reason for cullage prior to sunburn protectant applications. From 2019 forward, bird peck was the main reason for cullage on both sites (0.6% to 3.2%). The increase in the incidence of this defect is associated with the shift in harvest dates, from mid and late September to late September and mid-October.

Sunburn can be a concern because the apples need to be exposed to sunlight to develop good color coverage. However, sunburn mitigation techniques (overhead cooling in Quincy, chemical sprays in Prosser) had positive results in suppressing sunburn. Prosser has a higher incidence of sunburn (max. of 24% in 2018) due to the higher sunlight exposure provided by the tree training system. There, application of sunburn protectants was able to suppress severe sunburn damage by 1.2 to 2.4% (data not shown). Mild sunburn incidence oscillates annually, 5% to 20% in Prosser and 2% to 10% in Quincy. This type of sunburn typically colors over and might not be visible during storage.

Fungal disease and storage disorder incidence are low in this selection. No superficial scald, internal browning, or cavity was found during six years of evaluations. Soft scald has been observed mostly from Prosser on the last pick (maximum incidence of 3.2% in 2017) but does not occur every year. Russet is common but mostly located in the stem bowl (data not shown).

Lenticel breakdown was found for the first time in 2021, in only one lot (Prosser, 1st pick, CA). This fruit had lenticel markings at harvest, probably caused by heat exposure. An experiment was conducted to assess lenticel breakdown susceptibility, in which fruit was washed with organic wash or dish soap and left at room temperature (72°F) for 7 days (Figure 3). The symptoms started to appear after 3 days at room temperature. After 7 days, lenticel breakdown incidence was 9% for apples washed

with organic wash, and 56% for fruit washed with dish soap. The results indicate that even though the fruit was susceptible, the onset of symptoms can be controlled.

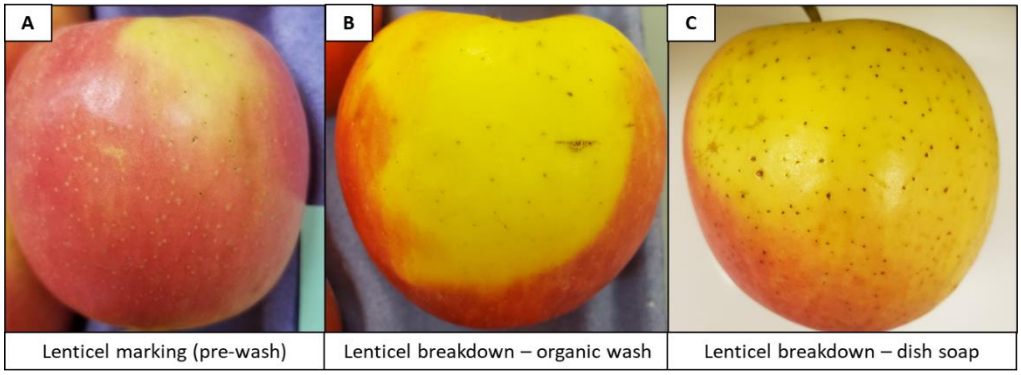


Figure 3. Selection L with lenticel marking before fruit wash (A), and lenticel breakdown symptoms after washing with organic wash (B) and dish soap (C) and left at room temperature (72°F) for 7 days.

Split incidence is low but increases as the apples mature. The overall incidence is up to 3.5%, but it can be higher when the fruit is harvested mid to late October (Figure 4). There is an annual variation of stem punctures and bruises; the overall range of both defects is from 0.5% to 5%. The apples are not stem-clipped at harvest. The incidence of these defects could be affected by the harvest methods; fruit is placed in 30lb. plastic crates at harvest and moved by hand in the field, during transport, storage, and quality analysis.

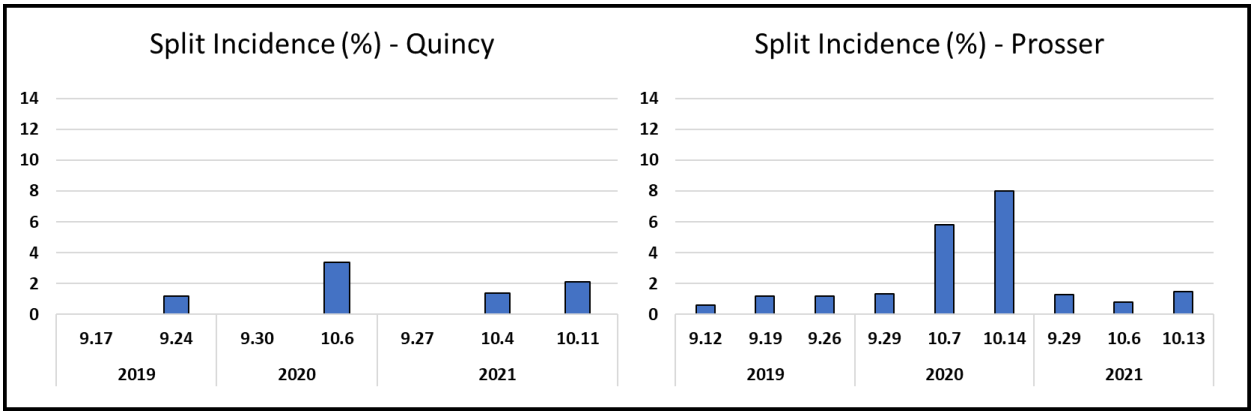


Figure 4. Percentage of split for selection L harvested at Quincy or Prosser, from 2019 to 2021.

- *evaluate packing line handling (waxing and bruising)*

The COVID-19 pandemic has delayed this activity. One packing line handling evaluation was conducted in early 2020. Data collected shows that fruit can hold wax well (high gloss), only losing some of the gloss when held at room temperature for 7 days (high to medium gloss). The apples were not bruising sensitive when run over a commercial packing line.

In addition to fruit collected for quality analysis in 2022, fruit from Quincy was harvested in bins and will be used for packing line handling evaluation, including glossiness, bruising, stem puncture, decay, storage disorders, and fruit flavor.

- *assess consumer acceptance*

Due to the COVID-19 pandemic, the consumer taste panels scheduled for the Spring of 2020 were canceled. Two formal consumer tastings were held in public events in 2022, with a total of 360 participants. Selection L was compared with two sets of Cripps Pink and one set of Honeycrisp apples (Figure 5). The standard selections were donated from local packing houses, and fruit quality reflects what would be available in the market at that time.

The fruit sampled in Yakima was Cripps Pink (premium) stored in CA and treated with 1-MCP, and selection L stored in CA. For the Wenatchee event, the samples were Cripps pink (WA extra fancy) stored in CA, Honeycrisp stored in CA and 1-MCP treated, and selection L stored in RA.

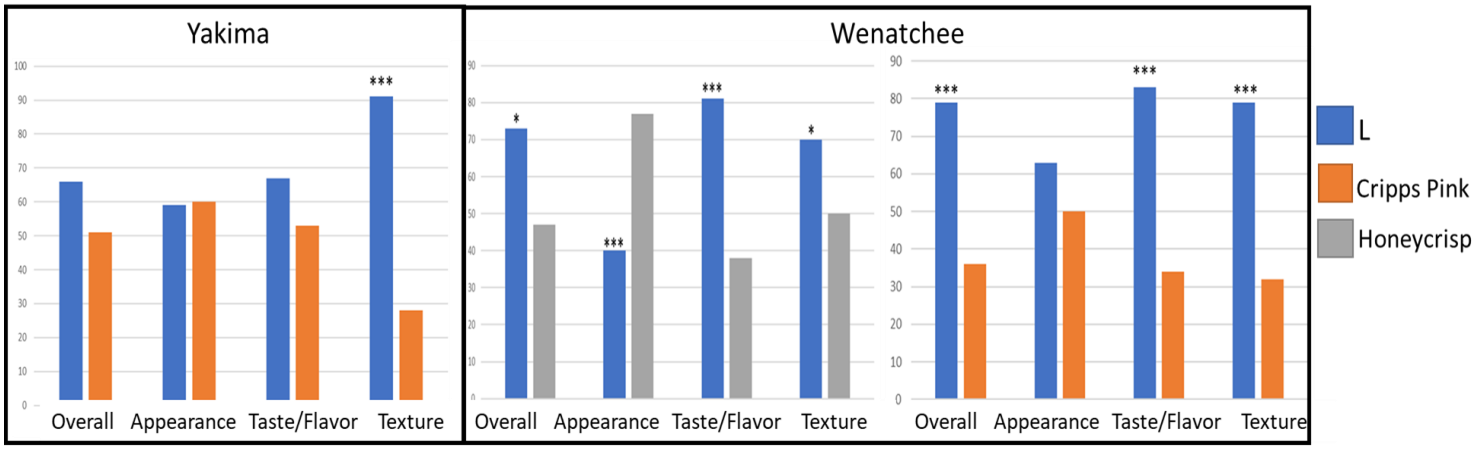


Figure 5. Results from consumer taste panels held in Yakima and Wenatchee. Selection L was compared with Cripps Pink or Honeycrisp for overall liking, appearance, taste/flavor, and texture. Comparisons with * are significantly different (* $p \leq 0.05$, ***$p < 0.01$).

In Yakima, selection L scored higher than Cripps Pink for overall liking, taste/flavor, and texture, but only texture was statistically significantly higher (***$p < 0.01$). In Wenatchee, Selection L scored significantly higher (***$p < 0.01$, * $p \leq 0.05$) than Cripps Pink and Honeycrisp for all traits, except appearance. Overall selection L is preferred for texture and flavor when compared with Honeycrisp or Cripps Pink. The texture was significantly preferred on all comparison sets.

<u>BPAC meetings and field visits:</u> 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 events were held in 2020 and 2021. The BPAC meeting was held via ZOOM in July, and fruit samples were sent to each member up to 2 weeks prior to the meeting.

In 2022, one field visit was held in Prosser and Quincy prior to harvest. The BPAC members also had the opportunity to visit the WSDA plantings of Selection L at Sunrise and Roza research stations. The BPAC meeting occurred in August in a hybrid format. Samples were available for tasting.

Project Title: Phase 3 Evaluations of Apple Breeding Program Selections

Executive summary

Keywords: apple breeding, new apple varieties,

Abstract

Currently, there are five selections in the WABP Phase 3 (P3). Selection Q, R, and S were top worked in Quincy and Sagemoor in 2020 and 2021, respectively. Both locations were defruited in the first year, and trees from Quincy were harvested for the first time in 2022. Storage evaluation is ongoing. Selection P is a bicolored apple, crispy, juicy, and with a unique tart-sweet flavor. It has a low incidence of field and storage disorders. Selection L is a bicolored apple that colors well when exposed to sunlight. It is slow to brown, easy to pick, and pre-harvest drop have not been observed. Its unique trait is high firmness retention during storage, which, combined with the low incidence of disorders and diseases in the field and during storage, grants this selection a long shelf-life potential. Two formal consumer tastings were held in public events in 2022, with a total of 360 participants. Selection L was compared with Cripps Pink and Honeycrisp apples. Overall, selection L is preferred for texture and flavor when compared with Honeycrisp or Cripps Pink. The texture was significantly preferred on all comparison sets.

Methods

The general methods are to determine the full bloom date, perform observations twice a month during the growing season and twice a week visits pre-harvest to evaluate tree growth habits and crop load, perform a minimum of two sequential picks/selection, store fruit under commercial conditions (w/o 1-MCP) in RA & CA (1% carbon dioxide, 2% oxygen; up to 10 months), perform fruit quality and cull analysis (in the field and after storage), determine the size profile, and organize fruit tastings (formal and informal). Specific evaluations are conducted based on each selection's unique characteristics.

Project Outcomes

1. In Quincy, selection P was typically harvested in mid to late September (Honeycrisp timing). Harvesting this selection in early October for two consecutive years did not negatively impact fruit quality, flavor, or storage disorder incidence.
2. Selection L has performed consistently well during 5 years of evaluations. It has a low incidence of field and storage disorders, and firmness is stable during long term-storage, granting this selection a long-shelf life potential.

Significant findings

1. Selections P and L have a good shelf-life potential granted by the low incidence of disorders and diseases in the field and during storage.
2. Selection L was preferred by consumers in two pairwise tastings in spring 2022 for overall liking, texture, and flavor when compared with Honeycrisp or Cripps Pink. Preference for texture was significantly higher, outperforming both selections.
3. Selections Q, R, and S were grafted in Quincy (2020), and Sagemoor (2021) and grew to the top wire in their first year. Quality analysis of fruit from Quincy is ongoing.

Future work

1. Determine fruit quality, yield, and storability of selections Q, R, and S.
2. Assess consumer acceptance and crop load management of Selection P.
3. Evaluate fruit set and packing line handling of Selection L.

Project Title: Apple genomes for postharvest fruit quality biomarkers

Report Type: Final Project Report, Year 4 of 4 (Including 1 year NCE) for AP-19-103

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

Project duration: 3 Year (+1 year NCE)

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

Other related/associated funding sources:

Funding Duration:
Funding Duration: annual congressional appropriation (USDA TFRL Base funds)
Amount: $85,000
Agency Name: USDA ARS
Notes: 3-year total $220,000: Personnel $100,000, RNA-Seq $90,000, Consumables $30,000,

Funding Duration: N/A
Amount: $86,000
Agency Name: WSU Ficklin Start-Up Funds
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.

Funding Duration: 2017-2022
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
Primary PI: Dr. Loren Honaas
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Item	2019	2020	2021
Salaries	33,000		
Benefits			
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	5,000	5,000	5,000
Travel			
Plot Fees			
Miscellaneous[1]	49,142		
Total	87,142	5,000	5,000

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

Budget 2
Co PI 2: Dr. Stephen Ficklin
Organization Name: WSU Department of Horticulture
Contract Administrator: Anastasia Mondy
Telephone: 509.335.6885
Contract administrator email address: anastasia.mondy@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			
RCA Room Rental			
Shipping			
Supplies			
Travel			
Plot Fees			
Miscellaneous[1]			
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

Budget 3: Co-PI Mattheis requested no budget

Objectives:

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

   NOTE: The 'Gala' genome was published by another group, so we diverted resources from the 'Gala' genome to the 'Granny Smith' genome.
2. **Exceeded:** Refine biomarker discovery pipeline using machine learning algorithms, comparative network analyses, and comparative genomics
3. **Complete:** Begin validation of biomarkers via PCR gene tests in multi-lot, multi-year surveys

Significant findings:

1. Assembled top quality apple genomes, posted to GDR for public access, published 'Honeycrisp'
2. Prototype biomarker models perform well
3. Insights into molecular response of 'Gala' apple fruit to CA - updated molecular model
4. Validation studies generally show expected results in other cultivars/orchards/years
5. Year 4 validation fruit samples obtained, ready for new project AP-22-101
6. New methods to quality check genomes enhance gene studies

Results and Discussion

New apple genomes *(Significant findings 1 & 6)*

The genomes for 'Honeycrisp,' 'WA 38,' and 'Granny Smith' are diploid assemblies, which means they are essentially ***two perfect*** apple genomes, containing the haplomes inherited from each parent - 1 each from pollen and ovule (see 'Honeycrisp' haplomes in Fig. 1). The field of genome assembly and annotation is rapidly evolving, and our team was particularly well positioned to leverage the state-of-the-art technology in genome sequencing. Improved genome resources will promote the identification of genes that drive important traits. In addition to leaving less data on the table during the analysis phase (as Honaas has previously reported, see reports for AP-19-103, PR-17-104), the exceptional quality of the genomes from this project opens new doors for genome scale analyses in apple. This effectively increases the number of genome features (e.g. genes, gene arrangements, etc.) we can use to build models that aim to predict maturity and future fruit quality. For example, we detected a structural rearrangement of a chromosome in 'Honeycrisp' that contains >100 genes; it was apparently inherited by 'WA 38' (Fig. 2). These kinds of structural changes can have massive impacts on genes that are in or near these regions, potentially explaining traits that are unique to a cultivar. Another example relates to the activity of alleles; each gene in the apple genome has two versions called alleles (one in each haplome). Until we built our genomes, this kind of allele-specific analysis was not possible for each project cultivar. Moreover, work in 'Gala' has shown that 1 in 5 genes shows allele activity differences during fruit development (Sun et al. 2020). This is important because if only one of two alleles plays a strong role in a trait (think "dominant" vs "recessive") we would not be able to detect this without our diploid genomes. Last, our sophisticated gene family analysis approach (recently published - (Zhang et al. 2022; Khan et al. 2022) has shown that there are potentially ~100 unique genes in the 'Honeycrisp' genome that are not detected in the other 6 apple genomes. We were able to detect this by carefully classifying all available apple genes into plant gene families (using our software PlantTribes2 - Wafula et al. In Press). These examples are important because unique gene and genome features might help explain unique cultivar traits. All of these examples illustrate new opportunities, as well as key resources that help us avoid pitfalls, as we search for important genes to monitor for risk assessment and maturity prediction tools.

Models for textural changes in 'Gala' apple fruit during storage *(Significant findings 2 & 4)*

There were two main experiments aimed at the development of technology for new risk assessment tools. The first was focused on textural changes in 'Gala' apple fruit during storage. In

this experiment we stored 'Gala' apple fruit in various conditions (that include commercially relevant schemes) and tracked changes in fruit quality. There are a few main lessons from this work. The first is that while we can identify genes that are relatable to fruit texture changes, the wide range of possible storage conditions poses substantial challenges to biosignature development. This means that potential future tests may 1) need to be developed with a much larger training data set if biomarkers are to be deployed across ***all possible storage conditions***, or 2) need to be developed in a ***condition-limited manner***, such as for CA vs. air storage. Following the first experiments, we conducted a validation experiment where we stored 'Gala' fruit from a different orchard/year in similar conditions as the first experiment. We tested genes from our models using qPCR to see if the patterns were consistent across orchard/years. The results of this validation show that our top genes show very similar patterns of activity in fruit from a different orchard in a different year (75% agreement among all genes and storage conditions/treatments - Fig. 3).

Important also are practical considerations for biosignature tests beyond model performance, such as good signal to noise ratio (high vs. low levels of gene activity), lack of highly similar genes that can dilute or confound the signal (apple genomes are full of duplicate genes and large, complex gene families), and large scale changes through time (making tests more sensitive). We applied these and other criteria to select genes from the model, and also randomly selected a similar number of genes from the top genes in the model. Both of these subsets had similar performance, R^2 of 0.754 vs. 0.705 respectively. This indicates that applying additional criteria that are meant to enhance performance of PCR tests do not substantially reduce the predictive value of the model. This is important because we can choose genes that are likely to be easier to measure without sacrificing predictive value. All-in-all, while the models for textural changes seem to require many genes for maximum performance, it is reassuring that most of the genes we validated show consistent patterns across orchard/years.

Insights into fruit responses to low oxygen environments *(Significant finding 3)*

Another strategy to enhance postharvest fruit quality revolves around understanding how fruit respond to postharvest environments. This can offer clues about how fruit respond at a molecular level to, for example, 1-MCP, low temperature, and/or low oxygen (i.e. CA - controlled atmosphere). Our 'Gala' storage experiment provided excellent opportunities to examine how molecular models (that were elucidated over decades of work in model plants like rice, Arabidopsis, and others) operate in pome fruit species. In these fruit tree species a necessary first step is a careful classification of genes because the genes are not present in clean 1:1 ratios across plants - especially across distantly related plants like rice and apple. Our team leveraged our evolutionary expertise to classify all known apple genes into gene families, and then by looking at gene family trees identify apple genes that belong in molecular models from model plants. Doing this, we identified apples genes that respond in unexpected ways to environmental stresses in the postharvest period (Fig. 4), providing clues about the role of ethylene in losses of quality in long term fruit storage. We are in the process now of updating the molecular models for apple, and will continue to pursue this new line of inquiry towards optimized storage conditions for apple fruit. This is important because we might be able to identify windows of opportunity to apply certain types of crop protectants or plant growth regulators (or even new combinations thereof) that could be useful to maintain fruit quality in the postharvest period.

Prototype biomarkers - Next Generation Maturity Indices (NGMIs) *(Significant findings 2, 4, & 5)*

Our NGMI prototype models can use gene activity alone to predict the harvest date of project samples. That is, when we impose a contrast of maturity by picking fruit at intervals, we can then use gene activity data to look back and predict the harvest order, essentially recapitulating the harvest order. During the course of optimization we improved model performance substantially, with the tests approaching the performance level of the training data set (Fig. 5, panels A & B). Furthermore, we can generally order samples by harvest date using gene activity data from a relatively small number of genes, that is, model performance approaches maximum performance fairly quickly as genes are

added (Fig. 6A). Additionally, we can see the strong positive effect of adding more data to the models (Fig. 6B), indicating that additional orchard/years of gene activity (i.e. sequencing) data will enhance model performance. In fact, a key feature of our prototypes is that they are updatable - as new data are added, we can update the gene targets that are the basis of potential future tools for risk assessment tools, like NGMIs. Therefore, our approach which differs in key ways from previous efforts (we use deep comparative and evolutionary genomics frameworks, for example), will benefit from USDA funded data that Honaas' group is adding to the models, plus data that the new AP-22-101A project will add from many orchards and cultivars. Overall, our results suggest that gene expression patterns are likely viable biosignatures for a new maturity index, and have possible utility within and across cultivars. Combined with mature RNA sample stabilization technologies, NGMI service models based on PCR are potentially possible.

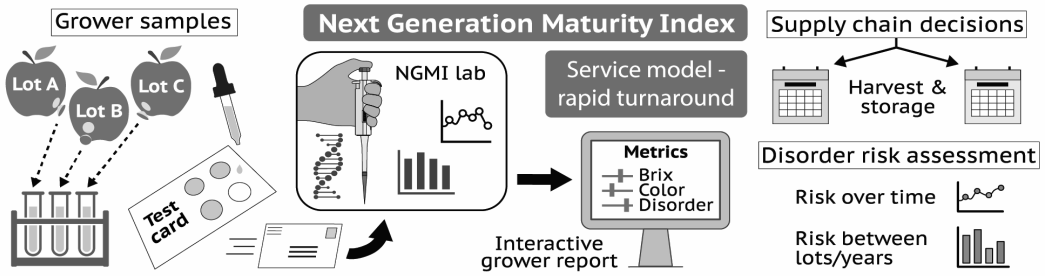
The next steps involve model optimization so we can understand how model performance changes with more data, plus other tweaks that are meant to account for multiple sources of noise. This is because we see clear examples of outliers where, for example, one year in one cultivar shows a divergent pattern - determining how to feed this information back into the model to improve the predictive power is a goal of Honaas' new project, AP-22-101A. The real-world outlook provided by our validation sample set suggests that we can make predictions in cultivars/years/orchards that were not part of model development. The patterns in our validation tests allow us to order fruit samples by pick date 92% of the time on average, but can vary from 70-100% depending on how the data are parsed (Fig. 7A vs. 7B). How to optimize the model to work with new data types and new cultivars remains to be explored - again, this is the goal of Honaas' new project AP-22-101A.

Long term outlook and industry impact

This project has established critical foundational resources and prototype biosignature workflows towards the development of commercially viable risk assessment tools. The compelling preliminary data that this project provides has helped elevate our SCRI proposal and has also helped coalesce a community of stakeholders and scientists around the possibilities of biosignatures for risk assessment in apple fruit. While our models can predict differences that we imposed in our experiments, the next steps involve model optimization to increase model performance. Our eventual aim is to differentiate ostensibly similar fruit before losses in quality occur - indeed our retrospective analysis of fruit quality in the project will show us which lots of fruit had differences in storage potential. Additionally, there are clear outliers in our models and validation tests: there are clear examples of a particular gene, year, orchard, or cultivar that do not always follow the model patterns. What this means is that more data and analyses are needed to understand the structure in the noise. For instance, we need more years of data to determine whether 1) the year or 2) the orchard location has a larger effect on a particular gene in a particular model. What is clear is that commercially viable NGMIs will likely require very sophisticated models based on multiple cultivars (or even species), multiple years, and multiple genes to make reliable predictions.

NGMI concept model

We envision a service based NGMI model based on our prototype biomarkers. The technology for tests in the field is mature and has been deployed commercially. We will use similar methods that include stabilization of fruit extracts on cards and gene measurements based on PCR.



Figures and Tables

Figure 1. The diploid 'Honeycrisp' genome shows high overall structural similarity with the 'Gala' apple genome (Sun et al. 2020). Ribbon plot showing high structural similarity, chromosome by chromosome, of the diploid 'Honeycrisp' genome assembly. A diploid assembly contains two apple genomes, each one called a haplome (abbreviated HAP below). This allows us to study both copies of every apple gene, which substantially increases the number of potential targets for biomarker model development and opens new doors for genetic analysis in apple that will shed light on important fruit traits.

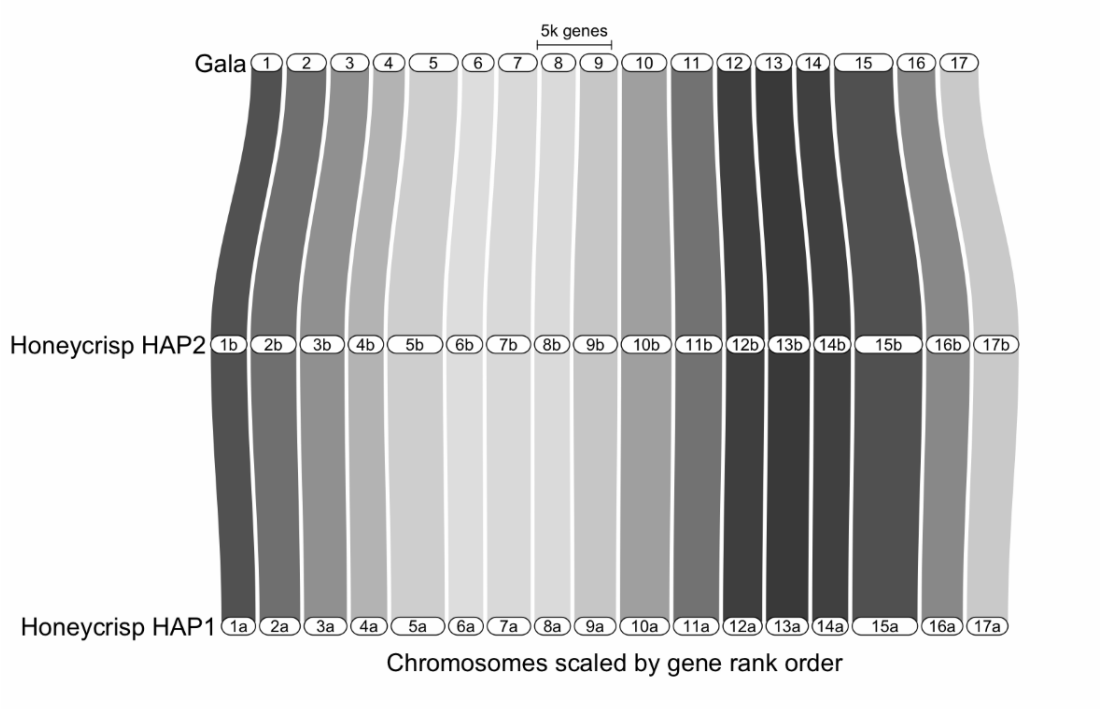


Figure 2. A structural difference in the 'Honeycrisp' genome was inherited by 'WA 38.' Synteny cartoon showing a chromosome inversion that contains ~120 genes (enlarged for detail). This inversion is only in 1 haplome of 'Honeycrisp,' and is therefore only in 1 haplome of 'WA 38.' We do not yet know the impact of this particular change, but it has been well documented that such changes can have large effects on gene activity in or near the inversion, and also on gene structure for genes at the boundaries of the inversion. Changes like this could potentially explain cultivar traits, but also represent potential pitfalls because this inversion is thus far only seen in 'Honeycrisp' and 'WA 38.' Genomes from this project are the first to have fully-phased, perfect, diploid assemblies for apple. This offers new glimpses into haplome structure variation in important apple cultivars.

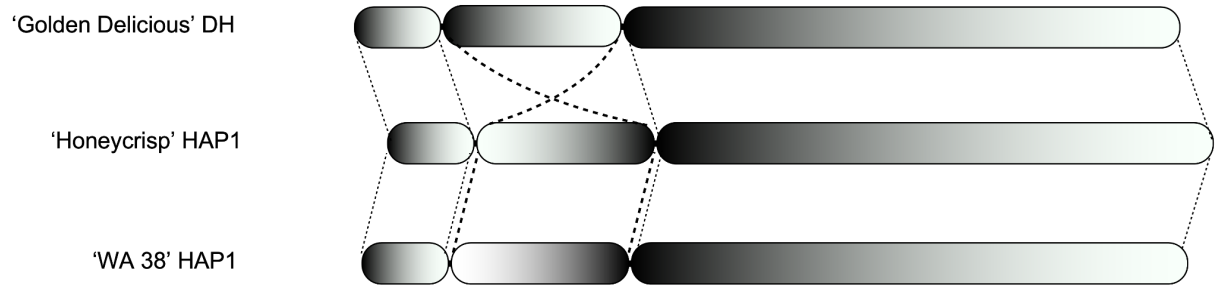


Figure 3. Models for prediction of fruit texture show consistent patterns across orchard/years. When we repeated the 'Gala' storage experiment (different year, orchard, and gene activity measurement methodology) the patterns were largely consistent for the example gene below. There were apparent differences in fruit maturity (estimated based on physiological indices; color, starch, texture), which could explain some differences between "Harvest" and "T1" timepoints in Conditions 4 & 5 between each experiment. Validation studies like these suggest our approach may eventually yield robust biomarkers, but at this early stage in development they primarily provide valuable information for model improvement.

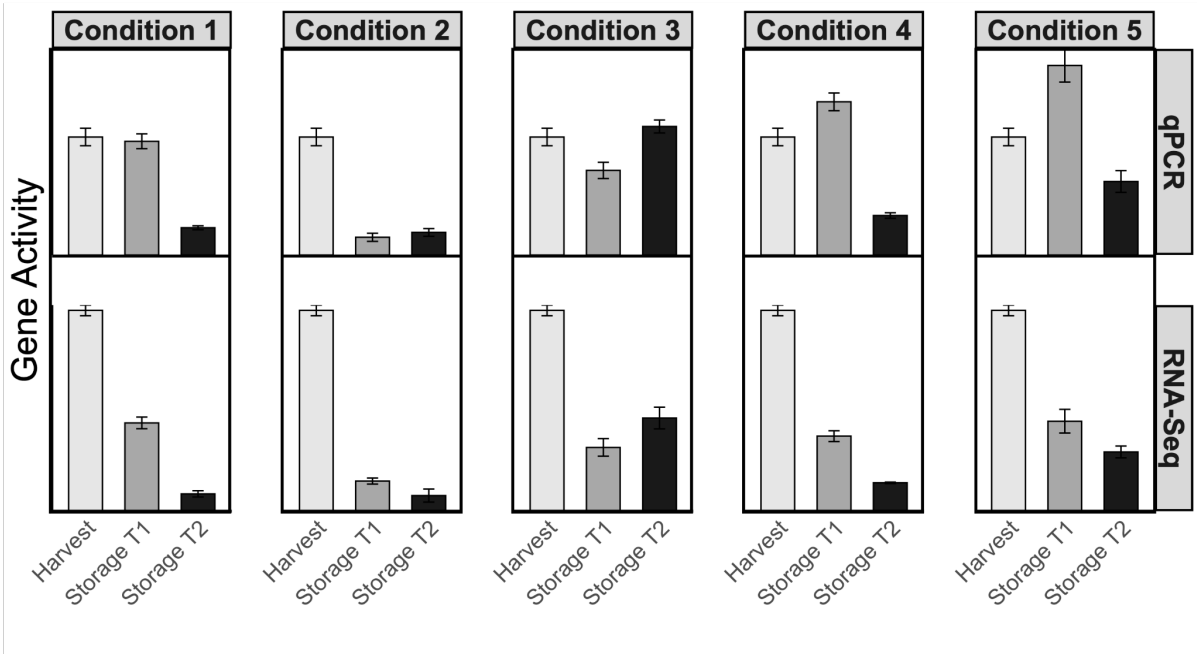


Figure 4. For two "twin" apple genes, a molecular schematic for how plants respond to low O_2 predicts activity for one gene, but not the other. A molecular signaling schematic that was developed in model plants (including Arabidopsis and rice) describes a molecular signaling mechanism that is activated by low oxygen, and may use ethylene as a signal molecule. We used gene family information to find the apple genes that correspond to genes in the published signaling schematic. Because of the complex history of the apple lineage, virtually all genes in apple are present in ratios other than 1:1 to other plant genes, like the ones below that are present in a 2:1 ratio. The experimental treatments that included controlled atmosphere (CA, i.e. low O_2) are shown as ***filled squares***, and all treatments in normal air are shown as ***open circles***. One of the "twin" genes was activated in fruit by low O_2, as expected for CA storage, with the fruit in normal air showing very low activity - **panel A**. However, the other "twin" (**in panel B**) showed an unexpected pattern that included activation in CA, but also in fruit that were stored in normal O_2 levels. This could represent activation of the plant stress pathway for low O_2, or perhaps another role for genes in the model that relates to ripening, rather than just low O_2. Insights like these may represent new opportunities to mitigate negative outcomes that are not well controlled, or are even exacerbated, by long term CA storage.

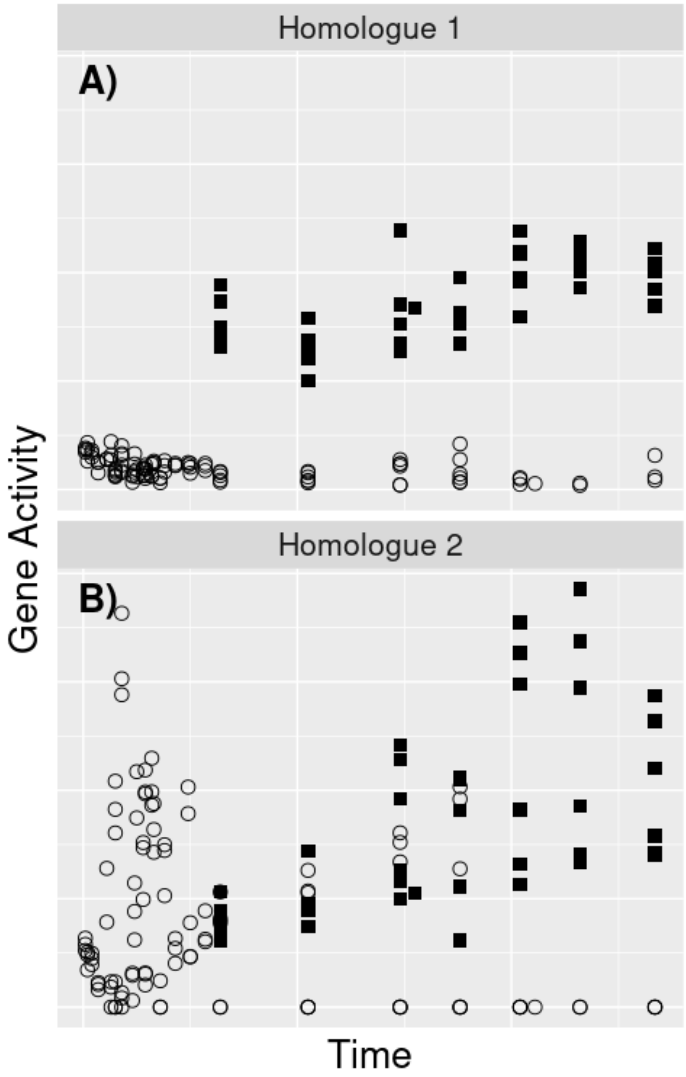


Figure 5. During our project, prototype NGMI model performance has increased. The performance of the NGMI prototypes is generally good enough for us to order picks by date, or even estimate harvest week, in our experiment using only gene activity data. The general scheme is to ***train*** models with a majority of the data, and then ***test*** the models with a portion of data that was set aside - this provides "new" data the model has not seen and allows us to estimate how the model will perform when we carry out real-world tests. Model performance is gauged by linear regression of actual vs. predicted pick date; $R^2=1$ would indicate a perfect set of predictions. The models performed more-or-less consistently during training (in the left column - **A, C, E**). Our optimizations improved the performance of the test cases starting at $R^2=0.786$ and increasing to $R^2=0.946$ (right column - **B, D, F**). The **test** data approached **training** data in model performance (see **B** vs. **A** for our latest model tests). This indicates that our models might be useful as NGMIs with sufficient development.

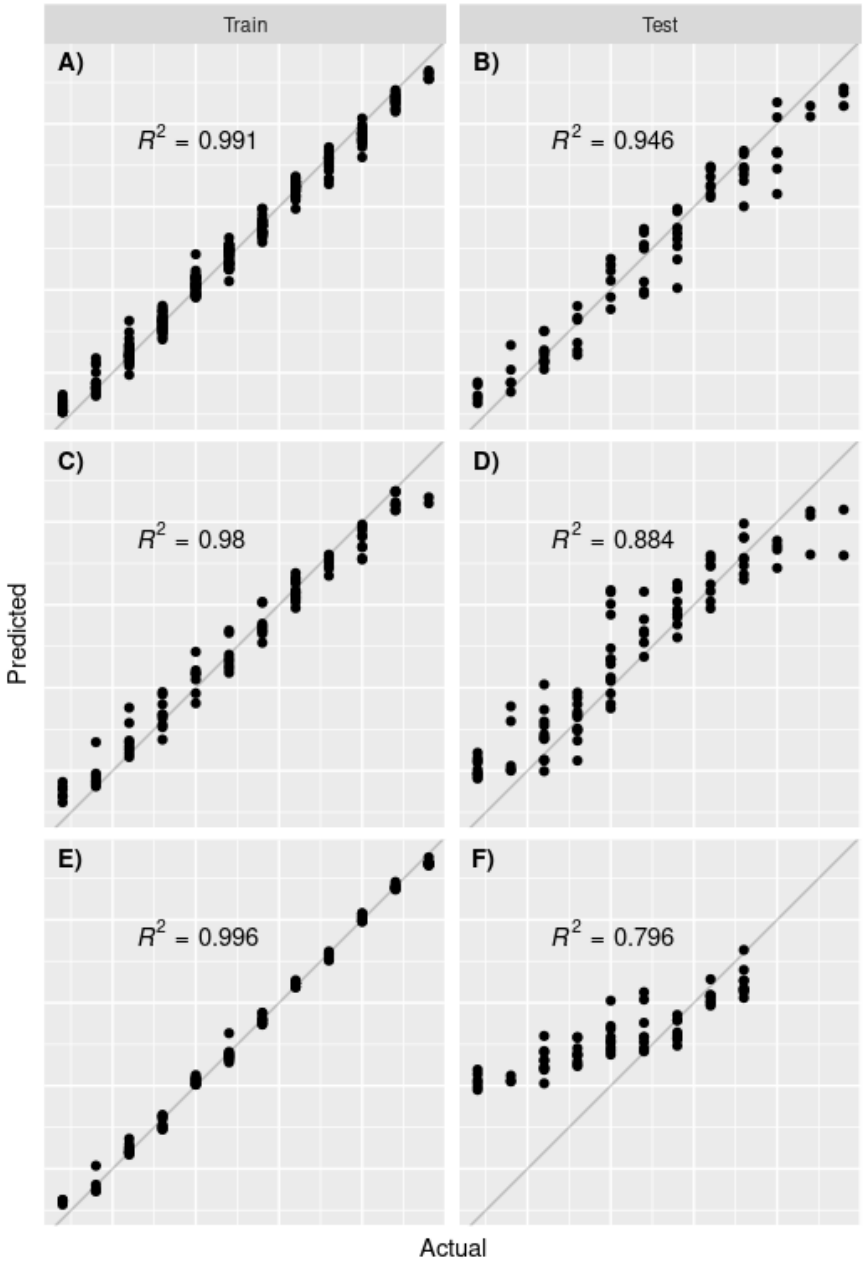


Figure 6. NGMI model performance rapidly increases as more genes and data are included in the model. We tested model performance with varying numbers of genes to explore where the NGMI prototype had changes in performance. The Y-axis shows model performance (i.e. R^2). We used 3 different amounts of training data (dotted, dashed and solid lines in **Panel A - Train** to explore performance as a function of the gene number we use to make predictions. We discovered that for the model, there was a sharp inflection point where model performance stabilized, and that more data increased model performance (indicated by higher stabilization points). Importantly, we saw a similar pattern in the "test" scenarios (**Panel A - Test**), indicating that the model was robust when feeding in new data, and seems to perform well with a tractable number of genes; both important considerations for a future test that is commercially viable. **Panel B** shows a zoomed in view to show detailed differences between **Train** and **Test** in **Panel A**. Note the y-axis scale differences, 0-1.00 in **A**, vs 0.80 - 1.00 in **B**.

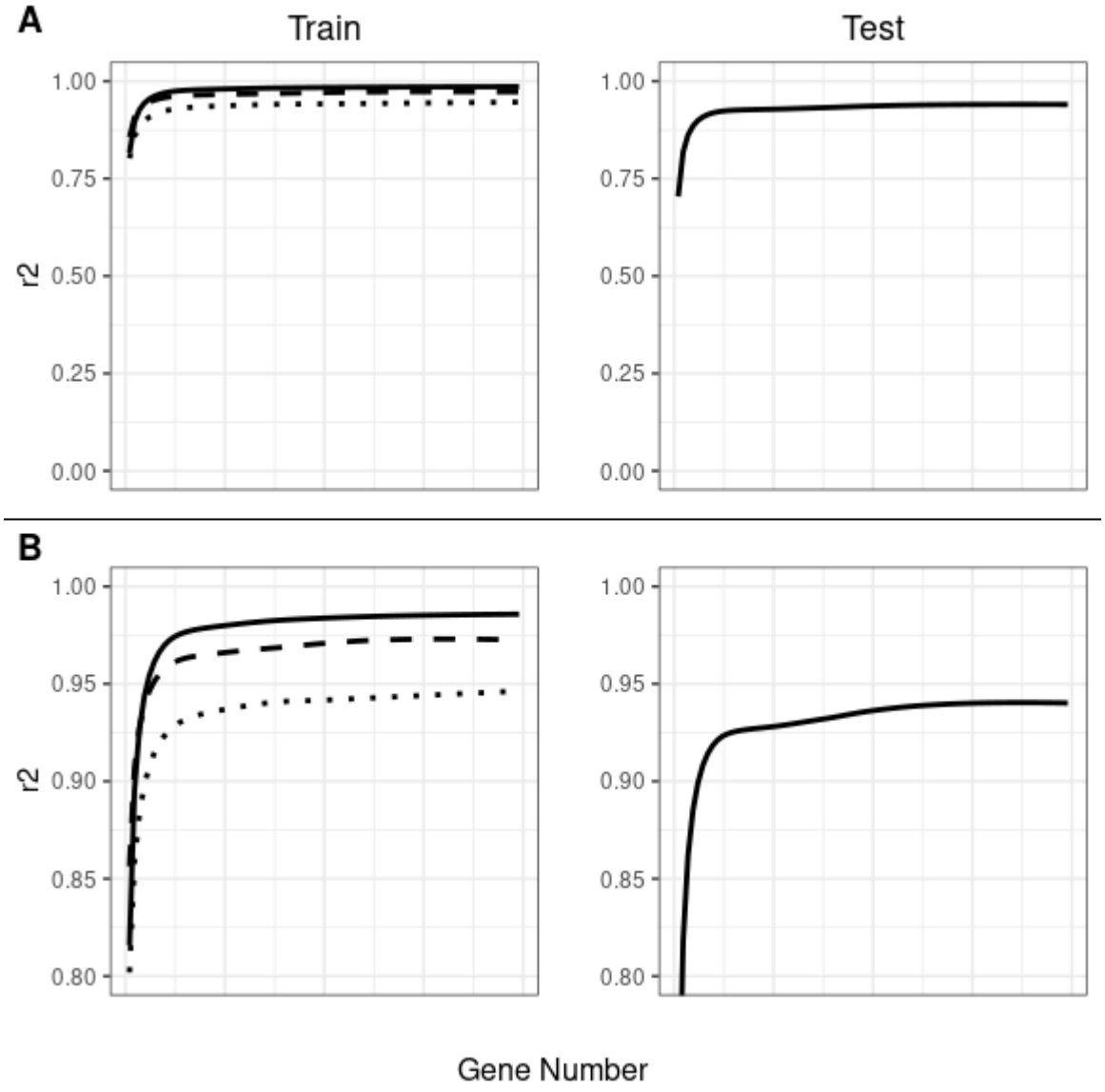


Figure 7. Real world analysis of the NGMI prototypes shows potential for use in many cultivars. When this project was started, we began building a validation sample catalog that now contains hundreds of cryopreserved RNA samples from many cultivar/orchard/years. After we built NGMI models, we tested activity from select fruit samples in our validation catalog to determine if we could recapitulate harvest order with NGMI prototypes. By-and-large, we can predict harvest order >92% of the time on average (panel A), though there are clear examples of outliers (panel B, orchard C). We are exploring how to score validation tests - our initial criteria are shown in Panel C. How these outlier cultivars, orchards, years, and genes can be used to enhance model performance, as well as how different types of data can be integrated into the models, are among the goals of our new WTFRC project AP-22-101A.

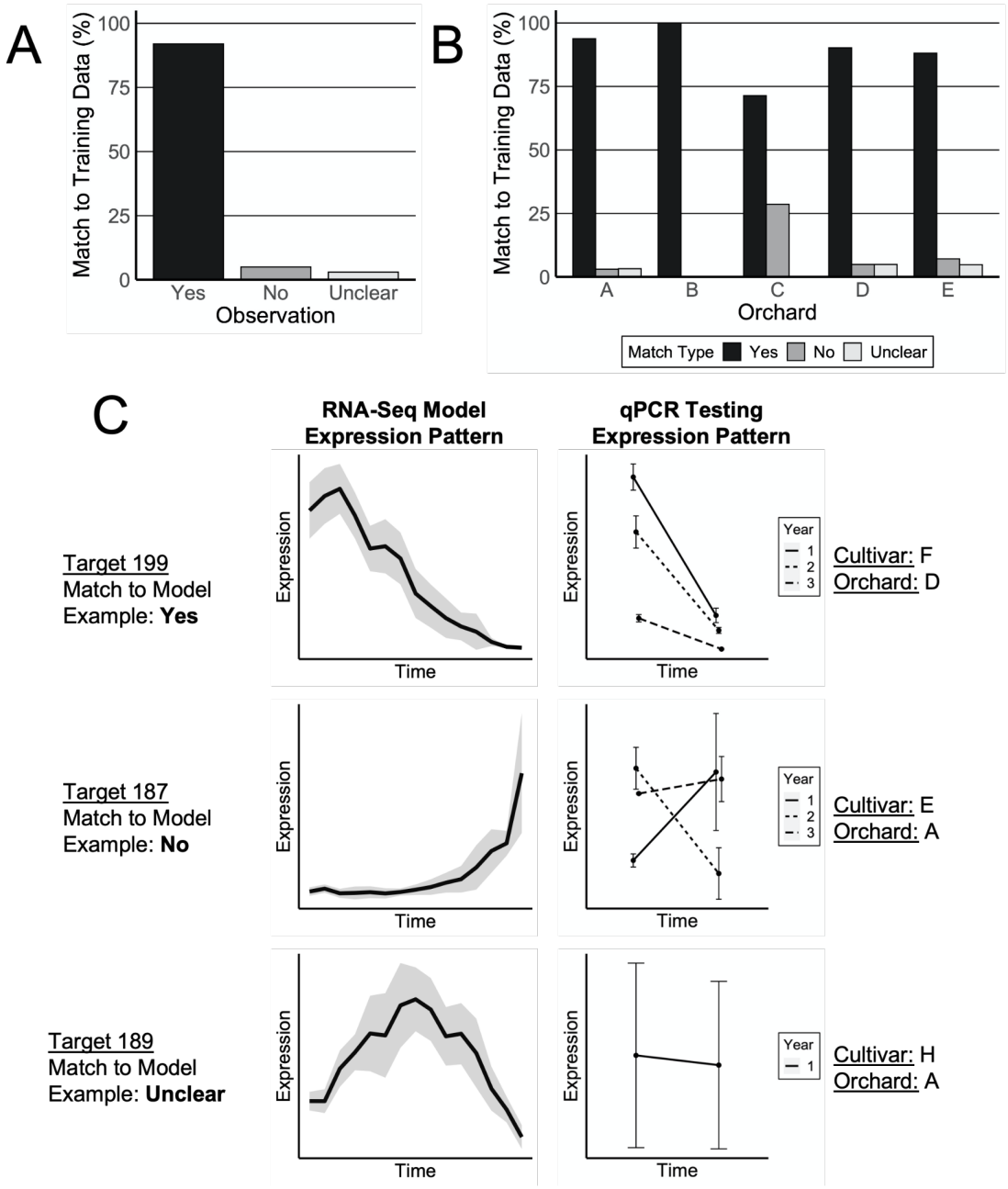


Table 1. Our high-quality apple genomes represent the state-of-the-art in plant genomes, and are likely the best apple genomes to date. Below are genome statistics for project cultivars, and another high-quality apple genome, 'Gala.' There are two general categories of genome statistics, ***Assembly*** and ***Annotation.*** Here, a map analogy is appropriate - think of the ***assembly*** as a satellite picture - it's the raw data for the landscape. Then, for the ***annotation***, imagine adding layers of information that include the boundaries and names of map features, like roads, buildings, rivers, and parks. Reported in millions of base pairs (A, T, G, Cs - abbreviated Mbp) the overall length of our genomes are consistent, yet our assemblies are in larger pieces (larger N50). BUSCOs are a widely used genome benchmark that stands for Benchmarking Universal Single Copy Orthologs; ***translation*** - these are roughly 2,300 plant genes we expect to see in a plant genome, so they are a good watermark for genome quality. Our finished 'Honeycrisp' and 'WA 38' genomes have the highest BUSCO scores of any apple genome. Our genome ***assembly*** is not much better than 'Gala,' but our genome markup strategy that creates the ***annotation*** is philosophically different, and allows us to identify more apple genes than other teams.

Assembly	Length Mbp	N50 Mbp	Assembly BUSCO %	Annotation BUSCO* %
'Gala' (Sun et al. 2020) Note: averages shown	673	14	98.4	95.0
'Honeycrisp' Haplotype 1	674	33	98.6	96.8
'Honeycrisp' Haplotype 2	660	33	98.7	97.4
'WA 38' Haplotype 1	678	36	98.7	95.9
'WA 38' Haplotype 2	667	37	98.7	97.4
'Granny Smith' Haplotype 1	666	38	98.9	90.8
'Granny Smith' Haplotype 2	665	37	99.0	90.3

*The annotation process is very labor intensive and requires many iterations that use additional kinds of evidence. The finished annotation results for 'WA 38' and 'Granny Smith' are expected to meet or exceed those in the 'Honeycrisp' genome. In fact, the topic *evaluation of genome quality* is an active area of research - Honaas' team recently published work that describes novel methods that can be used to evaluate and improve genome resources that have relatively poor assemblies and/or annotations (Zhang et al. 2022, Wafula et al. in press).

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Wafula, E.K., Zhang, H., Von Kuster, G., Leebens-Mack, J.H., Honaas, L.A., and dePamphilis, C.W. PlantTribes2: tools for comparative gene family analysis in plant genomics. *Frontiers in Plant Science*, In Press.

Executive Summary

Project title: Apple genomes for postharvest fruit quality biomarkers

Key words: machine learning, fruit maturity, fruit firmness, RNA-Seq

Abstract: New tools and technologies are needed to help sustain the viability of the tree fruit industry. A key area to innovate is enhancement of supply-chain decision making. By making more informed decisions, losses of fruit quality in the postharvest period could be reduced. Towards this goal, this project developed foundational resources, methods, and datasets that have been used to build prototype biomarker models, or more accurately ***biosignatures*** because multiple targets are required for reliable predictions. We focused on two areas that relate to postharvest fruit quality: at-harvest apple maturity and fruit textural changes during storage. We found that massive datasets (billions of measurements) can be leveraged with state-of-the-art computational methods to build models that are predictive of these two traits. Importantly validation experiments suggest that the models may work beyond the scope of the experiment, and reliable prototype models consist of a tractable number of gene targets.

Objectives:

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

   NOTE: The 'Gala' genome was published by another group, so we diverted resources from the 'Gala' genome to the 'Granny Smith' genome.
2. **Exceeded:** Refine biomarker discovery pipeline using machine learning algorithms, comparative network analyses, and comparative genomics
3. **Complete:** Begin validation of biomarkers via PCR gene tests in multi-lot, multi-year surveys

Significant findings:

1. Assembled top quality apple genomes, posted to GDR for public access, published 'Honeycrisp'
2. Prototype biomarker models perform well
3. Insights into molecular response of 'Gala' apple fruit to CA - updated molecular model
4. Validation studies generally show expected results in other cultivars/orchards/years
5. Year 4 validation fruit samples obtained, ready for new project AP-22-101
6. New methods to quality check genomes enhance gene studies

Future directions: Some of the next steps are outlined in Honaas' new project AP-22-101A. Briefly, we aim to refine and enhance the biosignature models by exploring new modeling techniques, adding new data, and testing ways to integrate model performance back into model development. The preliminary data that this project developed is used in a proposal to NIFA's Specialty Crops Research Initiative.

Project/Proposal Title: Evaluation of an alternative postharvest fungicide applicator

Report Type: Continuing Project Report Year 2

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Cooperators: Jason and Jordan Matson, Matson Fruit, Faith Critzer, University of Georgia; Clark Kogan, Statscraft LLC, Pace International LLC.

Project Duration: 3 Years
Total Project Request for Year 1 Funding: $132,793
Total Project Request for Year 2 Funding: $110,993
Total Project Request for Year 3 Funding: $4,500

Other related/associated funding sources: None

WTFRC Collaborative Costs: None

Budget 1
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Station manager/supervisor email address: cekruger@wsu.edu

Item	2021	2022	2023
Salaries	69,064	63,989	0
Benefits	21,563	18,520	0
Wages	6,483	5,411	0
Benefits	807	541	0
Equipment	0	0	0
Supplies	29,160	21,060	4,500
Travel	5,716	1,472	0

Miscellaneous	0	0	0
Plot Fees	0	0	0
Total	132,793	110,993	4,500

OBJECTIVES

1. Optimize coverage of fruit in alternative sprayer with fluorescent tracer and water sensitive paper (Hoheisel; yr 1).
2. Comparison of efficacy against postharvest decay organisms between drench and alternative fungicide application (Amiri; yr 1and 2).
3. Quantification of indicator organisms (*E. coli* and coliforms) in water and on fruit treated with fungicides applied in drench and alternative applications (Hoheisel; yr 1 and 2).
4. Communication of findings with the apple and allied industries and engage regulatory bodies for approaches for implementation of alternative fungicide application on farm (Amiri, Hoheisel; yr 1, 2 and 3).

Significant Findings

- Field drencher sub-optimized for spray coverage
- Coliform counts were higher in the field sprayer whereas *E. coli* recovery was higher in the warehouse drencher.
- Residue levels of thiabendazole (TBZ) were similar between the field sprayer and warehouse drencher but levels of fludioxonil (FDL) were higher on apples treated thought the field sprayer.
- Spores of *Penicillium* spp. (blue mold) were neither detected on apples nor in fungicide solutions of field sprayer or warehouse drencher
- Total microflora recovered from apples treated with fungicides through the field sprayer was significantly reduced compared to the control and fruit treated via warehouse drencher.
- Overall decay incidence after 8 months of storage at 37°F in regular atmosphere for the field drencher was equal to that of the warehouse drencher or lower.

METHODS

OBJECTIVE 1. Optimize coverage of fruit in alternative sprayer with fluorescent tracer and water sensitive paper. (Hoheisel; Year 1).

Alternative fungicide applicator. As previously mentioned, Matson Fruit has been field testing an alternative fungicide applicator which utilizes single-pass water to deliver fungicides given risk from cross-contamination from decay causing pathogens. Their initial work has resulted in spray systems which were utilized for the 2019-2020 growing seasons to treat in their operation as shown in Figure 1. A video depicting the sprayer in operation can be seen at https://www.youtube.com/watch?v=q685NrigZfw. Others in the industry are also interested in evaluating this applicator during the 2021 harvest. Jason and Jordan Matson are interested in partnering with WSU through this proposed work to further evaluate and optimize this system so that best efficacy can be achieved. Helping advance the number of alternative systems growers have at their disposal for controlling postharvest decays.

Figure 1. Novel fungicide spray applicator shown treating crop in 2020. The system is composed of a mix tank, pump, spray nozzles and catch basin to collect run-off with tank storage for disposal.

Expected outcomes. Deposition will be statistically compared for 27 different zones in a bin for both a new and traditional system and will be paired with biological efficacy and food safety (obj 2 and 3). Combining all three creates an extremely robust assessment of an alternative drenching system that may provide better control.
Potential pitfalls and limitations. We were very successful in this objective and believe it is completed unless results from fluorometry or efficacy study show large differences between top and bottom zones. Aside from that, in an engineering project constructing a novel system can lead to delays for the completion to the project. Biological efficacy studies suggested in Obj 2 and 3 should only be completed once a functional well-developed prototype is developed. In this study, prototype development has already been developed by Matson and therefore minimizes that pitfall and advances the total project. Also, because of COVID and tariffs, our lab has found difficulty ordering supplies that are no longer available in the US. For other projects, we have been able to order well in advance to ship internationally.

OBJECTIVE 2. Comparison of efficacy against postharvest decay organisms between drench and alternative fungicide application. (Amiri; Year 1 & 2)

Alternative sprayer application (Field sprayer). The optimized configuration for coverage as determined in objective 1 will be utilized for all subsequent studies. We will use Penbotec (pyrimethanil) and Scholar (fludioxonil) in coordination with Matson Fruit and will rotate fungicides from year 1 to year 2. The cultivars Honeycrisp and Gala were used for these trials.

Drench application. Matson Fruit will drench fruit, from the same cultivar used in alternative sprayer, following standard industry practices. The same antifungal compound evaluated in the alternative applicator will be utilized in the drench application.

Quantification of spores of fungal pathogens in fungicide water for alternative and drench applications. For the alternative sprayer, three-100mL fungicide solution samples will be aseptically captured during each spray. For drench application, fungicide solution samples will be collected at 0, 100, 200, and 500 bins treated with the same fungicide solution (same tank). Samples will be held at 4°C until further processed on agar media amended with triton x 100 (Amiri and Bompeix, 2005). Plates will be incubated for 5 days at 20°C (68°F) and fungal colonies will be counted and identified to the genus level. The concentration of fungal spores in fungicides solutions will be expressed as colony-forming unit (CFU). This experiment will be repeated in Year 2.

Quantification of fungal colonies on apples before and after fungicide application through alternative and drench approaches. Twelve individual apples will be sampled before and 30 min after treatment per replicate in a way to collect 4 apples from each bin (4 bins make one replicate) treated at the same time. Apples will be placed in a sterile plastic bag and held at 4°C until further analysis. An individual apple will be immersed in 100ml buffered peptone water with 0.1% Tween 80 and placed on rotary shaker for 30 minutes to suspend fungal spores in the buffer. Samples will be serially diluted and plated

in duplicate on agar medium amended with triton x 100 and plates will be incubated for 5 days at 20°C (68°F) and fungal colonies will be counted and identified to the genus level. The size of each fruit will recorder to estimate the area and the concentration of fungal spores/cm^2 of each fruit will be expressed as colony-forming unit (CFU). This experiment will be repeated in Year 2.

Determination of decay incidence and decay types in cold storage on fruit treated at harvest with fungicides through alternative and drench approaches. Four replicates of 100 apples each will be collected from different bins before fungicides are applied and four other replicates will be collected after the fungicides are applied via each method. Fruit will be placed in separate (each 100 fruit rep) labeled crates and stored at WSU-TFREC in regular atmosphere at 1°C (34°F). Fruit will be inspected every two months for decay incidence and decay type for up to 8 months. We will work with Matson fruit to conduct a second efficacy trial in commercial settings. At the time of harvest, 10 bins will be left untreated, 10 bins will be treated via alternative sprayer, 10 bins will be treated via traditional drencher. All bins will be labeled and stored in CA room at Matson's storage facilities for a period to be determined. At the end of the storage period, the bin will be run through the packing line to separate decayed from healthy fruit. Decayed fruit will be collected by Amiri' team to determine decay incidence and types in each bin set. Packout from each bin set will be obtained from storage facility manager.

Fungicide residue levels. In addition to the work outlined in Objective 1, we will evaluate fungicide residue levels generated by the alternative sprayed and traditional drencher. Two samples of 10 apples each will be sampled from individual bins, one sample on the top of the bin and the other sample will be from one foot deep from the top of the bin. A total of 4 replicate samples will be collected from each application methods and fruit will subjected to fungicide residue analyses.

Expected outcomes. We will determine if the alternative method of fungicide application has reduced risk for carrying-over fungal spores and is more effective in reducing fungal spores on fruit surface prior to storage. We will also assess the efficacy of this new alternative method in reducing decays in long-term storage. We should also obtain data on the fungicide residue level provided by this new alternative method and if those levels are adequate to provide protection against postharvest pathogens.

Potential pitfalls and limitations**.** Disease pressure may vary between seasons to obtain adequate or comparable data from the presence of the fungal spores on the fruit surface at the time of harvest. Some fungi may consist of endophyte (infections) that may not be detected by plating. Comparing the alternative method to the traditional spray method should take into consideration the number of bins treated via drencher to assess efficacy of a "clean" versus "dirty" tank. At the end of Year 2 (2023), we'll have 2 years of data and will be able to better compare the efficacy of the two sprayer models.

OBJECTIVE 3. Quantification of indicator organisms (*E. coli* and coliforms) in water and on fruit treated with fungicides applied in truck and alternative applications. (Hoheisel, Years 1 & 2)

Experimental design. A completely randomized design will be used for both water and apple analysis. There are four water samples per replicate and all treatments will be independently replicated eight times. There are twelve apple samples per replicate and all treatments will be independently replicated eight times. Populations of *E. coli* and coliforms will be the independent factor which will be evaluated to determine significant differences

Quantification of *E. coli* and coliforms in fungicide water for novel and drench applications. Three-100mL water samples will be aseptically captured during each spray or drench application. Samples will be held at 4°C until further processed utilizing the Colilert Quanti-Tray 2000 (Idexx, Wesbrook, ME). Samples will be incubated for 24h at 36°C. The wells in the Quanti Tray will be observed for their change in color from colorless to yellow (coliform detection) and presence of fluorescence (*E. coli* detection) using a fluorescence analysis cabinet Model CM-10A (Spectroline, Westbury, NY). Positive wells for *E. coli* and coliforms will be recorded and equivalent populations of Most Probable Number (MPN) for each organism per 100mL will be determined.

Quantification of *E. coli* and coliforms on apples before and after fungicide application through novel and drench approaches. Twelve individual apples will be sampled before and after treatment per replicate. Apples will be placed in a sterile plastic bag and held at 4°C until further processed. An individual apple will be immersed in 100ml buffered peptone water with 0.1% Tween 80 and rubbed by hand for 30 seconds to suspend bacteria in the buffer. Samples will be serially diluted and plated in duplicate on Petrifilm E. coli/Coliform Count Plates. Samples will be incubated for 24h at 35°C, after which colonies showing typical characteristics for *E. coli* and coliforms will be enumerated and used to calculate Colony Forming Units (CFU) per apple.

Expected outcomes. We will determine if there are differences in water quality and populations of indicator organisms on fruit in the novel, single-pass fungicide spray system compared to that of a traditional drench system. It is anticipated that the novel single-pass applicator will have improved water quality based upon populations of *E. coli* and coliforms compared to a recirculated drench system. If true, we would also anticipate a significant increase in cross-contamination from drench systems onto fruit. Ultimately, this information will help growers managing risk within their operation make informed decisions about the food safety benefits, if any, from this alternative fungicide application system.

Potential pitfalls and limitations. The authors do not foresee any significant pitfalls given past experiences enumerating *E. coli* and coliforms from postharvest water and on apples. Limitations to this approach are that the team is quantifying differences in indicator organisms and not foodborne pathogens. Therefore, any inferences will be with respect to indicator organism behavior and not that of foodborne pathogens (*Listeria monocytogenes,* Shiga-toxigenic *E. coli,* and *Salmonella*) directly. However, *E. coli* and coliforms are commonly used indicators and the most appropriate selection for this approach.

OBJECTIVE 4. Communication of findings with the apple and allied industries and engage regulatory bodies for approaches for implementation of alternative fungicide application on farm (Amiri, Hoheisel; yr 1, 2 and 3).

Communication with the Washington Department of Ecology. The team will also work with Marsha Porter at the WA Dept. of Ecology to outline specific criteria which must be adhered to when utilizing the novel applicator. This will help clearly communicate expectations to growers during outreach.

Communication with the apple industry. Each member of the WSU team has an extension appointment and regularly communicates with the Washington apple industry. Findings from this work will be communicated to the industry through grower meetings, newsletter articles, and factsheets to further disseminate knowledge gained. A detailed explanation of the sprayer parameters will be given for others to construct. Factsheets will be printed in both English and Spanish.

Results and Discussion

Objective 1. Optimize coverage of fruit in alternative sprayer with fluorescent tracer and water sensitive paper.

Optimization. Examination of the field drencher (FD) and prior residue analysis by Matsons showed that very little improvements needed to be made to the FD design. There are three nozzles (QCTF-VS20 Quick Turbo FloodJet Wide Angle Flat Spray Tip) across the spray bar and to reduce drift plastic guards have been installed on the side. The FD is run at 15psi to ensure large droplets and reduce drift. Time for each bin to be sprayed in the field drencher was 12 sec (n=16) and the packing house (PH) is standard 30 sec. The gallons per bin based on spray time and gpm of nozzle (FD) or water collected (PH) is FD=0.5g/bin and PH=1.5g/bin stack.

Apple bins were modified to have 4 slits in the top and bottom and rebar was inserted to form a rectangle that kept an 'apple-free zone' in which water sensitive paper (WSP) could be inserted on a pole. There were four collection zones in the top and four in the bottom. The WSP in both the single layer FD and stacked bin PH were complete coverage. This was expected and desired result of this type of chemical application (drench).

Evaluation. Fluorescent tracer (pyranine) was used to assess deposition of FD and PH. Pipe cleaners that are absorbent in their cotton fibers were placed in bins in the 'apple-free zones' so that 4 samples were on the top and bottom (n=8/bin). Based on the high volume of liquid applied by drenchers compared to standard field sprayers, we reduced the pyranine rate from 1000mg/L to 83mg/L=FD and 328mg/L. Tank samples were collected and differences in initial pyranine will be adjusted for in the calculations of pyranine parts per billion (ppb). Pipe cleaners were bagged, labeled, and stored in a dark cooler at 4°C (39°F) until laboratory analysis (currently being done). To each sample, deionized water will be added, and bags will be vigorously shaken for 30 seconds and allowed to settle. An aliquot of wash from each sample bag will be extracted and analyzed with a 10-AU fluorometer.

A linear mixed effects model was fit to characterize the tracer concentration (ng/cm^2) by zone and location for the packing house. Zone, location and the interaction between zone and location are included in the model as fixed effects, while the truck (or rep) is included as a random effect. An analysis of variance is performed to assess the effects of zone, location, and the interaction with Kenward-Rodger degrees of freedom. Least squares means and 95% confidence intervals are extracted for each zone x location combination. Pairwise differences with 95% confidence intervals are extracted between the top and bottom for each zone, with no family-wise adjustment for multiplicity. Pairwise differences between zones are extracted with a family-wise adjustment for multiplicity using the Tukey method.
Overall, there was no significant difference in coverage between packing house and field samples (Figure 2).

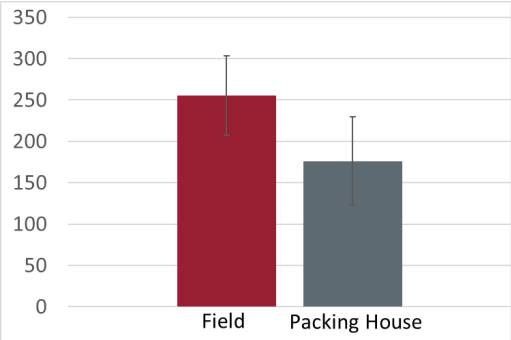

Figure 2. There is no significant difference (P-value: 0.2696) between the mean of the tracer concentration from the field and packing house, averaging across the experiment.

Deposition within the packing house was fairly uniform except for the upper most collection zone and location receiving more (Figure 3a). This is obviously due to the shower-down nature of the application. Nonetheless, it is positive that the lowest collection area (Lower, bottom zone) had similar deposition to other areas and is likely due to the extremely high flow rate in the packing house.

The field drencher (Figure 3b) is not stacked but goes under the spray bar with bin 1 going in first. After the last bin is sprayed, the driver waits 30 seconds and backs out with the bin 4 being the first under the spray bar. In this analysis there was a difference in deposition with the third bin receiving slightly less. We need to inspect possible differences in driving or patterns that could explain this difference. It contrasts with the regularity of time sprayed per bin (12 sec) which showed no significant difference in spray time among bins. Additional differences can be seen between the top and bottom zone of the bin, however, the impact of this would need to be assessed with efficacy data from storage rots. Meaning, there may be adequate deposition in the lower portion to control , but if not, rate should be increased to achieve more deposition in the bottom.

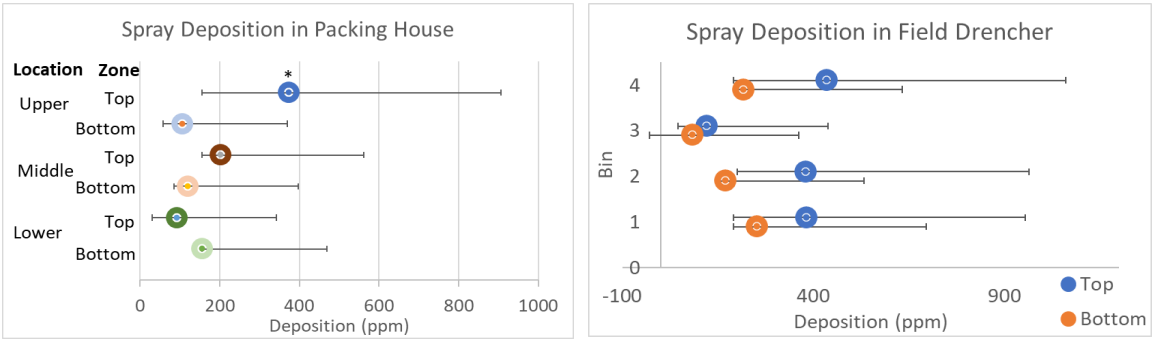

Figure 3. Spray deposition in the packing house and field drencher. In the packing house, bins are stacked (location) and there are two zones within a bin. Only the upper top collection area showed significant difference ($p<0.001$). In contrast, the field drencher is not stacked, but goes under the spray bar from bin 1 to 4. Significant differences were seen between the top and bottom of the bin ($p>0.0197$) and bin order ($p>0.047$).

Objective 2. Comparison of efficacy against postharvest decay organisms between drench and alternative fungicide application

<u>2.b. Quantification of spores of fungal pathogens on fruit treated via two drench applicators</u>

On Average, very a few *Penicillium* spores were recovered from the surface of the fruit and were generally less found in fruit drenched in the field than in the warehouse especially for Gala lots 1124 and 1113 (Table 1). Other minor fungi and often not pathogenic were recovered from fruit treated through both drenchers although slightly higher in the field-drenched fruit.

Table 1. Number of colonies of *Penicillium* spp. and other fungi recovered from the surface of the fruit treated through field (FD) and warehouse (WH) drenchers in September 2022.

Cultivar	Lot	Penicillium			Other fungi		
		Control	FD	WD	Control	FD	WD
Honeycrisp	1136	0.04	0.3	0.4	17.2	14.8	31.8
Gala	901	0.2	0.2	0.2	5.4	12.1	9.3
Gala	1124	0.04	0	0.3	2.4	6	4.4
Gala	1113	0.08	0	1.2	27.7	42	13

2.c. Fungicide residue levels

Residue levels of pyrimethanil on Honeycrisp apples collected from the top and the middle of the bins were equal between in the field and the warehouse drenchers, but residue levels were lower at the bottom of the bins drenched in the field (Figure 4right). Residue levels of pyrimethanil on Gala apples collected from the top, middle and the bottom of the bins were equal, for the same bin position, between in the field and the warehouse drenchers (Figure 4left). However, in the field drenched-bins, significantly lower residue levels were found at the bottom of bin compared to the top. This should not have an impact on decay management, since the minimum levels recommended for pyrimethanil are met (>1 ppm).

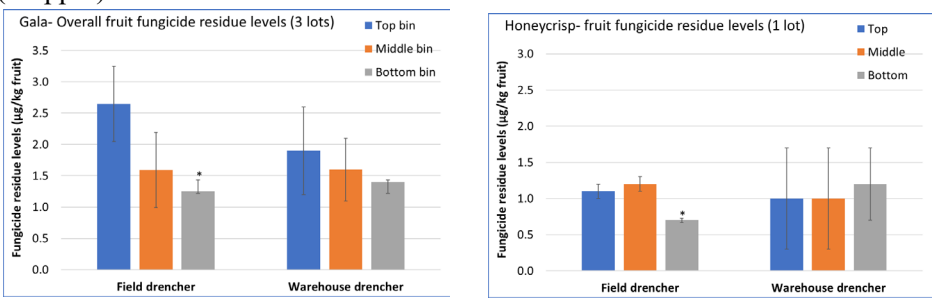

Figure 4. Residue levels of pyrimethanil on Gala apples (left) and Honeycrisp apples (right)

Concentration of pyrimethanil levels in solutions of the field sprayer were slightly bigger than those found in the solutions applied in the warehouse drencher (Figure 5). In the warehouse drencher, pyrimethanil concentrations were all above 200 ppm regardless of the number of bins treated with the same tank.

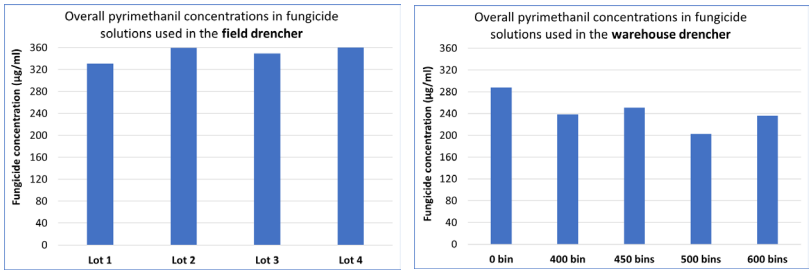

Figure 5. Concentrations of pyrimethanil in fungicides solutions of field (left) and warehouse (right) drenchers

2.d. Determination of decay incidence and decay types in cold storage on fruit treated at harvest with fungicides through alternative and drench approaches

Three hundred apples (100 apples/treatment) were collected from each lot and stored at 55°F for 2 weeks, then at 37°F in RA. Overall decay varied between lots and was either lower in field drencher after 9 months or equal to incidence recorded in warehouse drenched-fruit except in lot 1139 (Figure 6).

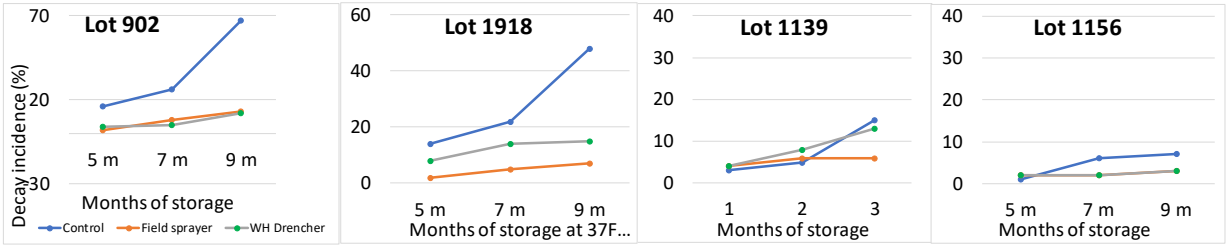

Figure 6. Overall decay incidence in four Honeycrisp lots untreated (control) or treated via field or warehouse drenchers in 2021 and stored in regular atmosphere at 37°F.

Objective 3. Quantification of indicator organisms (*E. coli* and coliforms) in water and on fruit treated with fungicides applied in truck and alternative applications

Very high levels of coliform and low levels of *E. coli* made it challenging to detect levels in a single test. Therefore, water samples were conducted in the harvest of 2021 and 2022. Lab analysis is complete and statistical analysis will occur over the winter of 2022. Due to the difference in testing procedures, it was possible to collect all apple samples in 2021. As seen in Figure 7a, approximately 94% (85-98%) of the apple samples in the packing house and 84% (70-93%) in the field are coliform free before any drench treatment. However, post drench treatment, 6% (2-17%) of the apple samples in the packing house and 94% (84-98%) of the apple samples in the field are coliform free after treatment. This is a significant ($p>0.001$) decrease for the packing house with a 87% (75-94%) decrease. Although there is a 9% difference (0.8- 20.5%) for the field drencher, pre and post treatments are not significantly different to each other.

Of the samples that tested positive for Coliform, some also showed *E. coli* populations. Nearly 100% (96-100%) of the packing house apple samples and 96% (86-99%) of the field apple samples were *E. coli* free on arrival. After the drench treatment, an estimated 93% (79-98%) of the packing house apple samples and 98% (92-99%) of the field apple samples were *E. coli* free. There was no significant different between pre- and post-spray application for either Drencher.

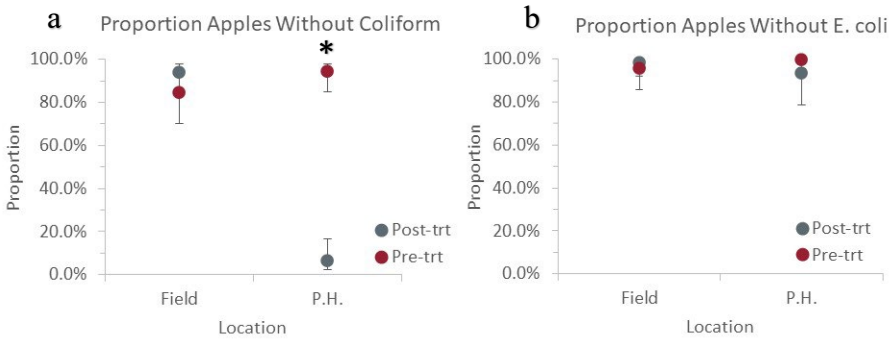

Figure 7. Proportion of apples without coliform (a) and *E. coli* (b) populations for apples pre- and post-drench treatment for Field and Packing House (P.H.) drenchers. There is a significant difference in apples with coliform (*=P-value>0.001) between the pre and post treatments in the packing house. While the field drencher showed no significant differences. And there was not a significant increase in apples with *E. coli* (b) pre or post drench for either treatment

For the subset of apples that did have contamination, the colony forming units (CFU) were compared pre and post spray applications. The mean CFUs for Coliform contaminated post application apple samples for field and packing house drenchers was 548 (127-2371) and 23899 (8255-69190), respectively (Fig 8a). For the field drencher, there is a non-significant 0.9-fold decrease in the CFUs for contaminated apples. In contrast, there is a 36.9-fold increase in the coliform CFUs for apples that tested positive for coliform. The mean CFUs for *E. coli* contaminated post application apple samples for field and packing house was 254 (51-1278) and 2288 (706-7417), respectively (Fig 8b). For apples from the field drencher, that is only 1.0 fold non-significant change in *E. coli* CFUs. Whereas apples from the packing house were nearly 100% free of coliform before treatment, the drench application introduces on average 2288 *E. coli* CFUs.

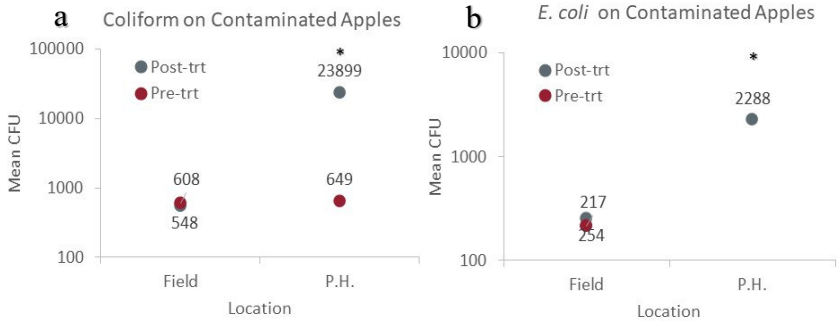

Figure 8. Considering only apples that were contaminated, this is the average colony forming units (CFU) of coliform (a) and *E. coli* (b). There was a significant difference (P> 0.001) between the mean pre and post applications in the packing house for both Coliform and *E. coli* CFUs. While the field drencher showed no change in coliform and *E. coli* CFUs

Future steps:

- By May 2023 — Obtain decay data from 2022 Honeycrisp and Gala apples
- 2023 — Conduct outreach activities
- Dec. 2023 — Provide a final report

Project Title: Understand and mitigate fungicide resistance in *Penicillium* spp.

Report Type: Continuing Project Report Year 3 NCE

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Cooperators: Prashant Swamy, Jonathan Puglisi, Rice Fruit, PA

Project Duration: 3 Years

Total Project Request for Year 1 Funding: $97,795
Total Project Request for Year 2 Funding: $92,068
Total Project Request for Year 3 Funding: $93,730

Other funding sources: Awarded
Amount: $9,643.20
Agency Name: State Horticultural Association of Pennsylvania
Notes: Awarded to co-PI Jurick II in 2018 entitled "Evaluating the efficacy of a new postharvest fungicide and developing tools to monitor fungicide resistance in blue mold populations."

WTFRC Budget: None

Budget 1

Primary PI: Achour Amiri
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Item	2020	2021	2022
Salaries[1]	40,925	42,562	44,264
Benefits[2]	13,464	14,003	14,563
Wages			
Benefits			
Equipment			
Supplies[3]	7,000	3,000	2,400
Travel[4]	885	885	885
Miscellaneous			
Plot Fees			
Total	$62,274	$60,450	$62,112

[1 & 2] Salaries for a Postdoc at 4872/month for 12 months at 0.7FTE and benefit rate of 32.9%. A 4% annual inflation is included for Year 2 and 3
[3] Supplies for lab work for fungal growth and maintenance, molecular reagent for detection and sequencing
[4] Travel to packinghouses for sampling and collaborative work for 1,500 miles a year at $0.59/mile

Budget 2:

Co-PI (2): Wayne M. Jurick II
Organization Name: USDA-ARS
Contract Administrator: Kristy Wallace
Telephone: 979-260-9659
Contract Administrator Email Address: Kristy.wallace@usda.gov

Item	2020	2021	2022
Salaries			
Benefits			
Wages[1]	$30,118	$30,118	$30,118
Benefits			
Equipment			
Supplies[2]	$4,900	$1,000	$1,000
Travel[3]	$500	$500	$500
Plot Fees			
Miscellaneous			
Total	$35,518	$31,618	$31,618

[1]Wages will be used to hire a GS-3 level employee to help conduct the research at USDA-ARS.
[2] Supplies for laboratory work including: genomic DNA isolation, library construction and whole genome sequencing, PCR, and media for fungal growth
[3] Travel for sampling packinghouses and collaborative work for 800 miles a year at $0.69/mile

OBJECTIVES

1. **Evaluate the pathogenic fitness of resistant populations having different fungicide resistance phenotypes.** Conidial germination assays indicated a fitness penalty to a lesser extent but the *in vivo* assay demonstrated that the resistant isolates were more aggressive in disease establishment. The objective is being accomplished and projected to be completed in 2022.
2. **Determine the genetic makeup of *Penicillium* species exhibiting various fungicide-resistant phenotypes to postharvest fungicides.** We identified single, double, and triple fungicide resistant *P. expansum* isolates, and single spore cultures were obtained. Each isolate was confirmed to be *P. expansum* by sequencing of three DNA bar code genes. Elucidation of genome sequences and analysis of resistant phenotypes is underway. One genome has been assembled with a fully sensitive phenotype and 3 other *P. expansum* isolates with single and double resistance to TBZ and FLU that have been sequenced. Assembly and annotation of these genomes is in progress.
3. **Assess the efficiency of various approaches to mitigate resistance in *Penicillium* spp.** Due to limited physical resources and access to commercial facilities due to the ongoing pandemic, we could not accomplish annual and two-year fungicide rotation experiments. However, we screened several chemo-sensitizing agents (CSA) to be used in mitigation strategies and identified four CSAs that are being further evaluated.

Significant findings

- 24 *Penicillium expansum* isolates with resistance to single-, double, or three fungicides were identified from commercial west coast packinghouses and their fitness cost was assessed *in vitro* and *in vivo*.
- Preliminary *in vivo* trials on Gala apples showed that fungicide- resistant isolates can outcompete sensitive isolates
- Three major *Penicillium* species, apart from *P. expansum,* are found to be abundant in the PNW packinghouses. Interestingly, these *Penicillium* species have different sensitivities to the current postharvest fungicides.
- Eight chemo-sensitizing agents (CSA) were screened, and four potential CSAs were selected for their use in mitigating fungicide resistance of *P. expansum* isolates with varying fungicide resistance profiles.
- All isolates identified from Mid-Atlantic and East coast are *P. expansum* as determined by whole genome sequence analysis except for three that were deemed *P. solitum*
- Whole genome sequence data has been obtained for a total of 28 isolates encompassing fully sensitive, single and double, and triple resistant *P. expansum* isolates. A mutation (E198K) was found to correlate with TBZ resistance in *P. solitum* and was not observed in our samples representing *P. expansum*.
- None of the isolates examined at the genome level contained known mutations in CYP51A1 that correlate with difenoconazole resistance.
- Known mutations in the Mrr1 or MDL1 genes, that correlate with multiple drug resistance phenotypes, were not discovered.
- The patulin gene cluster was observed in two of the isolates (data mining on all isolates not yet complete) but indicates their potential to produce patulin

METHODS

OBJECTIVE 1. Evaluate pathogenic fitness of resistant populations having different fungicide resistance phenotypes.

Activity 1.1 *In vitro* fitness parameters study: To determine if fungicide-resistant isolates exhibits reduced ability to grow, sporulate, and proliferate, their fitness will be evaluated using a variety of *in vitro* (artificial media) tests including: colony radial growth, spore production, and conidial germination. Isolates will be used to evaluate mycelial growth by measuring colony diameters using a digital micrometer and dividing by the total number of days (mm/day). Research has shown that different resistant phenotypes can be affected by osmotic stress (OS)and reactive oxygen species (ROS) that can be produced by the host during the infection. To test for OS, the isolates will be grown on PDA amended by 6% sodium chloride and growth will be recorded as described above after 6 and 10 days incubation. For ROS assay, isolates will be amended with 10, 20 and 40 mM of Paraquat and growth will be evaluated as described for OS effect. To test for conidial germination, conidia from 7-day old cultures growing on PDA plates will be harvested using sterile Tween-treated water (TTW) and conidial production determined using a hemacytometer. One hundred microliters of spore suspension from each plate will be adjusted to 100,000 conidia/ml and spread onto the surface of agar PDA plates in triplicate for each isolate. Following a 24-hour incubation at 20°C (68ºF) in a temperature-controlled incubator, one hundred conidia per plate will be assessed for germination under a light microscope. Only conidia with germ tubes 2.5 times the diameter of the spore will be considered germinated and percent germination will be calculated. It will be important to determine how persistent fungicide-resistant *Penicillium* spp. isolates can be in storage, where the fungus may get established and be exposed to other chemistries overtime. Hence, the stability of fungicide resistant phenotypes in *Penicillium* spp. will be carried out by culturing the fungus for 5-10 cultural generations (transfers) on defined medium without fungicide. The last generation will then be grown on unamended and medium containing discriminatory doses of each active ingredient (1ppm for PYR, 10ppm for TBZ, 0.5 ppm for FDL), for 7 days at 20°C (68ºF), to determine if the fungus is capable of retaining resistance in the absence of selection pressure (Jurick et al., 2019; Li and Xiao, 2008). Growth will be assessed and colony diameters on plates will be recorded.

Activity 1.2. *In vivo* fitness study: To assess the practical impact of fungicide resistance in *Penicillium* spp. to cause decay failures on fungicide-treated fruit after harvest, *Penicillium* spp. isolates (Table 1b) will be analyzed *in vivo* (on fruit) for their ability to incite decay and sporulate on fungicide-treated and untreated apple fruit. Organic apples and will be harvested a commercial maturity and stored at 1°C until the experiment is conducted. Apples will be surface disinfested for 5 min in 0.6% sodium hypochlorite solution, rinsed three times with sterile water, and air-dried. Each fruit will be wounded with the point of a 3-mm-diameter finishing nail to a 3mm depth. Approximately 1 hour after treatment, fruit will be dipped for 2 min with constant agitation in either sterile water as a positive control or in one of the three following fungicide solutions according to the labeled rate indicated by the manufacturer: Mertect® 340F (Syngenta Crop Protection, Greensboro, NC), Scholar® (Syngenta Crop Protection, Greensboro, NC), and Penbotec 400SC™ (Janssen Pharmaceutica, Belgium). Conidial suspensions (100,000 conidia/ml) will be obtained from 7-day-old PDA cultures (Table 1) grown at 20°C (68ºF) and used for inoculations. Each fruit will be inoculated by pipetting 25 µl of the conidial suspension from each isolate with a micropipette into each wound. Fruit will be air-dried for 30 min after dip treatment and then placed on fruit trays and stored in cardboard boxes at 1°C (34ºF) to mimic commercial storage conditions. Fruit will be assessed monthly for decay, and each treatment will have three replicate trays each containing 20 fruit. Lesion diameters will be measured with a digital

micrometer (% severity) and fruit with decay (% incidence) will be calculated and recorded (Jurick et al., 2011). Sporulation of each isolate will be assessed by swirling a sterile cotton swab around the lesion in a way to collect all spores. The swab will be washed in 5 ml of sterile water and the number of spores for each isolate/fungicide treatment will be assessed using a hemacytometer. All experiments will be conducted three times each having three replications. Data from all three experiments will be included in one analysis for differences between mycelial growth rate, conidial production, conidial germination, and lesion sizes between treatments by generalized linear analysis of variance in SAS (GLM ANOVA) and means will be compared by the Fisher's protected LSD at $P \leq 0.05$.

OBJECTIVE 2. Determine the genomic makeup of *Penicillium* species with various fungicide resistance phenotypes to postharvest fungicides.

Activity 2.1. Identification of new *Penicillium* species found in the PNW. Isolates stored in Amiri'sLab will be identified using a combination of classical mycological and genetic methods. Morphological methods will be carried out as described by Visagie et al., 2014. Briefly, isolates will be plated as conidial suspensions on 3 different media (CYA, MEA and YES) and incubated at 25, 30 and 37°C (77, 86, and 99ºF) for 7 days. Photos will be taken, and radial growth measured corresponding to colony diameter. Stipe morphology, spore size, and other characteristics specific to a given species will be analyzed. When appropriate, Ehrlichs test for alkaloids will be conducted as an additional diagnostic key to help with speciation. Five different and diagnostic molecular DNA loci corresponding to beta tubulin, calmodulin, RNA polymerase B, glyceraldehyde 3-phostphate dehydrogenase, heat shock protein 60 will be amplified by PCR, sequenced, and used for homology search to compare with known species. Individual sequences will be used to generate phylogenetic trees to assess new species relationships with known blue mold species that cause apple postharvest decay.

Activity 2.2. Elucidate whole genome sequences of *Penicillium* isolates with different fungicide resistance phenotypes. Next generation sequencing technology (Illumina) will be utilized to obtain the whole genome sequences of 16 *Penicillium* spp. isolates based on differences in their fungicide resistance profiles (Table 1a,b). The need for whole genome sequences is justified by the fact that assembled and annotated *Penicillium* spp. genomes will serve as platforms to investigate comparative transcriptomic (proteins) studies to ascertain the expression of specific genes involved in fungicide resistance in future studies. Such information can also provide global clues as to the strategies utilized by the fungus to overcome fungicide treatment, which can then be exploited to help maintain efficacy of current materials. It is expected that whole genome comparisons will provide specific information on genes involved in fungicide resistance, provide markers for designing detection strategies, and gain insights into the mechanism(s) of resistance so that new management strategies and materials can be applied to inhibit the development of fungicide resistant isolates. Such knowledge cannot be acquired by looking at partial sequences of the genome.

Individual PDA (Potato Dextrose Agar) plates will be inoculated with conidia of each isolate and grown in a temperature-controlled incubator at 20°C (68ºF) for 7 days. Conidia will be harvested from 7-day-old PDA Petri plates using 2 ml of 0.05% Tween 20-treated water and adjusted to 1 x 10^5 conidia/ml. One hundred microliters of conidial suspensions (approximately 1000 conidia) of each isolate will be added to 125 ml of Potato Dextrose Broth (PDB) in 250 ml flasks and grown at 20ºC (68ºF) for 7 days on a rotary shaker at 150 rpm. The mycelium will be harvested using a Büchner funnel and immediately frozen in liquid nitrogen. Genomic DNA will be prepared with DNeasy Plant Maxi Kit (Qiagen) according to manufacturer's instructions. Raw sequence reads will be generated by the Illumina MiSeq platform. The sequence is expected to reach 80X to 100X using 250 bp paired end reads. All reads will be used to generate *de novo* assemblies with Velvet Optimiser 2.2.0 and HGAP3. The quality of the

final assembly should be reflected by the resulting number of scaffolds, which is indicative of a high-quality assembly. In addition to *in silico* gene prediction, RNA-seq data from *P. expansum* will be used to validate the gene prediction models during genome annotation. Protein evidence from *P. expansum* (R19, Yu et al., 2014) will be used as references to assist the final annotation.

Comparisons between *Penicillium* spp. with differences in fungicide resistance will yield Single Nucleotide Polymorphisms (SNPs), insertions, and/or deletions in specific genes that can then be targeted to design nucleic acid-based detection methods. Whole genome comparisons will be accomplished using MCL clustering with default settings to generate unique gene and SNP sets between sensitive and resistant isolates. The fungicide-resistant specific genes will be annotated using Interproscan 5. Secondary metabolic gene clusters will be analyzed using SMURF and AntiSMASH to determine patulin, citrinin and penicillic acid genes to indicate the toxigenic potential of each isolate.

OBJECTIVE 3. Assess the efficacy of various approaches to mitigate resistance in *Penicillium* spp.

Activity 3.1. Effect of annual and two-year annual rotations on control efficacy and fungicide resistance development.

Progress on activity 3.1. This research activity is only possible in commercial storage rooms. It was not possible to find a collaborator who would allow us to carry-out this activity at their packinghouse. We will try to identify another collaborator in 2022, if not this activity will be canceled.

Activity 3.2. Efficacy of chemo-sensitizers in reducing or reversing sensitivity phenotypes of resistant *Penicillium* populations. Chemosensitization is a process used to render an organism, (like fungi or bacteria), that has developed resistance to a given fungicide or a drug more vulnerable to commercial fungicides. Our objective is to evaluate the ability of some known chemosensitizing agents (CSAs) to overcome resistance of *Penicillium expansum* to TBZ, PYR and FDL. Trials will include *in vitro* experiments to screen a number of these chemosensitizers from which the most efficient ones will be selected and tested *in vivo* on detached fruit in a second set of proposed experiments.

Activity 3.2.1. *In vitro* chemosensitization. The agents listed in Table 1 will be tested at different concentrations against the *P. expansum* isolates with different sensitivity phenotypes listed in Table 2. The CSAs will be tested alone and in combination with TBZ, PYR or FDL on PDA medium amended with the chemicals at different concentrations to be determined after preliminary tests in the lab have been completed. The amended plates will be inoculated with mycelial plugs of the 16 isolates listed in Table 1 and 3 plates per isolate will be used for each chemosensitizer and fungicide combination. The CSAs will be also tested for their efficacy to sensitize spores. For this, amended plates will be inoculated with three-100 microliter (µl) droplets of spore suspensions (100,000 spores/ml) of each isolate. Plates inoculated with mycelial plugs will be incubated at 20°C (68°F) and 1°C (34°F) for 5 and 30 days, respectively, after which the growth the mycelia will be measured. Plates inoculated with spore suspensions will be observed under a microscope after 24 hrs incubation. If no germination is observed, plates will be incubated longer and checked periodically for germination. Non-amended plates or plates amended with discriminatory doses of TBZ, PYR, or FDL without the CSAs will be used as controls for comparison.

Activity 3.2.2. *In vivo* chemosensitization on detached apple fruit. The most effective CSAs from the *in vitro* study (Activity 3.2.1) will be selected to be evaluated for their ability to increase efficacy of TBZ, PYR, and FDL on fruit. Commercially ripe 'Fuji' apples will be harvested from experimental orchards in Wenatchee and PA. Fruit will be surface sterilized in sodium hypochlorite then rinsed with

sterile water and air dried. Fruit will be wounded at the equator using a needle to make 3x3 mm wounds. Wounded fruit will be immediately dipped for 1 minute in solutions containing the following treatments:

- Control — Sterile water
- Fungicides alone — TBZ, PYR or FDL
- Chemosensitizers alone — CSAs
- Fungicides + CSAs — TBZ + CSAs/ PYR +CSAs / FDL + CSAs

After the dip treatment, fruit will be placed on trays to air-dry. After 2 hours, the wounds will be inoculated with 25 µl of a spore suspension at 50,000 spores/ml of each isolate from the ones listed in Table 1. Fruit will be stored in regular atmosphere at 34°F and monitored on a monthly basis to assess blue mold incidence and severity. If blue mold lesions are observed, the fungus will be re-isolated on PDA plates and will be re-tested for sensitivity to a given fungicide using established discriminatory doses to verify changes in sensitivity phenotypes.

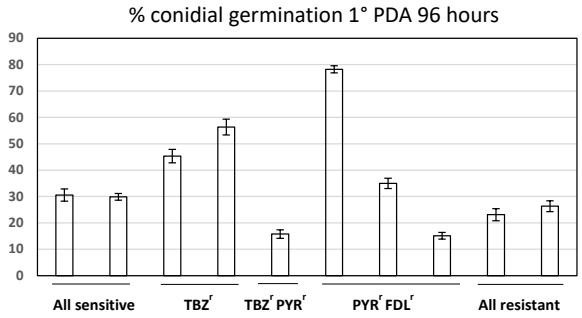
Table 1. List of chemosensitizer agents (CSAs) to be tested in this project

	Sensitizer name	Origin	Potential interaction/target
1	Cinnamaldehyde	Plant	Synergistic/cell membrane
2	Octylgallate	Food preservative	Synergistic/overcome resistance
3	Berberine	Plant	Synergistic/oxidative stress
4	2,5 dihydroxybenzoic acid	Plant	Synergistic/glutathione homeostasis
5	Carvacrol	Plant	Synergistic/ion homeostasis
6	Cinnamic Acid	Plant	Synergistic
7	Curcumin	Plant	Synergistic/oxidative stress
8	Thymol	Plant	Synergistic/drug efflux
9	CTBT	Synthetic	Synergistic/oxidative stress
10	Alkyl guanidine	Synthetic	Synergistic/oxidative stress/plasma membrane

Results and discussion

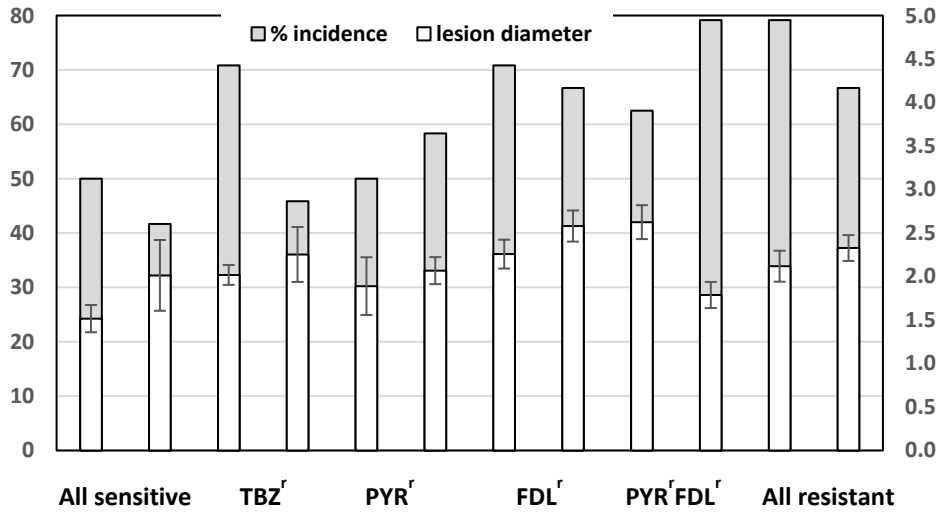
1. Fitness evaluations

We have identified 18 isolates of *Penicillium expansum* isolated from packinghouses in the pacific northwest (PNW) with resistance to thiabendazole (TBZ), pyrimethanil (PYR), and fludioxonil (FDL), as single, double, or triple resistant isolates. Isolate fitness was evaluated both *in vitro* and *in vivo*. *In vitro* experiments were carried out using spore suspensions (100,000 conidia ml^{-1}). Conidial germination assay was carried out on potato dextrose agar (PDA) at 1°C. Many dual- and triple-resistant isolates exhibited restricted germination compared to germination in controls (Fig 1).



Mycelial growth and response to oxidative stress were assessed at both 20° and 1°C. Osmotic stress was measured on PDA amended with 6% NaCl. While mycelial growth and response to oxidative stress among all isolates did not differ, the osmotic stress assay suggested a slight fitness penalty at 1°C.

In a preliminary experiment, the virulence of *P. expansum* fungicide-resistant isolates was assessed *in vivo* on Gala fruit by measuring lesion diameter after 60 days at 1°C. Results from the experiment indicated that resistant isolates grow more aggressively than fungicide-sensitive isolates in storage conditions (Figure 2).



Activity 2.1. The genetic makeup of *Penicillium* isolates

The *Penicillium* isolates that did not exhibit characteristics "*expansum-like"* symptoms when grown on fungal isolation media are referred to as "other" isolates. From a large collection of 967 isolates, we included a total of 644 isolates that grew within 7 days on the PDA medium. The characteristics of each isolate grown on PDA were used in subsequent evaluations. . Further, isolates were grouped according to several phenotypic characters, and a representative isolate from each group was obtained as pure culture (single spored) for molecular analysis. The underlying species identification of such isolates was carried out using classical mycological and molecular techniques. The visual distinctions used in the grouping of 'other' isolates included the predominant color of the media 10 dpi (colorless, green tint, or orange), the color of the fungal colony (dark green, tan/green, or cream), colony appearance (flat or raised), size of the colony (<2 cm, 2.5-3 cm or >3.5 cm), and the color of the colony on the reverse side of the plate. Based on these criteria, 166 isolates were chosen for DNA extraction and sequencing of marker genes for species identification. The isolates were grown on PDA, CYA, YES, and MEA for 10 days for comparison purposes. The phenotypic characteristics of major *Penicillium* species are shown in Figure 3.

The DNA sequencing results confirmed that the predominant *Penicillium* species in the PNW was *P. expansum* followed by *P. solitum, P. roqueforti, and P. commune.*

Overall, we identified at least three major species that are currently scrutinized for fungicide resistance against commonly used postharvest fungicides. Five different doses of FDL (0.1, 0.5, 1, 5 and 10 ppm), PYR (0.1, 0.5, 1, 5 and 10 ppm), and TBZ (1, 5, 10, 50 and 100 ppm) were used to assess fungicide sensitivities of major species. The results indicated that a large percentage of major non-expansum species developed a high level of resistance against three commercially applied fungicides

Activity 2.2. Elucidate whole genome sequences of *Penicillium* isolates with different fungicide resistance phenotypes.

We have identified isolates with varying levels of resistance to postharvest fungicides (Tables 2). These isolates were obtained from commercial packinghouses in WA, OR, PA and MD from infected fruit and cull piles. Single spore isolates were obtained, and glycerol stocks were preserved for each isolate. High quality genomic DNA was isolated for each isolate and quantified using gel and spectrophotometric methods. Intact DNA was then used to make libraries for NGS Illumina HiSeq 150bp paired end reads. Twenty-nine isolates have their genomes sequenced, assembled and annotated. Common mutations in B-tub locus have been identified and correlate 100% with resistance phenotypes. We have observed no mutations in the CY51A1 genomes of these 29 isolates, so they should be controlled by postharvest fungicides containing difenoconazole labeled for pome fruit (e.g. Academy). No mutations in common genes (MDL1, Mrr1) were detected as well.

Table 2. Isolates *P. expansum* obtained from commercial storage in the Mid-Atlantic (MD, PA, WV) and Pacific Northwest (WA, OR) regions for their fungicide phenotypes and whole genome sequence analysis.

Region	Isolate #	Phenotype	Total Number of Raw Sequences	GC% Total Reads	Mutation in B-tubulin
Mid-Atlantic	ARS1	$TBZ^{R}PYR^{S}FDL^{S}$	15,864,382	47.4	Yes (E198V, L240F)
	ARS2	$TBZ^{R}PYR^{S}FDL^{S}$	15,779,790	47.3	Yes (E198V, L240F)
	ARS3	$TBZ^{S}PYR^{S}FDL^{R}$	16,053,534	46.7	No
	ARS6	$TBZ^{S}PYR^{R}FDL^{S}$	16,070,506	47.1	No
	ARS11	$TBZ^{R}PYR^{s}FDL^{R}$	16,187,888	46.8	Yes (E198A)
	ARS15	$TBZ^{S}PYR^{S}FDL^{S}$	17,755,220	47.2	No
	ARS16	$TBZ^{S}PYR^{S}FDL^{S}$	16,460,740	46.6	No
PNW	219	$\mathbf{TBZ^{R}}PYR^{S}FDL^{S}$	16,053,974	47.0	Yes (E198V, L240F)
	184	$\mathbf{TBZ^{R}}PYR^{S}FDL^{S}$	16,178,330	46.9	Yes (E198V, L240F)
	23	$\mathbf{TBZ^{R}}PYR^{S}FDL^{S}$	16,280,196	47.1	Yes (E198V, L240F)
	2570	$TBZ^{S}PYR^{S}\mathbf{FDL^{R}}$	16,042,060	47.2	No
	2558	$TBZ^{S}PYR^{S}\mathbf{FDL^{R}}$	16,062,764	47.7	No
	2555	$TBZ^{S}PYR^{S}\mathbf{FDL^{R}}$	16,101,260	47.2	No
	2483	$\mathbf{TBZ^{R}PYR^{R}}FDL^{S}$	16,052,898	47.1	Yes (E198V, L240F)
	2311	$\mathbf{TBZ^{R}PYR^{R}}FDL^{S}$	16,301,890	47.5	Yes (E198V, L240F)
	8	$\mathbf{TBZ^{R}PYR^{R}}FDL^{S}$	16,306,660	47.0	Yes (E198V, L240F)
	2501	$TBZ^{s}\mathbf{PYR^{R}FDL^{R}}$	16,037,532	47.0	No
	153	$TBZ^{s}\mathbf{PYR^{R}FDL^{R}}$	15,117,556	47.6	No (G235G, silent)
	2517	$TBZ^{s}\mathbf{PYR^{R}FDL^{R}}$	16,045,202	47.6	No
	164-5-48	$\mathbf{TBZ^{R}}PYR^{s}\mathbf{FDL^{R}}$	16,029,930	47.4	Yes (E198K)
	164-4-39	$\mathbf{TBZ^{R}}PYR^{s}\mathbf{FDL^{R}}$	16,152,548	47.1	Yes (E198K)
	162-5-42	$\mathbf{TBZ^{R}}PYR^{s}\mathbf{FDL^{R}}$	16,000,486	47.4	Yes (E198K, L240F)
	3045	$\mathbf{TBZ^{R}PYR^{R}FDL^{R}}$	16,184,410	47.2	Yes (F167Y), G235G*
	2754	$\mathbf{TBZ^{R}PYR^{R}FDL^{R}}$	15,118,376	46.8	Yes (F167Y), G235G*
	1020	$\mathbf{TBZ^{R}PYR^{R}FDL^{R}}$	16,135,502	47.1	Yes (E198V, L240F)
	1267	$TBZ^{S}PYR^{S}FDL^{S}$	16,203,278	47.3	No
	40	$TBZ^{S}PYR^{S}FDL^{S}$	16,039,432	46.9	No
	3339	$TBZ^{S}PYR^{S}FDL^{S}$	16,024,584	47.1	No

*: silent mutation, not associated with fungicide resistance

Activity 3.2. Chemo-sensitizing approaches to mitigate fungicide resistance in *Penicillium* spp.

Eight CSA were tested for *in vitro* applications to overcome fungicide resistance (Table 1). *P. expansum* isolates with varying sensitivities to commercial fungicides (FDL, PYR, and TBA) were selected for chemo-sensitizing spores. Various concentrations of all CSA were used to determine optimum CSA efficiency. The efficacy of CSAs was determined based on the germination test and growth inhibition percentage. Based on the *in vitro* CSA study, four CSAs (cinnamaldehyde, carvacrol, octyl gallate, and thymol) were further chosen for *in vivo* testing on Gala apples. Preliminary i*n vivo* testing of representative isolates and four CSA were conducted on Gala apples in 2021. Initial screening indicated

good efficacy of carvacrol and cinnamaldehyde on two isolates. A more detailed study will be undertaken in 2022.

Projected experiments in 2022

- Complete and summarize *in vitro* and *in vivo* evaluation of pathogenic fitness of resistant *Penicillium* spp. populations.
- Complete *in vivo* analysis of short-listed CSAs and determine the efficacy of CSAs
- Phenotyping additional *Penicillium expansum* isolates to identify 1 remaining/elusive dual fungicide phenotype TBZ/FLU resistance category (need 2 total), for gDNA extraction, sequencing, assembly and annotation
- Submit assembled genome sequences to NCBI for public database establishment and BioProject development.
- Complete mining genome sequence data for SNPs, Indels and other sequence variants that correlate with fungicide resistance phenotypes.
- Develop Secondary Metabolite (SM) gene cluster profiles for each isolate to determine their toxigenic potential for producing patulin, citrinin and penicilic acid.
- Submit peer-review publications and communicate the results of the study with stakeholders.

Presentations describing research data from this project

1. Puglisi and Amiri. 2023. *In vitro* and *in vivo* phenotypic characterization of fitness cost in fungicide-resistant *Penicillium expansum* isolates. OPDMC. Portland, OR, Jan 12. 2023.
2. Puglisi and Amiri. 2022. Assessing Fitness cost in *Penicillium expansum* isolates with resistance to multiple fungicides. Annual meeting of the American Phytopathological Society. Pittsburgh, PA.
3. Puglisi, J., Swamy, P., Jurick, W., and Amiri, A. 2021. Evaluation of pathogen fitness in the context of fungicide resistance in *Penicillium expansum,* the causal organism of blue mold in pomes. PlantHealth 2021
4. Swamy, P., Sielaff, Z., and Amiri, A. 2021. Emerging *Penicillium* species in commercial apple packinghouses in the Pacific Northwest. PlantHealth 2021
5. Swamy, P. Four major *Penicillium* species cause blue mold of pomes in Washington packinghouses. Hort Show, WSTFA 2021
6. Puglisi, J. Assessing fitness cost in *Penicillium expansum* isolates with resistance to multiple fungicides. Hort Show, WSTFA 2021.

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

WTFRC Project Number: AP-18-104A

Report Type: No-Cost Extension

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

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $118,779
Total Project Request for Year 2 Funding: $121,797
Total Project Request for Year 3 Funding: $125,404

WTFRC Collaborative Costs:

Item	2018	2019	2020	2022
Salaries	4,141	4,224	4,308	
Benefits	1,367	1,394	1,422	
Wages	4,500	4,703	5,267	
Benefits	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

Budget 1
Primary PI: Meijun Zhu
Organization Name: Washington State University
Contract Administrator: Anastasia Kailyn Mondy
Telephone: 509-335-4563
Contract administrator email address: arcgrants@wsu.edu

Item	2018	2019	2020	2022
Salaries	37,124	38,609	40,154	
Benefits	12,412	12,909	13,424	
Wages	15,340	15,953	16,592	
Benefits	1,094	1,137	1,183	
Equipment	0	0	0	
Supplies	25,500	25,500	25,500	
Travel	2,000	2,000	2,000	
Miscellaneous	5,000	5,000	5,000	
Plot Fees	0	0	0	
Total	98,470	101,108	103,853	0

OBJECTIVES

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

SIGNIFICANT FINDINGS

1. The greater die-off rate of *Listeria innocua* on fresh apples was observed at the first 6 weeks regardless of apple varieties (Granny Smith and Red delicious) and 1-MCP application. There was a 1.5-2.0 log reduction after 6 weeks of cold storage under commercial RA or CA storage.

   The die-off rate of *Listeria* on apples was reduced after 6 weeks of RA or CA storage. There was a 2.2-3.0 log reduction after 36 weeks of CA storage.
2. A similar die-off rate of *Listeria* on apples under CA storage with ozone gas as that of RA/CA storage during 6 weeks of storage. During the first 6 weeks of storage, the concentration of ozone gas gradually increases to reach the target concentration.
3. The application of ozone gas facilitates the die-off during 6-24 weeks of storage regardless of 1-MCP pretreatment and apple varieties (Granny Smith, Red Delicious, and WA38).
4. A 36-week storage ozone gas application caused an additional 2.5~3.0 log CFU/apple reduction on Granny Smith and Red Delicious apples compared to CA alone.
5. For WA 38 storage study, gaseous ozone was only applied during the first 24 weeks of storage followed by regular CA for additional 12 weeks. It achieved a similar anti-*Listeria* efficacy as Granny Smith and Red Delicious apples treated with 36 weeks of ozone gas application. It indicates ozone gas application can be shortened to 24 weeks.
6. Ozone gas at 50-87 ppb showed similar antimicrobial efficacy for all apple varieties tested.
7. The initial indigenous yeast/mold count of uninoculated apples was 4.5-5.0 $\log_{10}$ CFU/apple of the apple varieties tested. The yeast/mold counts remained stable during the first 12 weeks of RA regardless of apple varieties. By 24 weeks of storage and beyond, the yeast/mold counts on apples under RA were higher (Granny Smith and WA 38 apples) or similar (Red Delicious apples) compared to those during CA storage. Low doses of ozone gas application decreased yeast/mold counts on apples.
8. Continuous low-dose ozone gas application for 9 months or 6 months at 50-87 ppb did not cause negative impacts on fruit quality, or internal and external disorders of apples for all tested varieties. Granny Smith apples under CA storage could develop ozone burn-like symptoms.
9. Results on Granny Smith apples and Red Delicious apples have been published (Shen et al., 2021; Sheng et al., 2022). We are currently evaluating the behavior of a non-*Listeria* surrogate, *Enterococcus faecium* on WA38 apples under the same storage conditions.

METHODS

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

Objective 1. Assess the 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 the apple surface

A 3-strain *L. innocua* cocktail was prepared by mixing equal numbers of each respective strain into a suspension. Unwaxed and unbruised apples 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. For WA 38 storage study, gaseous ozone was only applied during the first 24 weeks of storage followed by regular CA for additional 12 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

On 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, 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 the fate of natural microorganisms on apple fruit surfaces when stored in refrigerated air or 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 weeks of storage for total plate count and yeast and mold enumeration.

2. Survival microorganism analysis

On 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 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 the sun and shade side of the apples. Total soluble solids were evaluated using Atago PR-32 digital Brix refractometer. The 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. The sample size for both external and internal disorder analyses was 50 apples per replication per storage regimen, with 4 replicates for each analysis.

RESULTS AND DISCUSSION

1. Survival of *L. innocua* on WA38 apples under RA, CA, and CA treated with low-dose ozone gas for 24 weeks.

The initial level of *L. innocua* on WA38 apples before storage was ~ 6.70 log_{10} CFU/apple. During the first 3 weeks of cold storage, the populations of *L. innocua* on apples were reduced by 1.6-1.7 log_{10} CFU/apple under all storage conditions, before the ozone concentration got to the target concentration (Fig. 1), which was slightly more reduction than that on Red Delicious apple (Shen et al., 2021). There was 3.4-3.5 log_{10} CFU/apple reduction of *L. innocua* on WA 38 apples under RA storage or CA storage with or without 1-MCP treatment during 36 weeks of storage (Fig. 1), which was higher than reductions observed on Granny Smith and Red Delicious apple (Shen et al., 2021; Sheng et al., 2022).

The continuous low-dose ozone gas application in CA cold storage is effective in facilitating the die-off of *L. innocua* on WA38 apples. It caused an additional 2.4-2.8 log_{10} CFU/apple reduction at the end of 24 weeks of storage when the ozone gas was withdrawn. Anti-*Listeria* efficacy of 24 weeks of gaseous ozone application was similar to that observed on Granny Smith and Red Delicious apples treated with 36 weeks of ozone gas application (Shen et al., 2021; Sheng et al., 2022).1-methylcyclopropene (1-MCP) treatment before storage had a minor effect on *L. innocua* survival on WA38 apples ($P > 0.05$).

2. Fates of resident microbiota on WA38 apples at different storage conditions.

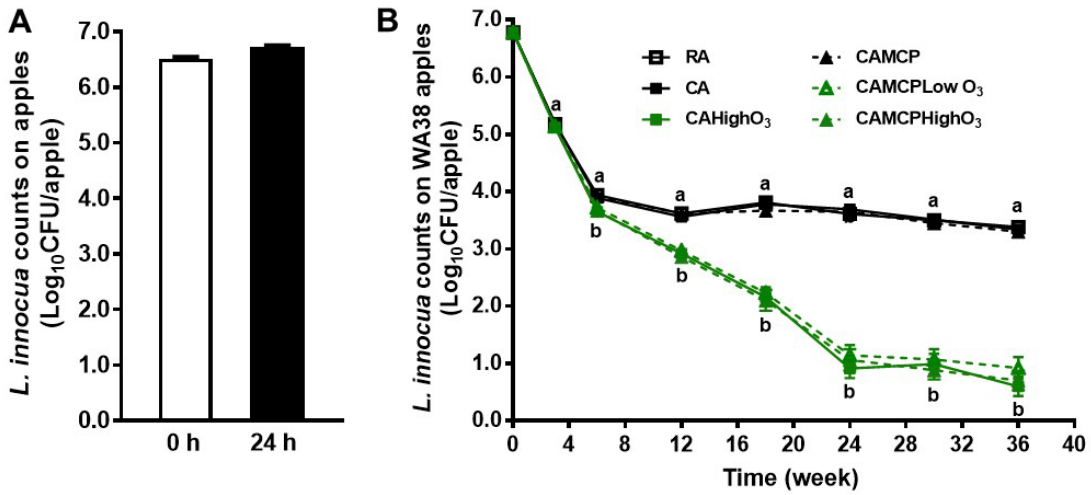
To evaluate the impacts of ozone gas application on the resident bacteria, mold and yeast counts on apples during storage, non-waxed and uninoculated apples were subjected to different storage conditions at the same condition as inoculated apples for total plate count (TPC) and yeast/mold (Y/M) enumeration. The initial apple resident microflora and indigenous yeast/mold count of the receiving WA38 apples were 3.75 log_{10} CFU/apple and 4.87 log_{10} CFU/apple, respectively, which are in the range of Granny Smith and Red Delicious apple (Shen et al., 2021; Sheng et al., 2022).

The resident bacteria on WA38 apples increased by 0.6-0.7 log_{10} CFU/apple under RA or CA storage by 12 weeks of storage (Fig. 2). Total plate counts on WA38 with low dose ozone treatment were first reduced by 1.2-1.3 log_{10} CFU/apple at 24 weeks of storage, then increased, but it was lower than that at harvest.

The yeast/mold counts of WA38 apples increased by 0.5-0.6 log_{10} CFU/apple after 36 weeks of RA or CA storages (Fig. 3). Yeast/mold counts reduced by 0.2-0.4 log_{10} CFU/apple on WA38 apples treated gaseous ozone for 24 weeks (Fig. 3).

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

The weight and TSS of apple fruits at 9 months of storage were not different from that at harvest regardless of storage conditions (Table 1). The firmness of WA38 apples after 9 months of CA storage with or without 1-MCP and gaseous ozone treatments was the same as that measured at harvest; however, the firmness of WA38 apples under RA storage was significantly reduced (Table 1). TA of WA38 apples after 9-month storage was significantly lower than that of apples at harvest regardless of storage treatments. Gaseous ozone application had no impact on TA (Table 2). No external disorder or internal disorder was observed on all WA38 apples at 9 months of storage (Data not shown).

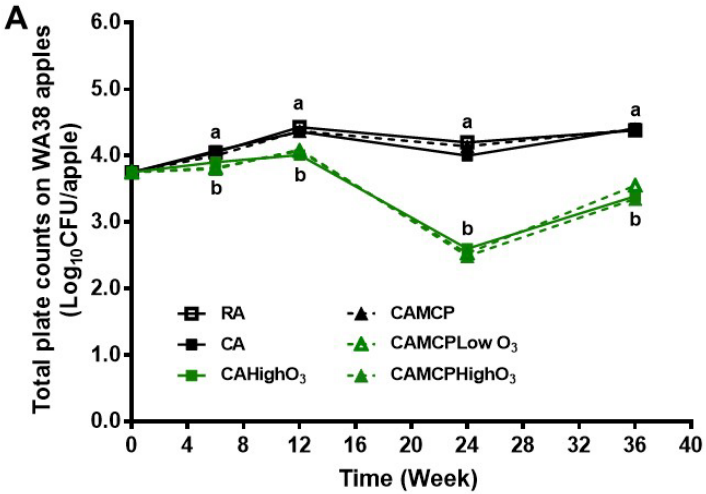


C

Bacterial reduction during storage on WA38 apples (log_{10}CFU/apples)

	RA	CA	CAHighO_3	CAMCP	CAMCPLowO_3	CAMCPHighO_3
Week	***L. innocua***	***L. innocua***	***L. innocua***	***L. innocua***	***L. innocua***	***L. innocua***
0	0.00 ± 0.00^A	0.00 ± 0.00^A	0.00 ± 0.00^A	0.00 ± 0.00^A	0.00 ± 0.00^A	0.00 ± 0.00^A
3	1.60 ± 0.07^A	1.59 ± 0.08^A	1.66 ± 0.07^A	1.61 ± 0.08^A	1.64 ± 0.07^A	1.64 ± 0.06^A
6	2.84 ± 0.05^A	2.89 ± 0.04^A	3.13 ± 0.04^A	2.87 ± 0.05^A	3.06 ± 0.03^A	3.11 ± 0.04^A
12	3.16 ± 0.05^A	3.22 ± 0.06^A	3.86 ± 0.07^A	3.16 ± 0.06^A	3.81 ± 0.06^A	3.92 ± 0.06^A
18	2.97 ± 0.06^A	3.00 ± 0.07^A	4.62 ± 0.14^A	3.11 ± 0.08^A	4.55 ± 0.10^A	4.68 ± 0.18^A
24	3.17 ± 0.05^A	3.09 ± 0.06^A	5.66 ± 0.18^A	3.13 ± 0.07^A	5.50 ± 0.20^A	5.72 ± 0.19^A
30	3.28 ± 0.05^A	3.27 ± 0.07^A	5.79 ± 0.18^A	3.33 ± 0.07^A	5.71 ± 0.18^A	5.90 ± 0.16^A
36	3.40 ± 0.05^A	3.44 ± 0.04^A	6.18 ± 0.17^A	3.48 ± 0.02^A	5.81 ± 0.19^A	6.08 ± 0.17^A

Figure 1. Fates of *L. innocua* on WA38 apples during 36 weeks of cold storage under different storage regimes. A. The initial bacterial population on apples; B. Survival of *L. innocua*; C. Reduction of *L. innocua*. RA: refrigerated atmosphere; CA: controlled atmosphere; MCP: apples were treated with 1-methycyclopropene prior to cold storage; CAHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 ppb; CAMCPHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 ppb, where apples were treated with 1-methycyclopropene prior to cold storage; CAMCPLowO_3: CA storage with continuous gaseous O_3 application at 55.5 ± 8.8 ppb, where apples were treated with 1-



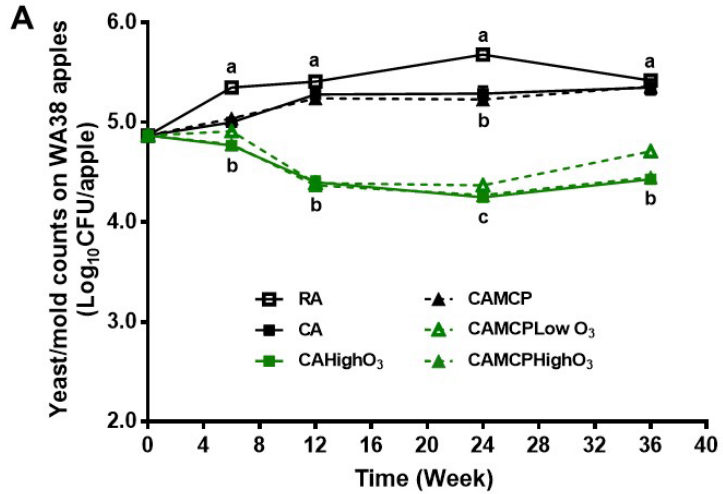
B

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

		CA		CA+1-MCP		
Week	RA	/	High O_3	/	Low O_3	High O_3
0	0.00	0.00	0.00	0.00	0.00	0.00
6	0.30	0.32	0.15	0.25	0.07	0.05
12	0.68	0.61	0.26	0.62	0.32	0.34
24	0.45	0.25	-1.15	0.39	-1.22	-1.26
36	0.63	0.66	-0.36	0.65	-0.20	-0.40

Negative values indicate a reduction of microbial counts.

Figure 2. Apple resident bacteria on WA38 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; CAHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 ppb; CAMCPHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 ppb, where apples were treated with MCP-1 prior to different storages;CAMCPLowO_3: CA storage with continuous gaseous O_3 application at 55.5 ± 8.8 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	/	High O_3	/	Low O_3	High O_3
0	0.00	0.00	0.00	0.00	0.00	0.00
6	0.48	0.13	-0.10	0.17	0.04	-0.09
12	0.54	0.41	-0.47	0.37	-0.48	-0.50
24	0.81	0.42	-0.62	0.36	-0.50	-0.60
36	0.55	0.48	-0.44	0.49	-0.16	-0.42

Negative values indicate a reduction of microbial counts.

Figure 3. Apple natural decay microorganisms on WA38 apples during 36 weeks 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 a 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 before cold storage; CAHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 ppb; CAMCPHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 ppb, where apples were treated with MCP-1 before different storages; CAMCPLowO_3: CA storage with continuous gaseous O_3 application at 55.5 ± 8.8 ppb, where apples were treated with 1-MCP before cold storage.

Table 1. Fruit quality attributes of WA38 apples at harvest and 9-month of cold storage under different conditions.

Treatment	Weight (kg)		Firmness (kg)		TSS (% Brix)		TA (% malic acid)	
	0-m	9-m	0-m	9-m	0-m	9-m	0-m	9-m
RA	0.26 ± 0.05^{A}	0.25 ± 0.04^{aA}	8.33 ± 0.16^{A}	6.22 ± 0.13^{aB}	NA	14.25 ± 0.25^{a}	0.77 ± 0.03^{A}	0.26 ± 0.02^{aB}
CA		0.26 ± 0.06^{aA}		8.00 ± 0.11^{bA}		15.05 ± 0.15^{a}		0.34 ± 0.02^{bB}
CAMCP		0.24 ± 0.03^{aA}		8.22 ± 0.08^{bA}		14.43 ± 0.17^{a}		0.41 ± 0.00^{bB}
CAMCPLowO_3		0.26 ± 0.04^{aA}		8.24 ± 0.09^{bA}		14.18 ± 0.15^{a}		0.37 ± 0.04^{bB}
CAMCPHighO_3		0.25 ± 0.05^{aA}		8.29 ± 0.10^{bA}		14.38 ± 0.27^{a}		0.41 ± 0.03^{bB}
CAHighO_3		0.26 ± 0.05^{aA}		8.12 ± 0.07^{bA}		14.55 ± 0.19^{a}		0.37 ± 0.09^{abB}

The average diameter is 78.3-81.8 cm, which is not different among treatments before and after storage. TSS: Total soluble solids; TA: titratable acidity. RA: refrigerated atmosphere; CA: controlled atmosphere; MCP: 1-methycyclopropene; CAHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 µg/L; CAMCPHighO_3: CA storage with continuous gaseous O_3 application at 78.2 ± 12.2 µg/L, where apples were treated with MCP-1 before subjecting to storage; CAMCPLowO_3: CA storage with continuous gaseous O_3 application at 55.5 ± 8.8 µg/L, where apples were treated with MCP-1 before subjecting to storage. $^{a-d}$Mean within a column of the selected quality attribute without acommon letter differ significantly ($P < 0.05$). $^{A-B}$ Mean the comparison of an individual quality parameter at 0-month (at-harvest) and 9-month storage without common letter differ significantly ($P < 0.05$). Mean ± SEM, n=40.

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Project/Proposal Title: Interaction of microbiome and *Listeria* on apples during cold storage

Report Type: Continuing Project Report

WTFRC Project Number: AP-20-100A

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Cooperators: Stemilt Growers LLC.; Allan Brothers Inc., Hansen Fruit

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $84,704
Total Project Request for Year 2 Funding: $86,853
Total Project Request for Year 3 Funding: $88,076

WTFRC Collaborative Costs:

Item	2020-2021	2021-2022	2022-2023
Salaries			
Benefits			
Wages	2,509	2,548	2,588
Benefits	1,280	1,272	1,284
RCA Room Rental	6,750	6,952	7,160
Shipping			
Supplies	500	525	550
Travel	650	700	725
Plot Fees			
Miscellaneous			
Total	11,689	11,997	12,307

Footnotes:

Budget 1
Primary PI: Meijun Zhu
Organization Name: Washington State University
Contract Administrator: Anastasia Kailyn Mondy
Telephone:
Contract administrator email address: arcgrants@wsu.edu

Item	2020-2021	2021-2022	2022-2023
Salaries	31,500	32,760	34,070
Benefits	11,010	11,451	11,909
Wages	3,200	3,328	3,461
Benefits	305	317	329
Equipment			
Supplies	16,000	16,000	15,000
Travel	2,000	2,000	2,000
Miscellaneous	9,000	9,000	9,000
Plot Fees			
Total	73,015	74,856	75,769

OBJECTIVES

1. Examine survival of resident microbiota on apple surfaces stored under RA and CA.
2. Characterize the dynamic change of dominant and differential bacterial and fungal populations in the microbiome of fresh apples in the co-occurrence of *Listeria* under RA or CA storage.

SIGNIFICANT FINDINGS

1. The *L. innocua* count was decreased by 1.5 $\log_{10}$ CFU/apple on apples under RA or CA storage.
2. *Enterobacteriaceae* had higher counts on apples contaminated with *L. innocua* than uninoculated control apples at RA or CA storage.

   The *Enterobacteriaceae* count was lower on apples under CA storage than those under RA storage.
3. The count of *Pseudomonas* decreased during 36 weeks of cold storage. Introducing *L. innocua* on apples increased the reduction of *Pseudomonas* on apples, especially under CA storage.
4. Lactic acid bacteria count on apples slightly increased after 36 weeks of cold storage regardless of storage condition.
5. Populations of native bacteria and yeast and molds, particularly *Penicillium*, were increased on apples with or without *L. innocua* inoculation after 36 weeks of RA or CA storage.
6. A 9-month CA or RA storage had a great influence on fungal community structure; these significant differences were found on the phylum level, family level, genus level, and species level.
7. Inoculation with *L. innocua* significantly impacts the fungal community on apples at the selected sampling time (before commercial storage or after 9 months of CA or RA storage).
8. *Basidiomycota* followed by *Ascomycota* are dominant fungal phyla of Fuji apples, regardless of *L. innocua* inoculation and storage condition.
9. The relative abundance of *Basidiomycota* of the non-inoculated apples decreased after 9 months of CA or RA storage while *Ascomycota* increased.
10. The relative abundance of *Basidiomycota* and *Ascomycota* in the inoculated apples remained stable after 9 months of RA storage; the relative abundance of *Basidiomycota* of inoculated apples decreased and *Ascomycota* increased after 9 months of CA storage.
11. *Bulleribasidiaceae* is the dominant family in non-inoculated apples followed by *Filobasidiaceae*. *Filobasidiaceae* is the dominant family in inoculated apples followed by *Bulleribasidiaceae* and *Pleosporaceae*. The abundances of these families changed after 9 months of CA or RA storage.
12. *Vishniacozyma* and *Filobasidium* are dominant genera in non-inoculate apples accounting for 52.7% and 25.6% of total fungal genera. *Filobasidium, Vishniacozyma*, and *Alternaria* are dominant genera in inoculated apples with 27.8%, 22.6%, and 21.6% relative abundance, respectively. These genera were changed after 9 months of CA or RA storage.
13. *Vishniacozyma victoriae and Filobasidium magnum* were the main species detected on non-inoculated apples and inoculated apples. The relative level of *Filobasidium magnum* content decreased after 9 months of CA and RA storage regardless of inoculation.
14. *Tausonia pullulans* level in apples was low regardless of the inoculation but was extremely elevated in the inoculated apples after 9 months of RA storage (Fig. 5), increasing from 0.1% to 26.2%.

METHODS

1. Apple cultivar selection

We acknowledge that the different varieties may behave differently in terms of bacterial adhesion and dynamic change of the microbiome on their surface during cold storage. Thus, four popular varieties, Fuji, Granny Smith, Cosmic Crisp, and Pink Lady apples were used in this study.

2. Strain selection and inoculum preparation

L. innocua is a widely used nonpathogenic surrogate for *L. monocytegenes* (Sheng, Shen, & Zhu, 2020). To elucidate the impact of strain variability on their survival under cold storage, *L. innocua* isolates from Bidart apple facility and other processing plants were used to prepare a 3-strain cocktail of *L. innocua* inoculum per our well-established method (Sheng et al., 2018).

3. Inoculation

Washed and unwaxed apples of selected varieties were individually and separately inoculated to establish 1×10^6 CFU/apple using a 3-strain cocktail of *L. innocua* per our well-established method (Sheng, Edwards, Tsai, Hanrahan, & Zhu, 2017; Sheng et al., 2018; Sheng et al., 2020).

4. Cold storage treatments

Unwaxed and uninoculated or inoculated apples of selected varieties were randomly divided and subjected to well-controlled RA or CA storage for 9 months. 1% CO_2 and 1.2% O_2 were used in CA storage following the practices of commercial packing facilities for the selected varieties. A storage temperature of 33 ºF (1°C) was chosen for the selected apples. All fruits were subjected to 1-methyl cyclopropane (1-MCP, a maturation inhibitor) treatment once before they are put in their respective storage rooms.

5. Sampling during cold storage

Fruits were sampled right before storage, at 3, 6, 12, 18, 24, 30, and 36 weeks of storage. Four replicates of 10 fruits each will be used on each sampling day at each storage condition.

6. Surviving Listeria analysis

On each sampling day, four sets of 10 apple fruits under the respective storage conditions were sampled and transported to the Food Microbiology Lab on the Pullman campus of Washington State University for microbial analyses. Upon arrival, *Listeria* survival of apple surfaces was analyzed immediately or within 24h per our well-established method (Sheng et al., 2017; Sheng et al., 2018). If survival of *Listeria* on apple fruits was below the detection limit, the suspension was enumerated for Presence/Absence after 48h enrichment in Buffered *Listeria* Enrichment Broth (BLEB) and streaking onto a selective *Listeria* agar plate. Presumptive positive colonies were further confirmed by PCR (FDA, 2015).

7. Resident microbiota enumeration

To enumerate *Enterobacteriaceae*, the detached microbiota suspension was plated on TSAYE overlaid with Violet Red Bile Glucose agar and incubated at 35°C for an additional 24 h.

To enumerate *Pseudomonas*, the detached microbiota suspension was plated on TSAYE plates overlaid with *Pseudomonas* selective agar supplemented with 10 µg/ml of cetrimide, 10 µg/ml of Fucidin, and 50 µg/ml of cephalosporin, and then incubated at 28°C for 5 days.

Total native bacteria were enumerated on TSAYE plates and incubated at 30°C for 3 days. Lactic acid bacteria were enumerated by pour plate method using de Man, Rogosa and Sharpe (MRS) agar and incubated at 35°C for 48 h.

Yeast and mold were plated on potato dextrose agar (PDA) supplemented with 100 µg/ml chloramphenicol and incubated at room temperature (~22°C) for 5 days. Colonies were classified into yeasts, molds, and *Penicillium* regarding morphological characteristics.

8. Next-generation sequencing analysis of microbiome on apple surfaces

1) <u>Microbial detachment from apple surface</u>

At each sampling day, 4 composite replications containing 16 uninoculated and/or inoculated apple fruits were collected. Microbial suspension detached from 16 apples was pooled together and used for DNA extraction as described in the following.

2) DNA extraction and purification

Genomic DNA was extracted from microbial samples collected above using commercial DNA extraction and purification kit from Qiagen (Valencia, CA) per our established method (Kang, Yang, Zhang, Ross, & Zhu, 2018). The concentration and quality of DNA will be measured using Nanodrop spectrometry (Thermo Scientific), while the quality of DNA will be monitored by DNA agarose gel.

3) Next-generation DNA sequencing

Next-generation sequencing of the microbiome was performed by the Initiative for Bioinformatics and Evolutionary Studies (IBEST) Genomics Resources Core at the University of Idaho using Illumina MiSeq dual-barcoded two-step PCR amplicon sequencing. To produce amplicons for sequencing, the V4 region of the bacterial 16S rRNA gene was amplified using universal primers (515F: GTGCCAGCMGCCGCGGTAA, 806R: GGACTACHVG GGTWTCTAAT) with flanking regions ACACTGACGACATGGTTCTACA or TACGGTAGCA GAGACTTGGTCT at F515 or R806, respectively, for the first PCR reaction. The PCR products obtained from the first PCR were diluted and used as the template for the second PCR to add barcodes and sequencing adapters. Equal amounts of amplicons were pooled to create a composite sample, which was then normalized, and denatured prior to sequencing per the Illumina protocol for a 2×301 MiSeq run (Illumina, Inc., San Diego, CA).

For fungal community, the internal transcribed spacer region (ITS1) of the fungal ribosomal RNA gene will be amplified using the prepared microbial DNA and universal primers of ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS2: 5'- GCTGCGTTCTTCATCGATGC-3' with flank regions ACACTGACGACATGG TTCTACA and TACGGTAGCAGAGACTTGGTCT at ITS1F and TIS2, respectively, for the first PCR. The second round of PCR (PCR2) will be performed to add sample-specific barcodes and Illumina adapters by priming the common tag sequences (Schlatter, Yin, Hulbert, & Paulitz, 2020; Schoch et al., 2012) and using the first PCR product as a template. Barcoded amplicons of PCR2 were quantified andcombined at equal amounts to construct the fungal ITS library (Schlatter et al., 2020; Schoch et al., 2012).

4) Bioinformatics analysis of apple microbiome under storage

Raw DNA sequence reads from the Illumina MiSeq will be demultiplexed and classified using the established method by bioinformaticist at IBEST (Kang et al., 2018).

9. Fruit quality analysis

At harvest or 36-week storage, fruit quality such as firmness, total soluble solids, and titratable acidity, as well as external and internal disorders, including superficial scald and lenticel decay, were assessed at the end of cold storage by the WTFRC quality lab using established methods (Sheng et al., 2018). A sample size of 10 apples per replicate with 4 independent replicates per wax type was used for internal and external disorder assessment.

10. Statistical analysis.

Data were analyzed with IBM SPSS 19.0 (Chicago, IL). Mean differences were compared by the one-way analysis of variance (ANOVA) followed by a Tukey multiple comparison test. P values less than 0.05 were considered significant differences.

RESULTS AND DISCUSSION

1. Fungal composition of apples inoculated with or without Listeria innocua at the phylum level

At the phylum level, the fungal population of Fuji apples, regardless of *L. innocua* inoculation and storage condition, was dominated by *Basidiomycota* followed by *Ascomycota* (Fig.1). In non-inoculated apples, the relative abundance of *Basidiomycota* decreased, and *Ascomycota* increased after 9 months CA or RA storage ($P< 0.05$) (Fig. 1BC). For inoculated apples, the relative abundance of *Basidiomycota* and *Ascomycota* remained stable for 9 months of RA storage; however, the relative abundance of *Basidiomycota* of inoculated apples decreased and *Ascomycota* increased after 9 months of CA storage ($P< 0.05$).

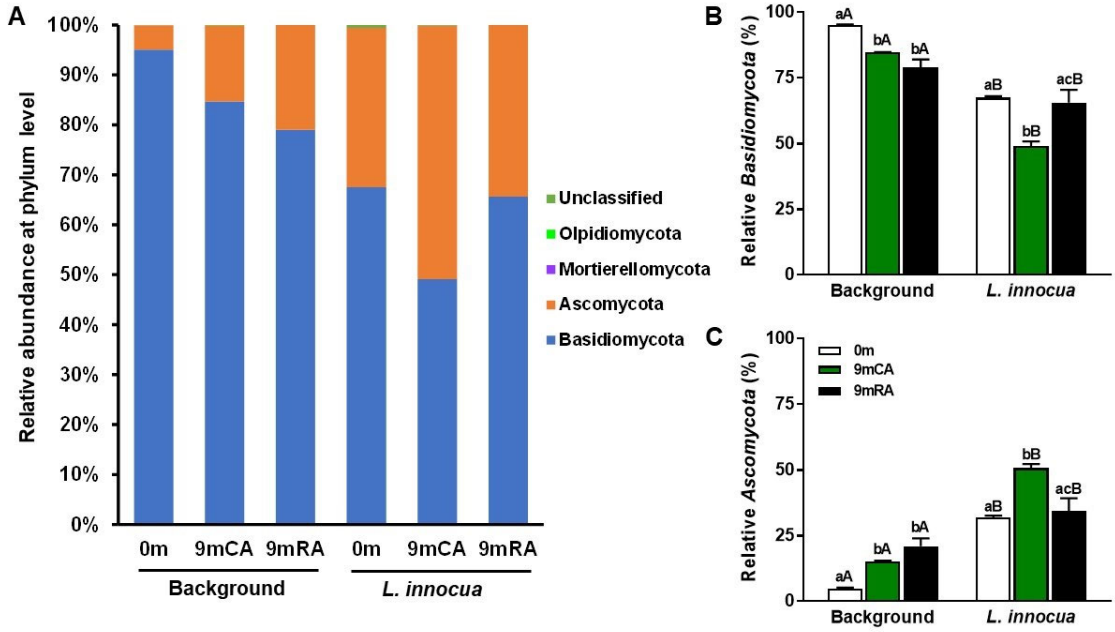

Figure 1. Relative abundance of fungal phyla detected in Fuji apples before and after 9 months of storage at commercial RA and CA room. [a-b]Mean among different storage without a common letter differ significantly ($P < 0.05$) for the inoculated apples (*L. innocua*) or non-inoculated apples (Background). [AB]Mean at each sampling point without a common letter differs significantly between the inoculated apples (*L. innocua*) and non-inoculated apples (Background) ($P < 0.05$).

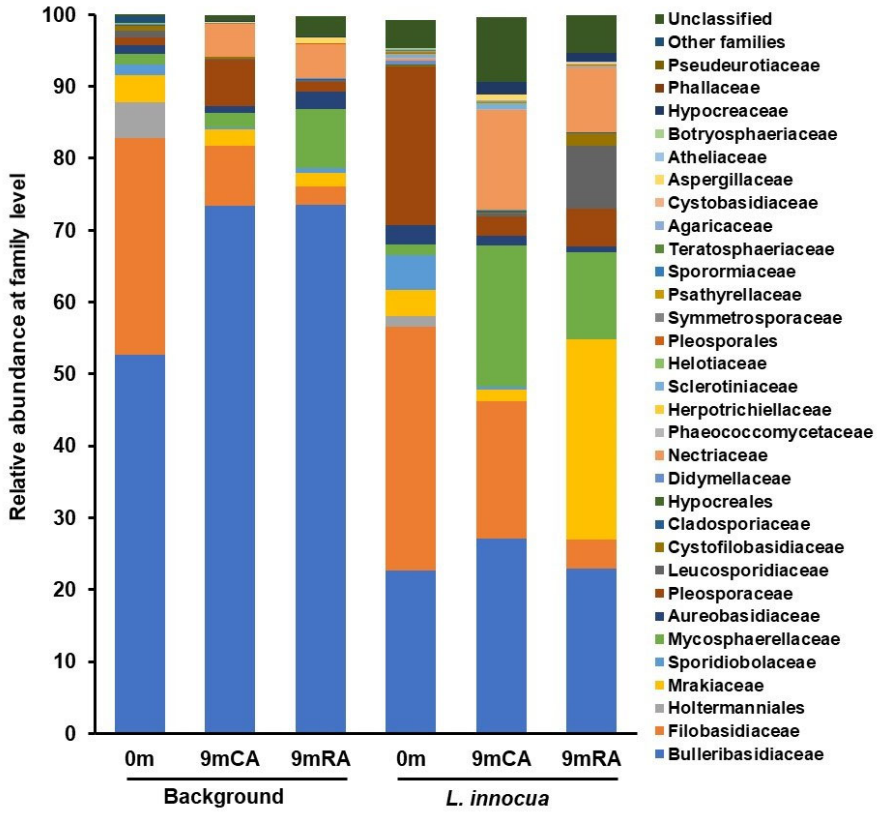

Figure 2. Relative abundance of fungal family detected in Fuji apple inoculated with or without *Listeria innocua* before and after 9 months of storage at commercial RA and CA room. Four replicates with each replicate contains 16 apples.

2. *Fungal families of Fuji apples inoculated with or without Listeria innocua before and after 9 months of CA or RA storage.*

For non-inoculated apples, *Bulleribasidiaceae* is the dominant fungal family, which accounts for 54.7%, followed by *Filobasidiaceae*, accounting for 30.1% of the fungal families (Fig. 2). The relative abundances of *Bulleribasidiaceae* increased from 54.7% to 73-74% after 9 months of CA and RA storage, while the relative abundance of *Filobasidiaceae* was decreased to 8.4% and 2.4% after 9 months of CA and RA storage, respectively. (Fig. 2, Fig.3AD). In *L. innocua* inoculated apples, *Filobasidiaceae* is the dominant family, accounting for 30.1% abundance which is similar to that in non-inoculated apples, followed by *Bulleribasidiaceae* and *Pleosporaceae*, which accounting for

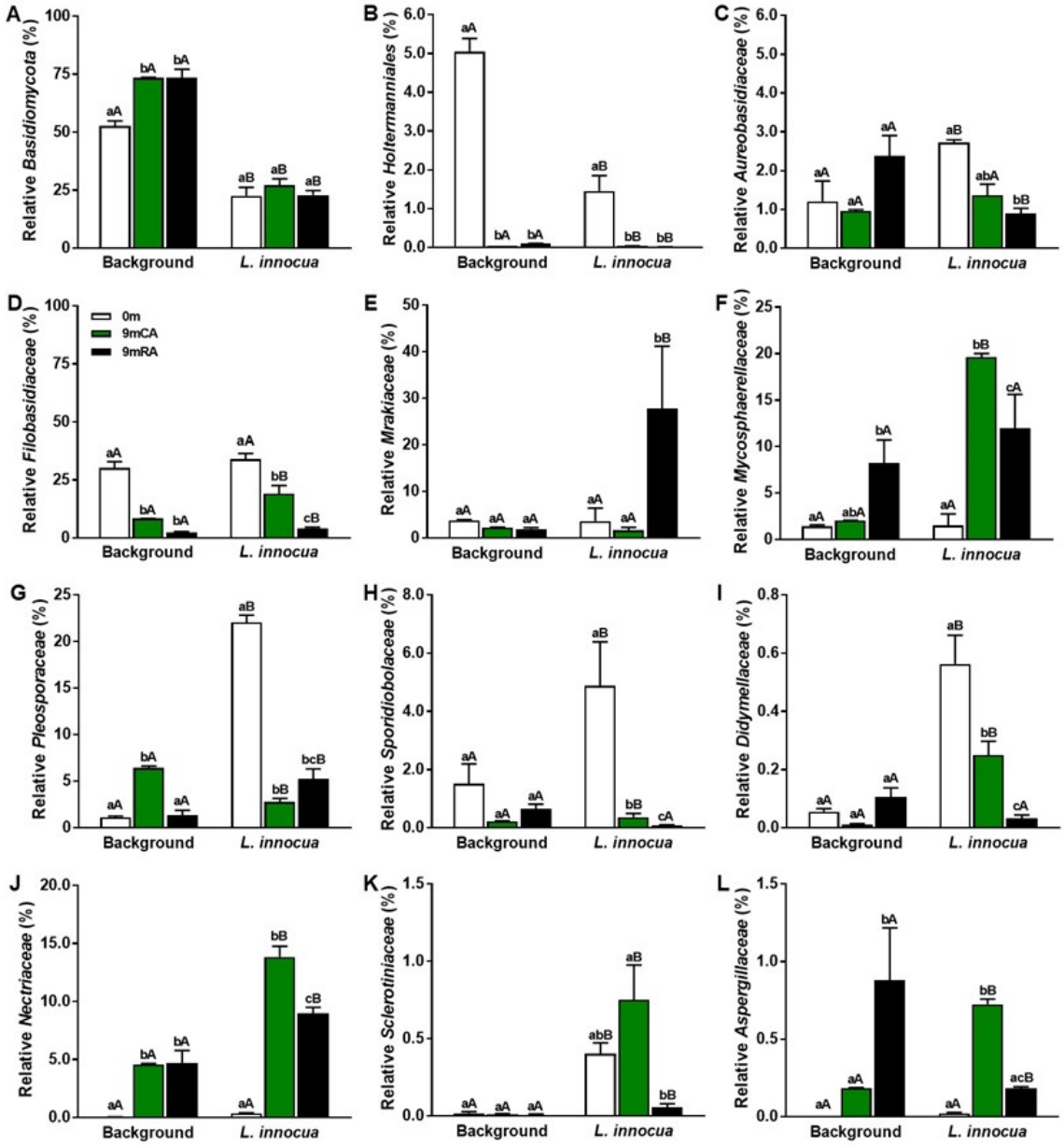

Figure 3. The selected fungal families of Fuji apples before and after 9 months of CA and RA storage with statistically significant differential abundances. Mean ± SEM, there are four replicates, each replicate has 16 apples. [a-b]Mean among different storage without a common letter differ significantly ($P < 0.05$) for the inoculated apples or non-inoculated apples. [AB]Mean at each sampling point without a common letter differs significantly between the inoculated apples (*L. innocua*) and non-inoculated apples (Background) ($P < 0.05$).

22.6% and 22.1%, respectively (Fig. 2, Fig. 3ADG). The population of *Bulleribasidiaceae* in the inoculated apples was lower than that in non-inoculated apples (P <0.05), while the counts of *Pleosporaceae* was significantly higher compared to the non-inoculated apples (P <0.05) (Fig. 3 AG). The relative abundance of *Bulleribasidiaceae* in the inoculated apples remained relatively stable after 9 months of CA and RA storage (Fig 3A). The count of *Filobasidiaceae* and *Pleosporaceae* in the inoculated apples decreased significantly after 9 months of CA or RA storage (P <0.05) (Fig. 3DG). Additional changes at the family level caused by *L. innocua* inoculation and 9 months of CA or RA storage were shown in Fig. 3.

3. *Fungal genera and species of Fuji apples inoculated with or without Listeria innocua before and after 9 months of CA or RA storage.*

Vishniacozyma is a dominant genus detected in non-inoculate apples, accounting for 52.7% abundance, followed by *Filobasidium*, accounting for 25.6% of total fungal genera (Fig. 4). Relative abundance of *Vishniacozyma* increased to 73-74% after 9 months of CA or RA storage (P <0.05), while *Filobasidium* count decreased to 6.4% and 1.8%, respectively, after 9 months of CA or RA storage (P <0.05) (Fig. 4). In *L. innocua*-inoculated apples, *Filobasidium, Vishniacozyma*, and *Alternaria* are dominated genera, which accounts for 27.8%, 22.6%, and 21.6% abundance, respectively (Fig. 4). Relative *Filobasidium* and *Alternaria* detected in the inoculated apples significantly decreased after 9 months of CA and RA storage (P <0.05), while *Vishniacozyma* remained stable across storage (Fig. 4). Relative abundance of *Penicillium* family increased after 9 months of RA storage (P <0.05), regardless of *L. innocua* inoculation. Relative abundance of Rhodotorula and Holtermanniella decreased after 9 months of CA or RA storage in both inoculated and non-inoculated apples (P <0.05). Gibberella had a low abundance and remained low in the inoculated apples after 9 months of CA and RA storage but was significantly increased in non-inoculated after 9 months of CA storage. More differential genera can be found in Fig. 4.

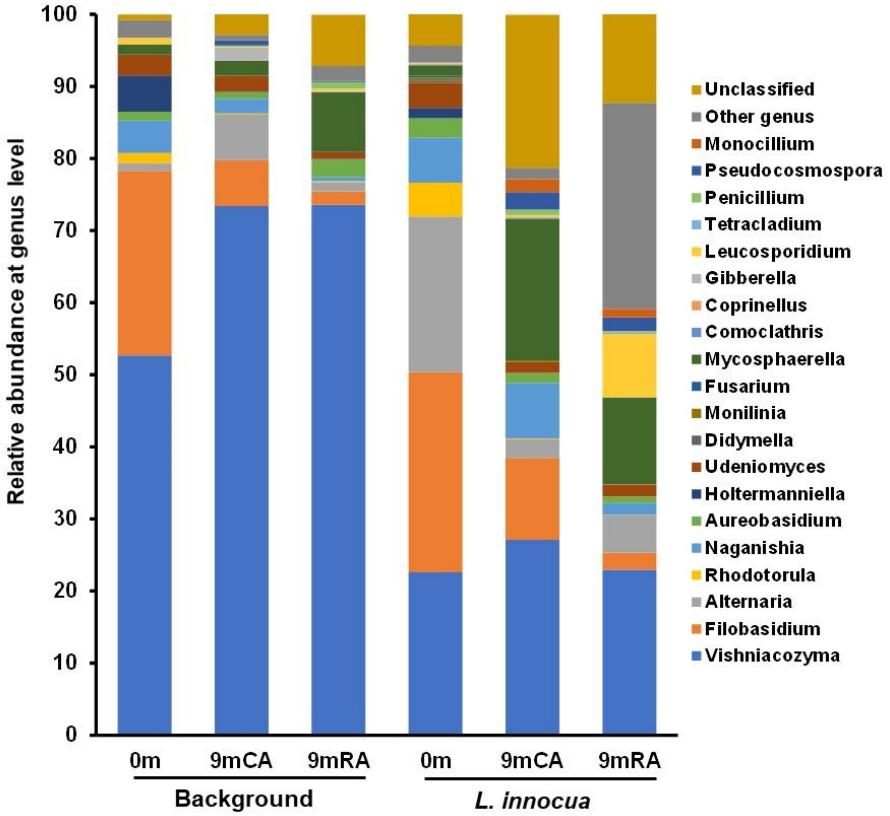

Figure 4. Relative abundance of fungal genera detected in Fuji apple inoculated with or without *Listeria innocua* before and after 9 months of storage at commercial RA and CA room. Four replicates with each replicate contains 16 apples.

At the species level, *Vishniacozyma victoriae* was the main species detected on non-inoculated apples, accounting for 48.2% of total species, followed by *Filobasidium magnum* with 25.1% relative abundance. Relative abundance of *Vishniacozyma victoriae* increased after 9 months of CA and RA storage, while *Filobasidium magnum* content decreased after 9 months of CA and RA storage ($P < 0.05$) (Fig. 5). In inoculated apples, both *Vishniacozyma victoriae* and *Filobasidium magnum* were main species detected with 18.8% and 26.2% relative abundance, respectively (Fig. 5). Relative abundance of *Vishniacozyma victoriae* in the inoculated apples were similar before storage and after 9 months of CA or RA storage. The relative *Filobasidium magnum* content in the inoculated apples decreased after 9 months of CA and RA storage ($P < 0.05$) as observed in the non-inoculated apples (Fig. 5). *Holtermanniella takashimae* level was higher in non-inoculated apples than that in inoculated apples and decreased after 9 months of CA or RA storage, regardless of the inoculation. Relative abundance of *Mycosphaerella tassiana* significantly increased after 9 months of RA storage (both inoculated and non-inoculated) and CA (inoculated apples) ($P < 0.05$) (Fig. 5). Relative abundance of *Tausonia pullulans* in apples was low regardless of the inoculation but was extremely elevated in the inoculated apples after 9 months of RA storage (Fig. 5), increasing from 0.1% to 26.2%.

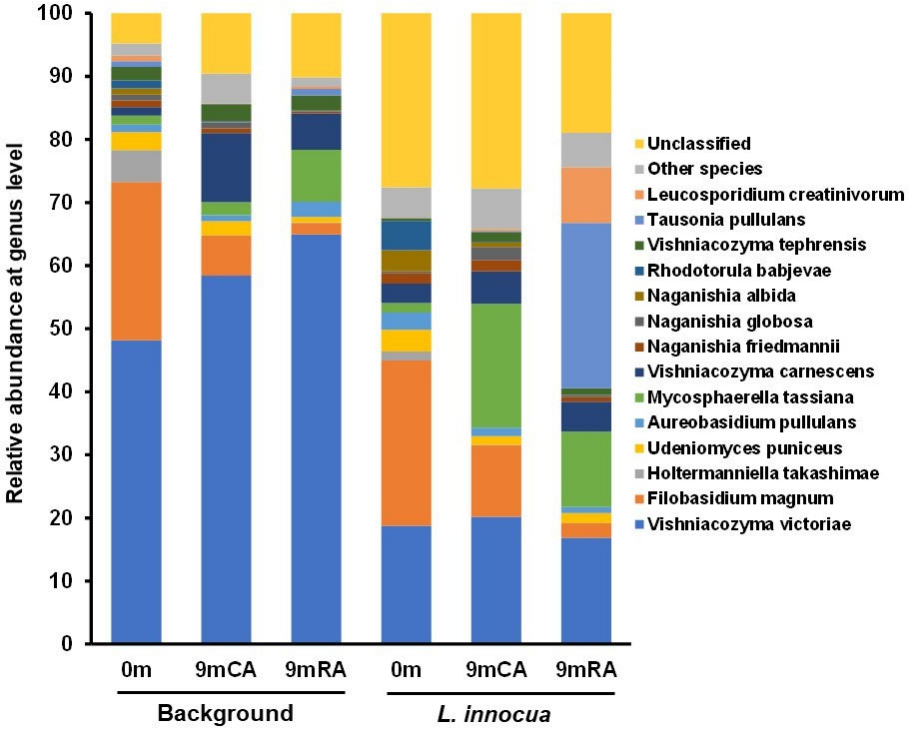

Figure 5. Relative abundance of fungal species of Fuji apple inoculated with or without *Listeria innocua* before and after 9 months of storage at commercial RA and CA room. Four replicates with each replicate contains 16 apples.

In conclusion, significant differences in the fungal community structure were found between apple samples taken from different sampling times (before the storage and after 9 months of CA or RA storage) for both inoculated and non-inoculated apples. Significant differences were also found between apples inoculated with or without L. innocua at the selected sampling time. These significant differences were found on the phylum level (Fig. 1), family level (Fig. 2), genus level (Fig. 4), and species level (Fig. 5).

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Project Title: Reducing carbon dioxide-related postharvest disorders **(AP-19-100)**

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Collaborators: Dr. Ines Hanrahan, Christine McTavish, Erin Tudor, Shae Milne, Emmi Klarer, Natalie Valdez

Report Type: Final Project Report

Project Duration: 3-Year with no cost one year extension
Total Project Request for Year 1 Funding: $79,314
Total Project Request for Year 2 Funding: $92,893
Total Project Request for Year 3 Funding: $95,036

Other related/associated funding sources: Awarded
Agency Name: USDA-ARS, In-house project
Amount: $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).

Other related/associated funding sources: Proposed
Amount: $540,888/4 yrs.
Agency Name: USDA-NIFA
Notes: Pre-proposal with complimentary objectives submitted to SCRI program.

Budget 1
Primary PI: David Rudell
Organization Name: USDA-ARS
Contract Administrator: Chuck Myers
Telephone: (510) 559-5769
Contract administrator email address: Chuck.Myers@usda.gov

Item	2019	2020	2021	2022
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	0

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. 'Honeycrisp' and 'Pazazz' are sensitive to CO_2 but also develop soft scald and soggy breakdown.
3. Incidence of symptoms related to CO_2 sensitivity were reduced or eliminated by DPA drenching.
4. Reducing CO_2 exposure concentration and delaying CA impacted CO_2 disorders albeit inconsistently among cultivars.
5. Peel and cortex chemistry of typically CO_2-related symptoms is different from other peel and cortex defects.
6. Cultivars can be screened for CO_2 sensitivity using an easy, inexpensive protocol.

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. Symptomatic 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 and Year 2 were re-tested in Year 3.

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

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. Activity 3) focused on developing low-cost and simple protocols for the industry to create a high CO_2 environment to screen their own cultivars.

For activity 1, 'Golden Delicious' was harvested 2 weeks before commercial maturity while 'Fuji', WA-38, '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. For activity 2, 'Pazazz', JUICI, 'Scilate', 'Honeycrisp', and WA-38 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 CA conditions of 0.6% O_2, 5% CO_2 at 33 °F utilized. Each cultivar had 108 apples per treatment combination. At 3 months, external disorder incidence was evaluated, and at 6 months, external and internal disorder incidence as well as fruit quality was evaluated. The remainder of cultivars determined to be CO_2 sensitive under Objective 1 were tested in Year 3, which included 'Fuji', 'Plumac', 'Golden Delicious', and JUICI (retested).

For the third activity, 360 apples each of a CO_2 sensitive cultivar (JUICI) and a non-sensitive cultivar ('Delicious') were harvested 7 days after first commercial pick. 108 apples from each cultivar were either drenched with an emulsion containing DPA (2000 ppm) or with a solution containing only the inactive ingredients from the DPA emulsion (DPA-free). The DPA and DPA-free treatment were each then separated into clear plastic bags with 36 fruits per bag. Each bag was tightly twisted shut and secured using two zip-ties to ensure the bag would not leak (Figure 4). The remaining untreated fruit were stored in boxes as a control. Bags were stored in 30 °F air with O_2 and CO_2 levels were evaluated daily, then all treatments were evaluated for external and internal CO_2 injury at 4 months.

Objective 3: Identify chemistry associated with CO_2 sensitivity

Our broad analysis of peel and cortex chemistry is ongoing and is the remaining activity we need to complete during the no cost extension. To develop a system for diagnosing peel and cortex browning caused by CO_2 sensitivity, symptomatic peel and cortex from activities outlined under objective 1 were sampled regularly with adjacent healthy tissue and healthy tissue from DPA treated fruit as control. Any browned tissue in or on DPA treated fruit was also 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 also determined 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 confirmed the chemistries that are specific to CO_2 sensitivity. 'Pazazz' was chosen for this activity, as it was one of the most CO_2 sensitive cultivars. 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, 8, and 16 weeks. This experiment was repeated in Year 3 with 'Fuji', as it was also one of the more sensitive cultivars but stores better than 'Pazazz'.

RESULTS AND DISCUSSION

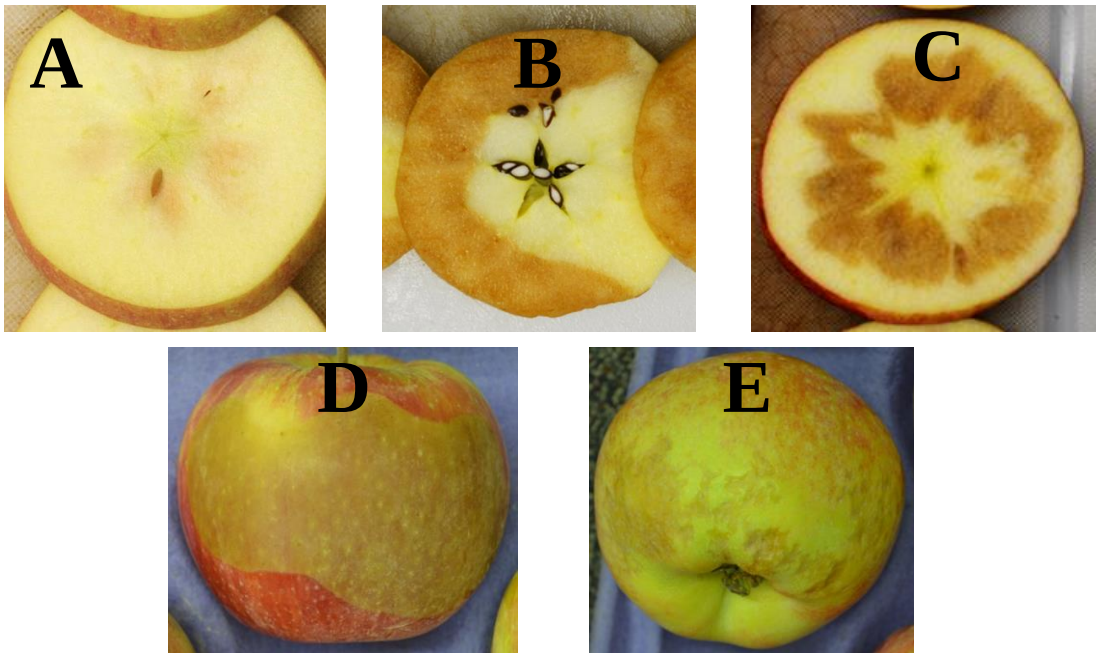

Figure 1. General categories (types) of observed disorders. (A) Lens shaped cavities ('Braeburn'), (B) non-radial browning (soggy breakdown 'Honeycrisp'), (C) radial browning ('Honeycrisp'), (D) peel browning (soft scald, 'Honeycrisp'), and (E) orange ("rugose scald") peel ('Pazazz'). C and E are typically 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 (Fig. 1). Symptoms were presented and discussed at a scientific roundtable to amend WSU online disorder databases in the summer of 2020.

All cultivars, other than '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', 'Plumac', 'Braeburn', 'Scilate', JUICI, 'Honeycrisp', 'Pazazz', 'Smitten', 'Gala', and 'Cripps Pink'. 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. Internal cavities were the least impacted by harvest maturity. We observed some symptoms that were less recognizable such as severe core and peel browning of 'Fuji' (Fig. 2).

Figure 2. Softened solid brown cortex on 'Fuji'. Incidence was not eliminated by DPA drench.

Table 1. Percent incidence of different internal and external disorders in Year 1 (see Fig. 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. This activity was repeated in Years 2 and 3 on cultivars with no disorders in Years 1 and/or 2. Only one harvest was tested in year 3.

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 (Year 3)		no DPA		**69**		**47**	
		DPA		**81**		**36**	
Cripps Pink (Year 3)		no DPA	8				**92**
		DPA					
Ambrosia	early	no DPA					3
	early	DPA					
	late	no DPA					
	late	DPA					
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 (Year 3)		no DPA	**14**		**31**		
		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					
WA 38	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, $p<0.05$).

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. 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', 'Plumac', 'Braeburn', 'Scilate', JUICI, 'Fuji', 'Honeycrisp', 'Pazazz', 'Smitten', and 'Cripps Pink' that were not drenched at harvest with DPA emulsion. 'Fuji' developed cavities, severe browning that had "radial" appearance, and softened solid brown cortex (Fig. 2) and incidence was lowered but not eliminated by DPA treatment. '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. None of the cultivars that did not develop disorders in Year 1 developed disorders when retested in Year 2. However, 'Smitten' and 'Cripps Pink' both developed CO_2-related disorders in Year 3. A high incidence of both external and internal browning was found this year in/on both control and DPA drenched 'Gala'. This peel (sometimes called "caramelization") and stem end flesh browning in this cultivar is not CO_2-related (not controlled by DPA) and has been attributed to climatic conditions and possibly the transition into cold storage.

Improving cold chain practices when CO_2 sensitivity is indicated

Activity 1 tested thresholds for O_2:CO_2 % atmosphere combinations. Lower CO_2 resulted in lower incidence of CO_2 related disorders for all cultivars, while lower O_2 levels increased disorder incidence along with elevated CO_2 although only in JUICI (Table 2). Apart from 'Golden Delicious' and WA-38, which were clean after 4 months, radial browning symptoms occurred in all other cultivars tested at 5% CO_2. At 1% CO_2, "radial browning" was not found in 'Scilate' and 'Fuji' and incidence was reduced in 'Braeburn', 'Pazazz'. In JUICI, incidence was higher 5% CO_2 than 1 % CO_2 at 0.6% O_2. Incidence in 'Honeycrisp' and 'Pazazz' was not different, potentially due to conflation of CO_2 related and non-CO_2 related disorders of which these cultivars are susceptible. "Orange peel" symptoms were found on both 'Honeycrisp' and 'Pazazz' at 5% CO_2. This further demonstrates these cultivars' high sensitivity to CO_2 injury. Disorders not related to CO_2 sensitivity (soft scald and soggy breakdown) were not influenced by differing combinations of O_2:CO_2. Soft scald was found in both 'Honeycrisp' and 'Pazazz' and "non-radial browning" in 'Scilate', 'Fuji', 'Honeycrisp', and JUICI. Cavities in the cortex tissue were found in all cultivars except 'Golden Delicious' and WA-38. Cavities were not present in 1% CO_2 for 'Fuji' and 'Honeycrisp', and reduced in 'Braeburn' and JUICI, but 'Scilate' and 'Pazazz' were not apparently linked with CO_2 % (Table 2).

Table 2. Reducing CO_2 reduces radial browning and cavities. Lower O_2 % enhances sensitivity to CO_2 in JUICI. Other disorders were not impacted by CO_2 or O_2 levels. 'Golden Delicious', 'Plumac', and WA-38 were also tested but did not develop disorders in this test. 'Cripps Pink' and 'Smitten' developed disorders in the screening test in Year 3 and were not included in this test. Radial browning, rough or "orange peel" peel texture, and cavities are typically associated with CO_2 sensitivity. Different letters indicate incidence of a symptom is different from other atmospheres for that cultivar according to a pooled z-test (n=36, $p<0.05$).

Cultivar	**O_2 (%)**	**CO_2 (%)**	**Soft scald %**	**Orange peel %**	**Radial browning %**	**Non-radial browning %**	**Cavity %**
Scilate	0.6	1			0.0 a	5.6 a	2.8 a
	1	1			0.0 a	0.0 a	0.0 a
	0.6	5			13.9 b	0.0 a	77.8 b
	1	5			8.3 ab	8.3 a	2.8 a

Fuji	0.6	1			0.0 a	0.0 a	0.0 a
	1	1			0.0 a	0.0 a	0.0 a
	0.6	5			11.1 b	0.0 a	44.4 b
	1	5			2.8 ab	5.6 a	13.9 c
Honeycrisp	0.6	1	19.4 a	0.0 a	2.8 a	25.0 a	0.0 a
	1	1	38.9 a	0.0 a	0.0 a	44.4 a	0.0 a
	0.6	5	38.9 a	8.3 a	8.3 a	44.4 a	2.8 a
	1	5	25.0 a	2.8 a	8.3 a	30.6 a	8.3 a
Braeburn	0.6	1			8.3 a		47.2 a
	1	1			2.8 a		16.7 b
	0.6	5			47.2 b		97.2 c
	1	5			66.7 b		52.8 a
JUICI	0.6	1			0.0 a	0.0 a	11.1 a
	1	1			2.8 a	0.0 a	5.6 a
	0.6	5			22.2 b	0.0 a	69.4 b
	1	5			5.6 a	2.8 a	38.9 c
Pazazz	0.6	1	63.9 a	61.1 a	5.6 ab		0.0 a
	1	1	50.0 a	61.1 a	0.0 a		25.0 b
	0.6	5	69.4 a	69.4 a	11.1 b		36.1 b
	1	5	50.0 a	36.1 b	8.3 ab		0.0 a

Most prior work indicates 1-MCP exacerbates CO_2 sensitivity, although it reduces ripening while apples are not in CA. We set out to test whether a delay of CA conditions would decrease CO_2 sensitivity after treatment with 1-MCP. We used CA conditions harsh enough to cause disorders (0.6% O_2: 5% CO_2) so as to detect even the slightest sensitivity. Results indicate that delaying CA can reduce CO_2 related disorders, albeit inconsistently across cultivars, but not soft scald, soggy breakdown, or the small cavities impacting JUICI cortex with no apparent relationship with CO_2 sensitivity. After 6 months of storage, 'Honeycrisp', 'Pazazz', JUICI, 'Fuji', and 'Golden Delicous' developed external CO_2 "orange peel" symptoms (Fig. 1; Table 3). Delaying CA establishment did not affect incidence on 'Honeycrisp', but a 4 week delay reduced incidence on 'Pazazz' compared with no delay and 2 week delay. Both 2 and 4 week delays eliminated 'Golden Delicious' orange peel although "ghosting" was prominent on peel stored in the reduced CO_2 atmosphere (1 % CO_2), especially if CA was established at harvest. Also, a superficial scald like symptom was prominent in both atmospheres if CA establishment was delayed 4 weeks.

With the exception of 'Fuji', internal CO_2 related disorder symptoms were found in all cultivars tested in this activity. 'Fuji' had a high watercore incidence which can exacerbate internal browning in less than optimal storage conditions. Most cultivars had lower incidence of radial browning with increased CA delay. For JUICI, a 2 week delay reduced incidence, while waiting an additional 2 weeks (4 weeks total) did not produce any further reduction (Fig. 3; Table 3). Incidence was reduced in 'Pazazz' only following a 4 week delay of CA but had no impact on radial browning of 'Honeycrisp'. This may result from a conflation of soggy breakdown and radial browning of which both cultivars are sensitive. Internal cavities in the cortex were detected in all cultivars tested in Year 2 and were cultivar-dependent regarding the impact of delayed CA. Delayed CA did not impact cavity incidence in 'Pazazz' or 'Scilate'. Delayed CA reduced cavities in 'Honeycrisp' and WA-38. Delaying CA actually increased cavity incidence in JUICI compared to no delay or a 4 week delay. Large cavities were reduced in this cultivar in a second Year 3 trial of this cultivar. JUICI also developed small cavities that were even present at harvest, in many cases. While large and small cavities were considered together in the first trial, large cavities were considered different from the small cavities in the second JUICI trial. CO_2 during storage was kept very high for these tests, and it is possible that combinations of delayed CA and reduced CO_2 during CA would be effective for

reducing symptoms on sensitive cultivars. Neither firmness nor titratable acidity were impacted by CA delay in any of the cultivars in this activity.

Figure 3. Radial browning on JUICI treated with 1-MCP with (from left to right) no delay, 2 week, and 4 week delay before CA storage at 0.6% O_2, 5% CO_2 for 6 months (Year 2)

Table 3. Delayed CA reduced orange peel in 'Pazazz' and 'Golden Delicious' and radial browning of JUICI, 'Pazazz', and WA-38 but not for other cultivars. Percent incidence of different internal and external disorders in Year 2 after treatment with 1-MCP and delays before CA storage (0.6% O_2:5% CO_2). Radial browning and orange peel symptoms are typically associated with CO_2 sensitivity. Different letters indicate incidence of a symptom is different from other atmospheres for that cultivar according to a pooled z-test (n=108, year 2 or n=90, year 3; $p<0.05$).

Cultivar	**Delay**	**Soft Scald %**	**Orange peel %**	**Radial browning %**	**Non-radial browning %**	**Cavities %**
Honeycrisp	0 weeks	11.1 a	1.9 a	16.7 a	4.6 a	13.9 a
	2 weeks	13.9 a	0.9 a	4.6 b	8.3 ab	1.9 b
	4 weeks	12.0 a	2.8 a	13.0 a	13.9 b	6.5 ab
Pazazz	0 weeks	11.1 a	45.4 a	16.7 ab		16.7 a
	2 weeks	17.6 a	50.9 a	21.3 a		17.6 a
	4 weeks	10.2 a	28.7 b	8.3 b		20.4 a
JUICI (Year 2)	0 weeks		0.9 a	88 a		16.7 a
	2 weeks		0.0 a	18.5 b		23.1 ab
	4 weeks		0.0 a	15.7 b		29.6 b
JUICI (Year 3)	0 weeks			76.9 a		30.6 a
	2 weeks			2.8 b		6.5 b
	4 weeks			5.6 b		8.3 b
Scilate	0 weeks	0.9 a		11.1 a		9.3 a
	2 weeks	0.0 a		9.3 a		3.7 a
	4 weeks	0.0 a		12 a		3.7 a
WA-38	0 weeks			4.6 a		12 a
	2 weeks			0.0 b		0.9 b
	4 weeks			0.0 b		0.0 b
Fuji	0 weeks		8.3 a	26.9 a		14.8 a
	2 weeks		2.8 a	22.2 a		9.3 a
	4 weeks		3.7 a	21.3 a		7.4 a
Golden Delicious	0 weeks		6.5 a			
	2 weeks		0 b			
	4 weeks		0 b			

An inexpensive protocol for establishing CO_2 sensitivity

Placing trays of apples in sealed bags during cold air storage (see materials and methods) appears to be a viable, inexpensive means to test for CO_2 sensitivity of a cultivar (Fig. 4). Sealing trays of JUICI (CO_2 sensitive) and 'Delicious' (CO_2 insensitive) in this manner reduced O_2 to around 9% for both cultivars and elevated CO_2 to 3.1% for 'Delicious' by 9 d and 3.4% by 14 d, respectively. 'Delicious' did not develop any symptoms. However, JUICI developed radial browning only in fruit sealed in bags without DPA (Table 4). Radial browning incidence did not differ among replications validating the precision of this protocol. This protocol is inexpensive (cost of garbage bags, zip ties, and existing O_2/CO_2 meters) appears an effective means to screen new cultivars.

Table 4. Bagging apples in sealed trash bags during air storage caused radial browning in JUICI, a CO_2-sensitive cultivar. Radial browning incidence in JUICI after 2 months of storage in bags. The same letter indicates the no difference. "Cavities" in this case are the very small type found in this cultivar.

	No DPA bagged			DPA bagged		
	Bag 1	Bag 2	Bag 3	Bag 1	Bag 2	Bag 3
Radial browning	5 a	6 a	7 a			
Cavities	0 a	1 a	0 a	1 a	3 a	0 a

Figure 4. A simple procedure for screening apples for CO_2 sensitivity seals apples in trash bags, seals them with zip ties and places them in 33 °F air storage for 2 months. O_2 and CO_2 can be verified periodically using an O_2/CO_2 meter.

Different chemistries are linked with different symptoms and causes of symptoms

Our screening of peel and cortex chemistry among symptomatic and asymptomatic periphery tissue from all cultivars yielded chemical markers that may be used distinguish CO_2-related peel and cortex symptoms from those related to other horticultural and storage factors. We compared related chemical differences and changes from all of the disorder symptoms in the test by screening 720 and 588 chemicals in symptomatic and asymptomatic tissue in the peel and cortex, respectively. Many of these natural chemicals point to processes associated with temperature adaptation and oxidation

which would be expected to be linked with tissue browning or processes leading up to symptom development.

Symptomatic cortex was determined to be related to CO_2 sensitivity if it was mostly eliminated by DPA drench and was reduced by reducing atmospheric CO_2. Cortex chemistry comparisons were made between symptomatic and asymptomatic tissue from all cultivars that developed internal browning or cavities. Initial evaluations focused on 'Honeycrisp' disorders as it also had soggy breakdown which could be distinguished from internal browning related to CO_2 sensitivity (Fig. 5, left). While there were many chemical differences overall, we found that certain chemicals were most indicative of tissue status and may provide the best metabolic markers. Browned tissue contained higher levels natural chemicals ostensibly associated with stress adaptation such as sphingolipids, diglycerides, phytosterol conjugates (ASGs), and vitamin E metabolism. Oxidation of vitamin E and production of some ASGs was specifically linked to CO_2-related cortex browning. This observation held true for nearly every sample with CO_2-related internal browning as supported by oxidized Vit E levels (Fig. 5, right).

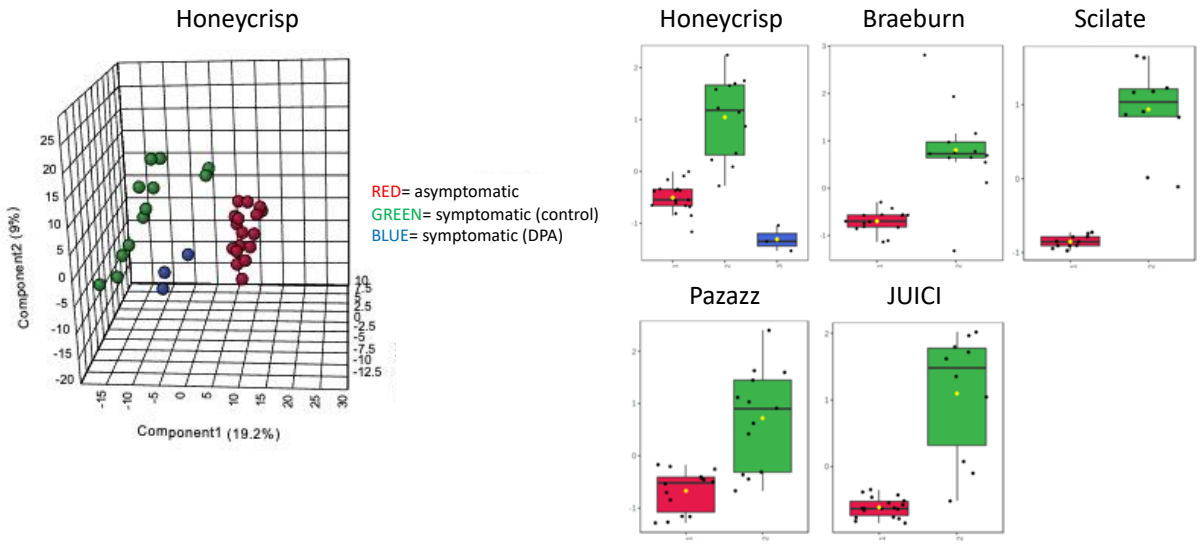

Figure 5. (Left) Analysis (principal components analysis) of 588 cortex chemicals show that asymptomatic (both control and DPA drench) as well as symptomatic tissue of control (CO_2 browning) and DPA drenched (soggy breakdown) 'Honeycrisp' all differ. There are many chemicals responsible for these differences in the multiple CO_2-sensitive cultivars analyzed. One example is an oxidized form of Vitamin E that is elevated specifically in symptomatic cortex linked with CO_2-sensitivity in 5 cultivars (Right). Vitamin E levels decrease alongside the increase of this metabolite indicating a potential process linked with symptom development.

To determine if any levels of any of these metabolites changed prior to symptom appearance, we stored DPA drenched and undrenched apples for up to 4 months under different CO_2 levels, sampling peel and cortex periodically. We studied 'Pazazz' in this way in Year 2. As disorder levels were extreme for this cultivar, we repeated this study with 'Fuji' in Year 3. Internal browning was first detected at 4 weeks especially in apples stored under 5 % with lesser amounts in those stored under 2.5 % CO_2 in control but not DPA drenched apples. As with in the initial selection, chemicals that were higher or lower in symptomatic compared with asymptomatic tissue were also lower in 'Fuji' in this study (not shown). Vitamin E oxidation was elevated in control fruit indicating that DPA impeded the process as is indicated by the ratio of oxidized Vitamin E to Vitamin E. However, this

process was only impacted in asymptomatic tissue by CO_2 levels once adjacent high levels of internal browning were present at 8 weeks. Similar increases of levels were observed with other highlighted chemicals. Monitoring these chemicals may distinguish different injuries but would not provide an early, pre-symptomatic indication of CO_2-sensitivity as they all appear to be directly linked with the symptoms.

We also can distinguish "typical" CO_2 sensitivity-related peel symptoms such as dimpled or "orange peel" from healthy (Fig. 6A) peel or peel with other defects such as soft scald (Fig. 6B). Further refinement of the search identified chemicals whose levels are higher or lower depending upon whether the symptoms are related to CO_2 sensitivity (Fig. 6C).

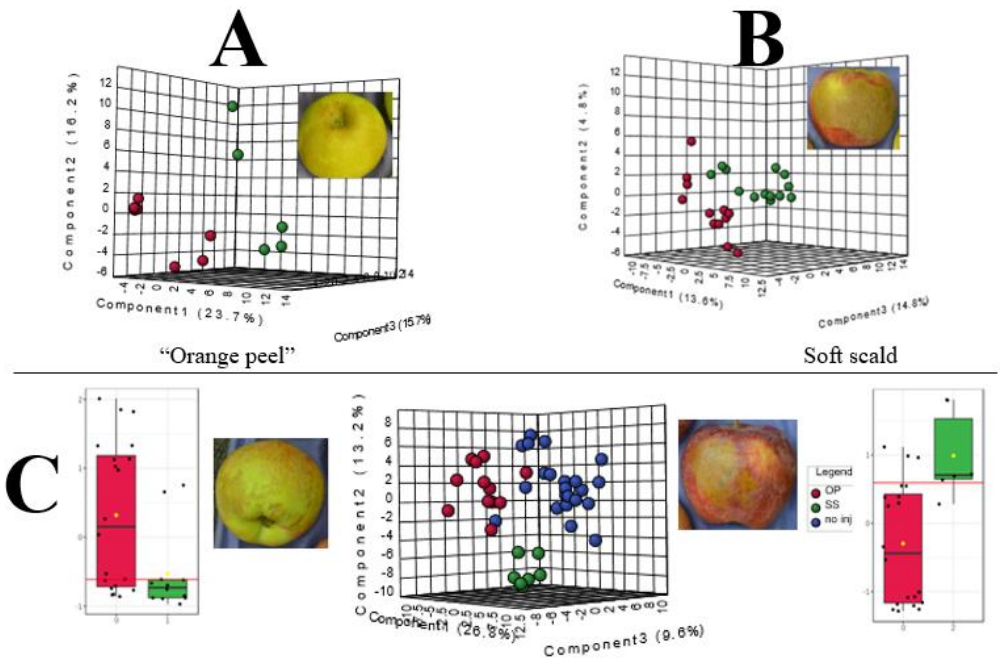

Figure 6. Peel chemistry of CO_2 sensitivity-related symptoms including A) orange peel (rugose scald) of 'Golden Delicious' B) soft scald of 'Honeycrisp' were different from periphery tissue. C) Chemistry of "orange peel" (red dots) and soft scald (green dots) were different in 'Pazazz' which developed both. Threonic acid, a natural peel chemical, is found in lower quantities in injured peel (green bar) than peripheral peel (red bar) in "orange peel" and in greater quantities than peripheral peel in soft scald.

To summarize, we have identified CO_2 sensitive cultivars as well as some potential means of mitigating CO_2-related disorders. As expected, DPA drenching was mostly effective at eliminating both internal and external symptoms of CO_2 sensitivity. The ineffectiveness of DPA for controlling some disorders also highlights where CO_2 sensitivity was not the cause of internal and external defects in some instances (soft scald and soggy breakdown), primarily for 'Honeycrisp' and its progeny, 'Pazazz', or for 'Gala' where similar symptoms have different causes. Symptoms of these disorders are often confused and are not mitigated using the same strategies. Other conditions that reduced CO_2-related disorders included reducing CO_2 during storage and, in the case of JUICI,

‘Pazazz’, ‘WA 38’, and ‘Golden Delicious’, delaying CA for up to 4 weeks following 1-MCP treatment at harvest. However, to date, this delaying CA establishment has inconsistently reduced disorders. Besides finishing CO_2 disorder mitigation storage studies, our no cost extension will entirely focus on finishing our peel chemistry analyses confirming peel chemistry specifically linked with CO_2-related disorder symptoms and the period leading to symptom development. To date, we have identified natural chemical differences that appear to be linked with symptoms related to CO_2 sensitivity. We are continuing to specifically confirm associations with CO_2-sensitivity. We expect that monitoring these chemicals during storage can indicate if CO_2 stress is occurring or if symptoms are related to CO_2 sensitivity.

Project Title: Reducing carbon dioxide-related postharvest disorders (AP-19-100)

Executive Summary

Keywords: cold chain, CO_2 sensitivity, postharvest disorder diagnosis, postharvest disorder mitigation, risk assessment

Abstract: Progenitors of many newer apple cultivars are sensitive to elevated CO_2 during storage. Consequently, there may also be an enhanced risk of CO_2-related disorders of the peel and flesh. The appearance of CO_2-related symptoms can vary by cultivar, and some cultivars can even develop similar symptoms not related to elevated CO_2 levels, confounding diagnosis and subsequent mitigation. We selected 15 cultivars to determine if they are sensitive to CO_2 during storage, symptom appearance, means for identifying CO_2-related disorders, and how to best mitigate CO_2-related disorders of these cultivars. Of those, 11 were sensitive, developing "orange peel" injuries and/or a variety of internal injuries. 'Honeycrisp' and a progeny, 'Pazaaz', develop internal and external disorders related to both CO_2-sensitivity and cold storage, alone. Diphenylamine drenches eliminated CO_2-related symptoms in nearly every case. Reducing CO_2 to below 1% also reduced disorder incidence, unless CO_2-senstivity was severe or other chilling-related disorders were also present. Delaying CA establishment for 2-4 weeks following 1-MCP treatment at the beginning of storage could reduce incidence of CO_2 related disorders without losing firmness or acidity. When multiple disorders developed, levels of specific peel chemicals were elevated when symptoms were related to CO_2 sensitivity. In summary, we determined that CO_2 sensitivity can cause disorders in many of these cultivars, and a combination of reducing levels of CO_2 during storage and delaying CA establishment from 2-4 weeks (only when 1-MCP can be used) can reduce these disorders in the most sensitive cultivars, especially when DPA drenching is not used.

Project outcomes:

1. Indication of CO_2 sensitivity of prominent cultivars.
2. Inexpensive protocol for specifically screening cultivars for CO_2 sensitivity.
3. Mitigation protocols for reducing disorders of CO_2 sensitive cultivars.
4. Identified chemistries specifically related to CO_2-related disorders.

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. 'Honeycrisp' and 'Pazazz' are sensitive to CO_2 but also develop soft scald and soggy breakdown.
3. Incidence of symptoms related to CO_2 sensitivity were reduced or eliminated by DPA drenching.
4. Reducing CO_2 exposure concentration and delaying CA impacted CO_2 disorders albeit inconsistently among cultivars.
5. Peel and cortex chemistry of typically CO_2-related symptoms is different from other peel and cortex defects.
6. Cultivars can be screened for CO_2 sensitivity using an easy, inexpensive protocol.

Future Directions:

1. Determining when CO_2 scrubbing is most necessary and to what degree for different cultivars.
2. Determining genetic factors associated with CO_2 sensitivity during storage.

Project Title: Postharvest system optimization for organic apple storage

Report Type: Final Project Report

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Project Duration: 3 Years

Total Project Request for Year 1 Funding: $ 50,000.00
Total Project Request for Year 2 Funding: $ 50,600.00
Total Project Request for Year 3 Funding: $ 56,000.00

Other related/associated funding sources:
Valent Biosciences (Retain OL), RipeLocker (vacuum units), WSU, USDA-ARS (CA chambers), Stemilt Growers & Zirkle Fruit (fruit for experiments), SCS (labpods maintenance)
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.

WTFRC Collaborative Costs: None

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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

1. All CA/DCA storage regimes evaluated, post conditioning at harvest, and a period in air (4 weeks) after CA/DCA opening, were suitable for long-term storage of Honeycrisp and Fuji apples. Nevertheless, preharvest managements (nutrition, pathogens, etc.) and seasonal climate greatly affected the amount of decay and incidence of physiological disorders during the storage period. The exploratory multivariate analyses including sites, bioclimatic indices, shoot length, fruit mineral content at harvest, crop load, and fruit maturity at harvest in all three seasons did not show consistent results to explain disorder's expression or softening rate postharvest in Honeycrisp.
2. In 2020, soft scald incidence in Honeycrisp was lower than in 2019 season, and it was significantly reduced by all CA/DCA storage regimes when compared to those observed in fruit stored in air for 4 months. Similar results were observed in 2021 season. Soggy breakdown only appeared in 2019 and 2021 seasons after 9 months in CA/DCA+4 weeks in air+7 days at 68°F, and mostly in one of the cool sites. Bitter pit was block-dependent all seasons. Incidence greatly increase during the air period (4 weeks) after CA/DCA.
3. Overall, the application of aminoethoxyvinylglycine (AVG- Retain OL) on Gala (2019 and 2020) and Honeycrisp (2019) apples effectively delayed fruit maturity progression preharvest, and maintained fruit firmness higher postharvest, although not always statistically significant and dose and timing-dependent, until 9 months in CA plus 7 days at 68°F when compared to the untreated control. Skin color development was negatively affected by AVG treatments preharvest in Honeycrisp.
4. Honeycrisp apples stored in low pressure (RipeLocker, RL) at 33°F were comparable in terms of fruit maturity to those stored in CA/DCA at 37°F (plus 4 weeks in air). Soft scald incidence was block-dependent the first year and slightly higher in RL-stored fruit in 2020 and 2021. Bitter pit (+lenticel blotch pit) was reduced by vacuum RL in most sites in 2019 and 2020 but not in 2021.

Similar results in fruit maturity for Fuji apples, as well as overall low disorder incidences, except internal browning in all CA/RL storage protocols in 2021 season.

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

Activities:
During 2019, 2020 and 2021 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. This was done by sampling homogeneous fruit from 20 trees per block, 3-4 times (every 7-8 days) before harvest (WBH). At commercial harvest, fruit quality was performed in 18 fruit per Block, and peel samples were collected for further mineral analysis. After conditioning Honeycrisp apples at 50°F for 7 days and Fuji apples by delaying CA imposition for 20 days at 34°F, fruit were placed in different dynamic storage regimes (Table 1). Postharvest evaluations for Year 3 are currently being carried out and will end in July 2022.

RESULTS
Fruit Maturity & Physiological disorders
Honeycrisp: In 2019, differences in fruit maturity between Blocks after storage (Table 3) followed the same trend observed at harvest (Table 2). In general, fruit in all DCA systems lost 1.5 lb firmness in average with slight differences between Blocks and storage regimes, after 6 and 9 months plus 4 weeks in air. In 2020, maturity indices differed in fruit from different Blocks or their interaction with storage regimes in the case of I_{AD} (data not shown) and firmness in some cases (Table 3). In general, TA decreased 0.09% in average after long term storage with differences mostly between fruit from different Blocks and only between storage regimes after 9m+4wk+7 days at 68°F (Table 3). Overall, TA in 2019 was higher than in 2020 throughout storage (Table 3). Although harvest dates were similar or earlier in 2020 compared to 2019, fruit was smaller and less firm throughout storage (Tables 2 & 3).
In general, decay incidence was below 10% in average after 6 months and 19% after 9 months, with differences between blocks and storage treatments (Table 4). Soft scald incidence was block-dependent (highest in C21), and it was higher in 2019 and 2021 than in 2020 (Table 4). Only in 2019 the interaction Block x Storage regime was statistically significant (Table 4). These effects were observed until the end of the storage period (Table 4). Overall, there was significantly less soft scald in all CA/DCA storage regimes than that observed in fruit from the same Blocks stored in air for 4 months (data not shown). Soggy breakdown followed the same trend as soft scald in 2019, with significantly higher incidences in Block C21 compared to the rest after 9m+4wk+7 days at 68°F (data not shown).
Bitter pit varied between blocks and storage regimes with the highest incidence observed in fruit from W25 and W42 during all three seasons and storage length (Table 4).
The exploratory multivariate analyses including sites, bioclimatic indices, shoot length, fruit mineral content at harvest, crop load, and fruit maturity at harvest in all three seasons did not show consistent results to explain disorder's expression or softening rate postharvest in Honeycrisp. A larger dataset is needed to increase correlations between variables.

Fuji: In general, there were no major differences in fruit maturity at harvest between blocks within years (Table 5). Similar trends were observed postharvest, with no major differences between storage regimes (Table 6). Overall, TA in Year 1 and Year 3 were higher than in Year 2 until 9m+4wk+1 d at RT (Table 6). Among postharvest defects and disorders, decay and internal browning were the most prominent ones. Decay incidence was the highest in Year 3, and it significantly increase after CA opening plus 4 weeks in air (Table 7). In most cases, there was a significant block-effect (Table 7). Internal browning appeared after 9m+4w+7d (shelf-life) in all three seasons with significant block differences (Table 7). Superficial scald also appeared at this time-point with incidences below 5.0% in average and only in Year 1. CO_2

injury were also observed in all three season, but with very low incidences (0.6-1.1%) and significant differences between Block x Storage interaction (data not shown).

Table 1. Orchard information includes location, variety, rootstock, year planted, and harvest dates for all seasons.

Block	Location	Variety	Rootstock	Year planted	Harvest date (Year 1)	(Year 2)	(Year 3)
W25	Rock Island	Honeycrisp	B-9	2012	8/31/19	8/27/20	8/26/21
W42	Othello	Honeycrisp	B118	2009	9/2/2019	9/4/20	9/7/21
C21	Royal City	Firestorm	M9 337	1996	9/10/2019	9/1/20	9/3/21
C802	Quincy	Honeycrisp	M9-Pajam2	2010	9/6/2019	9/9/20	9/7/21
W18	Rock Island	Aztec Fuji	M9 337	2009	10/7/2019	10/6/2020	10/1/21
W40	Othello	Fuji	B118/M9-Pajam2	2010	10/3/2019	10/6/2020	10/6/21
C4	Royal City	Aztec Fuji	M26	2006	10/9/2019	10/8/2020	10/6/21
C902	Quincy	Fuji	M9 337	2009	10/4/2019	10/8/2020	10/6/21

Table 2. Maturity indexes (weight, green background color, red coverage, I_{AD}, flesh firmness, soluble solid content, starch index, internal ethylene concentration, IEC, titratable acidity, and respiration) for Honeycrisp apples from different Blocks (W25, W42, C21, C802) at commercial harvest in Year 1 (2019), Year 2 (2020), and Year 3 (2021).

Year	Orchard	Weight (g)	Background color (1-4)	Red coverage (%)	I_{AD}	Firmness (lb)	SSC (°Brix)	SI (1-6)	IEC (ppm)	TA (% malic acid)	Respiration Rate (mL CO_2/kg/h)
2019	W25	226.6 b[Z]	2.6 ab[Y]	80.7 a[Y]	0.65 b	16.4 a	15.2 a	4.2	2.8 b	N/A	N/A
	W42	212.9 b	2.1 b	55.7 c	0.82 a	14.1 c	11.8 c	4.4	27.1 a	N/A	N/A
	C21	265.4 a	3.4 a	87.8 a	0.38 c	15.7 ab	13.4 b	5.1	0.0 b	N/A	N/A
	C802	219.4 b	2.2 b	65.8 b	0.81 a	15.0 bc	11.8 c	4.3	10.4 ab	N/A	N/A
	Sign.	**	*	*	**	**	**	NS	**		
2020	W25	169.4 b[Z]	2.9	78.1 b[Y]	0.95 a[Z]	13.2	12.4 ab[Z]	1.8 b[Z]	0.0	0.55 a[Z]	18.6
	W42	176.2 b	2.4	60.3 b	0.87 a	12.6	11.4 b	4.4 a	0.0	0.45 b	18.8
	C21	186.6 b	3.1	94.1 a	0.42 b	13.4	11.5 b	4.7 a	0.0	0.44 ab	5.7
	C802	268.4 a	2.7	63.6 b	0.60 b	13.0	13.5 a	4.4 a	0.1	0.50 ab	14.5
	Sign.	*	NS	*	*	NS	*	*	NS	*	NS
2021	W25	254.6	2.4 bc	70.8 b	0.82 a	14.4 ab	12.8 a	3.4	0.0	0.50	22.7
	W42	225.3	2.2 c	65.0 b	0.84 a	13.4 b	11.7 bc	3.6	0.0	0.53	18.0
	C21	220.5	3.2 a	85.6 a	0.41 b	15.2 a	11.0 c	3.2	0.3	0.59	20.2
	C802	199.7	2.7 b	65.3 b	0.77 a	13.7 ab	12.2 ab	2.8	0.0	0.44	16.7
	Sign.	NS	**	**	**	*	*	NS	NS	NS	NS

[Z]Means followed by different letters are statistically different (ANOVA, *= P≤0.05; **: P≤0.01; NS: non-significant).Tukey's mean separation test (P≤0.05). N/A: not available

[Y]Kruskall Wallis (P≤0.05) and Dunn's for mean separation.

Table 3. Maturity indexes (flesh firmness, soluble solid content, starch index, titratable acidity internal ethylene concentration) for Honeycrisp apples stored in Controlled atmosphere (**CA**: 3.0% O_2/ 0.5% CO_2; **CA-ILOS**: 0.5% O_2/ 0.5% CO_2- 10 days & 1.0% O_2/0.7% CO_2 thereafter; **CA-RQ**: 3.0% O_2/0.5% CO_2) from different Blocks (W25, W42, C21, C802) at commercial harvest in Year 1 (2019), Year 2 (2020), and Year 3 (2021).

Factors	Firmness (lb)			SSC(°Brix)			TA (% malic acid)			IEC (ppm)		
						6m+4w+1d						
Orchard (**A**)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W25	14.6 a[Z]	13.8 bc	14.6 b	15.1	13.6 a	12.8 a	0.55 a	0.34 a	0.44	86.0	0.0	15.0
W42	13.5 b	13.2 c	13.4 c	12.5	11.7 b	11.2 c	0.42 b	0.29 bc	0.44	25.9	0.0	27.2
C21	13.1 b	14.9 a	14.8 a	12.4	11.6 b	12.4 ab	0.42 b	0.27 c	0.54	40.3	0.0	65.6
C802	14.3 a	13.9 b	13.6 c	12.0	13.6 a	11.7 bc	0.53 a	0.34 a	0.44	41.5	1.1	5.6
Sign.	**	*	**	*	*	**	**	*	**	**	NS	NS
Storage (**B**)												
CA	13.7	13.8	14.6	13.0	12.2 b	12.1	0.46	0.31	0.46	47.29	0.7	49.2
CA-ILOS	13.7	14.0	14.7	13.0	13.1 a	12.3	0.51	0.32	0.50	54.79	0.0	0.0
CA-RQ	14.1	14.1	14.7	13.0	12.5 ab	11.7	0.45	0.30	0.46	43.22	0.0	35.8
Sign.	NS	NS	NS	NS	*	NS	NS	NS	*	NS	NS	NS
A x B	NS	NS	NS	*	NS	NS	NS	NS	**	**	NS	NS
						6m+4w+7d						
Orchard (**A**)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W25	14.6	13.8 a	15.1 b	14.8	13.9	13.0	0.47 a	0.28	0.49 ab	79.5	0.0	71.3
W42	12.5	12.9 b	13.1 c	11.6	11.5	11.4	0.32 b	0.24	0.43 b	160.1	2.5	88.1
C21	13.2	14.2 a	15.2 a	13.0	12.1	12.9	0.45 a	0.24	0.54 a	63.2	0.0	35.9
C802	14.4	13.8 a	13.5 c	12.7	13.1	13.3	0.47 a	0.29	0.44 ab	89.0	2.0	95.1
Sign.	*	*	***	*	*	NS	***	NS	*	NS	NS	NS
Storage (**B**)												
CA	13.8	13.8	15.1	13.0	12.4	12.3	0.38 b	0.27	0.48	84.5	0.2	82.1
CA-ILOS	13.8	13.4	14.5	13.1	12.9	13.7	0.47 a	0.26	0.51	136.8	0.8	76.4
CA-RQ	13.5	13.8	14.8	12.9	12.6	11.9	0.42 ab	0.25	0.46	72.6	2.4	59.2
Sign.	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS
A x B	*	NS	NS	*	*	NS	NS	NS	NS	NS	NS	NS
						9m+4w+1d						
Orchard (**A**)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W25	15.5 a	14.1	14.2 b	14.8	12.9	12.8 a	0.63 a	0.38 ab	N/A	0.00	0.00	58.8
W42	13.8 b	13.5	13.3 b	11.8	11.2	11.1 c	0.44 c	0.35 ab	N/A	0.00	0.00	31.1
C21	12.7 c	14.3	15.3 a	12.1	11.3	12.7 a	0.47 bc	0.34 b	N/A	0.00	0.06	39.3
C802	15.0 a	13.4	13.7 b	11.8	12.6	11.6 b	0.57 ab	0.39 a	N/A	0.00	1.44	55.4
Sign.	**	*	**	**	*	**	*	*		NS	NS	*
Storage (**B**)												
CA	14.2	13.8	14.8	12.6	11.9	12.3 ab	0.53	0.38 a	N/A	0.00	0.47	55.3
CA-ILOS	14.1	13.6	14.8	12.6	12.3	12.3 a	N/A	0.38 a	N/A	0.00	0.00	49.0
CA-RQ	14.3	14.0	14.7	12.7	11.8	11.9 b	N/A	0.34 b	N/A	0.00	0.66	40.2
Sign.	NS	NS	NS	NS	*	*	NS	*		NS	NS	NS
A x B	NS	*	NS	***	*	NS	NS	*		NS	NS	NS
						9m+4w+7d						
Orchard (**A**)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W25	14.7 a	13.3 b	14.5 b	15.0	12.6	12.8 a	0.39 b	0.28 b	N/A	0.00	0.26	103.5 a

W42	12.8 b	13.0 b	13.1 b	12.2	11.0	13.3 c	0.39 b	0.32 ab	N/A	0.07	7.41	62.1 ab
C21	12.0 b	14.3 a	15.9 a	12.3	11.5	12.2 ab	0.33 b	0.30 ab	N/A	0.02	0.79	34.0 b
C802	14.2 a	13.4 b	13.6 b	12.4	12.6	11.8 bc	0.50 a	0.35 a	N/A	1.01	7.53	81.3 ab
Sign.	***	*	**	**	*	**	**	*		NS	NS	**
Storage (**B**)												
CA	13.2	13.7	14.8	12.8	11.8	12.0	NA	0.31 b	N/A	0.78	1.84	75.1
CA-ILOS	13.6	13.5	14.6	13.2	12.3	12.2	0.397	0.36 a	N/A	0.02	3.88	70.1
CA-RQ	13.6	13.4	14.6	12.9	11.6	12.0	0.428	0.26 c	N/A	0.02	6.28	68.7
Sign.	NS	NS	NS	NS	*	NS	NS	*		NS	NS	*
A x B	NS	NS	NS	**	*	NS	NS	NS		NS	NS	NS

[Z]Means followed by different letters are statistically different (ANOVA, *= P≤0.05; **: P≤0.01; NS: non-significant).Tukey's mean separation test (P≤0.05). N/A: not available

Table 4. Fruit defects (incidence, average %) in Honeycrisp apples stored in Controlled atmosphere with different protocols (**CA**: 3.0% O_2/ 0.5% CO_2; **CA-ILOS**: 0.5% O_2/ 0.5% CO_2- 10 days & 1.0% O_2/0.7% CO_2 thereafter; **CA-RQ**: 3.0% O_2/0.5% CO_2) from different orchard blocks (W25, W42, C21, C802) at 6 months, 6 months plus 4 weeks in air plus 1 day or 7 days at room temperature (68°F) in Year 1 (2019), Year 2 (2020), and Year 3 (2021).

Decay (%)									
	6m			6m+4w+1d			6m+4w+7d		
Orchard (**A**)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W25	0.9	1.3	0.0	1.3	3.9	5.5	7.2	7.8 a	14.6
W42	1.3	0.9	0.0	6.2	3.9	2.9	8.3	6.1 ab	7.1
C21	0.4	0.9	0.0	5.3	1.1	0.0	7.2	1.1 b	6.8
C802	1.8	0.4	0.0	3.1	0.6	5.5	8.3	3.3 ab	9.9
Sign.	NS	NS		NS	NS	*	NS	*	NS
Storage (**B**)									
CA	1.7 a	0.6	0.0	5.3 a	1.7	3.8	10.0 a	3.8	6.1
CA-ILOS	1.7 a	0.6	0.0	4.3 ab	1.3	3.9	9.8 a	3.3	16.9
CA-RQ	0.0 b	1.3	0.0	2.3 b	4.2	2.8	3.6 b	6.7	5.7
Sign.	*	NS		*	NS	NS	**	NS	NS
A x B	NS	NS		NS	*	NS	*	NS	NS
Soft Scald (%)									
	6m			6m+4w+1d			6m+4w+7d		
Orchard (**A**)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W25	0.9	0.0	0.0	3.1	0.0	0.6 b	4.4	1.7	0.6 b
W42	8.0	0.0	0.0	8.0	0.0	0.6 b	10.6	0.6	0.6 b
C21	11.1	0.0	0.0	12.4	0.0	14.6 a	20.0	0.0	15.2 a
C802	0.0	0.0	0.0	0.44	0.6	0.5 b	0.6	0.6	0.5 b
Sign.	**	NS		**	NS	**	**	NS	**
Storage (**B**)									
CA	7.3	0.0	0.0	9.0	0.4	2.4	11.3	1.3	2.4
CA-ILOS	4.3	0.0	0.0	5.7	0.0	6.0	11.3	0.4	6.5
CA-RQ	3.3	0.0	0.0	3.3	0.0	3.8	4.2	0.4	3.8
Sign.	**	NS		**	NS	NS	**	NS	NS
A x B	**	NS		**	NS	NS	**	NS	NS
Soggy Breakdown (%)									
	6m			6m+4w+1d			6m+4w+7d		
Orchard (**A**)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0
W42	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.0

C21	0.0	0.0	0.0	0.0	0.0	0.0	8.9	0.6	0.0
C802	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0
Sign.	NS	NS	NS	NS	NS	NS	**	NS	NS
Storage (**B**)									
CA	0.0	0.0	0.0	0.0	0.0	0.0	6.3	0.0	0.0
CA-ILOS	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0
CA-RQ	0.0	0.0	0.0	0.0	0.0	0.0	1.3	1.3	0.0
Sign.	NS	NS	NS	NS	NS	NS	**	NS	NS
A x B	NS	NS	NS	NS	NS	NS	**	NS	NS
				Bitter Pit (%)					
		6m			6m+4w+1d			6m+4w+7d	
Orchard (**A**)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W25	0.0	7.3 a	0.0	1.8	10.6 a	3.5	3.9	14.4 a	4.6
W42	8.9	4.7 ab	0.0	11.6	9.4 a	0.6	17.2	10.0 a	2.3
C21	7.6	0.4 c	0.0	9.8	1.1 b	1.7	13.9	1.1 b	1.7
C802	0.4	0.9 bc	0.0	1.8	1.7 b	0.7	3.9	2.2 b	0.7
Sign.	**	*	NS	**	*	NS	**	*	NS
Storage (**B**)									
CA	2.7	3.5	0.0	4.7	5.0	0.0 b	8.3	6.3	0.0 b
CA-ILOS	5.3	2.6	0.0	7.0	5.0	1.9 ab	10.8	7.5	1.9 ab
CA-RQ	4.7	3.8	0.0	7.0	7.1	2.9 a	10.0	7.1	5.0 a
Sign.	NS	NS	NS	NS	NS	*	NS	NS	**
A x B	**	NS	NS	**	NS	NS	**	NS	NS

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

Table 5. Maturity indexes (weight, green background color, red coverage, I_{AD}, flesh firmness, soluble solid content, starch index, internal ethylene concentration, IEC, titratable acidity, and respiration) for Fuji apples from different Blocks (W18, W40, C4, C902) at commercial harvest in Year 1 (2019), Year 2 (2020), and Year 3 (2021).

Season	Orchard (A)	Weight (g)	Background color (1-4)	Red coverage (%)	I_{AD}	Firmness (lb)	SSC (°Brix)	SI (1-8)	IEC (ppm)	TA (% malic acid)	Respiration Rate (mL CO_2/kg/h)
2019	W18	237.3 b	3.0	93.9	1.13 a	16.6 ab	14.0 a	6.6	0.36	N/A	N/A
	W40	503.1 a	3.0	94.2	0.87 b	17.8 a	13.1 ab	6.1	0.19	N/A	N/A
	C4	244.9 b	3.0	95.0	1.06 a	17.0 a	13.6 ab	6.2	0.22	N/A	N/A
	C902	523.3 a	3.0	100.0	1.13 a	16.2 b	11.9 b	6.8	0.32	N/A	N/A
	Sign.	**	NS	NS	**	*	*	NS	NS	-	-
2020	W18	181.0	2.4	91.4	1.04 b	15.4	14.1 a	3.9	0.00	0.35	37.8
	W40	187.2	2.5	81.7	0.67 c	16.4	13.7 a	5.7	0.00	0.38	28.7
	C4	189.1	2.1	79.7	1.07 b	16.3	12.3 b	5.2	0.00	0.35	33.2
	C902	190.0	2.0	74.2	1.26 a	16.9	12.3 b	5.2	0.00	0.33	33.4
	Sign.	NS	NS	*	*	NS	*	NS	NS	NS	NS
2021	W18	222.8	4.0	89.4 b	0.99 a	17.7	15.1 a	3.9 b	0.00	0.51	33.6 a
	W40	226.1	4.0	98.8 ab	0.80 b	17.5	13.2 b	4.9 a	0.02	0.40	13.7 b
	C4	220.6	4.0	97.6 a	0.88 ab	18.6	15.6 a	3.8 b	0.29	0.41	17.4 ab
	C902	228.1	4.0	93.6 ab	0.92 ab	18.1	13.3 b	5.3 a	0.04	0.41	17.9 ab
	Sign.	NS	NS	**	*	NS	**	**	NS	NS	*

[Z]Means followed by different letters are statistically different (ANOVA, *= P≤0.05; **: P≤0.01; NS: non-significant).Tukey's mean separation test (P≤0.05).

Table 6. Maturity indexes (flesh firmness, soluble solid content, starch index, titratable acidity internal ethylene concentration) for Fuji apples stored in Controlled atmosphere (**CA**: 0.8% O_2/ 0.8% CO_2; **CA-ILOS**: 0.6% O_2/ 0.8% CO_2- 10 days & 0.8% O_2/0.8% CO_2 thereafter; **CA-RQ**: 0.8% O_2/0.8% CO_2) from different Blocks (W18, W40, C4, C902) at commercial harvest in Year 1 (2019), Year 2 (2020), and Year 3 (2021).

Factors	Firmness (lb)			SSC(°Brix)			TA (% malic acid)			IEC (ppm)		
							6m+4w+1d					
Orchard (A)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W18	14.8 a	16.0	17.0	14.9 a	15.8	16.4	0.372 a	0.299 a	0.469	0.0	0.0	0.0
W40	15.2 a	16.7	15.2	13.5 b	14.7	13.6	0.372 a	0.258 ab	0.314	0.6	0.1	0.1
C4	15.1 a	15.6	16.9	14.6 a	13.8	16.6	0.291 b	0.197 c	0.299	0.1	0.3	0.0
C902	14.0 b	14.7	16.8	13.4 b	14.4	14.8	0.376 a	0.243 bc	0.409	0.5	0.0	0.0
Significance	**	*	*	**	*	**	*	*	**	NS	NS	NS
Storage (B)												
CA	14.6	16.2	16.5	13.9	14.5	15.3	0.372 a	0.255	0.365	0.0 b	0.0	0.0
CA-ILOS	15.0	16.7	16.4	14.3	14.9	15.3	0.326 b	0.254	0.362	0.8 a	0.0	0.1
CA-RQ	14.7	16.0	16.5	14.1	14.5	15.4	0.361 ab	0.238	0.390	0.1 b	0.2	0.0
Significance	NS	*	NS	NS	NS	NS	*	NS	*	*	NS	NS
A x B	NS	*	**	NS	*	**	NS	NS	**	NS	NS	NS
							6m+4w+7d					
Orchard (A)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W18	15.2	16.4 b	17.2	15.1 a	15.9 a	15.5	0.352 a	0.293 a	0.387	0.0	0.0	0.0
W40	15.1	17.2 a	16.4	14.1 b	14.8 b	13.7	0.352 a	0.278 ab	0.293	0.0	0.0	0.2
C4	14.9	16.0 b	17.4	14.6 b	14.1 c	16.3	0.278 b	0.191 c	0.255	1.8	0.1	0.0
C902	14.7	17.0 a	17.5	13.4 c	14.3 bc	14.3	0.370 a	0.238 bc	0.345	1.2	1.4	3.0
Significance	NS	*	*	*	*	**	*	*	**	NS	NS	*
Storage (B)												
CA	14.6 b	16.6 ab	16.8	14.0 b	14.8 ab	15.0	0.339 ab	0.253	0.351	0.0	0.0	0.5
CA-ILOS	15.0 a	17.0 a	17.6	14.5 a	15.0 a	15.1	0.362 a	0.262	0.372	0.0	0.1	0.2
CA-RQ	15.2 a	16.4 b	16.8	14.4 a	14.5 b	14.8	0.313 b	0.234	0.237	2.3	1.1	1.7
Significance	**	*	NS	*	*	NS	*	NS	**	NS	NS	NS
A x B	NS	NS	**	NS	NS	**	NS	NS	**	NS	NS	*
							9m+4w+1d					
Orchard (A)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W18	14.8	16.5	16.7	15.2	15.3	15.9	0.290	0.289 a[Z]	0.437	0.1 b	0.0	0.0
W40	15.0	16.8	16.0	13.6	14.0	14.4	0.303	0.261 ab	0.481	1.1 ab	0.0	0.1
C4	14.8	15.8	16.7	14.3	15.3	16.7	0.242	0.187 c	0.251	2.2 a	0.0	0.0
C902	15.0	17.1	16.9	13.0	13.8	15.0	0.336	0.252 b	0.365	2.2 a	0.0	0.0
Significance	NS	*	NS	*	NS	**	NS	*	NS	*	NS	NS
Storage (B)												
CA	15.1	16.7	16.3	14.2	15.4	15.2	0.307	0.253	0.355	1.4	0.0	0.0
CA-ILOS	14.8	16.7	16.8	13.9	14.4	15.8	0.260	0.251	0.355	1.1	0.0	0.0
CA-RQ	14.8	16.1	16.1	14.0	14.0	15.8	0.312	0.237	0.456	1.7	0.0	0.0
Significance	*	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
A x B	*	*	NS	*	NS	**	NS	NS	NS	NS	NS	NS
							9m+4w+7d					
Orchard (A)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W18	15.5	16.5	17.2	15.4 a	15.2 a	15.8	0.255 a	0.283 a	0.378	0.1 b	0.0	0.0
W40	15.2	16.8	15.9	13.9 bc	14.2 b	14.3	0.244 a	0.236 b	0.293	0.1 b	0.0	0.0
C4	14.9	15.9	17.1	14.8 ab	13.9 b	16.9	0.170 b	0.199 c	0.253	15.2 a	0.2	0.0
C902	15.1	17.1	16.6	13.4 c	13.8 b	16.6	0.265 a	0.231 bc	0.278	0.3 b	0.0	0.0
Significance	NS	*	NS	*	*	**	*	*	**	*	NS	-
Storage (B)												
CA	15.4	16.9	16.4 b	14.4	14.3	15.2	0.250	0.251 a	0.289	3.2	0.0	0.0
CA-ILOS	15.3	16.9	17.4 a	14.2	14.5	15.6	0.222	0.245 a	0.327	5.9	0.0	0.0
CA-RQ	14.9	15.9	16.6 ab	14.5	14.1	15.6	0.230	0.215 b	0.287	2.6	0.2	0.0
Significance	NS	*	*	NS	NS	NS	NS	*	NS	NS	NS	-

A x B	NS	*	NS	NS	NS	**	NS	NS	**	NS	NS	-

[Z]Means followed by different letters are statistically different (ANOVA, *= P≤0.05; **: P≤0.01; NS: non-significant).Tukey's mean separation test (P≤0.05).

Table 7. Fruit defects (incidence, average %) in for Fuji apples stored in Controlled atmosphere (**CA**: 0.8% O_2/ 0.8% CO_2; **CA-ILOS**: 0.6% O_2/ 0.8% CO_2- 10 days & 0.8% O_2/0.8% CO_2 thereafter; **CA-RQ**: 0.8% O_2/0.8% CO_2) from different Blocks (W18, W40, C4, C902) at 9 months plus 4 weeks in air plus 1 day or 7 days at room temperature (68°F) in Year 1 (2019), Year 2 (2020), and Year 3 (2021).

	Decay (%)								
	9m			9m+4w+1d			9m+4w+7d		
Orchard (**A**)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W18	0.4	0.4	1.7	0.9	3.9	1.0	13.3	5.6 ab	11.7
W40	0.9	0.4	2.3	4.0	1.7	1.8	8.9	10.6 a	1.8
C4	0.0	0.4	2.0	2.2	2.2	8.0	6.7	1.7 b	10.8
C902	0.4	0.0	2.3	3.6	1.1	4.6	10.6	2.8 b	10.3
Significance	NS	NS	NS	*	NS	*	NS	*	NS
Storage (**B**)									
CA	0.7	0.0	1.6	2.3	2.1	3.8	7.5	5.8	7.1
CA-ILOS	0.7	0.3	2.0	4.0	2.5	2.9	11.3	4.6	8.9
CA-RQ	0.0	0.6	2.7	1.7	2.1	5.9	10.8	5.0	9.0
Significance	NS	NS	NS	NS	NS	NS	NS	NS	NS
A x B	NS	NS	NS	*	NS	NS	NS	NS	NS
	Internal Browning (%)								
	9m			9m+4w+1d			9m+4w+7d		
Orchard (**A**)	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W18	0.0	0.0	0.0	0.0	0.0	0.0	0.0 b	0.0 b	7.0
W40	0.0	0.0	0.0	0.0	0.0	0.0	17.2 a	3.9 a	8.4
C4	0.0	0.0	0.0	0.0	0.0	0.0	0.0 b	0.0 b	4.9
C902	0.0	0.0	0.0	0.0	0.0	0.0	0.6 b	0.0 b	11.1
Significance	-	-	NS	-	-	-	*	*	NS
Storage (**B**)									
CA	0.0	0.0	0.0	0.0	0.0	0.0	2.9	0.0	8.3
CA-ILOS	0.0	0.0	0.1	0.0	0.0	0.0	3.8	2.1	8.3
CA-RQ	0.0	0.0	0.0	0.0	0.0	0.0	6.7	0.8	6.8
Significance	-	-	NS	-	-	-	NS	NS	NS
A x B	-	-	NS	-	-	-	NS	NS	NS

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

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

Activities:
During Years 1 and 2 different Retain OL treatments were applied to Gala and Honeycrisp commercial blocks in Hood River, OR (Year 1), and Gala in a. commercial block in Manson, WA. In all experiments treatments consisted in 10 fl oz/acre applied 4 and 1 week before harvest (T2), 20 fl oz/acre. (Full dose), 1 week before harvest (T3) plus an untreated control (T1) in Year 1, and all of them plus full dose 3 (T4) and 1 day (T5) before harvest in Year 2. Fruit was harvested twice: at commercial harvest and 7 days later. Maturity indices were evaluated from 27 days before harvest (DBH) until harvest and after 3, 6, and 9 months in CA storage plus 7 days at room temperature (68°F).

RESULTS

Year 1: When treatments were harvested according to the untreated fruit (H1) optimum maturity, Retain-treated fruit (T2, T3) was only significantly higher after 9 months in CA plus 7 days at RT (9.1 lb versus 7.8 lb) in Gala. Conversely, when they were harvest at the optimum maturity in the Retain-treated fruit (H2, approx. 1 week later), T3 showed consistently (although not always statistically different) higher flesh firmness and SSC from 3 until 9 months of storage than the rest of the treatments, except at 9 months plus 7 days at RT. This was also true in Honeycrisp. Both, T2 and T3 significantly affected red skin color (% coverage) in Honeycrisp apples. There were no consistent differences between treatments in IEC, SI, SSC or I_{AD} in Gala throughout storage. In Honeycrisp apples, only the I_{AD} values were consistently higher (less ripen), but not always statistically different, in Retain OL-treated fruit in comparison to the Untreated control. There were no statistical differences between defects incidences between treatments in any of the experiments.

Year 2: In general, all Retain OL treatments affected flesh firmness, I_{AD}, starch degradation (index) and fruit respiration progression preharvest. Retain OL-treated fruit maintained higher I_{AD}, flesh firmness effectively delaying the commercial harvest. T2 maintained the highest flesh firmness in fruit postharvest, although not always significantly different from T3 in H1 and T3, T4 and T5 in H2. Similar results were observed for the I_{AD} (chlorophyll degradation) values, which were higher (less degraded) in T3 compared to the rest of the Retain treatments (Table 8).

Table 8. Maturity indexes (weight, chlorophyll degradation (I_{AD}), flesh firmness (lb) soluble solid content, starch index, and titratable acidity (% malic acid)) for Gala apples treated with Retain OL (1: Untreated Control; 2: 10 Fl Oz/Ac, 21 DBH+7DBH; 3: 20 Fl Oz/Ac, 7 DBH; 4: 20 Fl Oz/Ac, 3 DBH; 20 Fl Oz/Ac, 1 DBH) and stored in Controlled atmosphere (0.8% O_2/ 0.8% CO_2) for 9 months plus 7 days at 68°F. Year 2 (2020) season.

	Eval. Time	Trt	Wt (g)		I_{AD}		Firmness (lbs)		SSC (°Brix)		Starch Index (1-6)		Acidity (% malic acid)	
H2 Gala	3 mo	1	187.91		0.175	a[Z]	13.39	a	13.6	b	6.0	c	0.216	
		2	176.31		0.448	b	15.09	b	12.0	a	5.1	a	0.200	
		3	187.73		0.465	b	14.48	a,b	12.2	a	5.5	b	0.162	
		4	186.67		0.483	b	13.96	a,b	11.9	a	5.9	c	0.207	
		5	187.79		0.422	b	14.16	a,b	12.4	a	6.0	c	0.213	
	p-value		0.643		<0.001		0.003		<0.001		<0.001		0.173	
	3 mo +7d	1	189.84		0.164	a	12.83	a	13.6	b	6.0		0.224	a,b
		2	184.01		0.550	c	14.92	b	12.4	a	6.0		0.232	b
		3	194.74		0.390	b	14.32	b	12.9	a,b	6.0		0.177	a
		4	191.96		0.419	b	14.12	b	12.9	a,b	6.0		0.203	a,b
		5	184.32		0.367	b	14.33	b	12.6	a	6.0		0.218	a,b
	p-value		0.613		<0.001		<0.001		0.001		-		0.033	
	6 mo	1	169.77		0.271	a	12.76	a	12.3		8.0		0.105	
		2	175.46		0.509	b,c	14.73	c	12.7		8.0		0.110	
		3	175.66		0.526	c	14.28	b,c	12.4		8.0		0.138	
		4	184.53		0.379	a,b	13.66	a,b	12.7		8.0		0.109	
		5	182.68		0.456	b,c	13.89	b,c	12.4		8.0		0.102	
	p-value		0.404		<0.001		<0.001		0.444		-		0.626	
	6 mo +7d	1	-		0.274	a,b	11.97	a	12.9		8.0		0.094	
		2	-		0.531	c	14.88	b	13.1		8.0		0.105	
		3	-		0.496	c	14.14	b	12.7		8.0		0.096	
		4	195.92		0.321	b	13.97	b	12.7		8.0		0.114	
		5	194.56		0.183	a	14.76	b	13.0		8.0		0.121	
	p-value		0.865		<0.001		<0.001		0.625		-		0.704	
	9mo	1	184.84	a	0.172	a	10.50	a	13.4		8.0		0.171	
		2	208.89	b,c	0.400	b	14.72	b	13.0		8.0		0.159	

	3	197.11	a,b	0.418	b	14.59	b	12.9		8.0	0.168
	4	237.91	c	0.384	b	11.88	a	13.1		8.0	0.140
	5	199.51	a,b	0.370	b	13.73	b	13.0		8.0	0.148
p-value		<0.001		<0.001		<0.001		0.109		-	0.385
9mo +7d	1	198.46	a,b	0.147	a	9.59	a	13.8	b	8.0	0.156
	2	193.13	a	0.360	b	14.87	b	13.3	a,b	8.0	0.197
	3	212.49	b,c	0.327	b	14.00	b	13.2	a,b	8.0	0.190
	4	199.64	a,b	0.300	b	12.63	b	13.2	a,b	8.0	0.193
	5	195.69	a	0.269	a,b	13.35	b	12.8	a	8.0	0.193
p-value		0.020		0.001		<0.001		0.020		-	0.059

[Z]Means followed by different letters are statistically different (ANOVA, P≤0.05).Tukey's mean separation test (P≤0.05).

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

Activities:
After commercial harvest, fruit from all commercial blocks in Obj. 1 and both cultivars, Honeycrisp and Fuji, were placed in vacuum storage (approx. 10% of regular atmosphere) bins (RipeLockers, RL) at 37°F (only Year 1) and 33°F after conditioning (see Obj. 1). Postharvest evaluations for Year 3 are currently being carried out and will be finish in July 2022.

RESULTS
Honeycrisp: In 2019 season, there were no major differences in maturity between vacuum RL and regular CA storage. Nevertheless, fruit stored in RL33 had less chlorophyll degradation (I_{AD} value) and less respiration after 9 months+4 wks+7 days at 68°F in all three seasons. Differences in fruit volatiles, including ethanol, were observed right after 9 months of storage in season 2020 and 2021, but they tended to disappear after 4 weeks in air (data not shown). The effect of the storage regime over soft scald was block-dependent in Year 1, and slightly higher in fruit stored in RL33 in Year 2 (Table 9). The same for soggy breakdown in Year 2. Bitter pit (+lenticel blotch pit) was significantly reduced by vacuum RL in most sites, regardless of differences in lot susceptibility. Similar results were observed in Year 2 (Table 9).

Table 9. Physiological disorders (incidence, average %) in Honeycrisp apples from different orchard blocks (W25, W42, C21, C802) stored in Controlled atmosphere (3.0% O_2/ 0.5% CO_2) or vacuum/low pressure in Ripelockers at 37°F (RL37) or 33°F (RL33) for up to 9 months plus 4 weeks in air plus 7 days at 68°F in Year 1 (2019), Year 2 (2020), and Year 3 (2021).

	Soft Scald (%)								
	9m			9m+4w+1d			9m+4w+7d		
Orchard	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W25	0.0	0.0	0.4	2.6	0.0	0.0 b	0.9 b	1.7	0.0 b
W42	1.6	0.3	2.8	3.6	0.8	7.1 ab	11.9 a	1.7	9.3 ab
C21	5.0	0.3	3.5	6.6	0.8	11.0 a	13.5 a	0.8	11.7 a
C802	0.4	0.3	0.6	3.1	2.5	0.0 b	6.2 ab	2.5	0.9 ab
P value	*	NS	NS	NS	NS	*	*	NS	*
Storage									
CA	3.0	0.0	1.6	3.7	0.0 b	3.7	5.8	0.4 b	3.0
RL33	1.4	0.5	1.7	4.8	2.1 a	4.5	10.2	2.9 a	6.3
RL37	0.8	N/A	N/A	1.5	N/A	N/A	7.9	N/A	N/A
P value	NS	NS	NS	NS	*	NS	NS	*	NS
A x B	*	NS	NS	*	NS	NS	NS	NS	NS
	Soggy Breakdown (%)								
	9m			9m+4w+1d			9m+4w+7d		

Orchard	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W25	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.0 b	0.0
W42	0.0	0.0	0.0	0.0	0.0	0.0	0.7	2.5 a	0.0
C21	0.0	0.0	0.0	2.2	0.0	0.0	12.8	0.0 b	0.0
C802	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.0 b	0.0
P value	-	-	-	≤0.05	-	-	NS	≤0.05	-
Storage									
CA	0.0	0.0	0.0	1.7	0.0	0.0	7.5	0.0	0.0
RL33	0.0	0.0	0.0	0.0	0.0	0.0	2.1	1.3	0.0
RL37	0.0	N/A	N/A	0.0	N/A	N/A	1.0	N/A	N/A
P value	-	-	-	*	-	-	NS	NS	-
A x B	-	-	-	*	-	-	NS	*	-
				Bitter pit + Lenticel blotch pit (%)					
	9m			9m+4w+1d			9m+4w+7d		
Orchard	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
W25	0.0	4.8 a	2.2 ab	1.8	14.2 a	0.9	2.0	14.2 a	4.0
W42	6.1	6.1 a	3.3 a	9.4	7.5 a	2.8	14.4	9.2 a	3.7
C21	2.7	0.6 b	0.0 b	5.7	0.8 b	0.0	8.1	0.8 b	0.0
C802	3.3	1.3 ab	0.3 b	5.3	1.7 b	0.0	9.0	1.7 b	0.0
P value	*	*	*	*	*	NS	*	*	NS
Storage									
CA	2.4	5.1	1.3	6.4	6.7	0.6	7.9	7.5 a	0.0
RL33	3.7	1.3	1.1	5.3	5.4	0.7	7.4	5.4 b	2.9
RL37	3.0	N/A	N/A	3.0	N/A	N/A	9.4	N/A	N/A
P value	NS	*	NS	NS	NS	NS	NS	*	NS
A x B	*	NS	NS	NS	NS	NS	NS	NS	NS

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

Fuji: In Year 1, 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. The effect of the storage regime over its expression was block-dependent. No superficial scald was observed in Year 2 or 3. Internal browning, CO2 injury and bitter pit incidences were below 4% in average in Year 1. Only internal browning was observed in Year 3, in all Blocks and mostly during 9m+4w+7d. In this case, higher levels were observed in RL33 treatment.

Executive Summary

Project Title: Postharvest system optimization for organic apple storage

Keywords: Honeycrisp, Fuji, fruit quality, cold storage, DCA

Abstract:
In order to evaluate different postharvest technologies for organic apples, 'Honeycrisp' and 'Fuji' apples from four different orchards were picked at commercial harvest during 3 consecutive seasons (2019-2021) and placed into different controlled atmosphere regimes. For 'Honeycrisp' these were: 1. CA (3% O_2/0.5% CO_2); 2. CA-RQ (3% O_2/0.5% CO_2), and 3. CA-ILOS (Initial low oxygen stress; 0.5% O_2/0.5% CO_2 -10 days, 1.0% O_2/0.7% CO_2 thereafter) after conditioning fruit for 7 days at 50°F. For 'Fuji' apples CA regimes were: CA: 0.8% O_2/ 0.8% CO_2; CA-ILOS: 0.6% O_2/ 0.8% CO_2- 10 days, and 0.8% O_2/0.8% CO_2 thereafter; CA-RQ: 0.8% O_2/0.8% CO_2 with pre-conditioning of 4 weeks in air before CA imposition. Fruit maturity and physiological disorders development were assessed after six and nine months of storage plus four weeks in air (37°F or 34°C) and 7 days at 65°F ('shelf-life'). Overall, all CA/DCA storage regimes evaluated were suitable for long-term storage of organic Honeycrisp and Fuji apples. Nevertheless, preharvest managements (nutrition, pathogens, etc.) and seasonal climate greatly affected the amount of decay and physiological disorders development during the storage period.
In 2020, soft scald incidence in Honeycrisp was lower than in 2019 season, and it was significantly reduced by all CA/DCA storage regimes when compared to those observed in fruit stored in air for 4 months. Similar results were observed in 2021 season. Soggy breakdown only appeared in 2019 and 2021 seasons after 9 months in CA/DCA+4 weeks in air+7 days at 68°F, and mostly in one of the cool sites. Bitter pit was block-dependent all seasons. Incidence greatly increase during the air period (4 weeks) after CA/DCA. In general, Fuji had very low level of defects and disorders with the most prominent ones being decay and internal browning, both of which appeared during air storage after CA/DCA and after 7 days at 68°F, respectively.
Overall, the application of aminoethoxyvinylglycine (AVG- Retain OL) on Gala (2019 and 2020) and Honeycrisp (2019) apples effectively delayed fruit maturity progression preharvest, and maintained fruit firmness higher postharvest, although not always statistically significant and dose and timing-dependent, until 9 months in CA plus 7 days at 68°F when compared to the untreated control. Skin color development was negatively affected by AVG treatments preharvest in Honeycrisp.
Honeycrisp apples stored in low pressure (RipeLocker, RL) at 33°F were comparable in terms of fruit maturity to those stored in CA/DCA at 37°F (plus 4 weeks in air). Soft scald incidence was block-dependent the first year and slightly higher in RL-stored fruit in 2020 and 2021. Bitter pit (+lenticel blotch pit) was reduced by vacuum RL in most sites in 2019 and 2020 but not in 2021.

Project Title: Effect of dump tank composition on lenticel breakdown disorder

Report Type: Final Project Report

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Cooperators: Chelan Fruit, Allan Brothers (providing fruit for the trial).

Project Duration: 3 Years

Total Project Request for Year 1 Funding: $ 26,480.00
Total Project Request for Year 2 Funding: $ 13,403.00
Total Project Request for Year 3 Funding: $ 6,201.00

WTFRC Collaborative Costs: None

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Item	2019	2020	2021
Salaries	20,480	10,240	5,120
Benefits	325	163	81
Supplies	3,000	2,000	
Travel	2,500	1,000	1,000
Miscellaneous			

Total	26,305	13,403	6,201

Footnotes: Salaries: Temporary personnel to assist in fruit evaluations and analysis in years 1, 2, and 3.
Benefits: $325, $163, and $81 are requested for benefits tied to the temporary personnel.
Supplies: Supply costs of $3,000 in year 1, $2,000 in year 2 are requested to pay for boxes, trays and supplies for fruit maturity evaluation. Travel: $2,500, $1,000, and $1,000 is requested in years 1, 2, and 3, 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.

OBJECTIVES

1. Assess the progression of lenticel browning disorder (LBD) incidence and severity on different lots of fruit and packing operations with different water makeups.
2. Correlate mineral and organic composition of water sources from different packing operations with LBD development.
3. Evaluate the effect of chlorine, peroxyacetic acid, chlorine dioxide, and ozone concentrations on LBD development on apples under controlled environment.

SIGNIFICANT FINDINGS

- LBD incidence and severity kept increasing after 96 h at 68°F and it was the highest after 1 week in air plus 7 days at 68°F. Fruit from all lots developed LBD after this time and after being packed or presized (including the least susceptible fruit).
- Phosphorus accumulation in the processing water was positively correlated with high LBD incidences. Calcium, Boron, and Potassium may also be playing a role.
- High free chlorine was not correlated with elevated LBD incidence.
- There was no evidence that neither chlorine or peracetic acid (at 50 ppm) in simulated washing conditions (COD) can cause LBD development.
- Water management (filtering, replacement) is critical when processing susceptible fruit.

Objective 1. Assess the progression of LBD incidence and severity on different lots of fruit and packing operations with different water makeups.

Activities

Different commercial lots of apples cv. Gala were sampled between 1 and 3 months, 4-6 months, and 7-9 months during the storage season. For each Lot, fruit was retrieved prior and after processing in the packing line (presizer and/or confection line). LBD incidence (# fruit affected/# total fruit) and severity (0-3, where 1=mild, 1-3 lesions per fruit, 2=moderate, 4+ lesions per fruit, and 3=severe, 50% area affected by lesions; Picture 1) were evaluated visually after 24 h, 96 h, after 1week in air (33°F), and 1 week in air ply 7 days at room temperature (RT, 68°F) of retrieving the sample. Fruit quality was determined for each sample at the time of retrieval. Three replicates per lot were used with a sample of 100 fruit per replicate.

Picture 1. LBD severity

RESULTS

LBD incidence and severity increased from the evaluation at time zero (0 h, right after processing) until 1 week (in cold storage) plus 7 days at 20°C ('shelf-life') in presized and/or packed fruit from all lots and time-points during storage (Tables 1 & 2).

When fruit from the same lot was evaluated during the storage season (#6780, #7961, #6520), LBD incidence progressively increase over time (Table 1, Figure 1).

Fruit maturity at harvest and after the packaging or presizing is shown in Table 2. Maturity indices at harvest and postharvest (considering that all fruit was treated with 1-MCP) cannot explain differences on LBD susceptibility observed on pre-process LBD incidences. Preharvest factors such as, weather before harvest (dehydration pressure, etc.), nutritional levels, tree vigor and others affecting LBD development, were not considered in this study.

Table 1. Mean LBD incidence (%) observed at different evaluation times after warehouse sampling. Asterisks indicate significant statistical differences (Kruskal-Wallis, P≤0.05) between sample means (Pre-line/Post-line) at each evaluation time.

			Mean LBD incidence (%)				
Lot	Processing date	Sample	0 h	24 h	96 h	1wRA	1w+7d
	10/19/2020	Pre-line	0.0	1.5	3.0	3.0	4.0
#6780		Presized	0.0	2.3	4.0	3.0.0	4.0
	11/5/2020	Pre-pack	0.6	1.0	1.0	1.0	2.3
		Packed	3.0 *	4.0 *	15.0 *	21.0 *	24.0 *
	11/12/2020	Pre-pack	0.0	0.0	0.0	0.3	1.0
		Packed	1.0	1.0	3.0	14.0 *	23.7 *

	11/19/2020	Pre-pack	1.7	3.0	5.3	7.0	11.0
		Packed	9.3 *	21.3 *	27.0 *	37.0 *	49.7 *
	1/15/2021	Pre-pack	1.0	1.7	2.7	4.0	5.0
		Packed	1.7	1.3	2.0	2.7	6.7
	1/26/2021	Pre-pack	0.7	4.7	6.7	8.7	22.0
		Packed	22.7 *	35.0 *	48.7 *	53.7 *	64.7 *
	2/2/2021	Pre-pack	0.7	1.0	1.3	3.3	5.0
		Packed	8.7 *	15.0 *	20.3 *	22.7 *	31.3 *
#7961	12/7/2020	Pre-pack	0.0	0.0	0.0	3.0	4.0
		Packed	0.0	0.0	0.0	2.0	2.7
	12/11/2020	Pre-pack	0.0	0.0	0.0	0.0	0.0
		Packed	1.3 *	1.3 *	3.0 *	3.0 *	3.0 *
#6520	12/7/2020	Pre-pack	0.0	0.0	0.3	0.7	0.7
		Packed	0.0	0.0	0.3	1.3	1.3
	12/11/2020	Pre-pack	0.0	0.0	0.0	0.0	0.0
		Packed	0.0	0.0	0.3	2.3 *	3.0 *
#2750	12/11/2020	Pre-pack	0.0	0.7	1.3	2.3	3.3
		Packed	2.3	2.3	4.0	5.0	7.7
#2670	12/7/2020	Pre-pack	0.0	0.0	0.3	1.3	3.3
		Packed	0.0	0.0	0.6	1.7	3.3
#G-434	1/7/2021	Pre-pack	0.0	0.3	1.3	2.0	3.0
		Packed	0.0	0.5	0.5	0.8	1.5

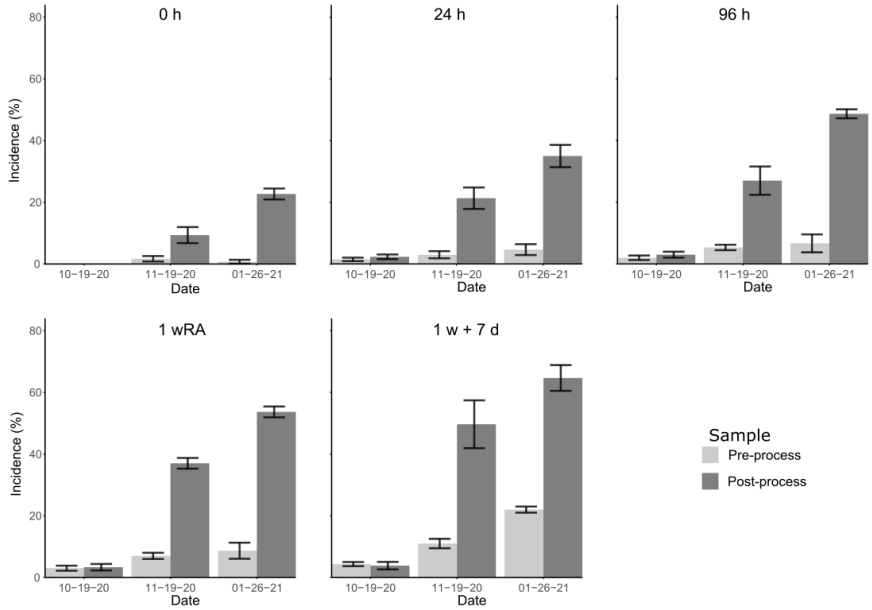

Figure 1. LBD incidence (%, average) pre and post-process in lot #6780 during different time during the storage season. Inside bars indicate standard error (n=3).

Table 2. Mean LBD severity (0-3) observed at different evaluation times after warehouse sampling. Asterisks indicate significant statistical differences (Kruskal-Wallis, $P \leq 0.05$) between sample means (Pre-line/Post-line) at each evaluation time.

Lot	Processing date	Sample	**Mean LBD severity**				
			0 h	24 h	96 h	1wRA	1w+7d
#6780	10/19/2020	Pre-line	0.00	0.00	0.02	0.03	0.05
		Presized	0.00	0.00	0.03	0.03	0.04
	11/5/2020	Pre-pack	0.03	0.04	0.04	0.04	0.10
		Packed	0.10	0.13	0.67*	0.97*	1.21*
	11/12/2020	Pre-pack	0.02	0.02	0.07	0.34	0.55
		Packed	0.00	0.00	0.00	0.01	0.04
	11/19/2020	Pre-pack	0.11*	0.33*	0.43*	0.67*	0.97*
		Packed	0.02	0.04	0.06	0.09	0.14
	1/15/2021	Pre-pack	0.01	0.02	0.02	0.03	0.06
		Packed	0.02	0.02	0.03	0.05	0.06
	1/26/2021	Pre-pack	0.27*	0.46*	0.69*	0.86*	1.11*
		Packed	0.01	0.05	0.08	0.10	0.26
	2/2/2021	Pre-pack	0.12*	0.21*	0.27*	0.30*	0.43*
		Packed	0.01	0.01	0.02	0.04	0.06
#7961	12/7/2020	Pre-pack	0.00	0.00	0.00	0.03	0.04
		Packed	0.00	0.00	0.00	0.05	0.06
	12/11/2020	Pre-pack	0.00	0.00	0.00	0.00	0.00
		Packed	0.03	0.05	0.05	0.06	0.06
#6520	12/7/2020	Pre-pack	0.00	0.00	0.00	0.02	0.02
		Packed	0.00	0.00	0.00	0.01	0.01
	12/11/2020	Pre-pack	0.00	0.00	0.00	0.00	0.00
		Packed	0.00	0.00	0.00	0.03	0.03
#2750	12/11/2020	Pre-pack	0.01	0.02	0.02	0.03	0.05
		Packed	0.03	0.05*	0.05*	0.06*	0.10*
#2670	12/7/2020	Pre-pack	0.00	0.00	0.01	0.02	0.03
		Packed	0.00	0.00	0.00	0.02	0.04
#G-434	1/7/2021	Pre-pack	0.00	0.00	0.01	0.01	0.03
		Packed	0.00	0.01	0.01	0.01	0.02

Table 2. Harvest maturity and postharvest treatments of fruit from different Gala apples (information provided by the warehouse).

Lot	Harvest	Treatments	Flesh firmness (lb)	Starch Index (1-8)
#6780	9/1/2020	1-MCP+Sch[Z] (9/3/2020)	19.46±2.28[Y]	2.3
#7961	9/6/2020	1-MCP+Sch (9/2, 3, 14/2020)	18.82±1.97	3.0

#6520	9/4/2020	1-MCP+Sch (9/2,13/2020)	19.49±1.72	2.3
#2670	9/1/2020	1-MCP+Sch (9/3,14/2020)	18.48±2.36	2.9

[Z] Scholar Max fogging, commercial rate.

[Y] Mean ± standard deviation

Table 3. Fruit maturity at the time of sampling during the storage period in Gala lots #6780, #7961, #6520, #2750, #2670, #G434.

Lot	Processing date	Sample	+1 days at 20°C			+7 d at 20°C		
			Firmness (lb)	SS (Brix)	SI (1-8)	Firmness (lb)	SS (Brix)	SI (1-8)
#6780	10/19/2020	Pre-line	15.3±1.7[Z]	13.6±0.7	6.0±0.0	15.0±1.6	14.2±0.8	7.7±0.4
		Presized	14.6±1.8	13.2±0.9	6.4±0.8	14.5±1.9	14.0±1.0	7.6±0.3
	11/5/2020	Pre-pack	12.8±1.2	13.4±0.8	7.6±0.7	15.0±1.6	13.9±0.6	7.9±0.2
		Packed	14.4±1.8	14.6±0.9	7.2±1.1	14.5±1.9	13.9±1.3	7.8±0.3
	11/12/2020	Pre-pack	15.9±1.4	13.1±1.1	7.5±0.8	14.8±1.8	13.6±1.4	8.0±0.1
		Packed	15.0±1.5	13.2±1.0	7.4±0.9	15.2±1.9	13.2±1.2	8.0±0.1
	11/19/2020	Pre-pack	14.9±1.4	13.5±0.8	7.9±0.3	14.2±1.6	13.8±1.1	8.0±0.0
		Packed	13.2±3.6	13.9±1.6	7.7±0.5	13.7±1.0	13.6±0.9	8.0±0.0
	1/15/2021	Pre-pack	16.0±1.2	14.8±0.7	6.0±0.0	15.9±1.8	15.1±0.9	6.0±0.0
		Packed	13.8±3.2	14.3±1.2	6.0±0.0	15.6±1.3	14.9±1.0	6.0±0.0
	1/26/2021	Pre-pack	14.0±2.1	13.6±1.0	6.0±0.0	13.2±1.6	13.9±0.6	6.0±0.0
		Packed	13.4±1.5	14.0±0.8	6.0±0.0	13.3±2.5	14.0±1.0	6.0±0.0
	2/2/2021	Pre-pack	15.9±1.4	13.6±1.6	6.0±0.0	15.2±2.2	14.7±0.8	6.0±0.0
		Packed	15.7±1.0	14.1±0.9	6.0±0.0	16.0±1.3	14.9±1.1	6.0±0.0
#7961	12/7/2020	Pre-pack	13.7±1.3	13.8±0.8	6.0±0.1	14.5±1.2	14.1±1.0	7.8±0.4
		Packed	13.4±0.9	13.2±0.9	6.0±0.1	13.3±1.9	13.4±0.9	7.9±0.3
	12/11/2020	Pre-pack	13.1±1.5	12.1±0.7	6.0±0.1	13.9±1.3	12.7±0.7	8.0±0.0
		Packed	13.8±1.1	12.9±1.0	6.0±0.1	13.5±2.2	12.5±0.9	8.0±0.0
#6520	12/7/2020	Pre-pack	13.1±2.3	12.7±1.1	5.9±0.2	13.0±2.1	12.5±0.9	6.0±0.0
		Packed	12.7±1.9	12.5±1.0	6.0±0.1	14.0±1.7	13.1±1.1	6.0±0.0
	12/11/2020	Pre-pack	12.7±2.4	11.9±0.7	6.0±0.1	13.0±1.7	12.3±1.0	6.0±0.0
		Packed	12.7±1.3	11.9±1.2	6.0±0.1	13.3±2.5	12.7±0.9	8.0±0.0
#2750	12/11/2020	Pre-pack	15.4±1.2	12.5±1.5	5.9±0.2	14.4±2.2	12.7±1.0	8.0±0.0
		Packed	13.9±1.1	12.1±1.0	5.9±0.2	15.1±1.8	12.8±1.2	7.9±0.2
#2670	12/7/2020	Pre-pack	14.8±1.3	15.9±2.1	6.0±0.1	14.7±1.6	12.7±1.1	8.0±0.0
		Packed	15.1±1.5	13.0±0.8	5.9±0.1	15.1±1.8	12.8±1.2	7.9±0.2
#G-434	1/7/2021	Pre-pack	15.1±0.5	13.5±0.8	6.0±0.0	13.7±1.2	13.4±0.8	6.0±0.0
		Packed	13.5±1.0	12.9±1.2	6.0±0.0	14.1±1.0	13.5±1.1	6.0±0.0

[Z] Mean ± standard deviation

Objective 2. Correlate mineral and organic composition of water sources from different packing operations with LBD development.

Activities

The water makeup (carbohydrate, protein, mineral content, chemical oxygen demand (COD), oxidation reduction potential (ORP), and turbidity) was determined for all water sources during the processing of each fruit lot, and later correlated with their LBD incidence differences (Δ LBD) between pre-process and post-process, both after 1 week in cold storage plus 7 days at 20°C.

RESULTS

The highest ORP, conductivity, temperature, turbidity, free chlorine and minerals were observed at the dump tank or first flume in the confection line in one of the operations. In the second operation, this was not the case and mineral content varied between flumes 1, 2 and 3 (data not shown).

Combining all lots, sampling dates, Δ LBD from each of them, and water in a multivariate statistical analysis (Principal Component Analysis, PCA; Figure 2), we were able to see four clusters which separation was driven by different water components, e.g. cluster 1, lot with high Δ LBD, was highly correlated with P content (highest content, Fig. 3). Furthermore, the level of P in the processing waters was also highly correlated with LBD incidence when all lots were combined (R^2=0.72; Fig. 4). Cluster 1 was also negatively correlated with free chlorine, Mg, Na, COD and pH. Although P content appeared to be critical for LBD development post processing, cluster 2, which has the same lot as cluster 1 but processed in different dates (with slightly less Δ LBD) was positively correlated with Ca, B, and K contents (Fig. 2). Nevertheless, this was also true for cluster 3 which grouped lots with low LBD incidence (Fig. 2). On the other hand, cluster 4 grouped the lot with the lowest Δ LBD and was positively correlated with free chlorine levels (highest levels, Fig. 3). The level of each water component for each cluster is shown in Figure 3.

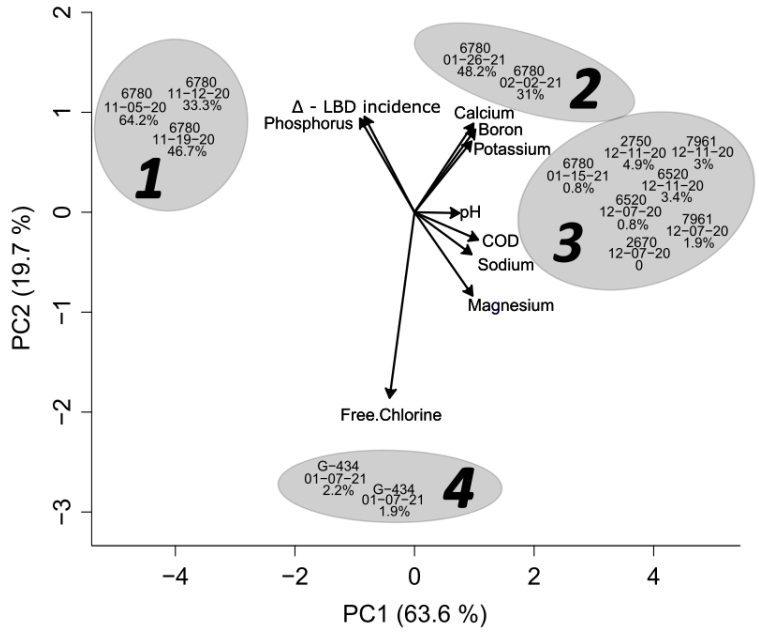

Figure 2. Principal Component Analysis (PCA) combining Δ LBD incidence (from different growers and pull-outs) and water chemistry in flume and pre-sizer. Each cluster component contains Lot number/ sample date/ LBD incidence difference (Δ LBD).

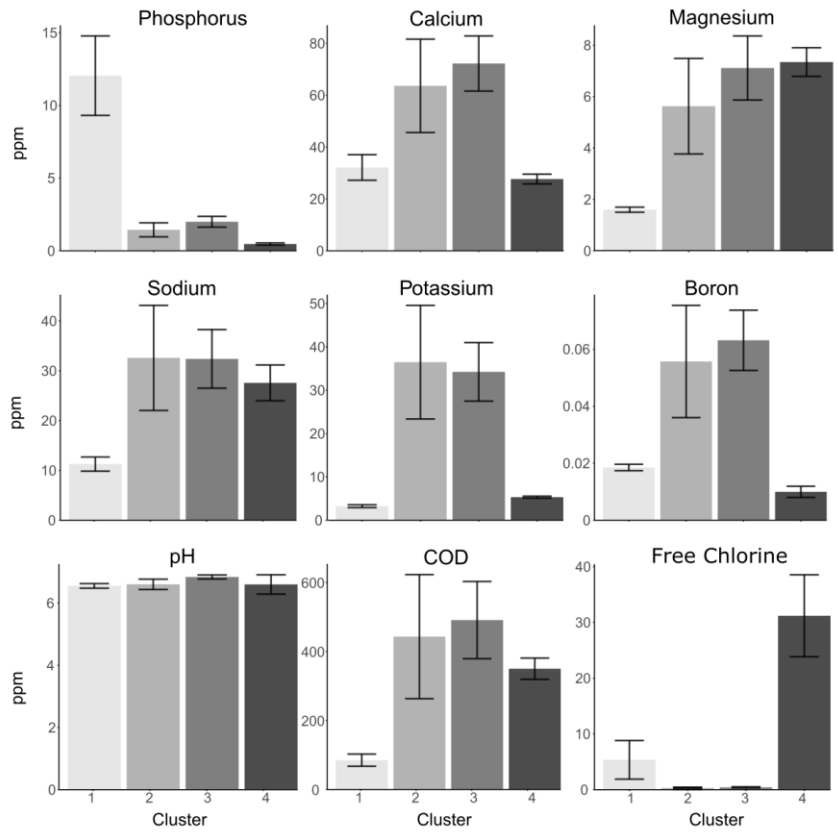

Figure 3. Mean water parameter for each cluster in PCA (Fig. 2). Inside bars indicate standard error within each cluster (n=3).

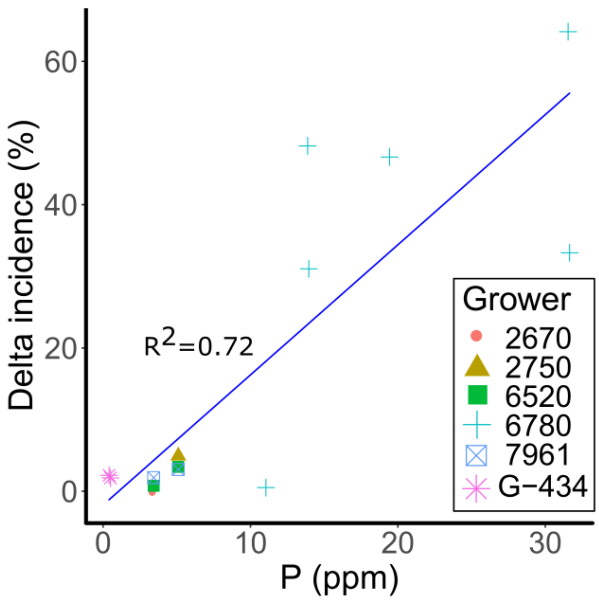

Figure 4. Mean water parameter for each cluster in PCA (Fig. 2). Inside bars indicate standard error (n=3).

Objective 3. Evaluate the effect of chlorine, peroxyacetic acid, chlorine dioxide, and ozone concentrations on LBD development on apples under controlled environment.

<u>Activities</u>

Different sanitizer treatments using simulated water make-up on apple packing lines (Table 4) were used on Gala apples from 3 'susceptible' commercial lots (#7610, #6560, #325). Temperature and pH of each treatment is shown in Table 5. Lenticel breakdown (LBD) incidence (# fruit affected/# total fruit) and severity (mild, moderate and severe, see Obj. 1) were evaluated as follows:

Storage condition→	7 days at 4°C			7 days at 22°C	
Evaluation time→	0h	24h (1d)	96h (4d)	168h (7d)	336h (14d)

Table 4. Treatments and composition of simulated experimental washing conditions (COD).

Treatments	**COD formula**
1) Untreated control 2) 500 ppm COD water only 3) 500 ppm COD + free chlorine 50ppm plus 5% (v/v) phosphoric acid to adjust pH to 6.5 4) 500 ppm COD + PAA 50ppm	**COD level** = 500 ppm **Silt loam soil =** 1.82±0.77 % (w/v)[a] **Unsweetened apple sauce:** 2.42±1.04 % (w/v)

[a] Mean % (w/v) ± standard deviation

Table 5. Temperature and pH of water and pH for each treatment.

Treatment	Temperature (°C)	pH
Control	-	-
Water only	14	6.7
Cl 50 ppm	11	6.5
PAA 50 ppm	16	4.4

A Fisher's exact test was used to analyze the categorical data of the incidence of lenticel breakdown damage based upon the following categorical variables: treatments [Chlorine (Cl) 50 ppm; Peracetic acid (PAA) 50 ppm; Water only, control], storage time (0, 1, 4, 7, 14 days), and lots (6560,7610,0325) A post hoc pairwise comparison was used to compare the levels of each categorical variable when a significant difference was observed. The significance level for all tests was $\alpha = 0.05$. Statistical analysis was performed in R (version 4.0.2) using RStudio (version 1.3.1056) (RStudio, Inc., Boston, MA, USA).

RESULTS

Overall, there were no significant differences ($p>0.05$) in LBD incidence or severity across lots and treatments (Table 6), but there were over storage time within each treatment (Fig. 5).

Table 6. Incidence and severity (Mild, Moderate, Severe) of LBD (%) over 14 days of storage time by lot (#7610, #6560, #325) and treatment (Control, Water only, PAA 500 ppm, Chlorine 50 ppm).

Treatment / Lot	Storage days	0			1			4			7			14		
	Scale	Mild	Mod	Sev	Mild	Mod	Sev	Mild	Mod	Sev	Mild	Mod	Sev	Mild	Mod	Sev
Control	7610	6.0%	0.0%	0.0%	8.0%	0.0%	0.0%	15.0%	0.0%	0.0%	20.0%	0.0%	0.0%	20.0%	0.0%	0.0%
	6560	2.0%	0.0%	0.0%	4.0%	0.0%	0.0%	5.0%	0.0%	0.0%	5.0%	0.0%	0.0%	8.0%	0.0%	0.0%
	325	3.0%	0.0%	0.0%	3.0%	0.0%	0.0%	12.0%	0.0%	0.0%	15.0%	0.0%	0.0%	15.0%	0.0%	0.0%
	Total	3.7%	0.0%	0.0%	5.0%	0.0%	0.0%	10.7%	0.0%	0.0%	13.3%	0.0%	0.0%	14.3%	0.0%	0.0%
Water only	7610	10.0%	0.0%	0.0%	10.0%	0.0%	0.0%	11.0%	0.0%	0.0%	12.0%	0.0%	0.0%	14.0%	0.0%	0.0%
	6560	5.0%	0.0%	0.0%	7.0%	0.0%	0.0%	9.0%	0.0%	0.0%	14.0%	0.0%	0.0%	16.0%	1.0%	0.0%
	325	4.0%	0.0%	0.0%	6.0%	0.0%	0.0%	11.0%	0.0%	0.0%	20.0%	0.0%	0.0%	24.0%	0.0%	0.0%
	Total	6.3%	0.0%	0.0%	7.7%	0.0%	0.0%	10.3%	0.0%	0.0%	15.3%	0.0%	0.0%	18.0%	0.3%	0.0%
PAA 50 ppm	7610	8.0%	0.0%	0.0%	9.0%	0.0%	0.0%	11.0%	0.0%	0.0%	17.0%	0.0%	0.0%	20.0%	0.0%	0.0%
	6560	4.0%	0.0%	0.0%	7.0%	0.0%	0.0%	10.0%	0.0%	0.0%	9.0%	0.0%	1.0%	15.0%	1.0%	0.0%
	325	5.0%	1.0%	0.0%	5.0%	2.0%	0.0%	11.0%	0.0%	2.0%	13.0%	2.0%	0.0%	15.0%	2.0%	1.0%
	Total	5.7%	0.3%	0.0%	7.0%	0.7%	0.0%	10.7%	0.7%	0.0%	13.0%	1.0%	0.0%	16.7%	1.0%	0.3%
Chlorine 50 ppm	7610	5.0%	0.0%	0.0%	6.0%	0.0%	0.0%	10.0%	0.0%	0.0%	14.0%	0.0%	0.0%	15.0%	0.0%	0.0%
	6560	3.0%	0.0%	0.0%	4.0%	0.0%	0.0%	13.0%	0.0%	0.0%	15.0%	0.0%	0.0%	20.0%	0.0%	0.0%
	325	2.0%	0.0%	0.0%	4.0%	0.0%	0.0%	8.0%	0.0%	0.0%	9.0%	0.0%	0.0%	17.0%	1.0%	0.0%
	Total	3.3%	0.0%	0.0%	4.7%	0.0%	0.0%	10.3%	0.0%	0.0%	12.7%	0.0%	0.0%	17.3%	0.3%	0.0%

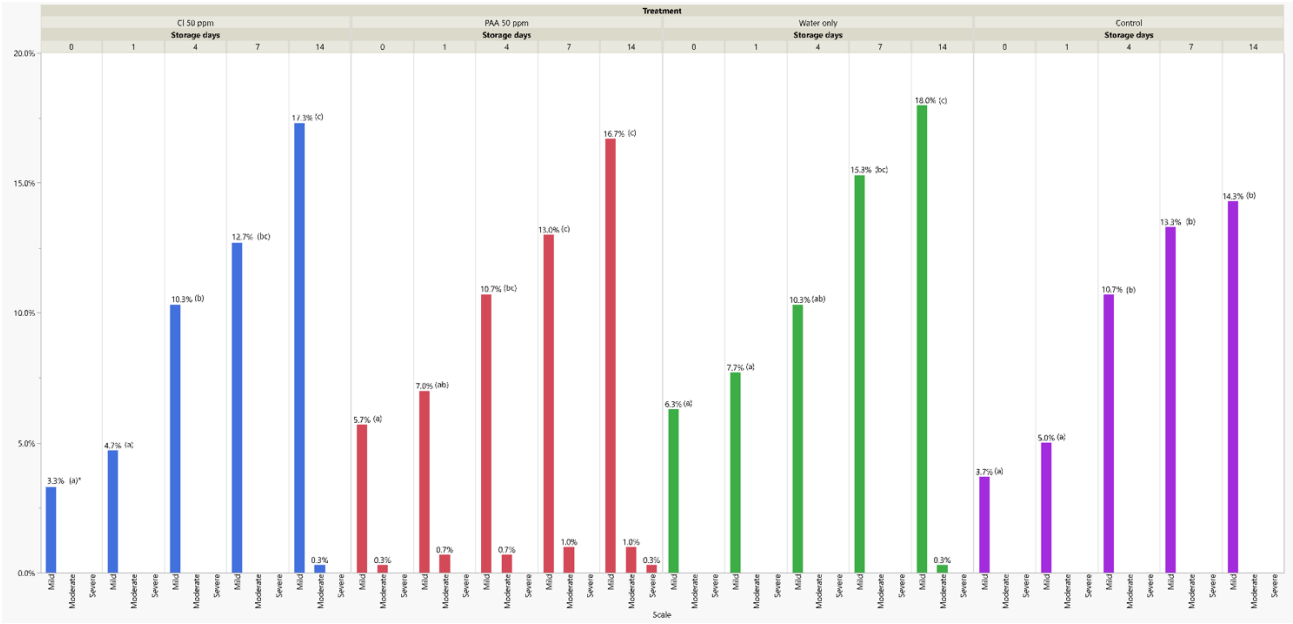

Figure 5. Incidence of LBD (%) over 14 days of storage time by treatment applied [Chlorine (Cl) 50 ppm; Peracetic acid (PAA) 50 ppm; water only, control]
*Value bars within treatments followed by the same lowercase letters in parenthesis are significantly different during storage time.

Executive Summary

Project Title: Effect of dump tank composition on lenticel breakdown disorder

Keywords: LBD, apple quality, Gala, heat stress, postharvest, packing

Abstract:
Lenticel breakdown (LBD) is an important physiological disorder on apples when growing in dry and hot environments. It appears mostly after fruit has been processed (packed and/or presized). Although it is of multi-factorial origin, processing conditions have a major influence on its development. The objective of this work was to assess the effect of water chemistry (carbohydrate, metals and minerals content, pH, ORP, conductivity, temperature, turbidity, COD, free chlorine) during processing on LBD development in commercial fruit lots throughout the storage period. Five different lots of Gala apple were sampled pre and post packaging/processing, along with water samples taken from different sections of the line (presizer, confection line: dump tanks, flumes) at the same time. Fruit from all lots developed LBD symptoms after processing, but only the most susceptible one's pre-processing. Symptoms started to appear 24 h after it and they continued to increase in number of fruit affected and severity until 1 week in air storage plus 7 days at 68°F. Phosphorus accumulation in the water was positively correlated with high incidences of LBD. Calcium, Boron and Potassium may also be playing a role in disorder's expression. High free chlorine was not correlated with LBD development. Neither chlorine (50ppm) or peracetic acid (PAA, 50ppm) solutions applied to susceptible fruit lots increased LBD incidence or severity.

FINAL PROJECT REPORT **YEAR:** No Cost Extension

Project Title: Critical limits for antimicrobials in dump tank systems

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Cooperators: WA packinghouses

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
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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

- Mean COD value (preliminary data) was 592 mg/L, with considerable variation amongst sites and over time.
- Chlorine efficacy was highly dependent on organic load and exposure time.
- *L. monocytogenes* was more resistant to PAA than *Salmonella* or STEC.
- PAA efficacy increased with exposure time, while chlorine remained unchanged.
- Chlorine dioxide and ozone were not as effective as PAA or chlorine and showed little or no efficacy at low COD conditions.

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 included data collection for only the 24hr of production. To encompass the full period water was 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 was used to evaluate significant differences of water attributes and nutritional compounds.

Objective 2. Determine the impact of free chlorine (FC), peroxyacetic acid (PAA), 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 were developed to represent standard features of washwater used in packinghouses in Washington. Three variations of dump tank water quality were used to represent postharvest water quality features which are inclusive of real-life conditions as determined by objective 1.

Microbial cultures. A five-strain cocktail of STEC, *Salmonella*, and *L. monocytogenes* associated with an outbreak were used for this objective. Bacterial strains 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* were 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 was incubated at 37°C (98.6°F) for 24 h to achieve a lawn of each strain. Each plate was flooded with 10 ml of Buffered Peptone Water (BPW) to harvest cells. *E. coli* and *Salmonella* strains were combined in equal volumes to create the five-species cocktail for inoculation. The same process was used for *L. monocytogenes*, with the exception that each strain was 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 was evaluated for chlorine and PAA, while one concentration plus no sanitizer control was evaluated for chlorine dioxide (3 ppm) and ozone (1 ppm). The upper limit was 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 was maintained at 6.5 with the addition of a 1 in 10 dilution of 50% (v/v) of phosphoric acid.

Determining impact of sanitizers on pathogen survival. Simulated washwater treatments were inoculated and bacteria enumerated to estimate survival after 15, 30 and 60 seconds of exposure. All samples were 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) was conducted. Post-hoc analyses was also conducted to determine significant differences between survival rates between and within treatments.

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 physicochemical 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. Distinct differences in inactivation curves between free chlorine (FC) and peroxyacetic acid (PAA) were seen for all organisms (Figures 1, 2, and 3). When bacteria were exposed to FC, there was a sharp significant (p<0.05) initial reduction in bacterial populations within the first 15 s, after which the resulting populations remained rather stable for the remaining exposure time. In contrast, when exposed to PAA, bacteria first exhibited a slower initial inactivation with a more rapid decline after 15 s. PAA is a peroxide of acetic acid generated

from the reaction of acetic acid and hydrogen peroxide (*11*). Having a large oxidation potential, even greater than chlorine, PAA's mode of antimicrobial activity is similar to other peroxides and oxidizers (*38*). It has been theorized that PAA oxidizes sulfhydryl and disulfide bonds located in the cell wall and in other cellular components (*9*, *16*). Through rupture of these bonds, the fluidity of the cellular membrane is altered, proteins are denatured, and enzymes and metabolite functions are disrupted, causing detrimental effects to the cell (*9*, *16*).

A two-slope inactivation was seen when bacteria were exposed to PAA (Figure 1 B, D, F, Figure 2 B, D, F, and Figure 3 B, D, F). PAA has been shown to oxidize organic compounds at a slower rate than FC, which could help explain the differences seen in inactivation curves (*20*). Additionally, it is thought that the cellular membrane of the bacterial cells may exhibit initial resistance to PAA diffusing into the cell when exposed for shorter periods of time, also resulting in a slower inactivation rate (*14*). Gereffi et al. (*10*) found that *Salmonella* populations in round green tomato flume water were undetectable (<1 log CFU/mL) after 30 s regardless of organic load when exposed to 25 ppm PAA. In the same study *Salmonella* populations were only recoverable 2 s after of exposure to 25 ppm FC demonstrating the quick inactivation mechanism of chlorine (*10*). A rapid decline in microbial populations when exposed to varying FC concentrations was also seen in this study for all organisms (Figure 1 A, C, E, Figure 2 A, C, E, and Figure 3 A, C, E), with the greatest decline in microbial populations achieved within the first 15 s of exposure.

Figure 4 demonstractes the efficacy of chlorine dioxide (3 ppm) when exposed to low COD conditions for target foodborne pathogens. This concentration is the highest which can be used for direct product contact based upon FDA regulations. It is known that chlorine dioxide can have excellent efficacy, but based upon current regulatory limits it is generally considered rather ineffective when COD increases in water systems. That trend was apparent in these results, with <1 log inactivation of *L. monocytogenes* (Figure 4B) and no significant reduction of STEC or *Salmonella* (Figure 4A and 4C).

Ozone was generated on site to achieve 1 ppm in solution. Similar to chlorine dioxide, limited efficacy was found under low COD conditions, but this concentration achieved a 1 to 3 log reduction of target organisms under these conditions, which was significantly different from water-only controls (Figure 5 A-C; $p<0.05$). Hilighting once more the impact organic load plays in efficacy and limited use for this compound in recirculated systems where organic load easily climbs.

When introduced into an aqueous solution, hypochlorite dissociates into sodium or calcium ions and hypochlorite (OCl^-) (*6*). OCl^- gains hydrogen atoms to become at equilibrium with hypochlorous acid (HOCl). In the pH range of 4-7, chlorine is predominantly in the form of HOCl (*17*). Due to its neutral net charge, HOCl penetrates through the bacterial cell's lipid bilayer membrane by passive diffusion, easily gaining access to the intercellular components and begins to quickly attack and oxidize multiple nucleophilic intercellular components (*4*, *6*, *8*). Due to its similar molecular size to water, HOCl can also attack the outer part of the cell, likely contributing to its quick inactivation rate (*8*). This rapid inactivation was also observed in a study conducted by Van Haute et al. (*6*), where inactivation of *E. coli* O157:H7 populations using a chlorine based sanitizer at 20, 35, and 50 ppm FC occurred within the first minute in standardized process water with a COD level of 500 ppm (*18*).

Results from this study also show the influence COD plays on sanitizer efficacy. In general, as sanitizer concentration and exposure time were kept constant an increase in organic loading decreased the efficacy of both sanitizers. Low organic loading conditions demonstrated that 30 ppm of FC was sufficient to cause a 5.08, 6.42, and 3.47 log-reduction in STEC, *L. monocytogenes* and *Salmonella* sp. populations, respectively, after just 15 seconds of exposure (Figure 1A, 2A, and 3A). However, increasing the amount of organic matter to reach a

COD level of 500 mg/L (mid), the same concentration of 30 ppm FC resulted in a 3.85, 4.65 and 2.81 log-reduction, for the same organisms (Figure 1C, 2C, and 3C). The efficacy of the FC was further decreased when the organic loading in the system was increased by approximately five times as much.

At the highest organic loading level, 30 ppm FC only decreased the initial microbial load by 2.46, 3.41, and 1.11 log CFU/mL in STEC, *L. monocytogenes*, and *Salmonella* populations, respectively (Figure 1E, Figure 2E and Figure 3E). The high COD level required 50 ppm of FC to achieve at least a 3-log inactivation within 60s for STEC, *Salmonella* and *L. monocytogenes*. In the presence of 50 ppm PAA, over a 3-log reduction was achieved within 60s for STEC and *Salmonella*, but not *L. monocytogenes* (1.81 log CFU/mL) (Fig. 1F, Figure 2F, and Figure 3F).

The impact of organic load on sanitizer efficacy in water systems has been previously reported (*7*, *10*, *11*, *13*, *15*). As the organic matter increases in the solution, a greater dose of sodium hypochlorite is needed to achieve appropriate FC concentrations, increasing the total amount of chlorine in the system. However, total chlorine is not indicative of an increase in efficacy of chlorine-based solutions (*17*). Total chlorine is the addition of both FC and combined chlorine present in the solution (*17*). Although combined chlorine compounds are a more stable compound than FC, their reaction kinetics to inactivate microorganisms is much slower (*17*). Furthermore, upon introduction of chorine into a system with high organic load, FC quickly reacts with the organic material, depleting sanitizer efficacy and generating toxic byproducts (*13*). Keeping FC concentration consistent at 30 ppm, a significant decrease ($p<0.05$) of microbial inactivation was observed in all three microorganisms as the organic load increased.

Conversely, in previous studies, PAA has been shown to be more resistant to organic matter compared to chlorine-based sanitizers due to its slower reaction with organic matter and greater resistance to self-decomposition in the presence of organic matter (*5*, *11*, 20). Resiliency to organic load was also seen in this study. After 60 s of exposure, 80 ppm of PAA was sufficient to reduce the microbial populations of STEC and *Salmonella* below the limit of detection regardless of organic load, demonstrating PAA efficacy even under high loading conditions (Figure 1 B, D, F and Figure 2 B, D, F).

A positive correlation was observed between bacterial inactivation and sanitizer concentration. Apart from organic load, increasing FC concentrations resulted in significant differences ($p<0.05$) in microbial populations. Significant reductions in STEC, *Salmonella* and *L. monocytogenes* populations were also seen after 30 s of exposure when PAA concentrations increased ($p<0.05$). Independent of COD, 25 ppm of PAA had little inactivation (<1 log) for any of the three microorganisms studied.

Currently there is little information available on the concentration of commercial antimicrobials that are needed to effectively mitigate cross-contamination risk in recirculation systems where water quality is constantly changing. Furthermore, the critical limits established for one commodity, may not have the same effectiveness in another commodity, leaving process operators guessing about adequate dosing strategies that should be used in their processing environment. Results from this study highlight the important roles water quality, sanitizer concentration and exposure time all play in inactivation of pathogenic microorganisms. These findings provide inactivation rates of target foodborne pathogens when exposed to PAA, FC, chlorine dioxide, and ozone using similar water quality parameters for apple packers. This information provides evidence to base their programs on in order to enhance the scientific basis of their food safety plan based upon their own water quality parameters.

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Figure 1. Bacterial survival (log CFU/mL) of a five-strain Shiga–toxigenic *Escherichia coli* (STEC) cocktail in simulated processing water with different sanitizer concentrations (ppm) of either free chlorine (A, C, E) or peroxyacetic acid (B, D, F) and varying levels of COD 30 mg/L (A, B), 500 mg/L (C, D), or 2500 mg/L (E, F). Data points represent the mean of log transformed STEC populations with three biological replications (n=18 per treatment). Error bars represent the standard deviations from the mean. Limit of detection was 1 log CFU/mL.

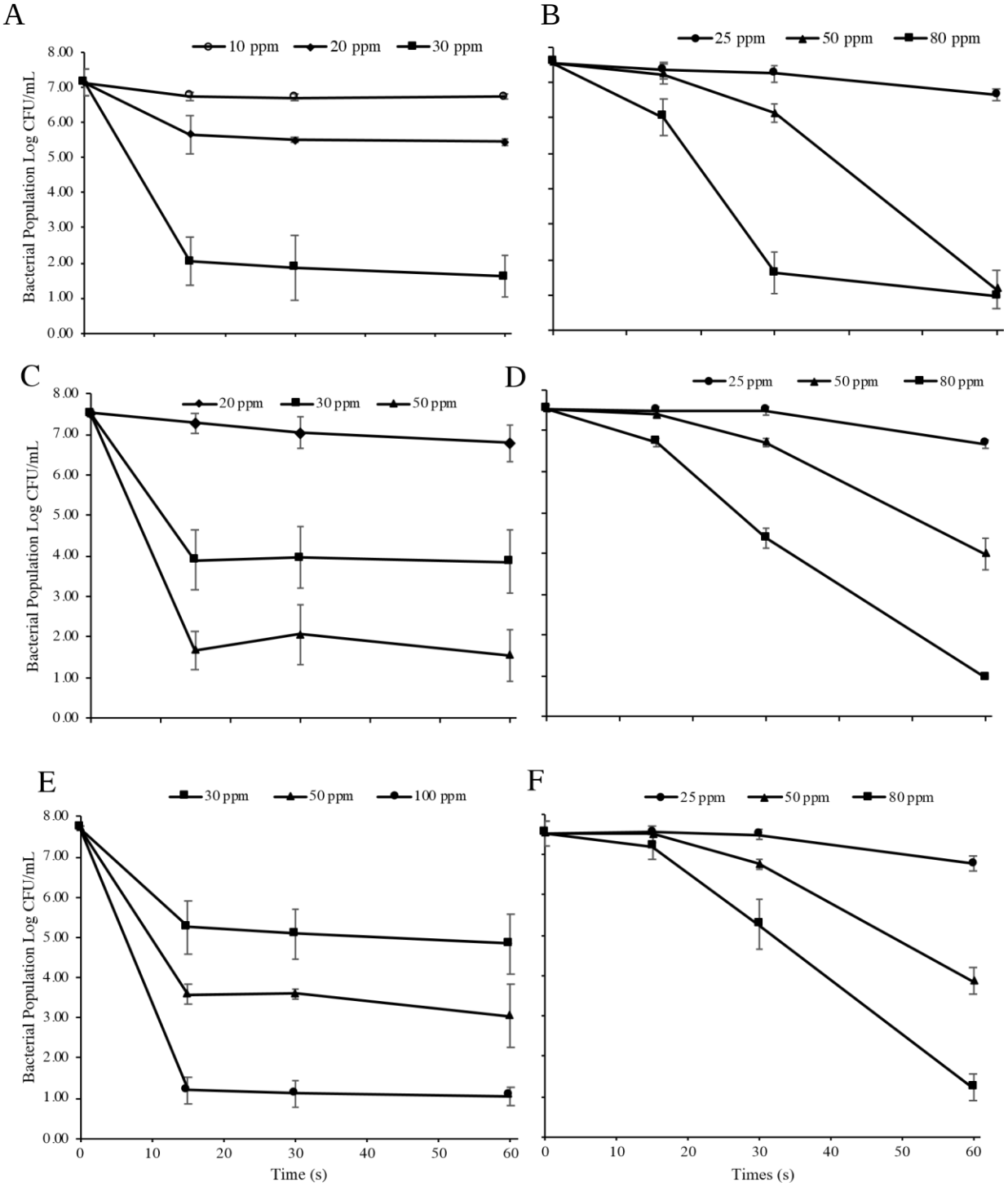


Figure 2. Bacterial survival (log CFU/mL) of a five-strain *Listeria monocytogenes* cocktail in simulated processing water with different sanitizer concentrations (ppm) of either free chlorine (A, C, E) or peroxyacetic acid (B, D, F) and varying levels of COD 30 mg/L (A, B), 500 mg/L (C, D), or 2500 mg/L (E, F). Data points represent the mean of log transformed STEC populations with three biological replications (n=18 per treatment). Error bars represent the standard deviations from the mean. Limit of detection was 1 log CFU/mL.

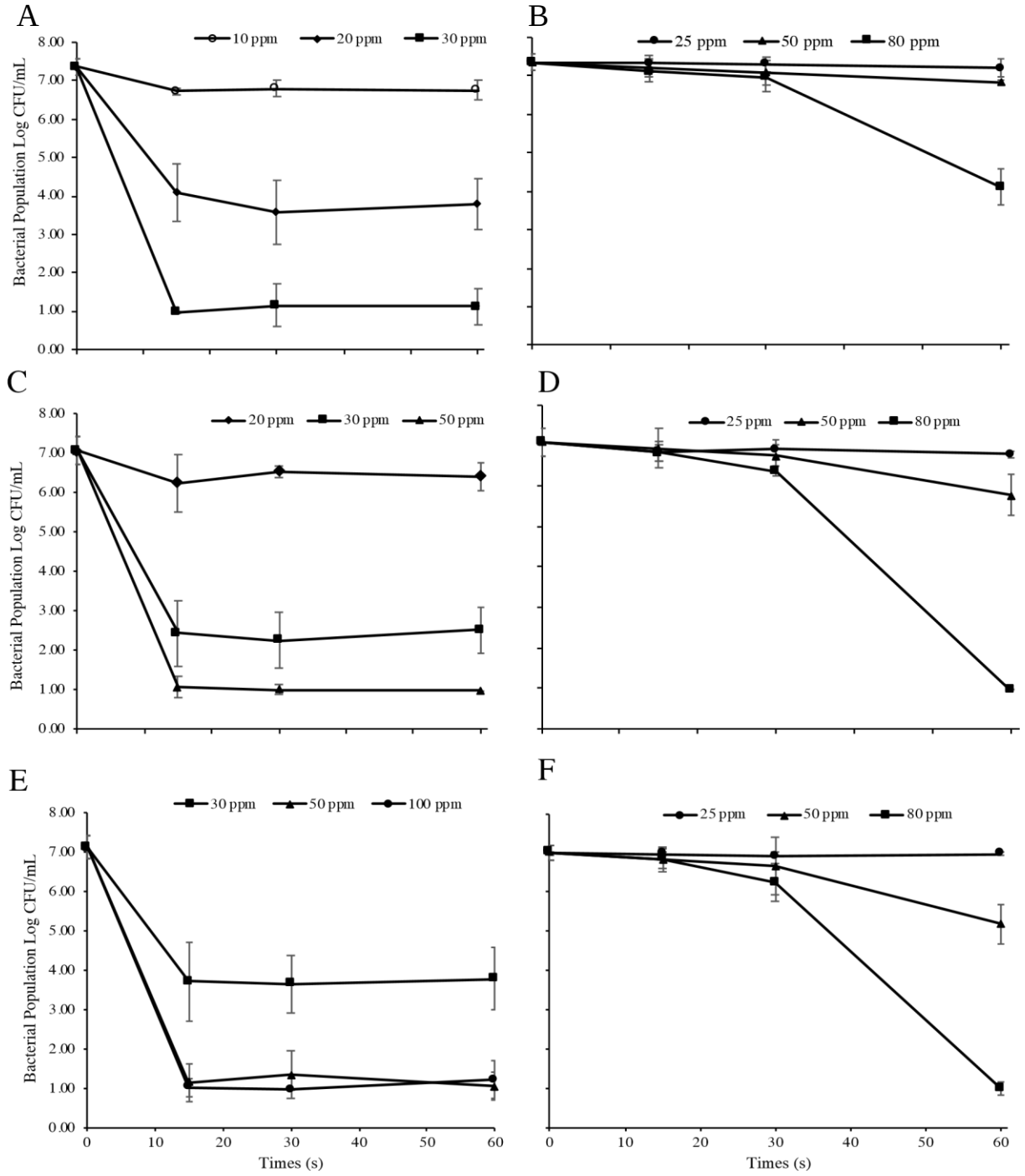


Figure 3. Bacterial survival (log CFU/mL) of a five-strain *Salmonella* spp. cocktail in simulated processing water with different sanitizer concentrations (ppm) of either free chlorine (A, C, E) or peroxyacetic acid (B, D, F) and varying levels of COD 30 mg/L (A, B), 500 mg/L (C, D), or 2500 mg/L (E, F). Data points represent the mean of log transformed STEC populations with three biological replications (n=18 per treatment). Error bars represent the standard deviations from the mean. Limit of detection was 1 log CFU/mL.

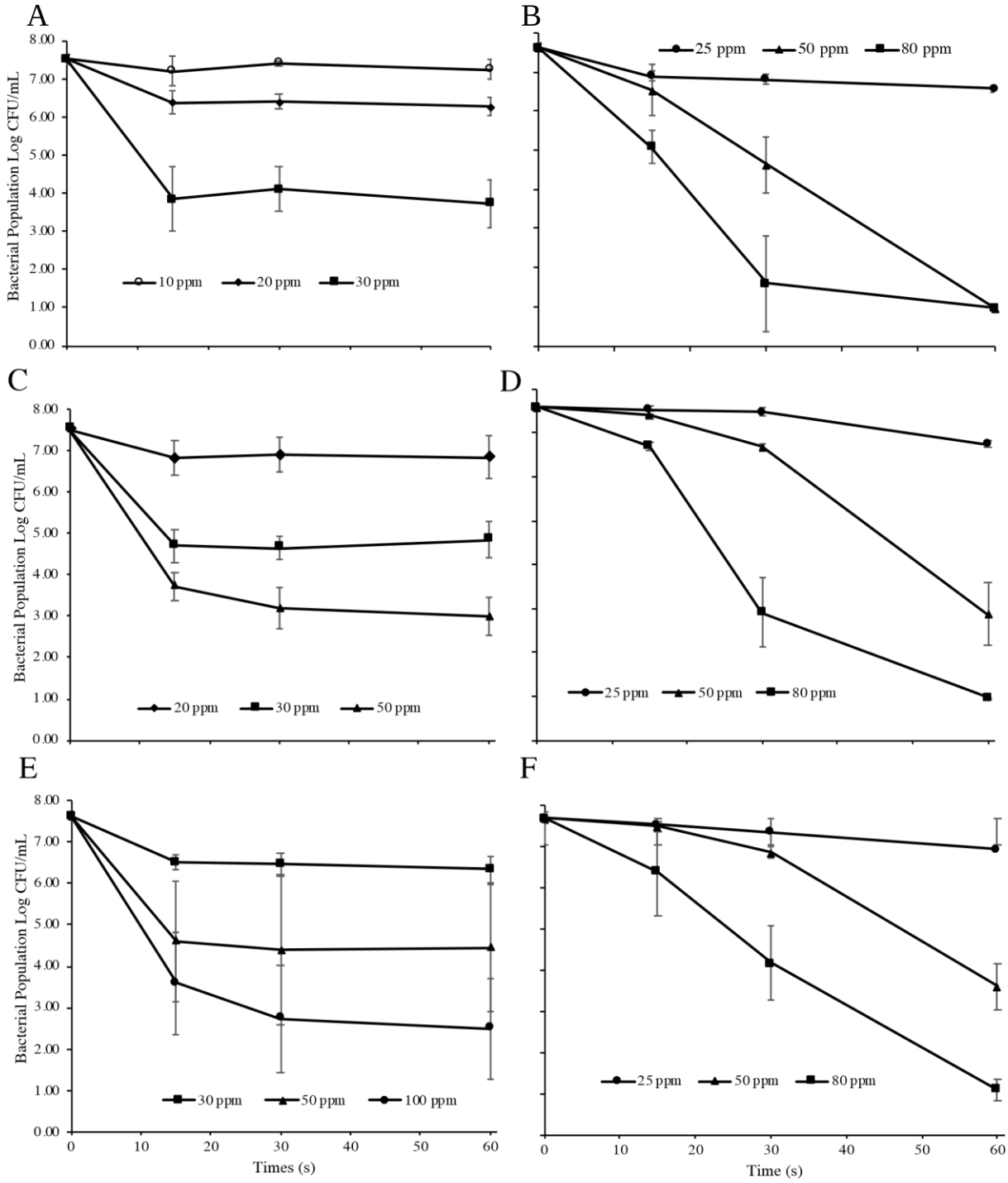


Figure 4. Bacterial survival (log CFU/mL) of a five-strain Shiga toxigenic *Escherichia coli* (STEC) (***A),*** *Listeria monocytogenes* (***B***) and *Salmonella* spp. cocktail (***C***) in deionized water (− ***)*** or simulated processing water with a COD level of 30 mg/L (----) when exposed to natural water conditions (●) or 3 ppm of residual chlorine dioxide (▲). Data points represent the mean of log transformed bacterial populations with two biological replications (n=12 per treatment). Error bars represent the standard deviations from the mean. Limit of detection was 1 log CFU/mL .

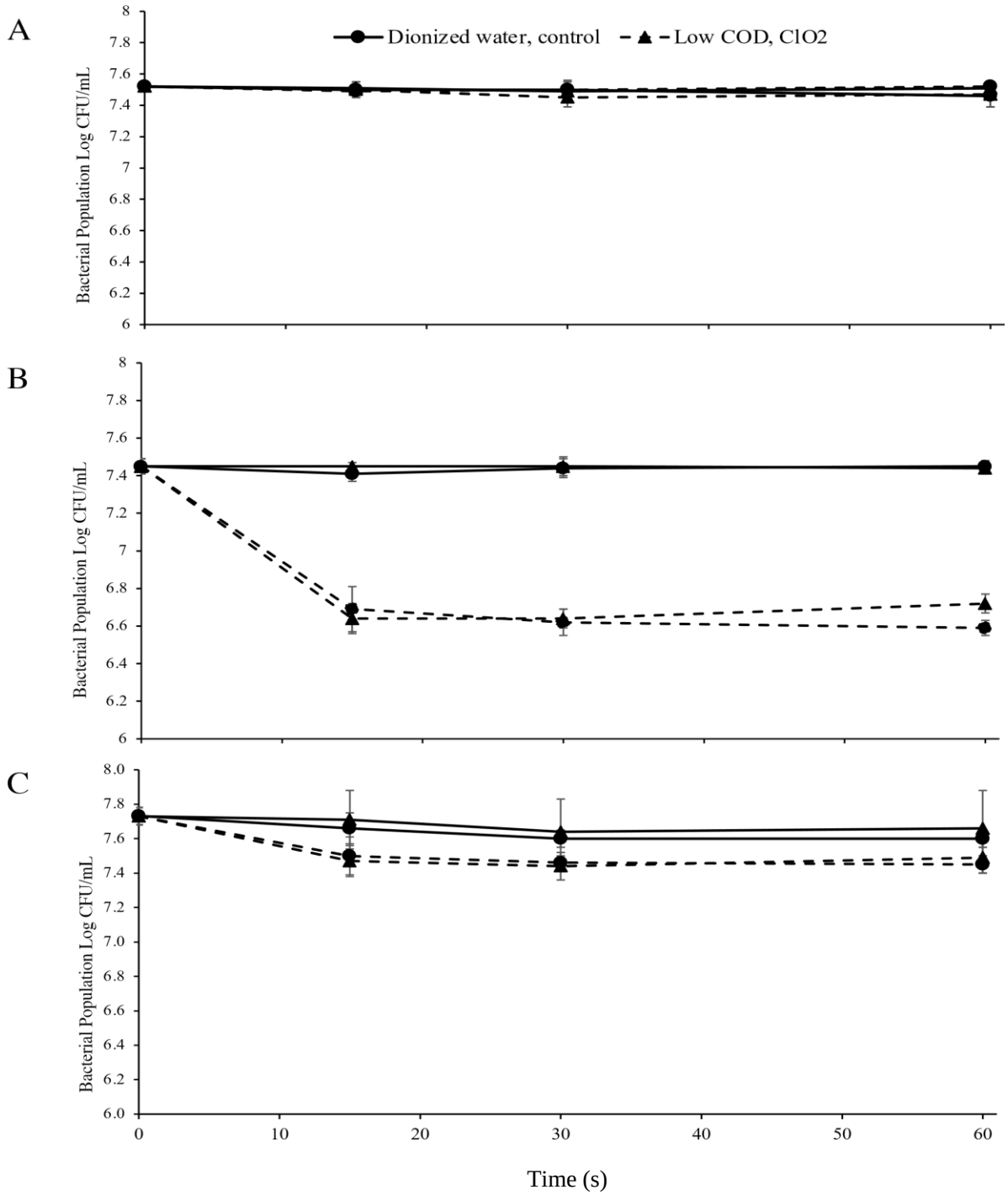
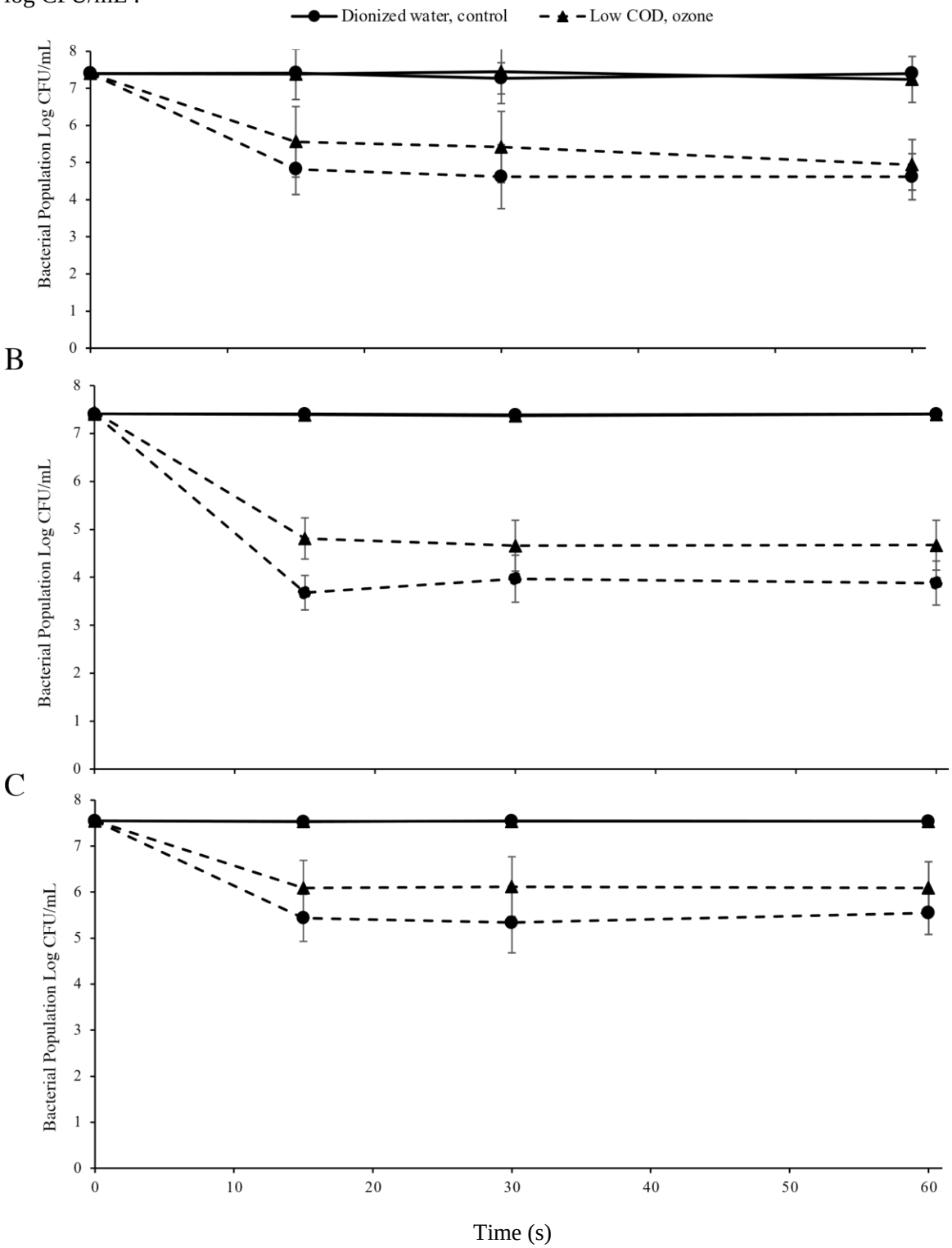


Figure 5. Bacterial survival (log CFU/mL) of a five-strain Shiga toxigenic *Escherichia coli* (STEC) (***A),*** *Listeria monocytogenes* (***B***) and Salmonella spp. cocktail (***C***) in deionized water (**−)** or simulated processing water with a COD level of 30 mg/L (----) when exposed to medical grade oxygen (●) or ozone (▲) when generated at 4 liters per minute for five minutes. Data points represent the mean of log transformed bacterial populations with two biological replications (n=12 per treatment). Error bars represent the standard deviations from the mean. Limit of detection was 1 log CFU/mL .



EXECUTIVE SUMMARY

Project Title: Critical limits for antimicrobials in dump tank systems

Key words: *Postharvest washing, organic load, chlorine, PAA, chlorine dioxide, ozone*

Abstract:

Studies have shown the risk of cross-contamination in fruit and vegetable recirculating washing systems (e.g., flumes and dump tanks) when improperly managed with commercial antimicrobials (e.g. sanitizers). However, there is little evidence regarding minimum concentrations needed to effectively inactivate target organisms and mitigate cross contamination risk. The majority of Environmental Protection Agency (EPA) labels for antimicrobials used in these systems do not include information on control for microorganisms that are a public health concern. The goal of this study was to determine the efficacy of commonly used antimicrobials [free chlorine, peroxyacetic acid (PAA), chlorine dioxide, and ozone] against Shiga-toxigenic *Escherichia coli* (STEC), *Listeria monocytogenes*, and *Salmonella enterica* in apple wash water with similar characteristics seen in industry. Three commercial apple packinghouses were visited during a packing season to obtain water quality data (n=104) from their recirculated washing systems. Water samples were collected from dump tanks with clean water (0 h) and throughout production (up to 84 h of recirculated water). Samples were analyzed for chemical oxygen demand (COD), turbidity, oxidation-reduction potential (ORP), conductivity, and pH. Based on the information collected from the packinghouses, simulated wash water with COD levels of 30, 500, and 2500 mg/L ppm were created in the laboratory. Sanitizers were added to the water to achieve 10, 20, 30, 50, or 100 ppm free chlorine; 25, 50, or 80 ppm PAA; 3 ppm chlorine dioxide; 1 ppm ozone. A five-strain cocktail of *Salmonella, L. monocytogenes*, or STEC was inoculated into the water, and aliquots were taken over 1 min to determine microbial inactivation. The efficacy of sanitizers was highly dependent on COD level, sanitizer concentration, and exposure time. Maintaining consistent the sanitizer concentration and time, increasing organic load resulted in a significant ($p<0.05$) reduction in efficacy of PAA and chlorine for all organisms evaluated. Exposure to 100 ppm free chlorine or 80 ppm PAA for 60 s resulted in at least a 3-log reduction for all microbial populations regardless of organic load. Limited efficacy was seen in chlorine dioxide or ozone under low COD conditions.

This study can be utilized as supporting documentation to base current postharvest sanitizer concentrations in recirculzted systems. Concentrations of PAA and chlorine have been determined which result in rapid inactivation of pathogens in water with similar properties to that observed during production. Chlorine dioxide and ozone, while fit for use in single-pass systems, are influenced substantially by organic load which will accumulate in dump tanks and flumes. This is especially important with the focus of HACCP-based 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.

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

Report Type: Final Project Report

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

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $98,447
Total Project Request for Year 2 Funding: $101,752
Total Project Request for Year 3 Funding: $105,882

WTFRC Collaborative Costs:

Item	2017	2018	2019
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

Budget 1
Primary PI: Meijun Zhu
Organization Name: Washington State University
Contract Administrator: Anastasia Kailyn Mondy
Telephone: 503-335-4564
Contract administrator email address: arcgrants@wsu.edu

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

OBJECTIVES

1. Assess antimicrobial efficacies of different commonly used chemical sanitizers against *L. monocytogenes* biofilm on the main food-contact surfaces.
2. Examine antimicrobial efficacies of steam against *Listeria* biofilm on different food-contact surfaces.
3. Evaluate the antimicrobial efficacies of steam in combination with the selected sanitizer against biofilm on the common food-contact surface using optimized parameters.

SIGNIFICANT FINDINGS

1. Efficacies of all tested sanitizers against aged (7-day-old) *Listeria* biofilm were reduced when compared to 2-day-old biofilm.
2. In general, efficacies against *L. monocytogenes* (*Lm*) biofilms on 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.
3. 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 *Lm* biofilms depending on surfaces.
4. Peroxyacetic acid (PAA) at 160 - 200 ppm and 1-5 min contact showed similar antimicrobial efficacies against *Lm* biofilms on all tested food-contact surfaces, causing a 4.0-4.6 $\log_{10}$ CFU/coupon reduction of *Lm* biofilms on tested surfaces.
5. The cell counts of *Lm* biofilm on SS were not impacted by finish type and wear degree of SS surface.
6. *Lm* counts on worn non-SS surfaces (LDPE, PET, PVC, and rubber) were significantly higher than that on new ones
7. Abrasion on surfaces reduced the efficacies of chlorine, QAC, and PAA against *Lm* biofilm
8. 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 reduction depending on the type of surface.
9. Saturated steam was more effective against *Listeria* biofilms on stainless steel surfaces than those on PET and rubber surfaces.
10. The effectiveness of both saturated and superheated steam in eliminating *L. innocua* biofilms decreased dramatically during prolonged steam treatment.
11. Organic matter soiling, regardless of sources, impaired sanitizer efficacies against *L. monocytogenes* biofilms independent of food-contact surfaces (new or worn) but did not negatively impact the efficacy of steam against *Listeria* biofilm on different surfaces.
12. Saturated steam exposure had no impact on the hydrophobicity and surface roughness of SS, PET, and rubber surfaces.
13. PAA at 40 ppm in combination with 6-sec saturated steam exposure provided > 6 log reduction of *L. innocua* biofilm on SS and PET surfaces.
14. PAA at 80 ppm and 6-sec saturated steam hurdle intervention resulted in ~ 5 log reduction on the rubber surface.
15. The efficacy of PAA and steam hurdle treatments was not impacted by the treatment order.
16. Organic soiling and/or surface defects, regardless of surface type, reduced the effectiveness of PAA and steam hurdle treatment in removing *Listeria* biofilm on surfaces.
17. Data on sanitizer interventions and saturated steam treatment have been published (Hua et al., 2019; Hua et al., 2021; Korany et al., 2018).

METHODS

Objective 1: Assess the antimicrobial efficacies of 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 *Lm* were evaluated. These *Lm* 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 the 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 *Lm* biofilm on different food-contact surfaces

1. Strain selection

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

2. Food-contact surface selection and biofilm formation

The surface selection and biofilm formation were the same as in the objective 1 studies.

3. Steam generator and temperature monitoring

The steam generator was located at the 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. 8AB).

4. Steam intervention

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.

5. Microbiological analysis

It was conducted as described in Objective 1.

Objective 3: Evaluate the efficacies of steam in combination with the selected sanitizer against biofilm on food-contact surfaces using optimized parameters.

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

RESULTS AND DISCUSSION

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

1. Impact of the age of biofilm on the efficacies of selected sanitizers against L. monocytogenes biofilm on polyester surface

We first compared the biofilm formation ability among the six *Lm* strains. There was no clear link between biofilm formation and the serotype of the selected strains (Fig. 1). NRRL B-33385, a 4b human clinic isolate had the lowest population density in the biofilm, while the *Lm* environmental isolate (NRRL B-33466) showed the highest biofilm forming ability among all strains tested (Fig. 1).

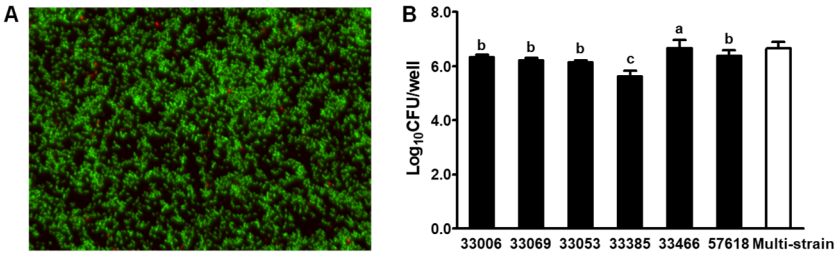


Figure 1. Biofilm forming ability of different *L. monocytogenes* strains on Polystyrene surface. A: BacLight Live/Dead staining B: *Lm* counts. Mean ± SEM. Bars topped with same letter are not different at $P < 0.05$.

Antimicrobial efficacies of all sanitizers except PAA against mixed strain *Lm* biofilm were reduced when compared to single strain *Lm* biofilm (data not shown). Antimicrobial efficacies of a sanitizers

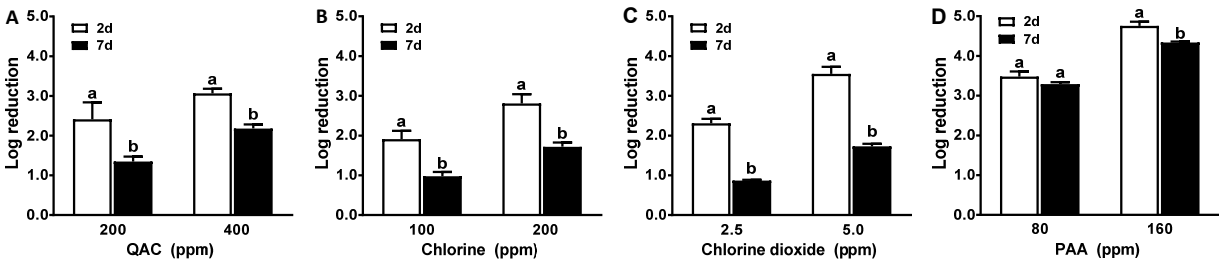


Figure 2. Efficacy of selected sanitizer intervention against mixed strain *L. monocytogenes* biofilm at different ages. 2d: 2-day-old biofilm; 7d: 7-day-old biofilm. A: QAC; B: Chlorine; C: Chlorine dioxide; D: PAA Mean ± SEM. Experiments were conducted independently three times, 6 replicates/treatment in each independent study.

against 7-day-old biofilm were reduced when compared to 2-day-old biofilm; antimicrobial efficacy of PAA was relatively less influenced by age of the biofilm (Fig. 2).

2. Efficacy of selected sanitizers against *L. monocytogenes* biofilms on food-contact surfaces

In general, increasing QAC concentration from 200 ppm to 400 ppm improved its efficacy against *Lm* biofilms on food-contact surfaces except for LDPE surfaces for both 1 min and 5 min exposures (Fig. 3). A 5 min exposure of QAC (200 or 400 ppm) showed a similar efficacy against *Lm* biofilms on SS coupons (Fig. 3A). Except for rubber surface, the efficacy of QAC against *Lm* biofilms on surfaces was enhanced when increasing treatment time increased from 1 min to 5 min (Fig. 3). Among all surfaces, QAC at 5 min exposure was the most effective on SS (Fig. 3), least effective on rubber (Fig. 3) and exhibiting a comparable efficacy against *Lm* biofilms on LDPE and PET (Fig. 3).

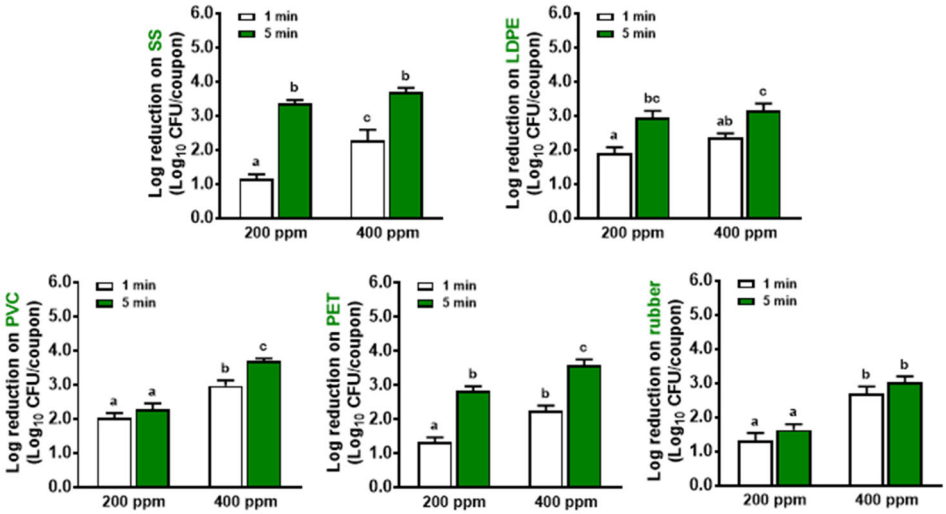


Fig. 3. Efficacies of QAC against *L. monocytogenes* biofilm on food-contact surfaces. 7-day-old biofilms were treated with 200 or 400 ppm QAC. [a-d] Bars topped with the different letters differ significantly at $P \leq 0.05$. Mean ± SEM. Studies were conducted independently three times, 6 replicated per treatment in each independent study.

Chlorine dioxide solution at 2.5 ppm exhibited a limited efficacy against *Lm* biofilms on all surfaces tested; 1 min exposure reduced ~ 1.1, 0.6, 0.9, 1.1, and 0.9 $\log_{10}$ CFU/coupon *Lm* biofilms on SS, LDPE, PVC, PET, and rubber surfaces (Fig. 4). Though the efficacy of chlorine dioxide was enhanced with increased concentration and contact time, it displayed limited potency to inactivate *Lm* biofilms on food-contact surfaces. A 5 min treatment of 5.0 ppm chlorine dioxide caused similar bactericidal efficacy against *Lm* biofilms on all surfaces with 2.4-2.7 $\log_{10}$ CFU/coupon reductions (Fig. 4).

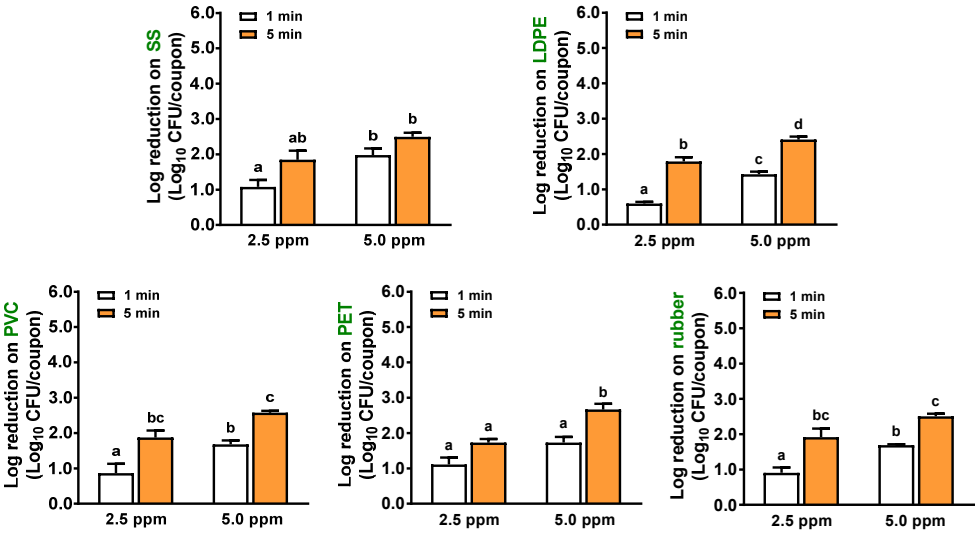


Fig. 4. Efficacies of chlorine dioxide against *L. monocytogenes* biofilm on food-contact surfaces. 7-day-old biofilms were treated with 2.5 or 5.0 ppm chlorine dioxide. [a-d] Bars topped with the different letters differ significantly at $P \leq 0.05$. Mean ± SEM. Studies were conducted independently three times, 6 replicated per treatment in each independent study.

The efficacy of chlorine against *Lm* biofilms on all surfaces was enhanced at increased concentration and extended contact time except LDPE surface (Fig. 5). A 1 min treatment of 100 ppm chlorine showed a similar efficacy against *Lm* biofilms as 1 min exposure of 200 ppm QAC (Fig. 3) and was more effective than 1 min treatment of 2.5 ppm chlorine dioxide (Fig. 4), causing 1.0 - 2.0 log CFU/coupon reductions of *Lm* biofilms. Chlorine at 200 ppm for 5.0 min exposure caused 3.8, 2.7, 3.3, 3.6, and 3.0 $\log_{10}$ CFU/coupon reductions of *Lm* biofilms on SS, LDPE, PVC, PET and rubber surfaces (Fig. 5).

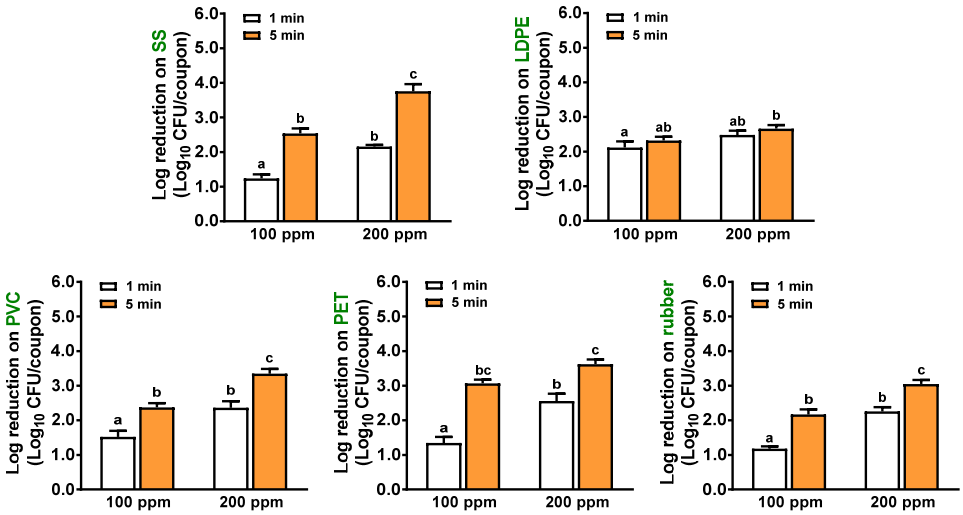


Fig. 5. Efficacies of chlorine against *L. monocytogenes* biofilm on food-contact surfaces. 7-day-old biofilms were treated with 100 or 200 ppm chlorine. [a-d] Bars topped with the different letters differ significantly at $P \leq 0.05$. Mean ± SEM. Studies were conducted independently three times, 6 replicated per treatment in each independent study.

Among all selected sanitizers, PAA was the most effective against *Lm* biofilms on all food-contact surfaces (Fig. 6). One min treatment of 160 ppm PAA reduced ~ 4.3, 3.5, 3.8, 4.1, and 3.7 $\log_{10}$ CFU/coupon *Lm* biofilms on SS, LDPE, PVC, PET, and rubber surfaces, respectively (Fig. 6). In general, bactericidal effects of PAA against *Lm* biofilms on all surfaces was not improved when concentration of PAA increased from 160 ppm to 200 ppm or when the treatment time increased from 1 min to 5 min (Fig. 6). A 5 min treatment of 200 ppm PAA caused 4.5, 4.0, 4.4, 4.3, and 4.4 log reduction of *Lm* on SS, PET, PVC, LDPE, and rubber (Fig. 6).

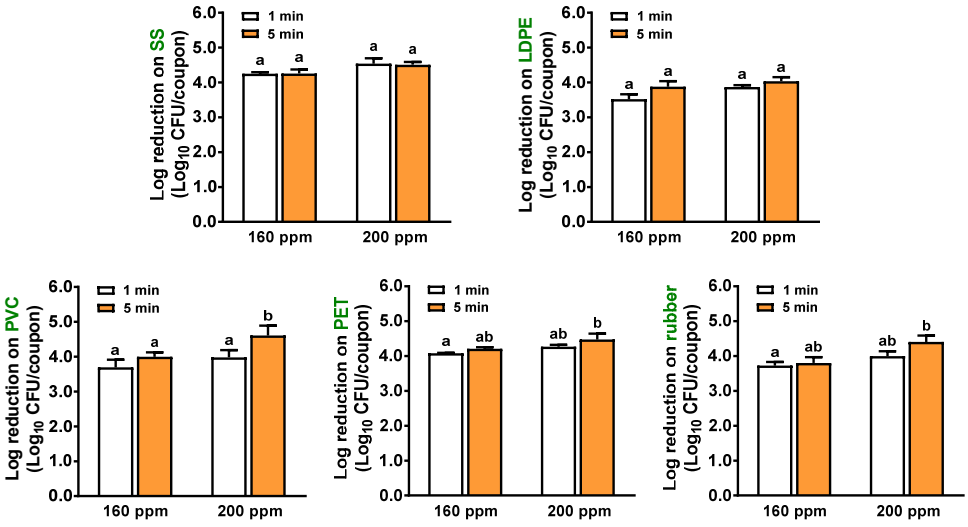


Fig. 6. Efficacies of PAA against *L. monocytogenes* biofilm on food-contact surfaces. 7-day-old biofilms were treated with 160 or 200 ppm PAA. [a-d] Bars topped with the different letters differ significantly at $P \leq 0.05$. Mean ± SEM. Studies were conducted independently three times, 6 replicated per treatment in each independent study.

3. Effects of organic matter on sanitizer's efficacy

The anti-*Listeria* efficacies of tested sanitizers were compromised by organic soiling regardless of surface types. Food residues from apple juice or milk comparably impacted efficacies of the sanitizers (Fig. 7). Thought PAA efficacy was impaired by organic soiling, it was still the most effective sanitizer, which caused 3.0-37 log CFU/coupon reductions of *Lm* biofilm on different surfaces (Fig. 7)

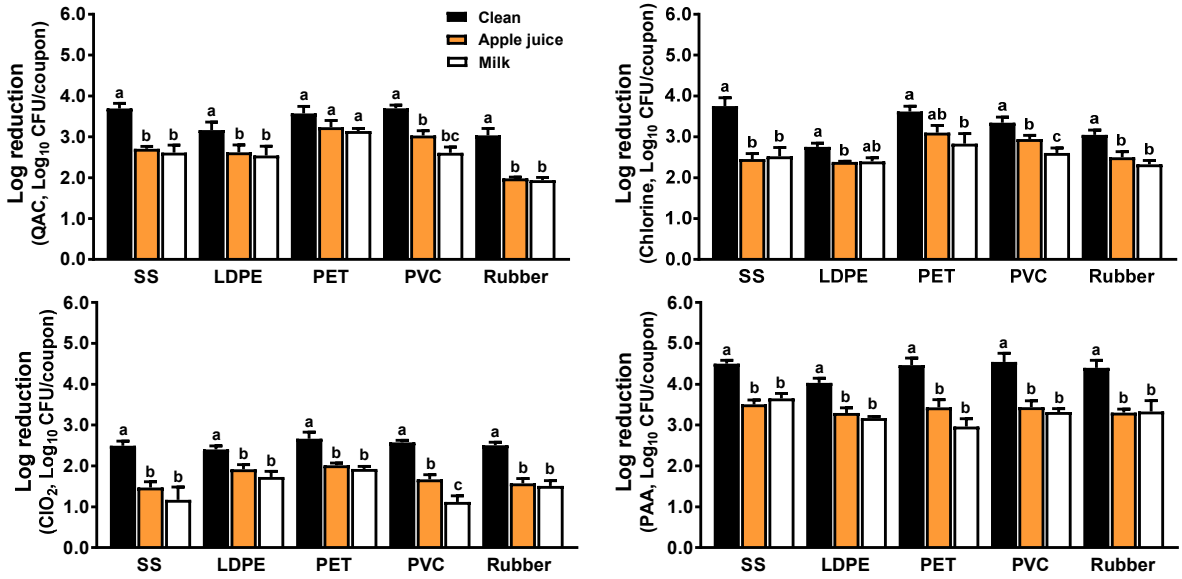


Fig. 7. Impacts of organic soiling on efficacies of tested sanitizers against *L. monocytogenes* biofilm on food-contact surfaces. 7-day-old biofilms were treated with 400 ppm QAC, 200 ppm chlorine, 5 ppm chlorine dioxide, and 200 ppm PAA. A. [a-d] Bars topped with the different letters differ significantly at $P \leq 0.05$. Mean ± SEM. Studies were conducted independently three times, 6 replicated per treatment in each independent study.

4. Sanitizers efficacies against *L. monocytogenes* biofilm on new and worn surfaces

The food-contact surfaces are subjected to natural aging and abrasion with usage and time. *L. monocytogenes* was found on worn rubber surfaces (Tompkin, 2002) and damaged plastic cutting boards (Berzins et al., 2010) in ready-to-eat meat facilities. Yet, limited information is available about the practical efficacies of sanitizers against *Lm* biofilm formed on worn food-contact surfaces. In this study, we first compared the count of *Lm* in biofilm formed on new and worn surfaces. *Lm* counts of SS were comparable on the new and worn surface, and there was a 6.93 – 7.10 $\log_{10}$ CFU/coupon of *Lm*. *Lm* populations on worn LDPE, PVC, PET, and rubber were significantly ($P < 0.05$) higher compared to the corresponding new surfaces: 7.89 – 8.64 (worn) vs 7.05 - 7.50 (new) $\log_{10}$ CFU/coupon.

Lm in biofilm on SS-2B exhibited higher resistance than that on SS-4, and the surfaces with defects or damage compromised the efficacies of sanitizers in removing *Lm* from SS coupons (Table 1). The 5 min exposure of 400 ppm QAC caused 2.38 and 2.88 log reductions on worn SS-2B and SS-4 surfaces, respectively, which was less effective than that obtained on new coupons ($P < 0.05$).

Table 1 Sanitizer efficacies against *L. monocytogenes* biofilm on the stainless-steel surface

Treatment	Reduction (Log_{10} CFU/coupon)			
	Surface	New	Defective	Worn
Chlorine	SS-2B	2.79 ± 0.14^{aA}	2.43 ± 0.16^{aA}	2.44 ± 0.11^{aA}
	SS-4	3.57 ± 0.09^{bA}	3.31 ± 0.13^{bA}	3.39 ± 0.16^{bA}
QAC	SS-2B	2.83 ± 0.21^{aA}	2.55 ± 0.18^{aAB}	2.38 ± 0.19^{aB}
	SS-4	3.65 ± 0.11^{bA}	3.17 ± 0.16^{bB}	2.88 ± 0.12^{bB}
PAA	SS-2B	4.05 ± 0.19^{aA}	3.53 ± 0.10^{aB}	3.41 ± 0.13^{aB}
	SS-4	4.32 ± 0.15^{aA}	3.85 ± 0.15^{aB}	3.83 ± 0.13^{bB}

SS-2B: stainless steel 2B finish, SS-4: stainless steel 4 finish. New: new surfaces. Defective: SS was bead blasted. Worn: SS was 80-grit sanded. [A-B] means within a row without the same letter differ significantly ($P < 0.05$). [a-b] means within a column without the same letter differ significantly for the same sanitizer treatment ($P < 0.05$). Mean ± SEM, n = 9.

Similarly, PAA at 160 ppm for 1-min contact is more effective against *Lm* biofilm on new SS surfaces than those on worn ones: 4.05 and 4.32 vs. 3.41 and 3.83 log reduction on SS-2B and SS-4 surfaces in new vs. worn conditions, respectively (Table 1). The effectiveness of sanitizer treatments on defective (moderate wear) and worn (severe wear) SS are comparable (Table 1).

The bactericidal effect of chlorine was significantly ($P < 0.05$) reduced on worn PVC, PET, and rubber surfaces compared to that on new surfaces, which removed 3.35 vs. 3.06, 3.23 vs. 1.84, 3.93 vs. 3.31, and 2.97 vs. 2.43 $\log_{10}$ CFU/coupon of *Lm* from LDPE, PVC, PET, and rubber in new vs. worn conditions, respectively (Table 2). QAC was more effective in removing *Lm* biofilm from new LDPE, PVC, and PET surfaces than from worn surfaces, but it caused a comparable reduction on new and worn rubber coupons (Table 2). PAA at 160 ppm for 1-min contact led to similar *Lm* reductions on new and worn LDPE, PVC, PET, and rubber surfaces (Table 2). Given that the population of *Lm* in biofilm on all tested worn surfaces is significantly ($P < 0.05$) higher than that on new surfaces, PAA was less effective in sanitizing the worn surfaces.

Table 2 Sanitizer efficacies against *L. monocytogenes* biofilm on non stainless surfaces

Surface	Condition	Initial levels	Reduction (Log_{10} CFU/coupon)		
			Chlorine	QAC	PAA
LDPE	**New**	7.05 ± 0.11^{a}	3.35 ± 0.11^{aA}	2.97 ± 0.15^{aB}	3.95 ± 0.15^{aC}
	Worn	7.89 ± 0.11^{b}	3.06 ± 0.14^{aA}	2.12 ± 0.12^{bB}	3.77 ± 0.19^{aC}
PVC	**New**	7.50 ± 0.12^{a}	3.23 ± 0.13^{aA}	3.37 ± 0.16^{aA}	3.80 ± 0.11^{aB}
	Worn	8.74 ± 0.03^{b}	1.84 ± 0.09^{bA}	2.05 ± 0.16^{bA}	3.93 ± 0.11^{aB}
PET	**New**	7.34 ± 0.12^{a}	3.93 ± 0.15^{aA}	3.66 ± 0.15^{aA}	4.64 ± 0.11^{aB}
	Worn	8.29 ± 0.09^{b}	3.31 ± 0.07^{bA}	2.47 ± 0.20^{bB}	4.35 ± 0.09^{aC}
Rubber	**New**	7.45 ± 0.07^{a}	2.97 ± 0.13^{aA}	2.51 ± 0.08^{aB}	3.68 ± 0.08^{aC}
	Worn	8.32 ± 0.19^{b}	2.43 ± 0.12^{bA}	2.55 ± 0.17^{aA}	3.95 ± 0.09^{aB}

New: new surfaces. Worn: surfaces were 80-grit sanded. $^{A\text{-}B}$ means within a row without the same letter differ significantly ($P < 0.05$). $^{a\text{-}b}$ means within a column without the same letter differ significantly for the same sanitizer treatment ($P < 0.05$). Mean ± SEM, n = 9.

Furthermore, the efficacies of QAC and PAA against *Lm* on worn SS and non-SS surfaces are compromised by organic matter conditioning. When the organic matter was present, QAC (400 ppm, 5 min) removed 1.69 and 1.38 $\log_{10}$ CFU/coupon of *Lm* on defective and worn SS-2B surfaces, 1.91 and 1.64 $\log_{10}$ CFU/coupon on defective and worn SS-4 surface, respectively, compared to 1.88 and 2.21 $\log_{10}$ CFU/coupon on new SS-2B and SS-4. PAA (160 ppm, 1 min) treatment reduced *Lm* by 2.78/2.58 and 3.11/2.93 $\log_{10}$ CFU/coupon on apple juice coated defective/worn SS-2B and SS-4 surfaces, respectively, compared to 3.24 and 3.50 $\log_{10}$ CFU/coupon reductions on new SS-2B and SS-4.

QAC (400 ppm, 5 min) decreased 2.12/1.37, 2.05/1.64, 2.47/1.00, and 2.55/1.52 $\log_{10}$ CFU/coupon *Lm* on clean/soiled worn LDPE, PVC, PET, and rubber surfaces. PAA (160 ppm, 1 min) removed 3.77/3.44, 3.93/3.80, 4.35/4.07, and 3.95/3.05 $\log_{10}$ CFU/coupon *Lm* on clean/soiled worn LDPE, PVC, PET, and rubber surfaces, respectively. Notably, up to ~7.0 and 4.5 $\log_{10}$ CFU/coupon of *Lm* were detected on all non-SS worn and soiled surfaces after QAC and PAA treatment, respectively.

In summary, the population of *Lm* in biofilms on all surface coupons except SS surfaces was significantly higher on the defective surfaces than on new ones. Worn food-contact surfaces reduced the effectiveness of all sanitizer treatments, especially when organic matter was present. Food residue/debris soiling, regardless of sources, reduced anti-*Listeria* efficacies of all sanitizers against biofilms on both new and worn surfaces regardless of types of surface coupons. Among all sanitizers, PAA was the most effective sanitizer against *Lm* biofilms on different surfaces. Data highlights the importance of surface maintenance and the importance of thoroughly cleaning food-contact surfaces prior to sanitizer interventions and effective cleaning and sanitization. Data also indicates that damaged/worn equipment and food-contact surfaces are more prone to *Listeria* contamination and could be persistent *Lm* contamination sources.

<u>Objective 2.</u> Examine antimicrobial efficacies of steam against *Listeria* biofilm on different food-contact surfaces.

Heating in the form of hot air, hot water, or steam is a traditional method for microbial reduction. A 6-min of hot water immersion treatment at 60 °C reduced 7-day-old *L. monocytogenes* biofilm on stainless steel (SS) by 3.2 $\log_{10}$ CFU (Tobin et al., 2020). A 15-sec of hot water treatment at 95-100 °C provided ~ 7 log reductions of *L. monocytogenes* attached to the inner surface of the model drainpipes (Berrang et al., 2014). Steam carries latent heat and is more efficient for microbial inactivation than hot air, or water. Steam application (> 93.3 °C for at least five minutes) has been approved by FDA to disinfect water-contact surfaces in bottled drinking water facilities (FDA, 2019). Steam offers various advantages over sanitizers and other intervention methods. It can heat surfaces/target materials quickly and reach into crevices/cracks while leaving no chemical residue on treated surfaces and it is environmentally friendly. Thus, the effectiveness of steam against *Lm* biofilm was further evaluated.

1. Steam and food-contact surface coupon temperatures

The steam temperature was maintained at 100 °C with a minor fluctuation. The temperature of the treated surface coupons rapidly reached 92 °C within 6 sec. The surface temperature of SS coupons at 6-sec of exposure was higher than that of PET, LDPE, PVC, and rubber surfaces. During subsequent steam exposure, the mean surface temperatures of treated surface coupons were similar for a 180-sec steam exposure, which was 98.1 ± 0.3 °C on SS, 97.8 ± 0.4 °C on PET, 96.6 ± 0.3 °C on LDPE, 96.9 ± 0.3 °C on PVC and 96.2 ± 0.3 °C on rubber surfaces (Fig. 8 CD).

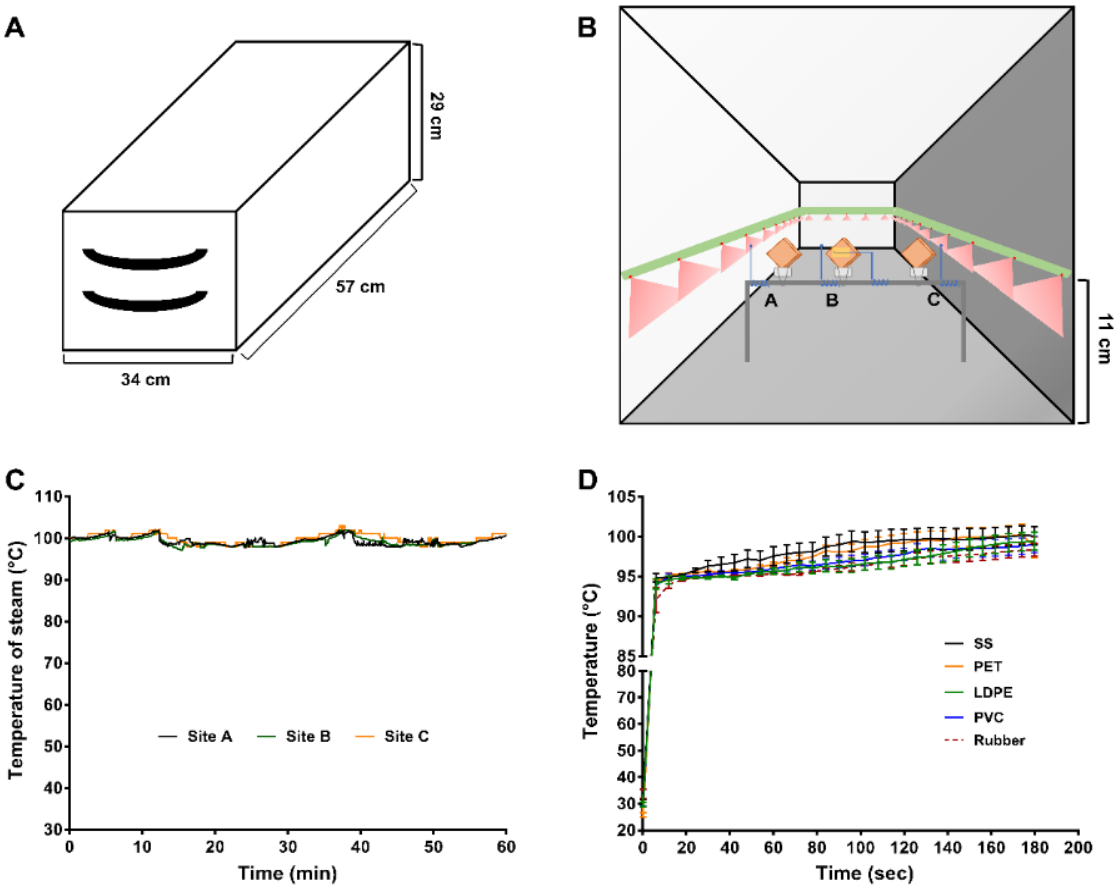


Fig. 8 Steam blancher apparatus and temperature profiles. A. Thaaaaaae dimension. B. Interior view of the steam blancher. Green: steam pipelines; red dots: steam nozzles, 25 in total. C. Temperature profile of steam during 60 min duration. D. Temperature profile of surface coupons during 180-sec treatment.

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

Steam had a quick bactericidal effect against 7-day-old *L. innocua* biofilms on all surfaces. A 6-sec exposure of steam provided 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. 9A). Fig 9B showed a representative image of Live/Dead staining of *L. innocua* cells in 7-day-old biofilms on SS before and after a 6-sec steam treatment, which further demonstrated the rapid bactericidal effect of steam.

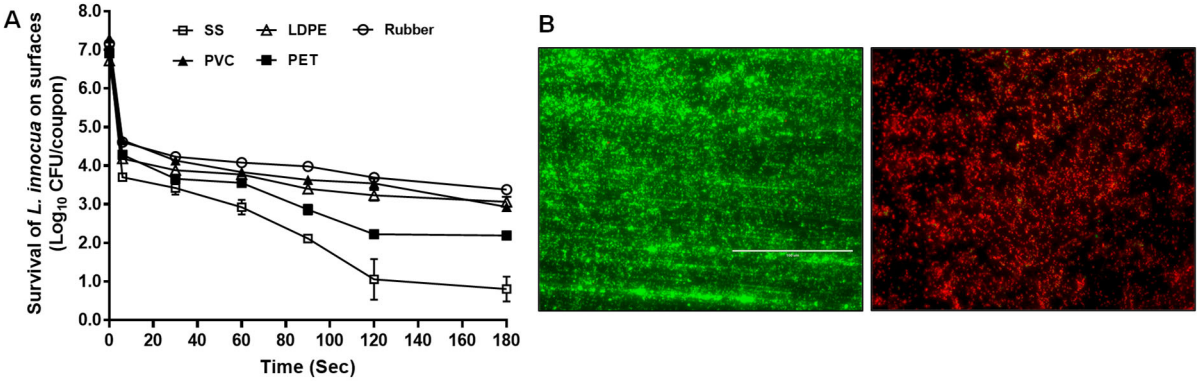
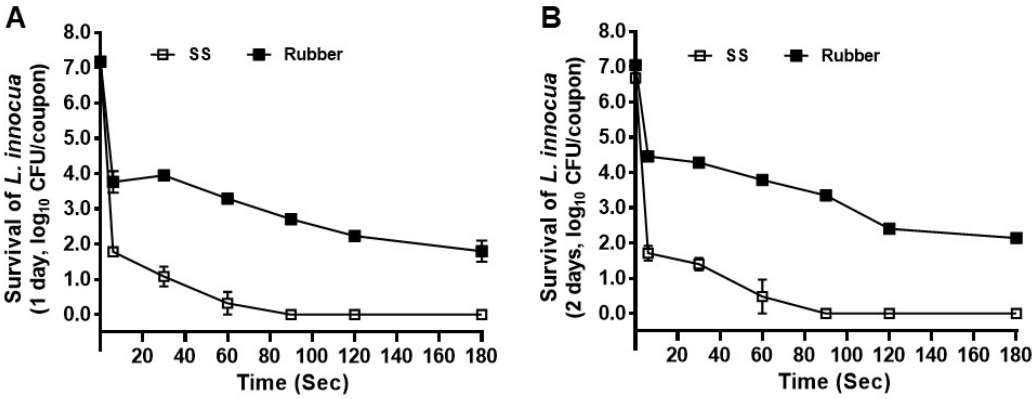


Fig. 9. Steam efficacy against cells in *L. innocua* biofilm on food-contact surfaces. 7-day-old biofilms were subjected to 100 °C steam for 0-180 sec. A. Representative survival of *L. innocua* biofilm on different food-contact surface coupons. B. Live/Dead staining of *L. innocua* cells in 7-day-old biofilm on SS surface. Left; *L. innocua* before steam treatment; right: *L. innocua* cells after a 6-sec steam treatment; Green: live cells; Red: dead cells; bar: 100 µm. Mean ± SEM, n = 9.



C

The reduction of *L. innocua* on surfaces ($\log_{10}$ CFU/coupon)

Time (sec)	1 day		2 days	
	Stainless steel	Rubber	Stainless steel	Rubber
6	5.5 ± 0.1^{aB}	2.7 ± 0.4^{aA}	5.1 ± 0.1^{aB}	2.6 ± 0.2^{aA}
30	6.1 ± 0.2^{abB}	2.9 ± 0.2^{abA}	5.6 ± 0.4^{aB}	2.5 ± 0.3^{aA}
60	6.7 ± 0.2^{bcB}	3.5 ± 0.2^{bA}	6.5 ± 0.2^{bB}	3.0 ± 0.1^{abA}
90	$>6.7^{cC}$	4.3 ± 0.3^{cB}	$>6.6^{bC}$	3.4 ± 0.4^{bA}
120	$>6.7^{cB}$	4.7 ± 0.1^{cA}	$>6.6^{bB}$	4.4 ± 0.2^{cA}
180	$>6.7^{cB}$	5.7 ± 0.4^{dA}	$>6.6^{bB}$	5.6 ± 0.5^{dA}

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

Fig.10. Steam efficacy against *L. innocua* cells on food-contact surfaces. The 1-day/2-day attached *L. innocua* on surface were subjected to 100 °C steam for 0-180 sec. Mean ± SEM was averaged from three independent studies where three replicates were used per treatment. [a-d] Mean within a column without a common letter differ significantly ($P < 0.05$). [A-C] Mean within a row without a common letter differ significantly ($P < 0.05$).

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. 9). Steam at 100 °C had comparable treatment efficacy on both LDPE and PVC surface; a 30-180 sec steam exposure caused 2.8 - 4.2 and 2.7 - 4.5 $\log_{10}$ CFU/coupon reductions on LDPE and PVC coupons, respectively (Fig. 9).

The exact mechanism for the tailing effects on different surfaces is not known. To evaluate the contributions of biofilm structures to the declined killing rate and the surviving tail during subsequent steam exposure, we evaluated the steam efficacy against surface-attached *L. innocua* or *L. innocua* in young biofilms on SS (most effective) and rubber (least effective) surface coupons (Fig. 10). In support of the role of biofilm architecture, steam is more effective against *L. innocua* attached on a surface or in young biofilm. A 90-sec of steam treatment reduced *L. innocua* in 1-day/2-day-old attachment/biofilm on SS to below the detection limit. There was an additional ~3 log reduction of *L. innocua* in 1-day/2-day-old attachment/biofilm on rubber surfaces (Fig. 10). However, there was still an obvious tailing effect of the inactivation curve of *L. innocua* especially on rubber surfaces, indicating factors other than biofilm structure contributed to the reduced effectiveness of steam for inactivating *L. innocua* in biofilms on rubber.

3. Impact of organic matter on the efficacies of steam pasteurization against *L. innocua* biofilm on different food-contact surfaces

Compared to clean surfaces, steam treatments were equally or more effective against 7-day-old biofilms formed on coupons that had been conditioned with diluted apple juice, a source of organic matter (Table 3). 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 3) (Hua et al., 2021).

Table 3 Impacts of organic matter on efficacy of steam

	Steam(sec)	Reduction (Log_{10} 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}

7-day-old biofilms on clean or soiled surfaces were treated with 100°C steam for 6 sec or 30 sec. $^{A-B}$ means within a row without the same letter differ significantly ($P < 0.05$). $^{a-b}$ means within a column without the same letter differ significantly for the same sanitizer treatment ($P < 0.05$). Mean ± SEM, n = 9.

4. 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. The detailed data can find in our published paper (Hua et al., 2021).

In summary, steam exhibited a fast killing kinetic against *L. innocua* biofilm on different food-contact surfaces; however, the killing rate of steam decreased dramatically during subsequent steam treatment and exhibited a tailing effect which was more pronounced on rubbers, PVC, and LDPE surfaces. Our data suggested that a short duration of steam exposure alone or in combination with chemical disinfection might be a promising sanitization strategy for removing *Listeria* biofilm or other foodborne pathogens on food contact surfaces, especially for SS, PET, and rubber surfaces.

<u>Objective 3.</u> Evaluate the antimicrobial efficacies of steam in combination with the selected sanitizer against biofilm on the common food-contact surface using optimized parameters.

Objective 1 study indicates that PAA was more effective than chlorine, QAC, and chlorine dioxide in removing *Lm* biofilms from commonly used food-contact surfaces. However, the anti-*Listeria* efficacies of PAA were compromised by the organic soiling and surface defects. Objective 2 study indicates that steam is an effective method for surface decontamination that incurs a quick inactivation of *Listeria* biofilms on stainless steel (SS) surfaces. A 6-sec treatment of steam at 100 °C reduced a 3.1 $\log_{10}$ CFU/coupon *Listeria* in biofilm on the SS coupons; however, the extension of steam treatment time beyond 6 seconds lowered the killing rate of the steam against *Listeria* in biofilms. Data indicated that steam treatment or PAA alone was insufficient to eradicate *Listeria* biofilms from food-contact surfaces. Therefore, the hurdle treatment in combination with short steam and PAA treatment was further evaluated.

1. Effectiveness of hurdle treatments against *L. innocua* biofilms on food-contact surfaces

The hurdle treatment combining saturated steam and PAA exhibited significantly (P<0.05) higher efficacy than PAA or saturated steam treatment alone (Fig. 11). The PAA (80 ppm, 1 min) followed by a 6-sec steam exposure lowered 6.75, 6.96, and 5.54 $\log_{10}$ CFU/coupon of *L. innocua* from SS, PET, and rubber surface coupons, respectively, compared to 2.63 - 3.34 $\log_{10}$ CFU/coupon reductions by saturated steam (100 °C, 6 sec) alone and 2.66 - 2.85 $\log_{10}$ CFU/coupon reductions by PAA (80 ppm, 1 min) treatment alone (Fig. 11).

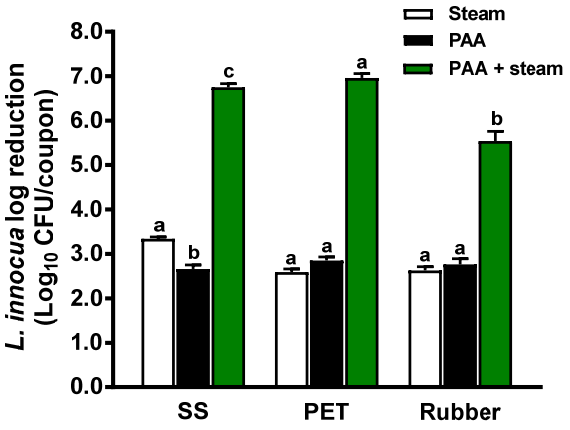


Fig. 11. Efficacy of PAA in the combination of saturation steam in removing *L. innocua* biofilms from the food-contact surfaces. The 7-day-old *L. innocua* biofilms on surface coupons were treated with PAA (80 ppm, 1 min), steam (100 °C, 6 sec), and their combination. Mean ± SEM was averaged from three independent studies, with four replicates per independent study. [a-c] Bars topped with different letters are significantly (P < 0.05) different for each surface type.

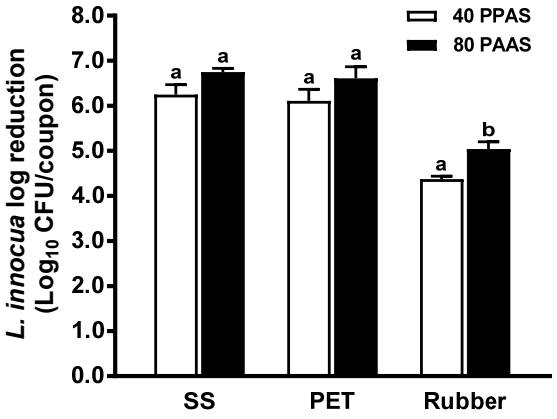


Fig. 12. Effectiveness of saturation steam with different PAA concentrations against *L. innocua* biofilms on food-contact surface. The 7-day-old *L. innocua* biofilms on surfaces treated with steam (100 °C, 6 sec) in combination with 40 ppm or 80 ppm PAA. Mean ± SEM was averaged from three independent studies, with four replicates per independent study. a-b Bars topped with different letters are significantly (P < 0.05) different for each surface type.

The hurdle treatment of 6 sec of steam in combination with 80 ppm or 40 ppm PAA had similar efficacies against *L. innocua* biofilm on SS and PET surfaces, which resulted in > 6 $\log_{10}$ CFU/coupon *L. innocua* within biofilms on both surfaces. However, the efficacy of 40 ppm PAA + steam against *L. innocua* biofilm on rubber surfaces was lower than that of 80 ppm PAA + steam treatment ($P<0.05$) (Fig. 12). Regardless of PAA levels, PAA + steam treatments had comparable efficacy on SS and PET, which was more effective than that on rubber surfaces. Furthermore, the efficacy of PAA and steam hurdle treatment was not significantly impacted by treatment order, whether treated with steam followed by PAA treatment or firstly treated with PAA followed by steam exposure (data not shown).

2. The impact of organic matter on the effectiveness of PAA + steam treatment against *L. innocua* biofilms on food contact surfaces

Surfaces of SS, PET, and rubber conditioned with apple juice reduced the effectiveness of 40 ppm PAA + steam treatment in removing *L. innocua* biofilm on SS and PET surfaces ($P<0.05$), but its efficacy on rubber surfaces was not impacted. The PAA at 40 ppm for 1 min treatment followed by 6-sec saturated steam exposure removed 5.56, 5.76, and 4.17 $\log_{10}$ CFU/coupon *L. innocua* on SS, PET, and rubber surfaces, respectively, in the presence of apple juice soiling.

3. The impact of surface condition on the effectiveness of PAA + steam treatment against L. innocua biofilm on surfaces

Compared to the new surface, the efficacy of 40 ppm PAA + steam treatment against *L. innocua* biofilm on worn SS and PET surfaces was significantly decreased (Table 4). Though we observed higher *L. innocua* reductions on worn PET and rubber surfaces compared to new ones after steam treatment alone. Given that the initial *L. innocua* level on worn PET and rubber was ~ 1 $\log_{10}$ CFU/coupon higher than on new PET and rubber, there were higher loads of *L. innocua* on worn PET and rubber after steam treatments than that on new ones. Collectively, the anti-*Listeria* efficacy of steam treatment, with or without 40 ppm PAA treatment, on all surface coupons tested was diminished by surface defects (Table 4).

Table 4 Efficacy of the hurdle treatment against *L. innocua* biofilms on worn surfaces

			Reduction (Log_{10} CFU/coupon)	
Surface	**Conditions**	**Initial levels**	**Steam**	**PAA + steam**
SS	New, clean	6.83 ± 0.05^{a}	3.34 ± 0.04^{aA}	$>6.53^{aB}$
	Worn, clean	7.22 ± 0.04^{a}	2.56 ± 0.04^{bA}	5.91 ± 0.27^{bB}
	Worn, soiled	7.15 ± 0.06^{a}	2.70 ± 0.12^{bA}	5.08 ± 0.12^{cB}
PET	New, clean	7.13 ± 0.09^{a}	2.59 ± 0.07^{aA}	6.61 ± 0.26^{aB}
	Worn, clean	8.28 ± 0.07^{b}	3.50 ± 0.07^{bA}	5.69 ± 0.22^{bB}
	Worn, soiled	8.18 ± 0.07^{b}	3.33 ± 0.05^{bA}	5.18 ± 0.08^{cB}
Rubber	New, clean	7.03 ± 0.09^{a}	2.65 ± 0.09^{aA}	4.37 ± 0.07^{aB}
	Worn, clean	8.00 ± 0.05^{b}	3.23 ± 0.10^{bA}	4.84 ± 0.04^{bB}
	Worn, soiled	7.97 ± 0.07^{b}	2.79 ± 0.13^{aA}	4.49 ± 0.04^{cB}

The 7-day-old *L. innocua* biofilms on were treated with steam (100 °C, 6 sec) with or without 40 ppm PAA. Mean ± SEM, n=12. $^{a-c}$ numbers topped with the same letters did not differ significantly ($P < 0.05$) within each column for the same surface material. $^{A-B}$ numbers topped with the same letters did not differ significantly ($P < 0.05$) within each row.

In summary, a low concentration of PAA in the combination with quick steam exposure was a viable sanitization intervention for food contact surfaces. PAA at 40 ppm in combination with 6-sec saturated steam exposure provided > 6 log reduction of *L. innocua* biofilm on SS and PET surfaces. PAA at 80 ppm and 6-sec saturated steam hurdle intervention resulted in ~ 5 log reduction on the rubber surface.

EXECUTIVE SUMMARY

L. monocytogenes forms biofilms on different food-contact surfaces, providing a continuous source of contamination to foods that encounter contaminated surfaces. Considering the caramel apple listeriosis outbreak, multiple food-contact surfaces including the polishing brush, drying brush, conveyor, and wooden bin inner surface was confirmed to be *L. monocytogenes* positive (Angelo et al., 2017). These types of contamination on commonly utilized surfaces highlighted the importance of effectively sanitizing food-contact surfaces. Direct food-contact surfaces have been required to be fully cleaned to prevent contamination/cross-contamination of "covered" produce and packing environments regulated under the Food Safety Modernization Act (FSMA) Produce Safety Rule (FSMA, 2016). The overall goal is to comprehensively evaluate the antimicrobial efficacy of commonly used commercial sanitizers at practical concentration and steam treatment against *Listeria* biofilm on different food contact surfaces. Given the food-contact surfaces are subjected to natural aging and abrasion with usage and time and contamination of food residues, we further evaluate the impacts of surface defects and organic soiling on the effectiveness of sanitization.

Our data indicated that all sanitizers at the concentrations commonly used in the food industry showed a stronger bactericidal effect against young (2-day-old) *L. monocytogenes* biofilm than old (7-day-old) biofilm. *L. monocytogenes* biofilm on stainless steel 2B finish exhibited higher resistance than that on stainless steel 4 finish. The population of *L. monocytogenes* in biofilms on all surface coupons except stainless steel surfaces was significantly higher on the defective surfaces than on new ones. Worn food-contact surfaces reduced the effectiveness of all sanitizer treatments, indicating damaged/worn equipment and food-contact surfaces are more prone to *Listeria* contamination. Food debris/organic soiling of food-contact surfaces reduced the anti-*Listeria* efficacies of all sanitizers against biofilms on both new and worn surfaces regardless of the types of surface coupons. Among all sanitizers, PAA was the most effective sanitizer against *L. monocytogenes* biofilms on different surfaces.

Steam exhibited a fast killing kinetic against *L. innocua* biofilm on different food-contact surfaces; a 6-sec steam treatment attained a 2.4 - 3.1 $\log_{10}$ CFU/coupon reduction depending on surface materials. However, the killing rate of steam decreased dramatically during subsequent steam treatment and exhibited a tailing effect which was more pronounced on rubbers, PVC, and LDPE surfaces, followed by PET and then SS surface. Organic matter soils did not compromise the bactericidal effects of steam against *L. innocua* biofilm on tested surfaces. Data indicated that steam treatment or PAA alone was insufficient to eradicate *Listeria* biofilms from food-contact surfaces. Therefore, the hurdle treatment in combination with short steam and PAA treatment was further evaluated. PAA at 40 ppm in combination with 6-sec saturated steam exposure provided > 6 log reduction of *L. innocua* biofilm on SS and PET surfaces. PAA at 80 ppm and 6-sec saturated steam hurdle intervention resulted in ~ 5 log reduction on the rubber surface. Our data suggested that a short duration of steam exposure alone or in combination with PAA or chemical disinfection might be a promising sanitization strategy for removing *Listeria* biofilm or other foodborne pathogens on food contact surfaces.

Data highlights the importance of surface maintenance and thorough cleaning of food-contact surfaces prior to sanitizer interventions and effective cleaning and sanitization. Results from this study also reflected the significance of the periodical application of sanitizers to avoid the establishment of the aged biofilm, which was much more difficult to be eradicated compared to the fresh one.

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Project/Proposal Title: Fate of *Listeria* on fresh apples as affected by commercial apple waxes

WTFRC Project Number: AP-20-104A

Report Type: Continuing Project Report

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Cooperators: Stemilt Growers LLC.; Hansen Fruit; Allan Brothers; Pace International LLC.; Jones-Hamilton Co.

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 83,842
Total Project Request for Year 2 Funding: $ 85,841
Total Project Request for Year 3 Funding: $ 86,419

WTFRC Collaborative Costs:

Item	2020-2021	2021-2022	2022-2023
Salaries			
Benefits			
Wages	2,104	2,135	2,168
Benefits	1,089	1,093	1,110
RCA Room Rental	2,250	2,316	2,385
Shipping		275	275
Supplies	250	275	275
Travel	350	375	400
Plot Fees			
Miscellaneous			
Total	6,043	6,469	6,613

Footnotes:

Budget 1
Primary PI: Meijun Zhu
Organization Name: Washington State University
Contract Administrator: Anastasia Kailyn Mondy
Telephone:
Contract administrator email address: arcgrants@wsu.edu

Item	**2020-2021**	**2021-2022**	**2022-2023**
Salaries	32,667	33,974	35,333
Benefits	6,632	6,898	7,173
Wages			
Benefits			
Equipment			
Supplies	28,500	28,500	28,500
Travel	2,000	2,000	2,000
Miscellaneous	8,000	8,000	6,800
Plot Fees			
Total	77,799	79,372	79,806

OBJECTIVES

1. Examine the fates of *Listeria,* resident bacteria, and yeast/mold on apples applied with commercial apple wax under subsequent cold storage.
2. Evaluate the fates of *Listeria* on waxed apples contaminated during wax application under subsequent cold storage.
3. Investigate the killing effects of residual sanitizers on the fates of *Listeria* and resident microbes on waxed apples under subsequent cold storage.

SIGNIFICANT FINDINGS

1. The dry temperature at ~22 °C/72 °F, 45 °C/113 °F, or 60 °C/140°F) had no impact on the survival of *L. innocua* on wax-coated apples.
2. *L. innocua* was reduced by 1.9 $\log_{10}$ CFU/apple on unwaxed apples during 18 weeks of refrigerated air storage.
3. *L. monocytogenes* reduced by 1.8-2.0 $\log_{10}$ CFU/apple on waxed apple during 12-week cold storage regardless of wax coating type.
4. Fates of *Listeria* on wax-coat apples were similar to that on unwaxed apples.
5. The die-off rate of *L. monocytogenes* on wax-coated apples contaminated during wax coating was not significantly different from that contaminated on apples before wax coating.
6. We observed a high cross-contamination risk of *L. monocytogenes* from inoculated apples to waxing brushes and from contaminated brushes to uninoculated apples during wax coating application.
7. Fungicides included in wax coating reduced yeasts and molds on wax-coated apples but not *L. monocytogenes*.
8. Wax coating had no impact on the survival of yeasts and molds on apples; there was 0.4-0.5 log10 CFU/apple increase after 18 weeks of cold storage regardless of wax treatment.
9. Wax coating increased the glossiness of apples regardless of wax treatment.
10. The application of wax, regardless of wax type, maintained total soluble solids (TSS) in apples after 18 weeks of cold storage, while TSS as significantly increased in unwaxed apples.
11. The titratable acidity (TA) was reduced on both unwaxed and waxed apples after 18 weeks of cold storage. Wax coating, regardless of type, had no impact on interior and exterior disorders.

METHODS

1. Strain selection

L. monocytogenes strains for BSL2 lab storage: To elucidate the impact of strain variability, a panel of *L. monocytogenes* serotypes consisting of serotypes 1/2a, 1/2b, and 4b was selected and used in this study.

L. innocua strains for commercial cold storage: *L. innocua* is a widely used surrogate for *L. monocytegenes,* which was used for determining the fates of *Listeria* during commercial cold storage. Three *L. innocua* isolates from an apple packing facility and other fresh produce processing plants were used to prepare a 3-strain cocktail of *Listeria* inoculum per our well-established method.

2. Apple inoculation

Apples were contaminated with *Listeria* prior to waxing application. Washed and unwaxed apples without cuts or bruises were individually and separately inoculated to establish 1×10^6 CFU/apple of 3-strain cocktail of *L. monocytogenes* or *L. innocua* per our well-established method. The inoculated apples were held at 22 °C for 24h before the wax coating was applied.

3. Waxing application

Wax selection: Three commercial apple fruit waxes including Prima Fresh 360 HS (PF360), Prima Fresh 606 EU (PF606) or Shield Brite AP-450 (AP-450) were used for Objective 1 studies. Prima Fresh 360HS was used for Objective 2 & 3 studies. Unwaxed and uninoculated apples will be included as a control for comparison.

Waxing application: Each wax solution was evenly spread on inoculated and uninoculated apple surfaces manually unless specified. To assess the fate of *Listeria* on waxed apples cross-contaminated during waxing, *L. monocytogenes* contaminated brushes were used to wax apples manually, while cross-contamination of *L. monocytogenes* to uninoculated apples.

4. Wax coating drying

To evaluate the impacts of wax coating drying conditions/temperatures on the survival of *Listeria* on waxed apples, apples right after wax coating were subjected to different drying temperatures (~22 °C/72 °F, 45 °C/113 °F, or 60 °C/140°F) for 2 min, followed by an additional 5-h drying at room temperature (~22 °C/72 °F) before being subjected to cold storage.

5. Cold storage treatments and sampling

BSL2 lab cold storage: Uninoculated or inoculated apples were subjected to 1°C/33°F storage for 16 weeks and sampled weekly/biweekly for enumeration of *L. monocytogenes* or resident microbiota (background bacteria or yeast/mold), respectively. Two independent trials with different lots of fruits were conducted sequentially. In each independent trial, twenty apples per treatment were sampled at each sampling day.

Commercial facility storage: Uninoculated apples or apples inoculated with a 3-strain *L. innocua* cocktail of different wax treatments were subjected to 1°C/33°F storage for 12-18 weeks in refrigerated air (RA) room of the commercial packing facility. Apples of each treatment combination were sampled after 2, 4, 6, 9, 12, and 18 weeks to enumerate the survival of *L. innocua* and yeast/mold. Studies were conducted for two consecutive years. Four sets of 10 fruits were used for each wax treatment in each sampling day for each independent/year study.

6. Survival microorganism analysis

Listeria enumeration: At each sampling day, *Listeria* survival on waxed apples under the respective storage (BSL2 or commercial facility) were detached and serially diluted. Appropriate dilutions were plated on trypticase soy agar supplemented with 0.6% yeast extract (TSAYE) plates overlaid with modified Oxford agar per our established method. All plates were incubated at 35˚C for 48 h and enumerated. If survival of *Listeria* on apple fruit is below the enumerative detection limit, the suspension was enumerated for presence/absence after 48h of enrichment in Buffered *Listeria* Enrichment Broth (BLEB) and streaked onto a selective *Listeria* agar plate. Presumptive positive colonies were further confirmed by PCR (FDA, 2015).

Resident microbiota: Rub solutions at appropriate dilutions were also plated on duplicate Potato Dextrose Agar plates supplemented with 0.1 g/l chloramphenicol for yeast and mold counts. The PDA plates were incubated at room temperature (~22°C) for 5 days.

7. Fruit quality analysis

At harvest or 18-week storage, fruit quality such as firmness, total soluble solids, and titratable acidity, as well as external and internal disorders, including superficial scald and lenticel decay, were assessed at the end of cold storage by the WTFRC quality lab using established methods (Sheng et al., 2018). A sample size of 10 apples per replicate with 4 independent replicates per wax type was used for internal and external disorder assessment.

8. Glossiness measurement

The gloss index of apples was determined at 60° with a glossmeter (Novo-Curve, Rhopoint Instrumentation, East Sussex, UK). The gloss units (GU) were measured directly on the fruit surface with 10 randomly selected spots per fruit. A total of 10 apple fruits per treatment condition was used for gloss analysis.

9. Statistical analysis.

Data were analyzed with IBM SPSS 19.0 (Chicago, IL). Mean differences were compared by the one-way analysis of variance (ANOVA) followed by a Tukey multiple comparison test. *P* values less than 0.05 were considered significant differences.

RESULTS AND DISCUSSION

1. Transfer of L. monocytogenes from apple-to-brush and brush-to-apple during wax application

To test the *potential of L. monocytogenes cross-contamination from apple-to-brush and brush-to-apple*, one waxing brush was used to coat one *L. monocytogenes* inoculated apple; then, this contaminated brush was used to wax five uninoculated apples in a sequence (Fig. 1A).

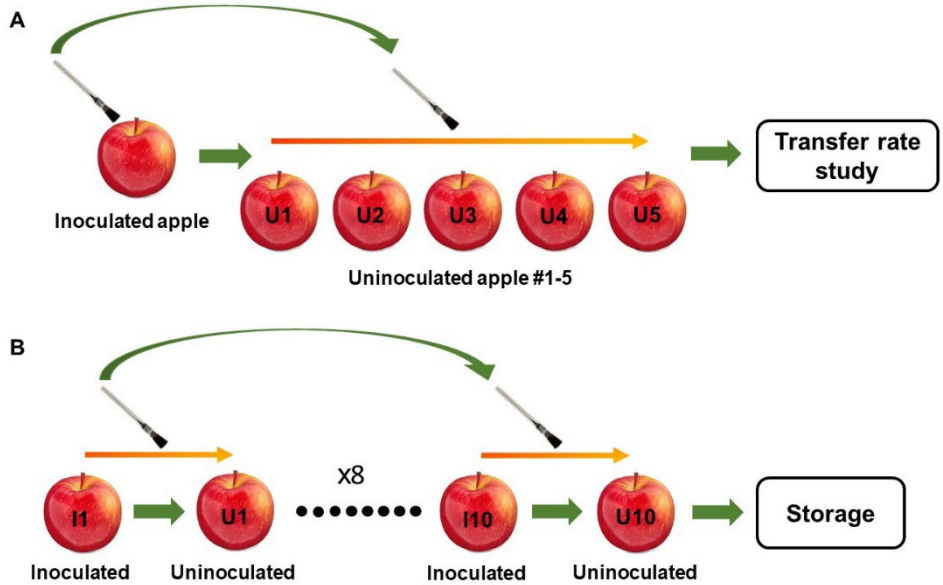

Figure 1. **Illustration for the preparation of waxed apples contaminated with *Listeria monocytogenes* during wax coating.** A. Wax-coated apples for the apple-to-brush and brush-to-apple transfer rate study. B. Wax-coated apples for the storage study. I: inoculated apple; U: uninoculated apple.

During PF 360 wax coating application, there were 3.7, 3.5, 3.3, 2.9, and 2.7 $\log_{10}$ CFU/apple of *L. monocytogenes* transferred from the inoculated apple (6.2 $\log_{10}$ CFU/apple) to uninoculated apple 1 to apple 5, respectively (Fig. 2A). After waxing of the 5th uninoculated apple, 3.6 $\log_{10}$ CFU/brush of *L. monocytogenes* was recovered from waxing brush (Fig. 2B). Similarly, for apples with a higher contamination level (8.4 $\log_{10}$ CFU/apple), 5.8, 5.6, 5.0, 4.8 and 4.6 $\log_{10}$ CFU/apple of *L. monocyto*genes were transferred to uninoculated apple 1 to apple 5 during wax coating application (Fig. 2C). After waxing of the 5th uninoculated apple, 5.5 $\log_{10}$ CFU/brush of *L. monocytogenes* was recovered from waxing brush (Fig. 2D). A similar transfer rate of *L. monocytogenes* from the inoculated apple to the waxing brush and uninoculated apples was found for AP-450, regardless of the initial contamination level (Fig. 2).

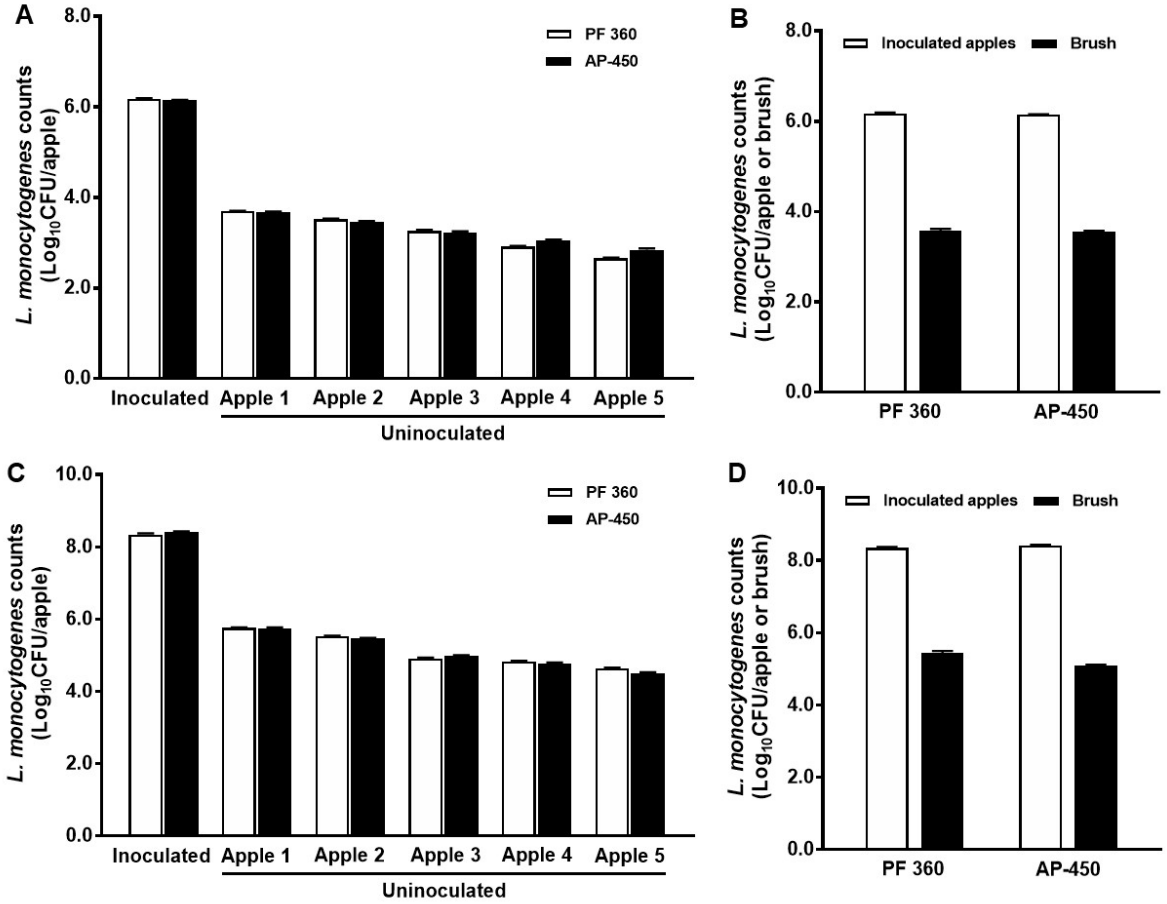

Figure 2. Transfer of *L. monocytogenes* from inoculated apples to uninoculated apples and waxing brushes during wax coating. A. Transfer from inoculated apples (~6 $\log_{10}$ CFU/apple) to uninoculated apples; B. Transfer from inoculated apples (~6 $\log_{10}$ CFU/apple) to waxing brushes. C. Transfer from high level inoculated apples (~8 $\log_{10}$ CFU/apple) to uninoculated apples; D. Transfer from high level inoculated apples (~8 $\log_{10}$ CFU/apple) to waxing brushes. Apple 1-5: *L. monocytogenes* on uninoculated apples transferred from contaminated waxing brushes. AP-450: Shield-Brite AP-450; PF 360: PrimaFresh 360. Data were presented with mean ± SEM, n = 24.

2. Survival of L. monocytogenes on waxed apples contaminated during different waxing schemes

To represent wax applications at apple packinghouses, three commonly used fruit wax coatings, PF 360, PF 606, and AP-450 were applied to the inoculated fruits, followed by up to 12-week storage. *L. monocytogenes* showed a similar trend on waxed apples under cold storage; there were 1.8-2.0 $\log_{10}$ CFU/apple reductions of *L. monocytogenes* on apples during 12 weeks of cold storage regardless of

wax coating type, though the reduction on AP-450 waxed apples was higher (P<0.05) at 2-9 weeks of storage (Fig. 3). The application of wax coating had a minor impact on the survival of *L. monocytogenes* on apples regardless of storage temperature (Fig. 3). Fates of *L. monocytogenes* on waxed apples under lab cold storage is mirrored to behaviors of *L. innocua* on waxed apples under commercial RA storage (Year 1 report).

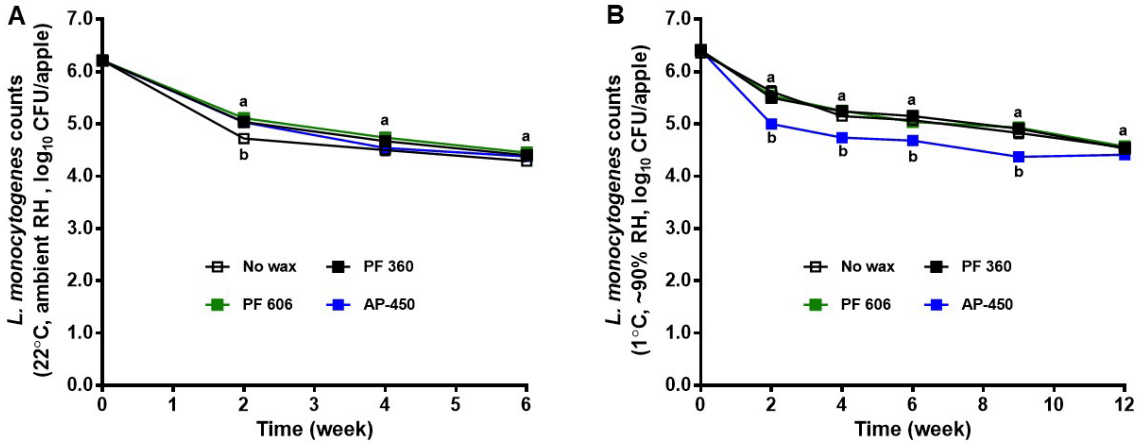

Figure 3. Fates of *L. monocytogenes* on wax-coated apples contaminated before wax coating application for up to 12 weeks of storage. A. 22°C and ambient RH. B. 1°C and ~ 90% RH; No wax: unwaxed control apples; PF 360: apple coated with PrimaFresh 360; PF 606: apple coated with PrimaFresh 606; AP-450: apple coated with Shield-Brite AP-450; RH: relative humidity. Mean ± SEM, n = 40. [a-b] Means at each sampling point without common letter differ significantly ($P < 0.05$).

Given the prevalence of *Listeria* species in waxing areas (Ruiz-Llacsahuanga, Hamilton, Zaches, Hanrahan, & Critzer, 2021; Simonetti et al., 2021), it is likely that *L. monocytogenes* can be introduced to wax-coated apples during the wax-coating process. Therefore, we next examined the fate of *L. monocytogenes* on PF 360 coated apples introduced during wax coating with the same contamination level as pre-contaminated apples at ~6 $\log_{10}$ CFU/apple (Fig. 1B). *L. monocytogenes* was reduced by 1.8 $\log_{10}$ CFU/apple after 12 weeks of cold storage (Fig. 4), which has a similar trend as *L. monocytogenes* introduced to apples before waxing application whether apple had an initial population of ~6 $\log_{10}$ CFU/apple (Fig. 3B) or ~8 $\log_{10}$ CFU/apple (Fig. 4).

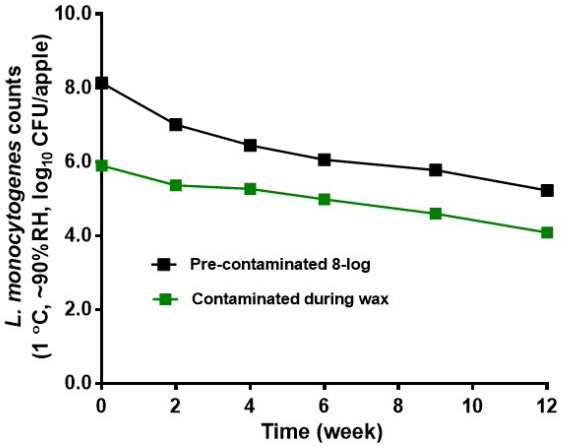

Figure 4. Fates of *L. monocytogenes* on wax-coated apples introduced during PrimaFresh 360 coating application for up to 12 weeks of storage. Source apples were inoculated with ~ 8 log10 CFU/apple of *L. monocytogenes* before wax coating (black line). Mean ± SEM, n = 40.

3. Yeast and mold counts on wax-coated apples during cold storage

The initial yeast and mold counts on apples immediately after wax coating were 4.7-4.8 $\log_{10}$ CFU/apple (Fig. 5). Application of wax coatings, regardless of wax type, did not impact (P>0.05) the survival of yeasts and molds on apples during 6-week ambient or 12-week cold storage (Fig. 5A, B). Yeast and mold counts of waxed apples under 6-week ambient storage gradually increased by 0.3-0.4

$\log_{10}$ CFU/apple with time (Fig. 5A). The yeast and mold population of apples under cold storage increased by 0.4-0.5 $\log_{10}$ CFU/apple during the first 4-week and maintained at 5.2-5.5 $\log_{10}$ CFU/apple during the subsequent 8 weeks of storage (Fig. 5B).

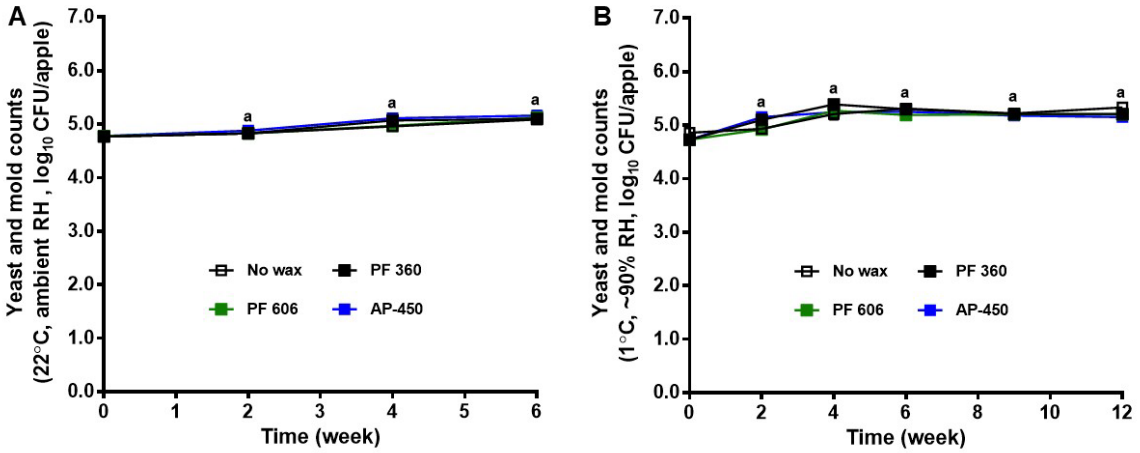

Figure 5. Yeast and mold counts on wax-coated apples contaminated before wax coating during up to 12-week storage. A. 22°C and ambient RH. B. 1°C and ~ 90% RH; No wax: unwaxed control apple; PF 360: apple coated with PrimaFresh 360; PF 606: apple coated with PrimaFresh 606; AP-450: apple coated with Shield-Brite AP-450; RH: relative humidity. Mean ± SEM, n = 40. [a]Means at each sampling point with a common letter did not differ significantly ($P > 0.05$).

4. *Impacts of Fungicide application in PrimaFresh 360 coating on fates of L. monocytogenes* and endogenous yeasts and molds *on waxed apples during 12 weeks of cold storage*

Fungicides can be incorporated into wax coating solutions under commercial apple waxing. To evaluate the potential impacts of fungicide applications during wax coating on the fate of *L. monocytogenes* on waxed apples, PF 360 wax coating was further applied in combination with two widely used fungicides, fludioxonil, and natamycin, followed by 12 weeks of cold storage. As shown in Fig. 5A, fludioxonil or natamycin in the fruit wax coating did not impact ($P>0.05$) the behavior of *L. monocytogenes* on waxed fruits. Populations of *L. monocytogenes* decreased by 1.7-1.8 log10

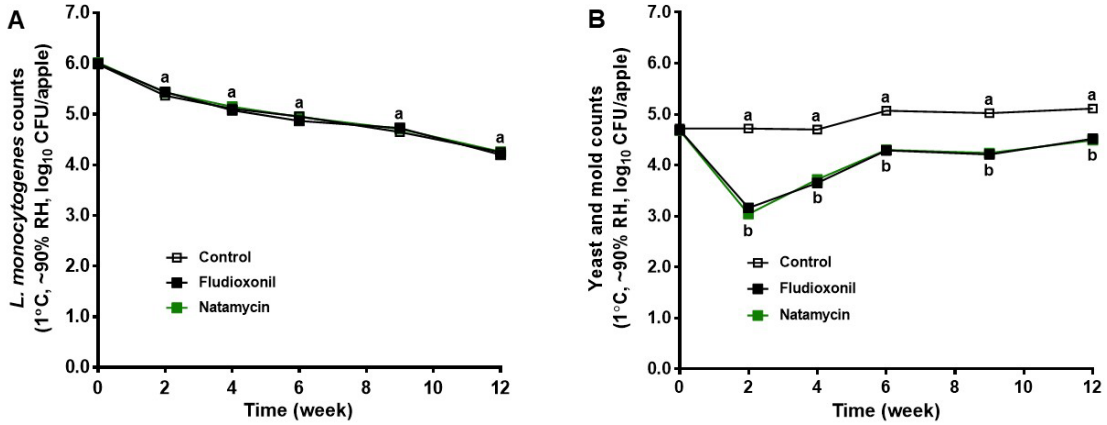

Figure 6. Impacts of fungicide application in PrimaFresh 360 coating on fates of *L. monocytogenes* and endogenous yeasts and molds on wax-coated apples during 12 weeks of cold storage. Apple coated with PrimaFresh 360 with or without fungicide. Mean ± SEM, n = 40. [a-b] Means at each sampling point without common letter differ significantly ($P < 0.05$).

CFU/apple on PF 360 coated apples regardless of fungicide application after 12 weeks of cold storage (Fig. 6A). Including fungicides in a wax solution reduced yeast and mold counts on waxed apples by 1.5-1.6 $\log_{10}$ CFU/apple at 2-week cold storage, but the counts then gradually increased to 4.5 $\log_{10}$ CFU/apple at 12-week cold storage (Fig. 6B). Fludioxonil and natamycin had similar effectiveness (P>0.05) in controlling yeasts and molds on waxed apples.

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CONTINUING PROJECT REPORT **PERIOD:** 2 year of 3 years

Project Title: **WA 38: SOP from planting to cropping**

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Cooperators: Several companies growing WA 38 in combination with different rootstocks have been contacted.

Project Request: **Year 1:** $90,860 **Year 2:** $88,523 **Year 3:** $87,292 **(Total $266,675)**

Other funding sources:
Project #AP14-103A: "WA 38 rootstocks and training systems" (2014-2016+1yr NCE) total funds $ 242,519 provided the support to maintain the orchard for this project.

BUDGET
Primary PI: Stefano Musacchi
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Budget

Musacchi-Serra-Lewis-Sallato			
Costs	Year 1 (2021)	Year 2 (2022)	Year 3 (2023)
Salaries[1]	$ 37,800	$ 39,312	$ 40,884
Benefit[2]	$ 16,760	$ 17,431	$ 18,128
Wages[3]	$ 12,000	$ 12,480	$ 12,980
Supplies[4]	$ 16,800	$ 11,800	$ 7,800
Travel[5]	$ 7,500	$ 7,500	$ 7,500
total	*$ 90,860*	*$ 88,523*	*$ 87,292*

Footnotes:
[1] Salary for a 75% Research assistant ($4,200/month) (Musacchi)
[2] Benefit on salary at 38.98%. Benefits on temporary at 22.4%
[3] Non-student temporary for 20 wks: 40hrs/wk at $15/hr (Musacchi/Lewis/Sallato).
[4] Labware/consumable, tree cost (Musacchi). Supplies include video recording and editing, printing material for outreach. Supply includes software fees for outreach material and translation. Supplies include video recording and editing, printing material for outreach.
[5] 13,043 miles/year for domestic travel ($0.575/mile) to go to the orchard. Travel to visit 10 blocks x 4 visits/year in Columbia Basin and North Central WA (Lewis). Travel to visit 10 blocks x 4 visits/year in south-central WA from Prosser (Sallato)

RECAP-OBJECTIVES:

1. Determine the rootstock effects on flower bud formation, fruit set, and spur extinction from planting to cropping (3rd year).
2. Investigate the WA 38 fruit set in the different types of bearing wood and assess the return bloom the following year.
3. Investigate the cultural management practices developed in WA 38 private orchards and summarize them in a list of recommended guidelines for growers.

SIGNIFICANT FINDINGS:

1. ***Determine the rootstock effects on flower bud formation, fruit set, and spur extinction from planting to cropping (3rd year) (Musacchi-Serra)***
   - WA 38 grafted on G213, G969, Bud10, G41 reported the lowest TCSA, while WA 38/M9-T337 and G890 showed the highest TCSA approximately 2 months after planting.
   - WA 38/G11 was the combination with the shortest portion of blind wood/branch - despite the longest branch length - significantly different from combinations with approximately double of blind wood, such as WA 38/M9-T337, G969, G213.
   - Average blind wood length of the unpruned trees was 15.3 cm, while it decreased to 5.5 cm when trees were subjected to click pruning soon after planting.
   - In the stubbed trees, a range of 64-85% of the nodes vegetated after the cut and significant differences emerged across combinations.
2. ***Investigate the WA 38 fruit set in the different types of wood and assess the return bloom on the different bearing woods (Musacchi-Serra)***
   - Largest part of WA 38 production in 2022 was held as single apple/cluster (65% avg. of 3 sites), followed by double apples/cluster (32% avg. of 3 sites) and the residual as triple apples/cluster.
   - As average across scenarios, 61% of the apples were produced on spurs, followed by 27% on brindilla and 12% on 1-year-old shoot lateral buds (ramo misto).
   - Apples on 1-year-old shoot lateral buds (ramo misto) were confirmed to be mainly small in size; while spurs produced apples with more variable sizes, from small to extra-large.
3. ***Investigate the cultural management practices developed in WA 38 private orchards and summarize them in a list of recommended guidelines for growers (Lewis-Sallato)***
   - WA 38 commercial sites were closely monitored for bloom density, crop load, and fruit quality.
   - In addition to the followed orchards, we developed a survey and evaluated additional 10 growers on their horticulture practices.
   - Harvest date in 2022 was between 6 to 20 days later than in 2021.
   - Fruit load varied between 63 and 143 fruit per tree, averaging a 70% increase as the trees mature and fill the space, with one site having reduced crop compared to 2021.
   - Crop load was highly variable among sites, ranging from 7.8 to 20 fruit per trunk cross section area (cm^2), influenced more by the year and site (management) than by rootstock.
   - Fruit size vary between 183 and 304 g, 15% smaller than in 2021, except for one orchard (in both rootstocks), where fruit size was 22% higher.
   - In 2022, shoot growth was generally reduced (average 4 cm short), except for orchard 2 (sites 3 and 4) where shoot growth was higher (9 cm larger). Well-balanced trees averaged 17 cm shoot length.

METHODS: Year 2 (2022)

Objective 1) Determine the rootstock effects on flower bud formation, fruit set, and spur extinction from planting to cropping (3rd year) ***(Musacchi-Serra)***

As written in the previous report, the block planted in 2021 was compromised, and the decision to replant was taken. The latest list of rootstocks in trial grafted with WA 38 is Bud9, Bud10, G11, G41, G935, G890, G969, M.9-T-337, and G213. In WSU-Sunrise farm (SRO) (Rock Island, WA), the new trees were planted on 4/27/2022 in the same rows as the previous planting, just shifting the holes to avoid replanting issues. The block was planted at 1,320 trees/A density with a 3 ft x 11 ft spacing. The ideal climate after planting (avg. 45°F in the following 4 weeks after establishment) allowed a satisfactory start of the new block. Thanks to the high quality of the trees well feathered from the nursery, we were able to impose the original treatments approximately 2 weeks after planting (5/10/22). The 2 main treatments applied at that time were: stubbed/scored (stub) and unpruned/not-scored (no stub). The stubs were carried out on half of the trees of each combination on all branches from top to bottom, and on average, the click-pruned branch was 11 cm long, while unpruned trees had an average branch length of 72 cm. The block maintained the established irrigation system from 2021. A standard operating procedure (SOP) customized with step-by-step instructions is part of this objective; therefore, we kept track of each of the orchard management practices, including training the trees to be able to well describe optimal management after planting.

On 6/30/2022, all tree-tops were singularized, removing the competitors and selecting the straightest apical shoot; the same day, the trees were tied to bamboo to the trellis to promote straight growth and avoid breakage from wind. At the same time, 9 trees/rootstock/treatment (total 162 trees= 81 stubs and 81 no stubs) were selected in the block; in each tree, 4 basal branches were tagged (total branches 648) and numbered (as branch 1 the most proximal to the ground).

To assess the response of early stubbed trees in comparison with no stubbed-unpruned trees, several vegetative parameters were assessed 2 months after the imposition of the treatments. The following parameters were measured or counted for each of the 4 branches/tree in the no stubs (unpruned) treatment: total length of each branch from insertion to the tip, the number of the first vegetated node in the branch, and the length of blind wood (distance from the insertion to the first vegetated node). From those parameters, the proportion of blind wood/branch was calculated. In the stubbed trees, we measured the same parameters as in the unpruned trees, but also the total nodes/stub and how many were vegetated were counted to calculate a proportion of vegetated nodes.

Objective 2) Investigate the WA 38 fruit set in the different types of bearing wood and assess the return bloom the following year *(Musacchi-Serra)*

At harvest 2021, we explored 5 different scenarios, and crop was harvested keeping discriminated apples originating from the different bearing woods [S=Spur (fruiting 2-3-years-old wood), RM=ramo misto (1-year-old shoot bearing on lateral buds), B=Brindilla (tip bearing shoot)] and fruit type based on fruit occupancy within a cluster (S=single, D=double, T=triple, Q=quadruple). Despite the apple quality of different bearing wood was not in the original plan of the present project, we perceived interest in understanding the impact of bearing wood on fruit quality. For this reason, we compared 2 combinations in 2 locations (WA 38/NIC29_Spindle_Quincy and WA 38/M9337-GS_Spindle_SRO) where the trees were trained as a spindle (despite different rootstocks, age, and top grafted/regular graft) for the 3 bearing woods. The sorting criteria for those apples were: only single (S) apples in the cluster, all best color, absence of defects, and size between 65-90 mm. The fruit quality analysis was performed after 6 months of cold storage in March 2022.

At harvest 2022, we narrowed down the "bearing wood" investigation to the 3 most interesting combinations (WA 38/NIC29_Spindle_Quincy, WA 38/G935_V system_Royal City, WA 38/M9337-GS_Spindle_SRO) to explore their productivity by fruiting wood. A total of 9 trees were part of the 2022 survey, and up to 12 possible groupings were potentially present in each tree (3 wood types ×4 cluster occupancy types). Yield per tree as number of apples/tree and kg/tree was logged, then all apples harvested from each tree were boxed and stored in regular air at 33 °F until grading. Approximately 4 weeks after harvest, all apples from each tree were separated into the 12 possible combinations and independently graded by a sorting line based on color and size. The same program utilized in 2021

sorted apples into 4 size categories: small (≤ 215 g), medium (216-263 g), large (264-339 g), and extra-large (≥ 340 g), corresponding to ≥88, 80, 72+64, ≤56 apples/box, respectively.

Objective 3) Investigate the cultural management practices developed in WA 38 private orchards and summarize them in a list of recommended guidelines for growers *(Lewis-Sallato)*
Rootstock selection in commercial orchards was based primarily on orchard management practices, and some priority was given to locations with more than one rootstock that could provide comparative data. A total of 5 orchards with two rootstocks each, except site 5, which has one rootstock (Table 1), were monitored during bloom, after June drop, maximum shoot growth (August), and at harvest (end September-beginning October). On each site, we collected information regarding bloom density, fruit set, shoot growth, crop load, and general orchard management practices. On each block/stage, we collected visual materials (photos and videos) to develop a comprehensive virtual database to share with WA growers.

Table 1. *Sites of WA 38 monitored for Objective 3.*

Site	Rootstock	Planting year	First crop?	Soil History	Training	Leader spacing (inches)	Irrigation	Heat mitigation	Fill space
1	G41	2018	3rd	Replanted	Vertical – Bi-axis	30	Single Drip + Sprinklers	Overhead Cooling	2/3
2	M9	2018	3rd	Replanted	Vertical – Bi-axis	30	Single Drip + Sprinklers	Overhead Cooling	2/3
3	G890	2018	3rd	Replanted	Vertical – Bi-axis	30	Single Drip	Netting	yes
4	M9	2018	3rd	Replanted	Vertical – Bi-axis	30	Single Drip	Netting	2/3
5	Bud10	2018	3rd	Replanted	Y Angle – Bi-axis	24	Double Drip	Sprays	50%
6	G11	2017	3rd	New	Vertical-Bi-axis	30	Single Drip + Sprinklers	Overhead Cooling	2/3
7	M9	2018	3rd	New	Vertical-Bi-axis	30	Single Drip + Sprinklers	Overhead Cooling	2/3
8	G41	2019	2nd	Replanted	Vertical-Bi-axis	30	Single Drip + Sprinklers	Sprays	50%
9	M9	2019	2nd	Replanted	Vertical-Bi-axis	30	Single Drip + Sprinklers	Sprays	50%

Note: all sites were planted at 12 ft row spacing and two leaders (Bi-Axis) training system

RESULTS AND DISCUSSION

Objective 1) Determine the rootstock effects on flower bud formation, fruit set, and spur extinction from planting to cropping (3rd year) *(Musacchi-Serra)*

The combinations in trial reported significant differences in terms of vigor since few weeks after planting (Table 2). The 4 most comparable combinations of rootstocks with WA 38 as scion with the lowest TCSA were G213, G969, Bud10, and G41. On the contrary, M9-T337 and G890 were the two rootstocks conferring the highest vigor. At the time of trunk measurements, the two treatments did not statistically differ yet, and the interaction rootstock × treatment was also not significant (Table 2). Table 3 reports results for "no stub" tree measurements. No significant differences in the length of blind wood per branch, but differentiations between original branch length in the unpruned trees coming from

the nursery emerged; the latter is ranging from 61.8 to 78.6 cm (on average), respectively in WA 38/G969 and WA 38/G11 (Table 3).

Moreover, some significant differences were found between the proportions of blind wood affecting basal branches (as a portion of the branch with no vegetative bud break over the total length of the branch, then averaged between the 4 selected branches/tree) across the 9 rootstock combinations with WA 38 scion (Table 3). Worth noting, WA 38/G11 was the combination with the shortest portion of blind wood/branch (14%) - despite the longest branch length - significantly different from combinations with approximately double of blind wood, such as WA 38/M9-T337 (25%), G969 (27%), G213 (30%; Table 2). The other rootstocks (Bud9, G935, G890, Bud10, G41) reported intermediate values for proportion of blind wood in the branch. Looking at the first node that was able to vegetate in each branch, WA 38/M9-T337 was the combination with the highest number of "blind nodes" (comparable statistically with G969, G213, G41, G935, Bud10) since the first one vegetating was on average the ninth node with respect to WA 38/G11 that, on the contrary, had the least number of blind nodes (first node to vegetate was on average between the fourth and the fifth, Table 3).

Table 2. *WA 38 trunk cross sectional area (TCSA, cm²) for trees grafted on 9 different popular rootstocks for WA regardless of the treatment imposed (on the top, significance root: *** = p≤ 0.001) on 6/24/22. On bottom, the comparison was done between the treatments regardless of the rootstock and then the interaction rootstock × treatment (NS= not significant).*

Rootstock for WA38	N trees	Avg. TCSA (cm^2) on 06/24/2022	
G213	68	2.05	E
G969	60	2.06	E
Bud10	69	2.12	DE
G41	67	2.17	DE
Bud9	67	2.26	D
G11	69	2.39	C
G935	68	2.47	C
M9T337	68	2.62	B
G890	67	3.70	A
Significance root		***	
TRT			
No Stub	299	2.45	
Stub	304	2.41	
Significance trt		NS	
*Significance root*trt*		NS	

Table 3. *WA 38 combinations with 9 different rootstocks in 2022: unpruned (No Stub) trees (N=9/combinations with 4 branches/tree) measurements on blind wood carried out in July 2022. Parameters reported are: the average length of blind wood (cm) and the total average length of unpruned trees, the proportion of the branch affected by blind wood expressed as % and average of 4 basal branches/tree. Moreover, the ordinal number [n^{th}] of the first vegetated node at time of measure presenting leaves from the branch insertion point in the trunk outwards (expressed as average of 4 branches/tree), and internode length (cm) in the blind wood portion of the branch is calculated dividing the length of blind wood by the number of blind nodes (expressed as average of 4 branches/tree). Significance: ** = p≤ 0.01, *** = p≤ 0.001 and letters of separation within each treatment were provided by SNK test.*

Trt	Rootstock combinations with WA 38 (2022)	N trees (N branches)	Avg. length of blind wood (cm)	Avg. length of unpruned branch (cm)		% Blind wood		Ordinal number of first node with leaves from the point of insertion outwards		Internode length (cm) in the blind wood portion	
			(avg. of 4 branches/tree)								
No Stub (=unpruned)	**G11**	9 (4)	11.0	**78.6**	A	**14**	B	**4.4**	B	**2.9**	A
	Bud 9	9 (4)	14.4	**73.4**	AB	**20**	AB	**5.5**	B	**2.6**	AB
	G935	9 (4)	14.2	**71.3**	AB	**21**	AB	**6.4**	AB	**2.3**	CD
	G890	9 (4)	14.4	**76.6**	A	**22**	AB	**5.6**	B	**2.6**	AB
	Bud 10	9 (4)	15.6	**69.8**	AB	**23**	AB	**6.1**	AB	**2.7**	AB
	G41	9 (4)	15.7	**70.1**	AB	**23**	AB	**6.4**	AB	**2.5**	BC
	M9-T337	9 (4)	18.0	**77.4**	A	**25**	A	**9.0**	A	**2.1**	D
	G969	9 (4)	15.4	**61.8**	B	**27**	A	**7.1**	AB	**2.2**	CD
	G213	9 (4)	19.1	**66.4**	AB	**30**	A	**6.7**	AB	**3.0**	A
	Significance		NS	**		**		**		***	

The 9 combinations, when left unpruned, also showed differences in the length of the internodes in the portion of blind wood. WA 38/G213 and WA 38/G11 (similarly also combinations on Bud9, G890, Bud10) were characterized by longer internode of blind wood in comparison with WA 38/M9-T337, WA 38/G969, WA 38/G935 (Table 3). The proportions of blind wood in the 4 branches independently are not presented here, but no constant pattern of decreasing or increasing blind wood seems to be present from the branch proximal to the ground to the more proximal to the tree-top (data not shown). While on average, the blind wood length of the unpruned trees was 15.3 cm, the same measurement decreased to 5.5 cm across all the combinations when investigating the response of trees subjected to click pruning soon after planting. This means that the general reduction of blind wood as a consequence of the cut was 64% (data not shown). Table 4 shows the measured and counted parameters related to the 81 stubbed trees (average of 4 basal stubbed branches). WA 38/M9-T337 unpruned trees showed the highest number of "blind nodes", but when the same combination was stubbed after planting, the number of blind nodes decreased to 3 (Table 3 and 4). One combination that responded very well to the pruning was WA 38/G213, which had the lowest number of blind nodes before the first vegetated node in the branch (statistically similar to G11, Bud9, G935, G890, Bud10, G41). The ordinal number of the first node presenting leaves in WA 38/G213 changed from 6.7 (avg.) to 3.5 (avg.), respectively, in the unpruned and pruned scenarios (Table 3 and 4). In the stubbed trees, we also counted the number of vegetated nodes after the cut, and a significant difference emerged; WA 38/G890 and WA 38/G213 reported 85-84% of vegetated nodes across the different combinations statistically different from WA 38/M9-T337, WA 38/G969 and WA 38/G41 with respectively 64%, 69% and 74% (Table 4).

Table 4. *WA 38 combinations with 9 different rootstocks in 2022: Stubbed trees (=pruned trees on 5/10/22) (N=9/combinations with 4 branches/tree) measurements on blind wood carried out in July 2022. Parameters reported are: the average length of stubbed branches (cm), the proportion of the branch affected by blind wood expressed as % and average of 4 basal branches/tree. Moreover, the ordinal number [n^{th}] of the first vegetated node at time of measure presenting leaves from the branch insertion point in the trunk outwards (expressed as average of 4 branches/tree), and internode length (cm) in the blind wood portion of the branch is calculated dividing the length of blind wood by the number of blind nodes (expressed as average of 4 branches/tree). Significance: ** = p≤ 0.01, *** = p≤ 0.001 and letters of separation within each treatment were provided by SNK test.*

Trt	Rootstock combinations with WA 38 (2022)	N trees (N branches)	Avg. length of stubbed branch (cm)	% Blind Wood	Ordinal number of first node with leaves from the point of insertion outwards		Internode length (cm) in the blind wood portion		avg. N nodes in stubs		Avg. % nodes vegetated in the stubs	
			(avg. of 4 branches/tree)									
Stub	G11	9 (4)	12.2	43	1.9	BC	3.5	A	4.4	B	77	AB
	Bud 9	9 (4)	11.2	42	2.0	BC	3.0	A	4.9	B	79	AB
	G935	9 (4)	11.3	43	2.0	BC	3.2	A	5.1	B	79	AB
	G890	9 (4)	10.5	42	1.7	BC	3.4	A	4.3	B	85	A
	Bud10	9 (4)	10.6	45	1.8	BC	3.4	A	4.2	B	80	AB
	G41	9 (4)	10.7	43	2.2	BC	2.7	AB	4.9	B	74	ABC
	M9-T337	9 (4)	11.7	43	3.0	A	2.1	B	6.2	A	64	C
	G969	9 (4)	10.5	47	2.3	B	2.7	AB	4.5	B	69	BC
	G213	9 (4)	11.3	37	1.5	C	3.5	A	4.1	B	84	A
	Significance		NS	NS	***		***		***		***	

Objective 2) Investigate the WA 38 fruit set in the different types of wood and assess the return bloom on the different bearing woods *(Musacchi-Serra)*

Several of the quality parameters resulted in being different across the locations (Table 5). Apples harvested in SRO – regardless of the bearing wood – showed to be larger, heavier, and riper, with higher firmness, soluble solids content (SSC), and dry matter (DM) than apples from Quincy (Table 5).

This can probably be attributed to the different crop loads recorded in 2021, with Quincy combination having a yield of 29.3 kg/tree and SRO combination of just 16.5 kg/tree (see Table 3 report year 1). No significant differences between sites for number of mature-healthy seeds/apples and TA. When comparing the 3 bearing woods – major interest for this analysis – some differences emerged. Apples from ramo misto had a larger diameter, higher SSC, DM than apples from Brindilla (Table 5). Apple maximum diameter, apple mass, and SSC showed a significant interaction (location × wood) that is reported in Table 5.

Table 5. *WA 38 quality analysis after 6 months of regular air 34°F storage from harvest 2021 for the locations WA 38/NIC29_Spindle_QUINCY and WA 38/M9337-GS_Spindle_SRO investigated in the 2021 survey for objective 2. Sorting criteria for those apples were: only single (S) apples in the cluster, all best color, absence of defects and size between 65-90 mm. Significance: *= p≤ 0.05, ** = p≤ 0.01, *** = p≤ 0.001, NS= not significant and letters of separation within each treatment were provided by SNK test. DM% and TA have a different number of replications not corresponding to N apples reported here (see methods).*

WA 38 harvest 2021 (obj. 2 survey)	*N apples*	Apple maximum diameter (mm)		Apple mass (g)		I_{AD} at 6M		Firmness (lb)		N mature-healthy seeds/tree	N underdeveloped seeds/tree		SSC (Brix)		DM%		TA (% malic ac.)
QUINCY	*83*	75.1	B	198	B	0.92	A	13.97	B	8.7	1.2	B	13.5	B	13.93	b	0.38
SRO	*62*	79.8	A	243	A	0.13	B	14.82	A	8.4	1.8	A	14.2	A	14.58	a	0.38
Significance location		***		***		***		***		NS	*		***		***		NS
B	*43*	76.0	B	212	A	0.57		14.27	AB	8.4	1.7		13.6	B	13.98	b	0.38
RM	*42*	78.2	A	224	A	0.67		13.89	B	8.8	1.3		14.0	A	14.41	a	0.37
S	*60*	77.1	AB	216	A	0.54		14.69	A	8.5	1.5		13.8	B	14.38	a	0.39
Significance wood		*		NS		NS		*		NS	NS		*		**		NS
Significance location*wood		**		**		NS		NS		NS	NS		*		NS		NS
QUINCY_B_S	*23*	72.2	D	178	D	0.94	A	14.04	BC	8.4	1.6		13.3	B	13.68	c	0.39
QUINCY_RM_S	*30*	77.2	BC	213	BC	0.88	A	13.53	C	8.8	1.1		13.9	A	14.08	bc	0.39
QUINCY_S_S	*30*	75.2	C	200	C	0.96	A	14.35	AB	8.9	1.1		13.3	B	14.03	bc	0.37
SRO_B_S	*20*	80.5	A	252	A	0.15	B	14.54	AB	8.5	1.8		14.1	A	14.29	ab	0.37
SRO_RM_S	*12*	80.6	A	253	A	0.14	B	14.80	AB	8.8	1.8		14.2	A	14.74	a	0.36
SRO_S_S	*30*	79.0	AB	232	AB	0.12	B	15.02	A	8.1	1.9		14.2	A	14.73	a	0.41
Significance combo		***		***		***		***		NS	NS		***		***		NS

The 2022 survey focused on 3 WA38 sites with different rootstocks, planting densities, years of planting, and locations (Table 6) to investigate the role of bearing woods in WA 38 production in different settings. In Table 6, N apples/tree (N=3), yield (kg/tree), tree density (trees/A), yield (Mton/A), and average fruit weight (g) are reported for each combination. The three scenarios in 2022 differed for average number of fruit/tree and average fruit weight, but not for yield/tree or per Acre (Table 6). WA 38/NIC29_Spindle _Quincy reported the highest number of apples per tree (161), while WA 38/G935_V system_Royal City the lowest (66); this crop load difference had repercussions on average fruit weight that respectively was 179 g (≃ 113-100 apples/box) and 242 g (≃ 80 apples/box).

Table 6. *Productive data for WA 38 combinations investigated in the 2022 survey for objective 2. Number of apples per tree, net yield in kg/tree, and average apple weight in g are reported as an average of 3 trees per combination. Significance: *= p≤ 0.05, NS= not significant and letters of separation within each treatment were provided by SNK test.*

Combination 2022	N apple/tree	Yield in kg/tree	Density (trees/A)	Yield in Mton/A	Avg. apple weight (g)
WA 38/NIC29_Spindle_Quincy	161 A	28	1584	45	179 A
WA 38/G935_V system_Royal_City	66 B	16	1980	31	242 A
WA 38/M9337-GS_Spindle_SRO	109 AB	22	1499	33	202 AB
Significance	*	*NS*		*NS*	*

Both sites, WA 38/NIC29_Spindle_Quincy and WA 38/G935_V system_Royal City (planted in 2018), showed to be quite consistent in production between 2021 and 2022 (Table 3 report year 1 and Table 6 current report).

The pack-out of apples from the 3 combinations in 2022 was higher than in 2021 (Figure 1). WA 38/G935_V system_Royal_City was the site with the highest proportion of marketable fruit, while WA 38/NIC29_Spindle_Quincy was significantly affected by a hail event on 06/05/2022 and WA 38/M9337-GS_3-axis_SRO recorded mainly mechanical damage and green spot (data not shown).In Figure 2, we report apple distribution at harvest by fruit occupancy within a cluster (single, double, triple, quadruple/cluster) for each of the 3 scenatios. Data 2022 confirmed that the greater part of WA 38 production is held as single apple/cluster (65% avg. of 3 sites), followed by double apples/cluster (32% avg. of 3 sites) and the residual as triple apples/cluster (Figure 2).

Data on proportions of apples harvested from each bearing wood per combination partially confirmed 2021 results (Figure 3). The majority of the apples are produced on spur (range 51-71%), followed by brindilla (19-36%) and ramo misto (5-22%). While WA 38/M9337-GS_Spindle_SRO reported a very similar distribution of the production across the 3 bearing woods than in 2021, WA 38/G935_V system_Royal_City had the tendency to hold more fruit on brindilla in the current season (Figure 3). On the other hand, WA 38/NIC29_Spindle_Quincy that in 2021 reported 81% of apples held on ramo misto, showed a change in 2022 (Figure 3); the proportion of fruit on ramo misto decreased in 2022 due to the aging of the tree-bearing structures (ramo misto structures in 2021 became spurs in 2022).

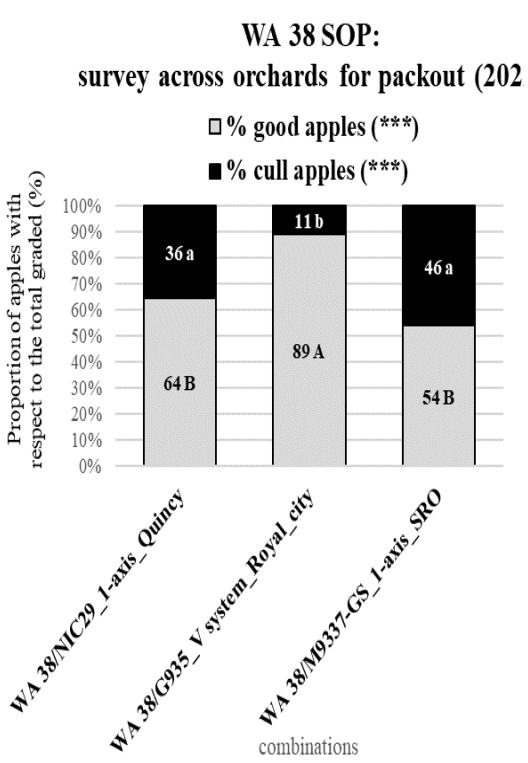

Figure 1. *WA 38 packout across the 3combinations surveyed in 2022.The proportion of cull apples versus the good (=marketable) apples was obtained by visual rating before grading on a sorting line.*

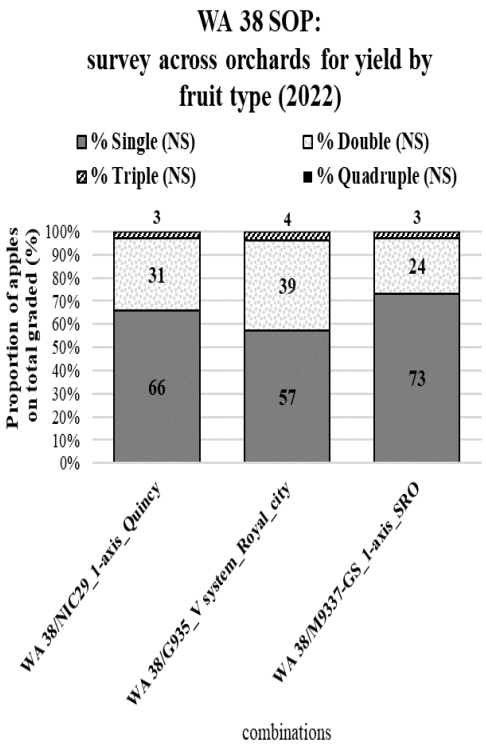

Figure 2. *WA 38 distribution of yield as sorted by type of apple cluster occupancy at harvest (single, double, triple, quadruple/cluster) for each of the 3 combinations in the 2022 survey.*

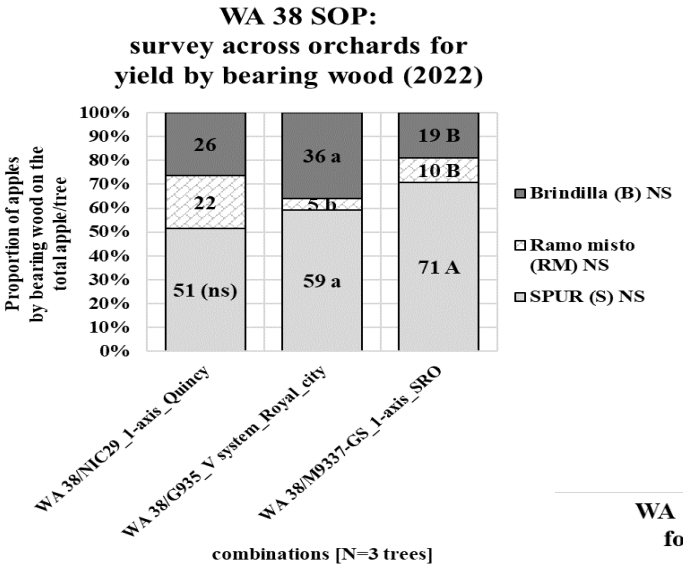

Figure 3. *WA 38 proportion of apples harvested by bearing wood types for each combination surveyed (N=3 trees/scenario) in 2022. The three bearing woods used to classify fruit based on their point of attachment to the tree are: Brindilla, Ramo Misto, and Spur. Not significant (NS) differences across the 3 combinations within each bearing wood (horizontally). Significant differences for 2 combinations out of 3 between different bearing wood within each site. Small letters are discriminating proportions in Royal City and capital letters in Sunrise (vertically) based on SNK test for p=0.05.*

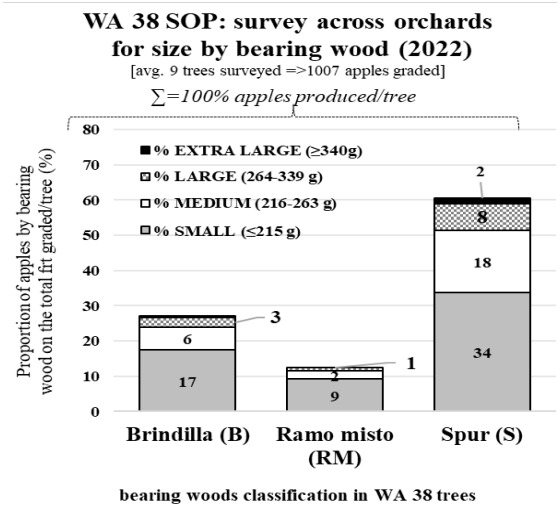

Figure 4. *WA 38 distribution of apples based on bearing wood type and size classes in 2022. These proportions of apples summed up together represent the total apples yielded/graded per tree in 2022 (N=9). The proportion of apple by size and wood represented here are averages across 9 trees. Num total apples graded = 1007.*

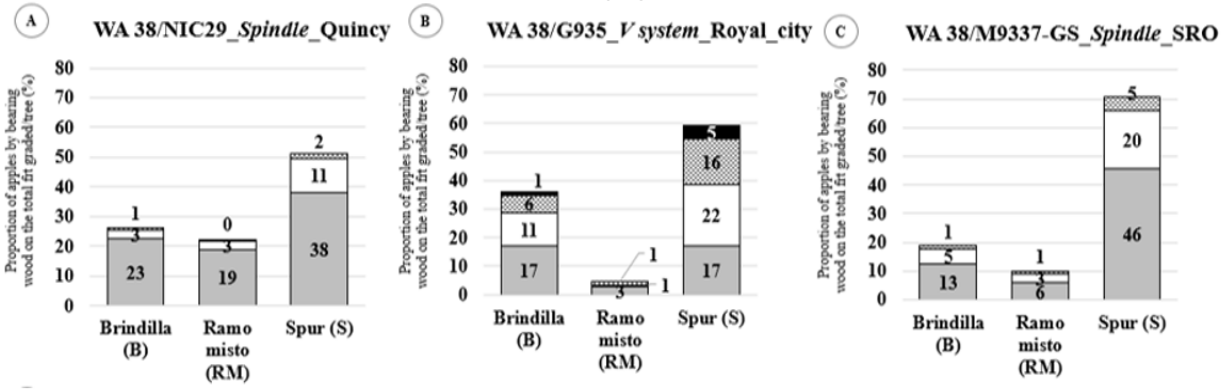

Figure 5. *WA 38 distribution of apples based on bearing wood and size class for each combination: A) WA 38/NIC29_Spindle_Quincy, B) WA 38/G935_V system_Royal_City, C) WA 38/M9337-GS_Spindle_SRO. These proportions of apples summed up together represent the total apples yielded per tree in 2021. Trees averaged here are 3 for each combination. Num total apples graded per combination: A) = 483, B) =198, C) =326.*

■ % EXTRA LARGE (≥340g)
▨ % LARGE (264-339 g)
□ % MEDIUM (216-263 g)
▩ % SMALL (≤215 g)

Averaging all 3 combinations to picture the overall variability in apple size, we observed in 2022 that apples from brindilla and ramo misto were mainly small (≥ 88 apples/box); the apples from spur reported higher proportions also in medium (80 apples/box) and large size (72-64 apples/boxes; Figure 4). Figure 5 (A-C) shows the breakdown of apple proportions by size in each bearing wood for each combination. WA 38/NIC29_Spindle_Quincy presented the predominance of smaller fruit across the 3 kinds of bearing wood (Figure 5A). Royal City apples from brindilla and spur belonged to the major 3 size classes corresponding to the range of ≥ 88 apples/box to 72-64 apples/box (Figure 5B). WA 38/M9337-GS_Spindle_SRO reported the majority of the apples on spur and in the small-medium size classes (Figure 5C).

Objective 3) Investigate the cultural management practices developed in WA 38 private orchards and summarize them in a list of recommended guidelines for growers *(Lewis-Sallato)*

In 2022 bloom density was generally high (with more than 150 clusters per tree). However, it varied between orchards (Table 6). Fruit load varied between 63 and 143 fruit per tree. When compared with 2021, seven of the nine sites had increased fruit per tree, averaging a 70% increase, except for two sites (sites 1 and 7, where there was no difference in fruit number per tree). The increased fruit load relates to the enlargement in tree size (filling the trellis space). Higher crop load (Figure 6) reduced shoot growth. Crop load was highly variable among sites, ranging from 7.8 to 20 fruit per trunk cross sectional area, influenced more by the year and site (management), than by rootstock (Figure 7).

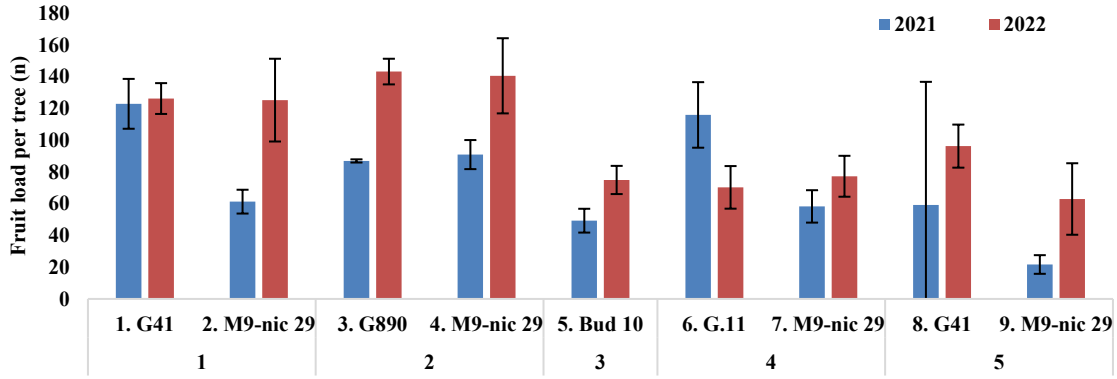

Figure 6. *Fruit load per tree for 2021 and 2022 by orchard (number) and rootstock. Bars indicate the mean value of 6 representative trees; error bars indicate standard deviation.*

Fruit size varies between 183 and 304 g, 15% smaller than in 2021, except for one orchard (in both rootstocks), where fruit size was 22% higher. As the tree ages, shoot growth is reduced (vigor control). In 2022 shoot growth was generally reduced (average 4 cm shorter than in 2021), except for orchard 2 (sites 3 and 4) where shoot growth was higher (9 cm larger). Balanced trees averaged 17 cm shoot length (Table 6)

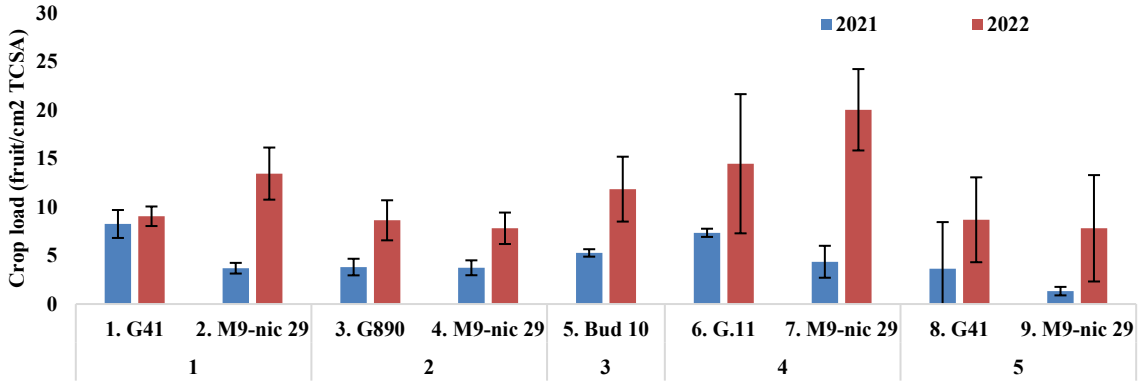

Figure 7. *Crop load (fruit per trunk cross sectional area) for 2021 and 2022 by orchard (number) and rootstock. Bars indicate the mean value of 6 representative trees, error bars indicate standard deviation.*

Table 6. *Bloom density, fruit mean weight, yield and shoot growth in WA38 monitored orchards.*

Site ID	Harvest date	Bloom Density 2021	Bloom Density 2021	Fruit Weight (g)	StdDev	Estimated Yield (bins/acre)*	Shoot Length (cm)	StdDev
1. G41	6-Oct	M	H	213	2.8	47	18.5	2.4
2. M9-nic 29	6-Oct	L	H	182	33.7	40	16.9	2.1
3. G890	3-Oct	M	M	221	16.7	55	27.2	1.1
4. M9-nic 29	3-Oct	M	M	222	15.2	54	28.6	2.8
5. Bud 10	17-Oct	H	M	276	13.1	59	15.8	0.3
6. G.11	14-Oct	H	L	263	16.1	32	17.1	1.7
7. M9-nic 29	14-Oct	H	L	304	21.3	41	20.9	5.0
8. G41	6-Oct	L	L	263	16.1	44	20.0	2.0
9. M9-nic 29	6-Oct	M	M	304	21.3	33	23.7	5.9

*bins/acre calculated based on fruit number x size x trees per acre, considering a bin weight = 925 lbs.

Outreach activities

In 2022, we organized and/or participated in five field days. Four in Spanish and one in English.

- Día de Campo WA 38- Polinización y cuaja de fruta (Quincy and Royal City) / WA38 field day in Spanish - Pollination and fruit set. **Sallato presenter.** April 21 @ 10:00 am - 1:00 pm
- Día de Campo WA 38- Polinización y cuaja de fruta (Roza) / WA38 field day in Spanish -Pollination and fruit set. **Sallato presenter.** April 19 @ 10:00 am - 1:00 pm
- Día de campo en WA38: Nutrición, vigor y estrés por calor. WSU- Huerta la Roza, IAREC. (WA38 Field Day in Spanish: Nutrition, vigor and heat stress). **Sallato presenter.** July 5 @ 10:00 am - 12:00 pm
- WA38 Preharvest Field Day. September 15 @ 8:30 am - 12:00 pm. **Hosted by Musacchi, Serra, Lewis, Sallato.** Invited presenter: L. Kalcsits. At the preharvest field day, we had 89 attendees. Of 22 respondents, over 93.4% indicated they were satisfied with the content. Learnings included: pruning, harvest timing, and maintaining vigor.
- Actualización de conocimientos en WA 38 (Español). October 19 @ 8:30 am - 11:30 am. **Hosted by B. Sallato**. We had 33 people attending the field day and 23 responses to the survey. 81.6% indicated increased knowledge, and 83.3% intention to apply some of the learnings during the session. E.g., means for vigor control, summer pruning and irrigation, when to apply calcium. The highest-rated topics are soils-root-tree and general characteristics of WA 38.

The surveys also identify the remaining challenges with growing WA 38. These included: reducing green spots, maintaining vigor, producing fruit (low production), pruning, and filling the trellis space.

FINAL PROJECT REPORT **PERIOD:** 2 year of 2 years
(AP-21-109A)
Project Title: **Maximize pollination window to improve fruit set in WA 38**

PI: Sara Serra
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Co-PI: Tory Schmidt
Organization: WTFRC
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Email: tory@treefruitresearch.com

Cooperators: Chelan Fruit - Monument Hills Orchard (Quincy, WA), Columbia Reach (Yakima, WA), AgroFresh, Extenday USA, Valent Biosciences.

Other funding sources/in-kind support:
We will use commercial orchards established by growers in the Royal City and Quincy areas in WA. The WA 38 orchard blocks available cover approximately 2 acres for an estimated value of $80,000 to be considered as in-kind support for the current project. Valent Biosciences is donating ReTain® and providing technical support for this project. AgroFresh has agreed to donate Harvista™ as well as technical staff support for material application for the two years of research. Extenday USA has donated reflective ground cover and technical support for material installation for this project.

Total Project Request: Year 1: $ 72,022, Year 2: $ 73,813 (Total: $ 145,835)

BUDGET
Primary PI: Sara Serra
Organization Name: Washington State University
Contract Administrator: Stacy Mondy/ Jason Hansen
Telephone: 509-335-6881/ 509-335-2885
Contract administrator email address: arcgrants@wsu.edu/ gary.hansen@wsu.edu
Station Manager/Supervisor: Chad Kruger
Station manager/supervisor email address: cekruger@wsu.edu

Budget 1

WSU: Serra-Musacchi		
Costs	Year 1 (2021)	Year 2 (2022)
Salaries[1]	$ 33,840	$ 35,194
Benefit[2]	$ 11,306	$ 11,758
Wages[3]	$ 4,800	$ 4,992
Benefit[4]	$ 1,076	$ 1,119
Supplies [5]	$ 4,000	$ 4,000
Fruit reimbursement for sampling	$ 5,000	$ 5,000
Travel[6]	$ 5,000	$ 5,000
Serra-Musacchi Total	$65,022	$67,063

Footnotes:
[1] Salary for a 50% Assistant Research Professor (Serra-Musacchi)
[2] Benefit on salary at 33.41%
[3] One non-Student temporary for 8 wks: 40hrs/wk at $15/hr (Serra-Musacchi).

[4] Benefits on temporary at 22.4%
[5] Labware/consumable, field products (Serra-Musacchi)
[6] 8,696 miles/year for domestic travel ($0.575/mile) to go to the orchards. Adjusted for COVID19 distancing (independent cars).

WTFRC Collaborative Expenses

WTFRC: Schmidt		
Costs	Year 1 (2021)	Year 2 (2022)
Wages/Benefits	$4,000	$3,750
Supplies[1]	$500	$500
Equipment costs[2]	$1,000	$1,000
Travel[3]	$1,500	$1,500
WTFRC Total	$7,000	$6,750

Footnotes:
[1] Spray suits, lab supplies for fruit quality analysis
[2] Fuel, maintenance, wear and tear on trailer, tractor, sprayer
[3] In-state travel to research plots

RECAP OBJECTIVES:

1. **Determine the AVG and 1-MCP effect on fruit set in WA 38**
2. **Explore the effect of pre-bloom deployment of reflective fabric on WA 38 fruit set**

SIGNIFICANT FINDINGS:

1. Determine the AVG and 1-MCP effect on fruit set in WA 38

a. *No significant differences in flower bud return and tree growth across the 5 treatments in 2022*
b. *At 8 weeks after full bloom, the fruitlet shedding ended at 91% in 2021 with no differentiation between ethylene inhibitor treatments or application timings and at 82% in 2022 with Retain treatments at both application times, reporting the lowest fruit drop.*
c. *While average number of apples per tree, production per tree, and crop load at harvest were all comparable across treatments in 2021, in 2022, both ReTain treatments reported the highest number of apples per tree, and crop load significantly differed across the five treatments, despite similar yield per tree.*
d. *"ReTain 56% bloom" and "ReTain +7 days" significantly penalized the average fruit weight in 2022 (~150 apples/box) with respect to untreated control trees ("CTRL"), and "Harvista 56%" fruit (~113 apples/box). In 2021, only "ReTain +7 days" penalized the average fruit weight.*
e. *For two consecutive years, the natural crop load in the orchard of untreated control trees was very high with limited potential for further enhancement (10.2 and 9.9 fruit/cm^2 TCSA).*
f. *"ReTain 56% bloom" showed 55% of the production as a single apple/flower cluster (lowest proportion), 30% as a double and 13% as a triple (significantly higher than the other treatments), and a 2% as a quadruple, suggesting an improved fruit set on a flower cluster basis.*
g. *"ReTain 56% bloom" produced 99.7% of 2022 apples in the small size (almost 17% more than control), while "Harvista 56% bloom" performed very similarly to "CTRL". In 2021, "ReTain +7 days" negatively impacted the packout towards small fruit (+13.4% smaller than in "CTRL").*
h. *The triple fruit/cluster category reported the highest proportions of smaller fruit in all treatments except for "ReTain 56% bloom", suggesting considering the option of a mid-summer hand thinning of triple apples at least down to double/cluster when the crop load in medium-high to minimize the smaller fruit.*

2. Explore the effect of pre-bloom deployment of reflective fabric on WA 38 fruit set

a. *Reflective material deployed in 2021 did not impact tree trunk growth, the vegetative growth of 1-year-old shoots, or the number of flower buds per tree when compared to control.*
b. *An abnormal frost event occurred in April 2022, which may have contributed to a confounding effect in the fruit retention assessment along the season as some compromised flowers abscised due to cold damage.*
c. *The last fruit retention assessment reported similar fruit drop for "RM until harvest", "no RM (CTRL)" and "RM for 2M" (90%, 90%, and 91%, respectively), while "RM for 1M" showed the lowest proportion of fruit left on tree (7%).*
d. *The reflective material employed for 2 years in this trial proved to alter the tree microclimate towards a drier and hotter canopy but a cooler and wetter soil.*
e. *Monthly photosynthesis measurements from May to September of both years did not show significant differences in carbon assimilation rates for trees with reflective material from early bloom and "no RM (CTRL)" trees.*
f. *Across the four treatments, yield/tree was not significantly different, ranging from 15.0 kg/tree to 17.9 kg/tree for "no RM (CTRL)" and "RM until harvest", respectively (similarly to 2021).*
g. *"RM until harvest" trees presented the highest proportion of 2022 apples belonging to large size class (46%), followed by "RM for 1 M" (41%), while "no RM (CTRL)" showed the largest proportion in the smallest size class (38%). The season-long deployment of RM from early bloom to harvest promoted fruit size and red coloration rather than fruit retention.*

Objective 1) Determine the AVG and 1-MCP effect on fruit set in WA 38

RESULTS AND DISCUSSION

The return bloom assessment was carried out in March 2022 in the same WA 38/Nic29 orchard (planted in 2018) in Quincy as the first year of this study. The number of flower buds per tree was counted before pruning in a subsample of 9 trees/trt (out of 12) and did not produce significant differences (p=0.0705) between the 5 treatments imposed in 2021. Despite the lack of statistical discrimination, "ReTain 31% bloom" showed the highest average, equal to 201 flower buds/tree, while "Harvista +7 days" produced the lowest average with 171 flower buds/tree (Figure 1). Similarly, no significant difference emerged in terms of TCSA annual growth across the 5 treatments (Figure 1).
In the second year (2022), we repeated the same experiment as in 2021 in the same orchard rows keeping the same treatments on the same plots to account for a 2 year-cumulative effect. Each treatment was represented by 12 trees (4 trees/row × 3 rows × 5 treatments) chosen in the same plots treated in 2021, for a total of 60 trees in the trial. The experimental trees were selected in spring 2022 to ensure a uniform starting point for the second season. The selection criteria were similar TCSA and a narrow range of flower buds (FB)/TCSA.TCSA was measured 4/6/2022 on a new set of trees and averaged 16.4 ± 2.2 per cm^2 with no differences across treatments. The count of flower buds/tree was done after pruning on 4/18/22, and the calculated flower bud loads averaged 11.1 ± 1.7 FB/ cm^2.

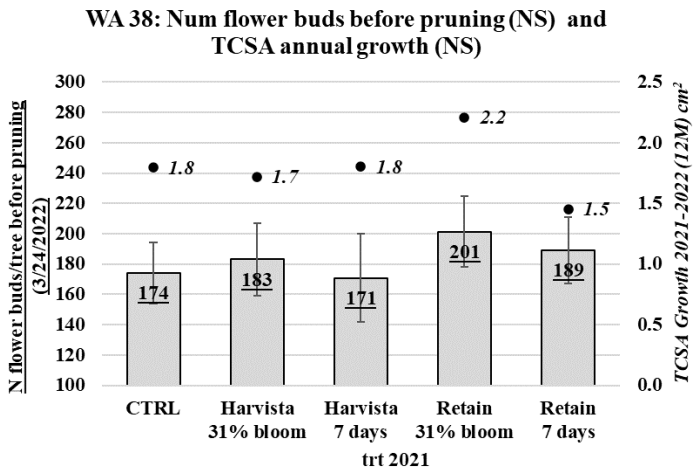

***Figure 1**. WA 38 return bloom in 2022 on trees in trial in 2021 (as N flower buds/tree before pruning) and annual trunk cross sectional growth (TCSA, cm^2) in 12 months in Quincy block across the 5 treatments in trial: "CTRL", "Harvista 31% (king) bloom", "Harvista +7 days" and "ReTain 31% (king) bloom" and "ReTain +7 days". (The "+" in the two treatments at 7 days is omitted in the x-axis from now on). Each column represents the mean yield of 9 trees per trt and the error bar indicates the standard deviation. Each circle marker related to the secondary Y axis represents the TCSA growth of 12 trees per trt. Differences reported are not significant (NS, p>0.05).*

A severe frost event hit the Wenatchee and Quincy areas during the second and third weeks of April 2022. On 04/15/22, a minimum air temperature of 26.9 °F was reported in Quincy (avg. daily temperature for April 2022 was 44.6 °F, while 52.9 °F in 2021). A survey on green cluster-early pink tip flowers conducted on 4/19/2022 by dissecting them to examine the potential impact of cold damage did not show significant browning of styles nor ovaries that would lead to a possible significant loss, indicating the frost protection system in the block was effective. For each experimental tree, 5 branches were labeled (total 300 branches) and tracked for their phenology from swollen flower bud stage until harvest, following the same protocol reported for 2021 (with minor modifications). The precise tracking of the phenology evolution was crucial to target the suitable time for the chemical applications. In 2022, the first spray of ReTain® (Valent Biosciences) and Harvista™ (AgroFresh) was on 5/3/2022, corresponding to 56% king flower open (or 17% of total flower open). The chemical application followed the same methodology described for the previous year. The utilized doses complied to the recommended label rates for both products: 1 pouch of 333 g/acre (123.4 g AI/ha; AI= active ingredient) of ReTain® and 60 g AI/acre (148.2 g AI/ha) for Harvista™. Seven days later, on 5/10/2022 (full bloom), the second spray ("ReTain +7 days" and "Harvista +7 days") was administered to the designated plots for each of the two ethylene inhibitors at 89% total flowers open (97% king open and

king petal fall had begun). To investigate the effect of the two ethylene inhibitors and their application time in improving the fruit set and on-tree retention of WA 38 fruitlets, fruitlet retention assessment on the 300 branches was carried out from June through October 2022. Fruitlet retention assessment began on 6/1/2022 (=21 days after full bloom (DAFB), avg. fruit diameter = 14 mm) and was repeated on 5 subsequent dates until harvest (6/21, 7/8, 7/18, 8/4, 10/7/2022). At 3 weeks after full bloom (6/1/2022), the "ReTain 56% bloom" showed 80% of fruitlets still on tree – a significantly higher proportion relative to the other 4 treatments, with the lowest being "CTRL" and "Harvista 56% bloom" with only 50% and 57% retention, respectively (data not shown). At 41 DAFB, on 6/21/2022 (avg. fruit diameter = 33 mm), the overall fruitlet drop reached 79% across the 5 treatments with significant differences between them. "ReTain 56% bloom" and "ReTain +7 days" showed 26 and 25% retention, respectively, while "CTRL" showed only 18% drop, statistically similar to both "Harvista" treatments (data not shown). Figure 2 (A to D) reports fruit retention/fruit drop for two-time points for each year of the trial. At approximately 8 weeks AFB (7/8/2022, avg. fruit diameter = 42 mm), the overall drop reached 82%, and the retention of fruit showed significant differentiation across the treatments (Figure 2). In fact, "Retain 56% bloom" and "Retain +7 days" maintained the highest proportions of apples on tree (21 and 19 %, respectively, Figure 2B), while "CTRL" recorded 16%. Compared to 2021, at 56 DAFB, the general fruit drop was 9% more intense (91%) than in 2022 at similar DAFB (Figure 2A and B) but did not report significant differences across the treatments. Weather conditions, in particular temperature, in the first 8 weeks AFB (avg. 2022: min 50.5 °F, avg. 60.5 °F, max 70.8 °F and avg. 2021: min 52.2 °F, avg. 63.2 °F, max 74.4 °F, Figure 3) could have had a meaningful impact on the fruit shedding dynamics and retention in the two years. In the two years of this project, we are able to corroborate that WA 38 natural shedding lasts 8 weeks after full bloom, as previously observed during the project on WA 38 pollination and fruit development (# AP-19-10) and published in Serra *et al.*, 2022.

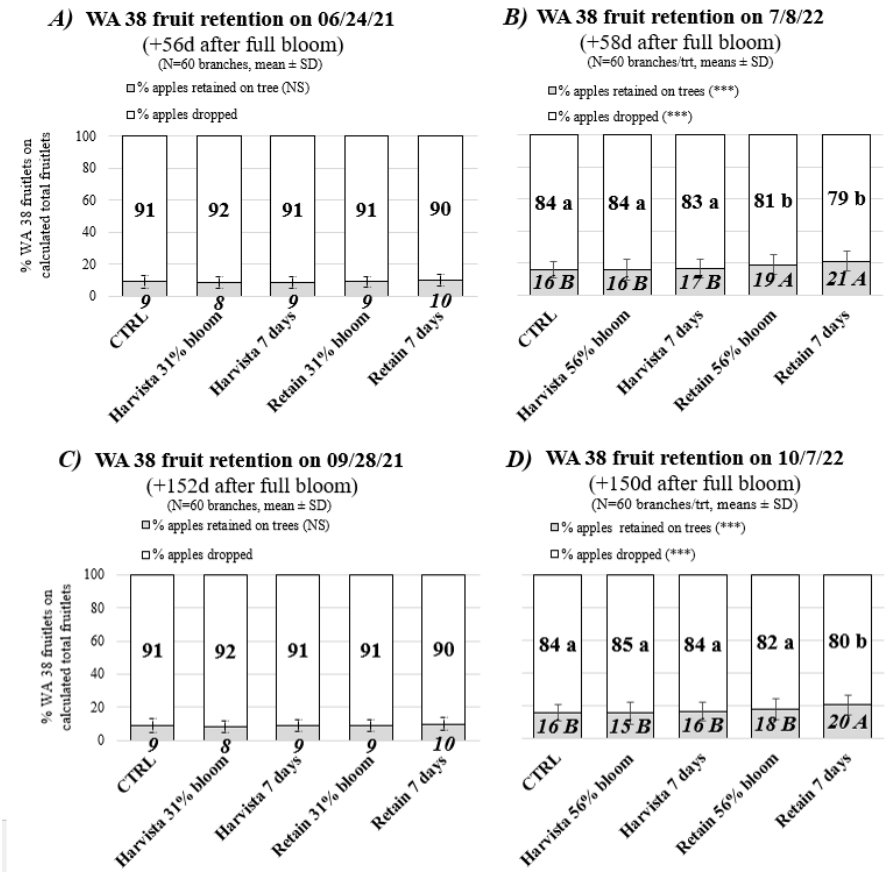

Figure 2*. WA 38 fruit retention assessments in the Quincy block across the 5 treatments in trial: "CTRL" (control, no treatment), "Harvista 31 or 56%¥ king bloom", "Harvista +7 days", "ReTain 31 or 56% king bloom", "ReTain +7 days" on 2 different dates in the 2 seasons in June 2021 (A= 8 weeks after full bloom), July 2022 (B=8 weeks after full bloom), September 2021 (C=22 weeks after full bloom) and October 2022 (D=22 weeks after full bloom). Each bar represents the mean of fruit retention (%) and fruit drop (%) in 60 branches/treatment each year. Error bars represent standard deviation of retained fruit percentage (in gray). NS= not significant, ***p≤0.001. ¥ The two percentages indicate the proportion of king flowers open in the tree at time of first chemical spray each year (31% in 2021 and 56% in 2022).*

At 10 weeks AFB (7/18/2022, avg. fruit diameter = 49 mm), once the natural shedding period typical of the variety had ended, the pattern of retention was similar to the 7/8/2022 assessment, confirming the highest proportions of retained apples in "ReTain +7 days" (+5% than "CTRL") and "ReTain 56% bloom" (+3% than "CTRL"), with significant differences with "CTRL" (data not shown). The following assessments on 8/4/22 (~12 weeks AFB; avg. fruit diameter = 57 mm; data not shown) and 10/7/2022 (~21 weeks AFB; avg. fruit diameter = 76 mm; Figure 2D), were also comparable to previous assessments, with the **highest retention of fruit in "Retain +7 days**" which was significantly different from all other treatments. The final assessment before harvest (10/7/2022) showed an overall average fruit drop of 83%, the same percentage of dropped fruit as reported in Serra *et al.*, 2022 for the year 2020 in a different block. Compared to 2021, which had higher temperatures during the shedding period, the fruitlet drop was higher at 91% (Figure 2C and 3). "ReTain +7 days" in 2022 contributed to a +4% retention with respect to "CTRL" (20% and 16%, respectively) in the experimental branches tracked along the season (Figure 2D). A survey conducted on a subsample of the branches (N=251) in 8/4/2022 in this block regarding the type of fruit present on tree by cluster occupancy showed 57% being single king apples, 26% single lateral apples, 9% king+lateral, 7% double laterals, and 1% king+2 laterals (data not shown).

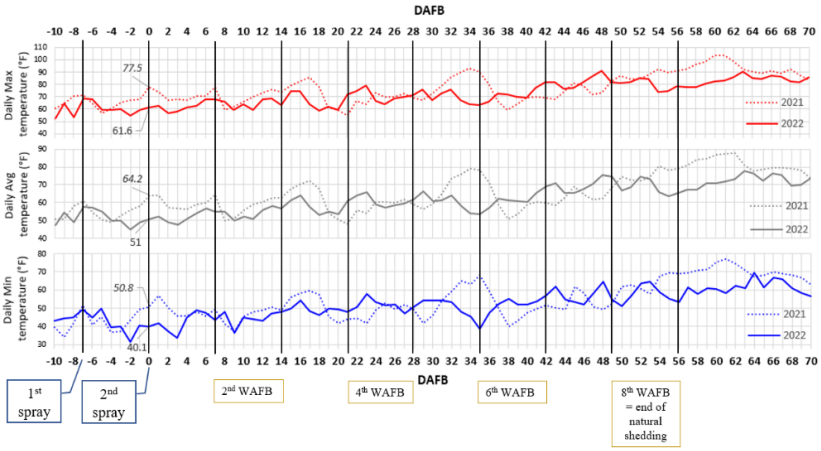

Figure 3. *Comparison between daily minimum, average, and maximum air temperature (°F) in Quincy in 2021 and 2022 from AWN station. Temperatures are presented in DAFB (x-axis), where 0 DAFB indicates "full bloom" for both years in the WA 38 experimental block. In both years, the first Harvista™ and ReTain® sprays were applied at -7 DAFB (corresponding to 4/22/2021 and 5/3/2022), while the second spray at 0 DAFB (corresponding to 4/29/2021 and 5/10/2022). WAFB= weeks after full bloom.*

The trial was harvested as a single pick on 10/13/2022 for the entire commercial block. The 5 treatments resulted in a significant difference in the number of apples picked/tree, with 223 apples on average in "Retain 56% bloom", 183 for "Retain +7 days", 169 in "Harvista +7 days", and 158 for both "CTRL" and "Harvista 56% bloom" (letters for mean discrimination: A, B, BC, C, C respectively; data not shown). The number of apples per tree in 2022, regardless of treatment, was on average 22 apples higher than in 2021. Similarly to 2021, production per tree did not reveal any significant differences across the 5 treatments with an average yield/tree ranging from 26.0 kg/tree to 27.1 kg/tree (57-60 lb/tree, Figure 4). However, average fruit weight in 2022 did significantly differ across treatments ($p \leq 0.001$), with "CTRL" and "Harvista 56% bloom" showing the highest and most similar fruit masses (173 g, 167 g, respectively, ~113 apples/box), followed by "Harvista +7 days" (157 g, ~125 apples/box). In the lower spectrum of fruit size, "Retain +7 days" yielded a smaller average fruit weight (142 g, ~125-138 apples/box), while "ReTain 56% bloom" produced the smallest fruit (121 g; ~150 apples/box, Figure 4). All treatments in both years had very high crop loads associated with general reductions in average fruit size in an optimum crop load scenario for WA 38 (5-6 apples/TCSA cm^2, 80-64 apples/box). While the crop load at harvest was not statistically different across treatments in 2021 (avg. 10.6 apples/TCSA cm^2), in 2022 the highest crop load of 13.3 apples/TCSA cm^2 for "ReTain 56% bloom", followed by "Retain +7 days" with 11.8. The other 3 treatments were similarly lower ("Harvista +7 day" 10.5, "CTRL" 9.9, and "Harvista 56% bloom" 9.6 apples/TCSA cm^2, data not shown).

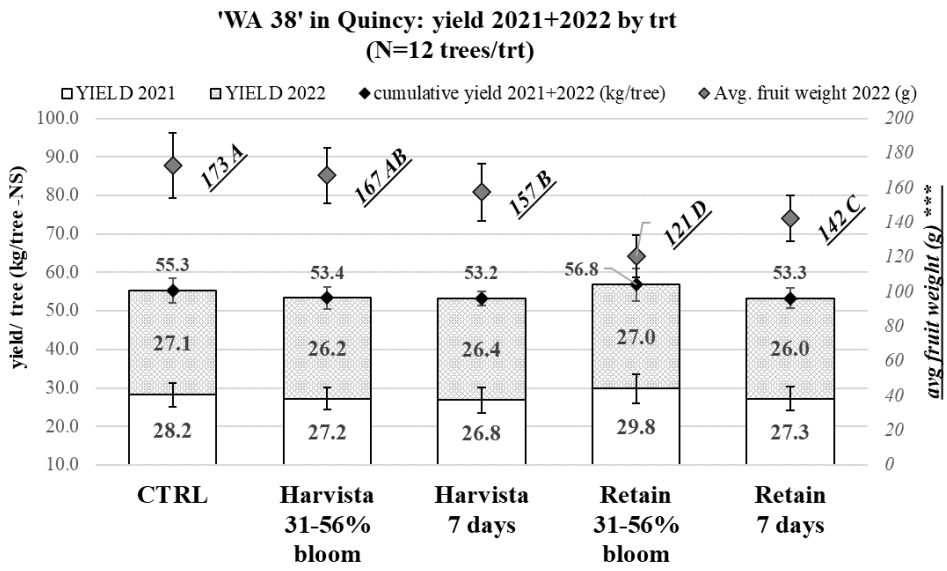

Figure 4. *WA 38 yield in kg/tree in 2021 and 2022 and 2022 average apple weight (g) in the Quincy block across the 5 treatments in trial: "CTRL" (control, no treatment), "Harvista 31-56% bloom", "Harvista +7 days", "ReTain 31-56% bloom", "ReTain +7 days". The 2 treatments at bloom are labeled with the 2 real percentage of king flowers that were open at time of spray in 2021 and 2022 (the target was in theory 50%). Each chemical spray was applied to the same plots as in 2021.Each column represents the mean yield of 12 trees/trt and the error bar indicates standard deviation. Each gray diamond marker related to the secondary Y axis represents the mean fruit weight of 12 trees/trt and the error bar indicates standard deviation. The 5 treatments are presented in X-axis in order of application time for each product after CTRL. ***=p<0.001, NS=not significant.*

A hailstorm hit the experimental block on 06/05/2022 causing significant damage to the small fruitlets (~ 16-21 mm size), impacting the final packout at harvest. In general, 33% of graded apples were culled, and both treatments at "+7 days" showed the highest proportion of culled fruit, while "Retain 56% bloom" and "CTRL" showed the lowest (data not shown). As performed in 2021, each apple harvested in 2022 was labeled at the time of pick based on the cluster pattern of origin as being single (S=1 apple/cluster), double (D=2 apples/cluster), triple (T=3 apples/clusters) or quadruple (Q=4 apples/cluster). All 2022 production was graded tree by tree, keeping apples separated from the 4 different cluster patterns within each tree. Significant differences in the proportion of fruit by cluster occupancy emerged across the treatments (Figure 5). "CTRL" and "Harvista 56% bloom" presented 70% of the crop as a single apple/cluster, which was shown to be significantly different from both treatments with ReTain. "ReTain 56% bloom" showed 55% of the production as single, 30% as double, and significantly higher proportion in the triple category, 13%, in comparison with the other treatments, and 2% in the quadruple (Figure 5). The same analysis of apple by cluster pattern in 2021 resulted in no significant differences.

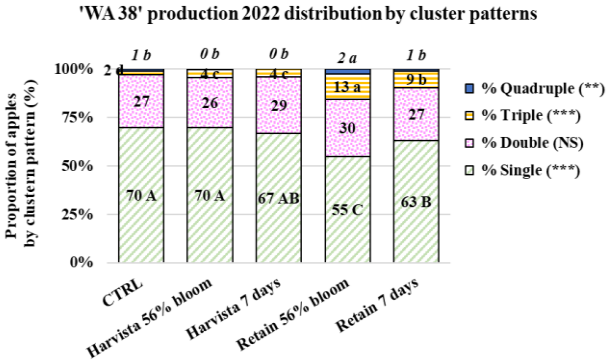

Figure 5*. WA 38 fruit grading by cluster patterns (or cluster occupancy) for the whole crop of Quincy trees (N=60) across the 5 treatments in 2022. Each tree production was graded by a sorting machine and apples were run separately among cluster categories: single, double, triple, quadruple. NS= no statistically significant differences, **=p≤0.01, ***= p≤0.001.*

Fruit size distribution was reported in proportion (%) of apples belonging to each of the three size categories for each of the treatments in trial (total 60 trees were graded= 10,685 apples, Figure 6). “ReTain 56% bloom” produced significantly more apples in the small classes (<215 g) than the other 4 treatments with 99.7% (Figure 6), while “CTRL”, “Harvista 56% bloom” and “Harvista +7 days” reported higher proportions of medium apples (216-263 g, ~80 apples/box) in comparison to both treatments with ReTain (Figure 6). In 2022, the “ReTain 56% bloom” significantly penalized the fruit size leading to almost 100% of the crop in the smallest category, while in 2021, the application of ReTain at bloom (31% king bloom) led to the highest proportion of medium-sized fruit.

Many factors might have contributed to the contrasting results between the two years, in particular, the weather conditions at the time of application (for stigmatic receptivity and ovule longevity), as well as flower phenological stage, pollination, flower cluster quality, and resource availability. Machine grading by color criteria (Extra-Fancy & Fancy = red overcolor >50%, Grade 1 = red overcolor 30-50% and utility = red overcolor < 30%) highlighted a significant difference among the treatments. “ReTain 56% bloom”, indeed, produced the lowest proportion of fruit in the Extra-Fancy & Fancy class (78% versus 92% in “CTRL”) and the highest percentages in grade 1 and utility apples (data not shown).

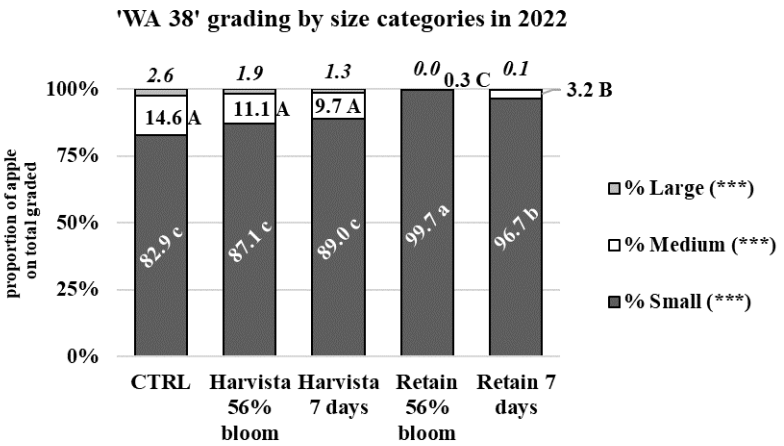

Figure 6. *WA 38 fruit grading in size categories for the Quincy block across the 5 treatments in 2022. Each tree production was graded by the sorter machine and apples were divided in 3 size categories: small (≤ 215 g), medium (216-263 g), and large (264-339 g) corresponding to ≥88, 80, and 72-64 apples/box, respectively. WA 38 fruit in the extra-large (≥ 340 g) class were absent from this trial. ***=p<0.001 for significant differences.*

Additionally, in this second year, we can confirm that differences in terms of proportions of fruit in size categories are strongly influenced by cluster patterns (S, D, T) within each treatment (data not shown). The triple fruit/cluster (T) category reported the highest proportions of smaller fruit in “CTRL”, “Harvista 56% bloom”, “Harvista +7 days”, and “ReTain +7 days” with respect to S (or S and D), while single fruit/cluster (S) showed significantly higher proportions of fruit in the medium size class than what was found in T (data not shown). These results corroborate 2021 findings and sound support in considering mid-summer hand thinning of triple apples when the crop load is medium-high.

Objective 2) Explore the effect of pre-bloom deployment of reflective fabric on WA 38 fruit set

RESULTS AND DISCUSSION

In March 2022, the number of flower buds before pruning was counted on the experimental trees selected for 2021 in the WA 38/G945 block (planted in 2018) to assess the effect of treatments on return bloom. As reported in Figure 7, the average number of flower buds ranged between 131 in the no reflective material ("CTRL") to 161 in "RM until harvest" with no significant differences. Despite the lack of statistical significance, "RM until harvest" tended to have 23% more flower buds than "CTRL". This same tendency was found for TCSA annual growth (in 12 months), where no significant difference emerged across the 4 treatments (Figure 7). Since the deployment of reflective material for longer duration, such as the treatment "RM until harvest", could have had an impact on the tree physiology, such as growth, the total length of 1-year-old shoots was measured on 3 trees/treatment before pruning and the number of shoots counted. Results on vegetative growth of 1-year-old shoots did not reveal significant differences across treatments, with an average of 23 m (~75 ft) of shoot growth/tree and 127 shoots of approximately 17 cm (6.7 inches) length (data not shown). The "RM until harvest" average of the total length of 1-year-old shoots was 5 m (16 ft) higher than "CTRL" (NS, data not shown).

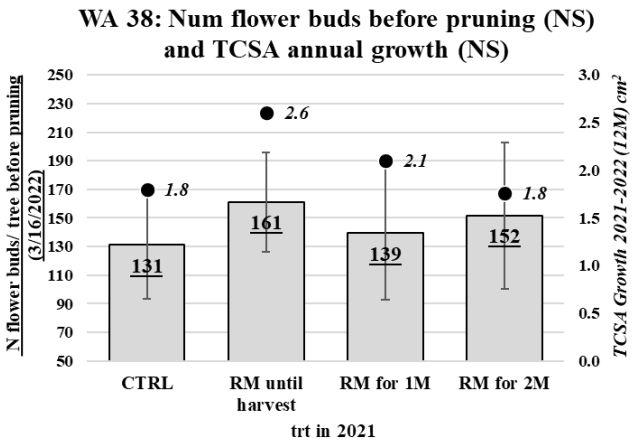

***Figure 7**. WA 38 return bloom in 2022 on trees in trial in 2021 (as N flower buds/tree before pruning) and annual trunk cross sectional (TCSA) growth (cm^2) in 12 months in Royal City block across the 4 treatments in trial: "CTRL", "RM until harvest", "RM for 1M" and "RM for 2M". Each column represents the mean yield of 12 trees per trt and the error bar indicates means ± standard deviation. Each circle marker related to the secondary Y axis represents the TCSA growth of 12 trees per trt and the error bar indicates standard deviation. Differences reported are not significant (NS =p>0.05).*

The plots designated for each treatment in 2021 were maintained the same in 2022, but new trees were selected for each treatment (4 east-facing trees/row × 3 V system rows) in April 2022 for a total of 48 trees to guarantee a uniform starting point for the second season of trial. Trees were selected for their similar number of flower buds (9.4 ± 2.1 FB/cm^2 TCSA) and TCSA (14.2 ± 1.9 cm^2) on 4/8/2022. Abnormally cold temperatures were recorded throughout April, including 25.3°F on 04/15/22 (avg. daily temperature for April 2022 was 44°F). While a frost protection system was activated, the orchard suffered a bit of cold damage, particularly in the lower canopy of the trees. A few days following the frost event, a subsample of flower clusters at green cluster-early pink tip stage were sampled and dissected. Approximately 46% of flowers dissected showed some browning in the styles, and 12%, some browning in the ovary (data not shown). This survey gave us more awareness about branch selection for 2022, knowing that the lower part of the canopy could have been compromised. Five branches/tree were tagged before bloom and described by position in the canopy and type of bearing wood. In each branch, the number of flower clusters was recorded following the 2021 protocol with minor modifications. Reflective material was installed on 4/28/22, when bloom was at ~10% king flowers open (~2% total flowers open). The reflective material used in the trial was the same as utilized in 2021 (open weave reflective fabric provided by Extenday® with 80% diffuse reflection). The 4 treatments evaluated in trial were: "RM for 1M" = RM deployment for 1 month (from 4/28/22 early bloom to 5/26/22), "RM for 2M" =RM deployment for 2 months (from 4/28/22 early bloom to 6/28/22), "RM until harvest" = RM deployment for all-season until harvest (from 4/28/22 early bloom to 10/12/22 harvest), and no reflective material ("CTRL"). Full bloom for the experimental block was 5/07/2022 (9 days later than 2021). Recording of fruitlet retention started 19 DAFB (on 5/26/22) as the

first assessment before the “RM for 1M” removal on 5/26/22, followed by 5 subsequent assessments before harvest. Fruitlet retention was calculated as described in the 2021 project report. On 5/26/22 (avg. fruit diameter = 13.3 mm), an average of 49% of fruitlets were still retained on tree with significant differences across the treatments; “RM for 2 M” showed the lowest percentage of fruit on tree significantly smaller than the other 3 treatments (data not shown). Twenty days later, on 6/15/2022 (6 weeks AFB, avg. fruit diameter = 29.9 mm), the average fruit retention dropped to 18% with “RM until harvest” producing the highest proportion of apples on tree (21%) with respect to the other treatments (16% and 17%; data not shown). Thirteen days later, on 6/28/22 (8 weeks AFB, date of “RM for 2 M” reflective material removal, avg. fruit diameter = 40.0 mm), the average fruit retention was 10%, with “RM for 1M” conferring the lowest proportion of fruit on tree (7%) - significantly different from “RM until harvest” and “CTRL” (12 and 11% respectively; Figure 8B). Figure 8 reports a visual comparison between two similar timings for fruit retention assessments in the two years; in general, the fruit drop tended to be more intense in 2022 (91%) than in 2021 (86%).

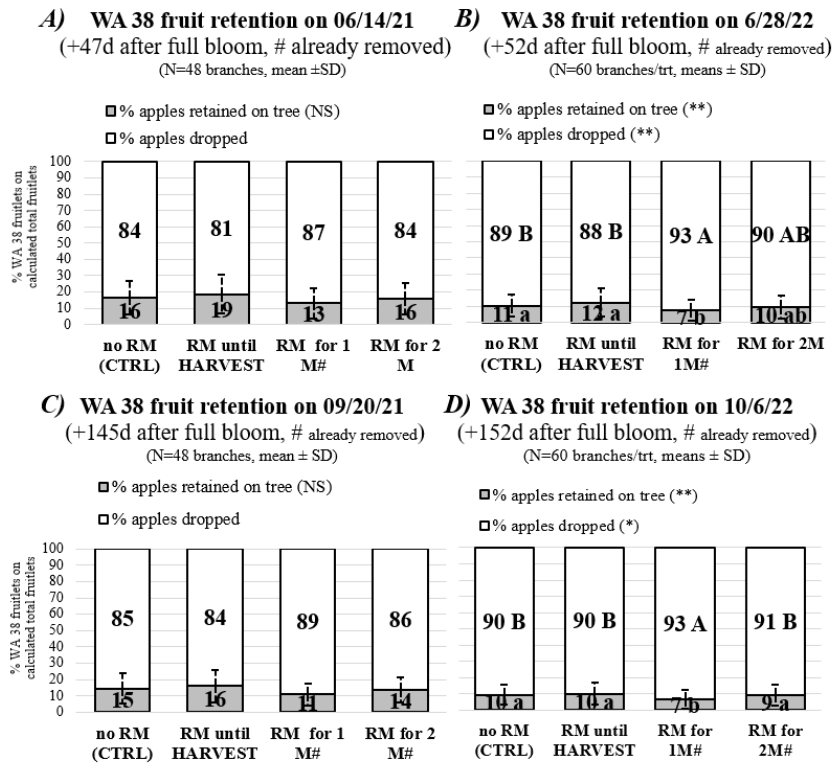

***Figure 8**. WA 38 fruit retention assessments in Royal City block across the 4 treatments in trial: no reflective material (“CTRL)”), RM deployed at bloom until harvest (“RM until harvest”), RM deployed for 1 month from bloom (“RM for 1M”) and RM deployed for 2 months from harvest to June (“RM for 2M”) on 2 different dates in the 2 seasons in June 2021 (A =7 weeks after full bloom), June 2022 (B= 8 weeks after full bloom), September 2021 (C=21 weeks after full bloom), and October 2022 (D=21 weeks after full bloom). Each bar represents the mean of fruit retention (%) and fruit drop (%) in 48 or 60 branches/treatment depending on the year. Error bars represent standard deviation of retained fruit percentage (in gray). NS= not significant, * $p \leq 0.05$, ** $p \leq 0.01$.*

The same statistical differences across the 4 treatments were maintained in the following 3 assessments: 7/12/22, 8/2/22, and 10/6/2022 (avg. fruit diameters = 50.4, 61.6, 80.4 mm, respectively). The last assessment reported similar fruit retentions for “RM until harvest”, “CTRL”, and “RM for 2M” (10%, 10%, and 9%, respectively), while “RM for 1M” showed the lowest proportion of fruit left on tree (7%, Figure 8D). In both years of trial, the treatment “RM for 1M” always recorded the lowest average of fruit retention relative to the other treatments (difference significant only in 2022; Figure 8C and D). The early removal of the reflective material after 1 month was confirmed to have negatively impacted fruitlet retention, probably perceived as a sudden shading or deprivation of resources altering the tree microenvironment. **Season 2022 ended up with an average of 9% of total fruit retained (regardless of the treatment); in 2021 the final proportion was 14%.** This may be attributed to the cold event in April 2022, increasing fruit drop in a magnitude not possible to discern from the natural shedding.

The second year of the trial confirmed some significant differences in canopy temperature/RH found in 2021 between “CTRL” trees and “RM until harvest”. While the two treatments did not differ for daily minimum temperature in the 6 months, the average daily temperature was 0.9 °C (~34 °F) higher in “RM until harvest” than “CTRL” in May (as in 2021), but not in the other months (Figure 9). Moreover, daily maximum temperatures were significantly higher in “RM until harvest” from May to September compared to “CTRL” (Figure 9). The installation of the reflective material affected the mid-canopy microclimate with +3.3 °C (~37.9 °F) daily maximum temperature, similar to the +3.5 °C (~38.3 °F) recorded in 2021. These treatments also differed for daily minimum RH in May and June 2022 (data not shown), with “RM until harvest” canopies recording respectively 5.4 and 3.4 lower RH% than “CTRL”. In July and August, the daily maximum RH was significantly higher in “RM until harvest” than “CTRL”. September 2022 showed significant differences in average daily RH% with 78.1% in “RM until harvest” and 75.5% “CTRL” (data not shown). Additionally, significant differences were found for soil daily average, minimum, and maximum temperatures, and soil moisture comparing the two treatments (Figure 10). “RM until harvest” experienced cooler daily average soil temperatures than “CTRL” soil (ranging between 1.1 and 1.6 °C cooler, depending on the month) at a depth of 20 cm (8”), 40 cm (16”) from the trunk on the east aspect.

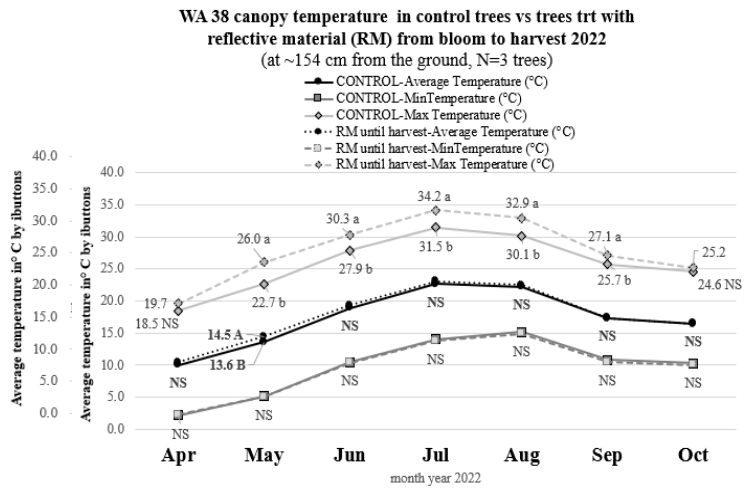

***Figure 9**. WA 38 monthly averages of daily average canopy temperatures (average, max, and min) and relative humidity (average, max, and min) measured in 2022 by iButtons hanging in the canopies at 150 cm from ground in Royal City. Three trees for “CTRL” and three trees for “RM until harvest” were monitored. NS =not significant. Letters discriminate means within each month for p=0.05*

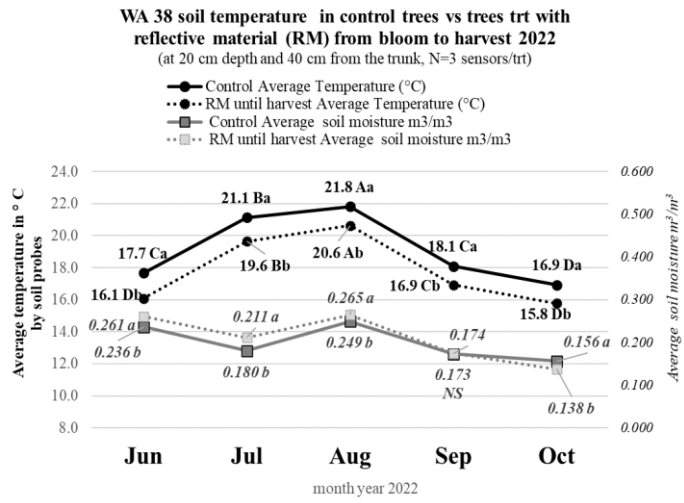

***Figure 10**. WA 38 monthly averages of daily average soil temperatures and soil moisture (VWC in m^3/m^3) measured by Meter Teros-11 sensors in 2022 in Royal City. 3 probes were buried in “CTRL” and 3 in “RM until harvest” at 20 cm (8”) depth and 40 cm (16”) from the east-sided trunks. NS =not significant. Lowercase letters discriminate means vertically pairwise for p=0.05, while capital letters discriminate means within trt along the season (comparison between months) within each month.*

This difference was also observed for average soil minimum and average maximum temperatures for each month (data not shown). Similarly, “RM until harvest” soil moisture was significantly higher than “CTRL” in the warmer months of the year (June to August, Figure 10). Overall, the reflective material utilized for 2 years modified the tree microclimate towards a drier and hotter canopy but a cooler and wetter soil. Therefore, warmer temperature and drier conditions during

bloom (here in May 2022) could have negatively impacted the flower longevity speeding the flower senescence.

Photosynthesis measurements were taken monthly on one leaf/tree on 9 trees/treatemtn following the same protocol as in 2021 to evaluate the impact of reflective materials installed at bloom on foliar carbon assimilation. Across the 5 months of measurement, no significant differences emerged among the treatments, with net photosynthesis rates ranging on average from 13.2 µmol CO_2 m^{-2} s^{-1} in May to 18.6 µmol CO_2 m^{-2} s^{-1} in June, while the other months reported intermediate values (data not shown).

At harvest 2022 (10/12/22), the average number of apples per tree was very similar across treatments, ranging between 63 for "RM for 1M" and 68 for "RM for 2M", with yield/tree varying from 15.0 kg/tree to 17.9 kg/tree, for "CTRL" and "RM until harvest", respectively. No significant differences emerged from the statistical analysis (Figure 11). Anyway, despite the lack of statistical significance among the treatment, probably related to the high variability between trees, we want to point out that RM maintained until the harvest was ~20% more productive than control (direct comparison between the two treatments resulted in p=0.0839). The lack of discrimination between average fruit weight for the 4 treatments, when analyzing just "CTRL" and "RM until harvest", a significant difference emerged for fruit mass in 2022. Indeed, "RM until harvest" were 29 g on average larger than "CTRL"; this difference can be translated in 72-64 apples/box and ~ 80 apples/box, respectively. The crop load at harvest 2022 did not differ across treatments and averaged 4.7 fruit/TCSA cm^2, while in 2021 was 5.3 fruit/TCSA cm^2. This lower crop load could be partially due to the frost event that hit the orchard in April 2022. Despite the lack of significance between productivity/treatment across the 2 years, numerically, "RM until harvest" reached 35.1 kg/tree (77 lb), while "no RM (CTRL)" just 29.2 kg/tree (64 lb, Figure 11), corresponding to a difference of 11 Mton/Acres in the two years (data not shown).

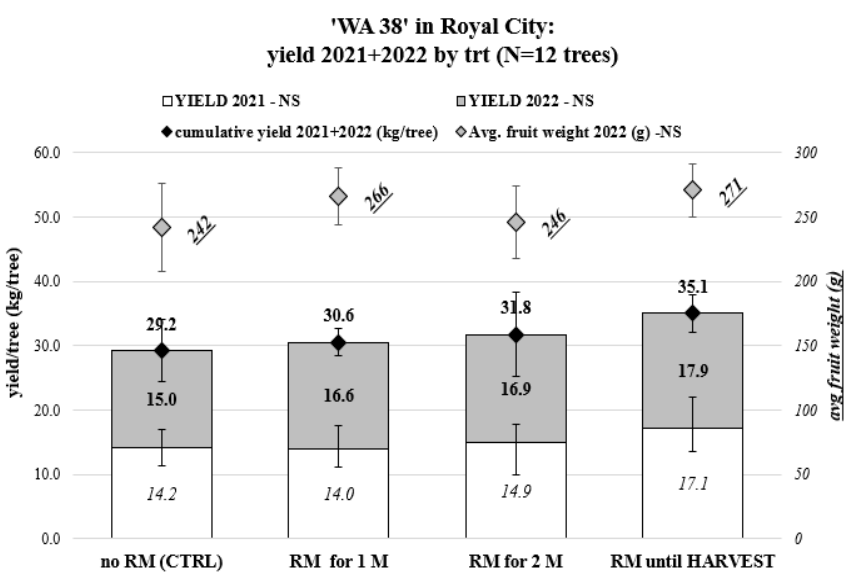

***Figure 11**. WA 38 yield in kg/tree in 2021 and 2022 and 2022 average apple weight (g) in Royal City block across the 4 treatments in trial: "CTRL", "RM for 1M", "RM for 2M"and "RM until harvest" (sorted by ascending cumulative yield in 2 years. Each column represents the mean yield of 12 trees per trt and the error bar indicates standard deviation. Each diamond marker related to the secondary Y axis represents the mean avg. fruit weight of 12 trees per trt and the error bar indicates means ± standard deviation. Differences in average fruit weight are reported as not significant (NS).*

Fruit grading for the 2022 crop revealed significant differences in the proportion of culled and marketable apples across the 4 treatments; the proportion of good-markatable apples was overall 83% (data not shown) with the best treatment being "CTRL". Similar to 2021, no major differences were found in the proportions of production by cluster pattern as single (S), double (D), triple (T) among the treatments, while a significant difference emerged for quadruple (Q) with "RM for 2 M" reporting slightly higher percentage of fruit in that category than the other three treatments (data not shown). **As an average of all treatments, 67.4% of apples were harvested as singles, 28.9% as doubles, 3.4% as triples, and only 0.3% as quadruple** (data not shown). Our grading program sorted apples into four size categories: small (≤ 215 g), medium (216-263 g), large (264-339 g), and extra-large (≥ 340

g), corresponding to ≥88, 80, 72-64, and ≤56 apples/box, respectively. Fruit size distribution for each of the treatments in the trial was expressed as a proportion (%) of apples belonging to each of the 4 size categories (total 48 trees were graded= 3,146 apples). In contrast to 2021, where no significant differences were found in the size distribution, the grading in 2022 showed a significant difference in the large class (~72-64 apples/box, Figure 12). Indeed, "RM until harvest" trees showed the highest proportion of apples belonging to large size (46%), followed by "RM for 1 M" (41%), while "CTRL" the smallest (26%; Figure 12). Moreover, "RM until harvest" and "RM for 1 M" reported the lowest proportion of fruit (17%) in the smallest size class (≥88 apples/box), significantly different from "CTRL" (38%; Figure 12).

Fruit color was assessed as 3 major categories: Extra-fancy & fancy, grade 1, and utility (respectively with red overcolor >50%, 30-50% and < 30%). In general, color was very satisfactory, ranging from 94% to 98% for Extra-fancy & fancy, respectively, for "RM for 1 M" and "RM until harvest" (data not shown), though there were so significant differences among treatments (same as in 2021).

Grading data were collected for each tree, keeping apples separated based on cluster pattern (S, D, T, Q) to identify differences in fruit size by type of fruit. While in 2021, 73% of triple (T) apples (regardless of the treatment) belonged to the small size class, representing the highest proportion in that category and significantly different from single (S) and double (D), in 2022, the difference was not quite significant (p=0.074, data not shown). Despite the lack of significance, there was a confirmed tendency of T apples to have a higher proportion in the smallest apple category (≥88 apples/box). In 2022, single (S) produced the highest proportion of extra-large fruit (6%), significantly greater than the proportion found in D and T (2 and 1%; data not shown). Another significant difference is worth reporting when looking at each treatment independently from the others: the proportion of triple apples in the medium size category (~80 apples/box) was 15% in comparison to 32 and 33% for double and single, respectively, in "CTRL". Based on the two-year data, it could be advisable in case of a high crop load year to thin down the triple clusters to double clusters, as single and double clusters indeed presented similar size distribution at harvest.

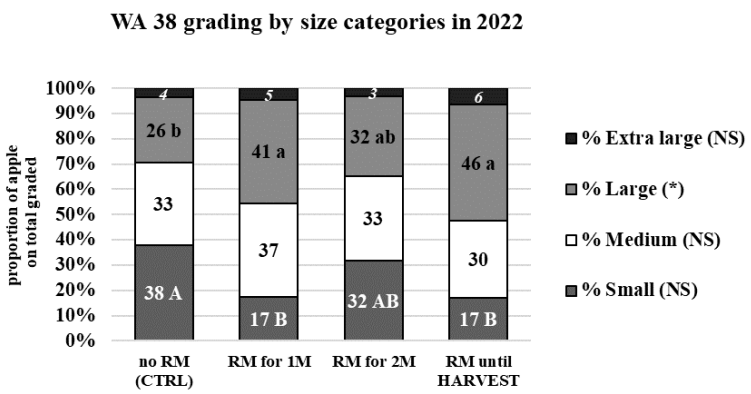

***Figure 12**. WA 38 fruit grading in size categories for Royal City block across the 4 treatments in 2022. Each tree production was graded by a sorting machine and apples were divided in 4 size categories: Small (≤ 215 g), Medium (216-263 g), Large (264-339 g) and Extra-large (≥ 340 g) corresponding to ≥88, 80, 72-64, and ≤56 apples/box, respectively. NS= no statistically significant differences and *=p≤0.05.*

FIELD DAYS

Two WA 38 field days were organized by WSU researchers and the Tree Fruit Extension Team before harvest in September 2021 and 2022. The participants were 109 and 89 in the 2 years (not counting speakers and WSU/WTFRC organizers). The tour was planned in different stations to cover different topics, not only the present project. At the Quincy station (location of obj.1), significant findings of this project were presented by Serra S. for both objectives (the Royal City block was not visited). Field day evaluations indicated that part of the attendees (in 2021) found information relating to this project useful. The Good Fruit Grower published about the events: A) "WSU leads Cosmic Crisp field days as harvest approaches" by Prengaman K., Courtney R., Mullinax

TJ // September 23, 2021, B) "Cosmic Crisp field day focuses on horticulture research and commercial experience" by Prengaman K.//September.

Executive Summary

Project title: **Maximize pollination window to improve fruit set in WA 38**

Keywords: *fruit retention, ethylene inhibitors, fruit abscission, reflective material, photosynthesis*

WA 38 is demonstrated to be a variety with abundant and prolonged bloom, though these characteristics do not necessarily translate to a satisfactory fruit set. The variety is self-thinning, naturally abscising 83-91% of fruitlets within 8 weeks from bloom. Some historical data on pilot trials reported inconsistencies in annual yield. Based on these aspects, the present project aimed to maximize WA 38 fruit set by testing different ethylene regulators and reflective material applications to manage post-bloom fruit drop. The first approach explored was the adoption of ethylene inhibitors to disrupt ethylene signaling at bloom. We tested AVG (ReTain®) and 1-MCP (Harvista™) at different timings in bloom to determine their effectiveness in improving WA 38 fruit set. Their mode of action is different: 1) AVG blocks ethylene biosynthesis, while 2) 1-MCP reduces ethylene receptor sensitivity – both with the effect of reducing fruit senescence initiated by ethylene. Because the timing of these applications can be critical in influencing the fruit set, we tested an early bloom application (~50 % king bloom) and another application 7 days later (+7 days, petal fall). The two phenological stages of application could delay the beginning of natural floral senescence, extending the pollination period (~50 % king bloom) and reducing the ethylene signaling responsible for early green fruitlet drop (+7 days, petal fall). The natural WA 38 crop load of the experimental block was ~10 fruit/ TCSA cm^2 across two consecutive years (2021 and 2022) with no artificial pollination implemented. This scenario limited the understanding of the full potential of applying both ethylene inhibitors due to a high fruit set already established naturally (recommended crop load of WA 38 is ~ 5-6 fruit/ TCSA cm^2). In the second year of the trial, both treatments with Retain showed the highest number of fruit/tree and crop load but similar yield with respect to the control (no ethylene inhibitor). This increased fruit number corresponded to a less desirable average fruit weight for both Retain treatments with an average size equal to 138-150 apples/box (control was, on average 113 apples/box). Harvista applied at bloom reported similar performances as control with a slight decrease in fruit size.

The second approach studied in this project investigated the magnitude of fruitlet abscission driven by competition between fruitlets, where the ones dropping precociously are those with a lower sink strength demonstrated by decreased growth rate. For this aspect, we utilized reflective material deployed at early bloom (<30% king open) in the orchard inter-rows with the aim to increase diffuse light in the canopy to increase carbon assimilation and photosynthate availability to support greater fruitlet retention. The reflective material was tested for 3 deployment durations from early bloom (1 month, 2 months, and until harvest, ~5 months). No significant improvement in either photosynthetic assimilation rate or fruit set was recorded in the two trial years across any reflective material applications concerning untreated control. RM maintained until harvest increased by 20% yield compared to the control, despite this difference resulted not statistically significant. However, we demonstrated that the reflective material utilized in the study did modify the tree microclimate towards a drier and hotter canopy but a cooler and wetter soil. The warmer canopies during bloom could have negatively impacted the flower longevity without gaining any benefit from an enhanced light environment to mitigate fruitlet competition. The season-long deployment of reflective material from early bloom to harvest did, however, result in improved fruit size and red coloration.

PROJECT OUTCOMES

- Outreach: 2 field days: September 2021 and 2022
- Literature (generated from project award #AP-19-102 but relevant for the present study as well):

Serra, S., Sheick, R., Roeder, S. and Musacchi, S. (2022). 'WA 38' abscission and fruit development in an open pollination scenario. Acta Hortic. 1346, 129-138.

- Presentations:

Serra S., Sheick R., Schmidt T., Musacchi S. "Preliminary results on the effect of AVG (ReTain®) and 1-MCP (HarvistaTM) applied at bloom on fruit set, yield, and apple size in WA 38 cultivar" (Poster presented by Serra S.). 31st IHC (International Horticultural Conference), S16 Innovative Perennial Crops Management, August 19, 2022.

FUTURE DIRECTIONS

Assess the effect of both ethylene inhibitors tested in 2021-2022 in a 3-tier-pruning severity trial to seek potential, improvement of fruit set in scenarios with different crop loads, in particular in a low crop load study case that did not occur in the present 2-year trial.

Project Title: WA38 applied research and demonstration block

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Cooperators: Keith Oliver, Garret Henry, Derek Hill, David Gleason, Valent BioScience, Bleyhls Co-op, Burrows tractor Inc, Drape Net, Corsi Consulting.

Report Type: Continuing Project Report

Project Duration: 3 -Years

Total Project Request for Year 1 Funding: 5,733
Total Project Request for Year 2 Funding: 9,605
Total Project Request for Year 3 Funding: 9,907

Other funding sources: **Awarded**
Amount: $152,938
Agency Name: 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.
Notes: Costs associated with objective 1 and wages for hourly support during sample collection will be covered by this proposal.

Other funding sources: **Awarded**
Amount: $50,000
Agency Name: IAREC – WSU
Notes: Funding support for five years (2020 – 2025) provided by Naidu Rayapati, IAREC Director for tree fruit orchards maintenance and plot fees.

Other funding sources: **Awarded**
Amount: $15,000
Agency Name: Valent BioScience
Notes: Costs associated with ReTain and Pollen spray for fruit set.

Organization Name: Washington State University
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Contract Administrator: Anastasia Mondy
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Item	2021	2022	2023
Salaries			
Benefits			
Wages	3,000	6,520	6,781
Benefits	673	1,025	1,066
Equipment			
Supplies[1]	2,060	2,060	2,060
Travel			
Miscellaneous			
Plot Fees			
Total	5,733	9,605	9,907

Footnotes: [1] Wages and benefits to support data collection. Supplies include tissue samples and chemical analyses if peel and fruit for nutrient differences in apples with and without physiological disorders (activity a and b), and fruit quality supplies: sampling bags, iodine, flagging tape, etc.

OBJECTIVES

1. Evaluate horticultural practices on WA 38 grown on G41 and M9-Nic 29 for better production and fruit quality.

We continue to manage, monitor, and experiment in the WSU WA 38 Roza orchard. Parallel projects include a heat stress monitoring and mitigation project, led by PIs Khot, and "Ca-related disorders management for vigorous conditions", led by PI Sallato. Information regarding these projects will be reported separately. Within the scope of this proposal, in 2022 we continue monitoring root growth differences between G41 and M9-Nic 29 (Obj 1.1), finalized the analysis for nutrient differences between GS in G41 and M9-nic 29 (Obj 1.2), provided outreach and extension on the use of ReTain ® and supplemental pollen application on fruit set (Obj 1.3), continued evaluating summer pruning timing on fruit set and vigor response (Obj 1.4) and finalized fruit maturity variability (Obj 1.5).

2. Utilize the WA 38 Roza farm as a demonstration block for community engagement and outreach.

The WA 38 Roza farm provided a venue for community engagement and outreach. In 2022, the block became the tree fruit Demo farm for the AgAID project, which has enabled the upgrade of plant, soil and weather based sensors. The Roza Farm hosted several field days, workshops and visitors in 2022. On May 30th, Sallato led a full day workshop for the WSTFA – WSDA - WSU collaborative "Agricultural Leadership Program", covering areas of Plant physiology (M. Whiting), Crop load management (D. Gleason), Irrigation (A. Moreno), IPM (T. DuPont) and Soil and Nutrient management (B. Sallato), in English and Spanish, for 33 students of the program. Co PI Bolivar hosted 3 field days in Spanish with industry and WSU speakers, where we shared updates on fruit set, heat stress management, green spot and vigor management, plant nutrition and maturity assessment. We finalized the season with a pre-harvest WA 38 field day, led by Sallato to provide updates on research and demonstration, and developed a needs assessment for our Hispanic community.

SIGNIFICANT FINDINGS

- In 2021 WA 38 was overcropped in bi-axis and V-trellis system, and both G.41 and M9-nic29 (over 100 bins/acre). This led to biennial bearing.
- Optimum load per tree, based on fruit load and fruit quality, ranged between 90 and 110 fruit per tree on bi-axis, and between 65 and 80 fruit per tree on V-trellis. We have not reached optimum yield and quality in the Spindle (at 1210 trees per acre).
- Root growth continues to be greater on G41, compared to M9, which translates in higher nutrient uptake, vigor and green spot incidence. In 2022, root growth rate was reduced during the cold temperatures during the spring.
- Nutrient levels in the soil have reached adequate range, except for P and B, while in leaves, all nutrients were within range, except for K, being above adequate range.
- In 2022, nutrient uptake in leaves and fruit were reduced compared to 2021.

METHODS

1. Evaluate horticultural practices on WA 38 grown on G41 and M9-Nic 29 for better production and fruit quality.

The WSU WA 38 block was planted in 2013 in a 0.8-acre block, to evaluate rootstock and training systems. The orchard is divided in three training systems: Spindle 3 x 12 ft (rows 1 to 4), V trellis with spindle training at 1.5 x 12 ft (rows 5 to 8) and bi-axis at 3 x 10 ft spacing (row 9 to 11), on two rootstocks, Geneva 41 (G.41) and M9-NIC29 (Figure 1). Rootstocks are randomly distributed within each training system in blocks of 10 or 22 trees. More details in Evans et al., 2013, final report.

Figure 1. WA 38 at WSU Roza Farm with three training systems; spindle (3 x 12 ft), V-trellis (1.5 x 12 ft.) and bi-axis (3 x 10 ft).

Initially the pollinizers were Granny Smith and Chehalis at density approximately of 14% (9% in V-Trellis and 18% in Bi-axis) on M-26 rootstock. In 2017, the Roza Farm was affected by a hail event during bloom accompanied by favorable conditions for fire blight development. Consequently in 2018, 24% of the WA 38 on M9-nic 29 and 11% of the pollinizers died due to fire blight infection and trees were removed. In 2020, we replaced the removed trees with WA 38 on Geneva 11 (G.11) and added missing pollinizers Snowdrift and Mt Evereste ™.

Soil conditions: The block is located on a silt loam soil, corresponding to the Warden series (most representative series for tree fruit production in the Yakima valley) over basalt rock. The depth varies slightly between 2.5 feet of effective soil depth to more than 4 ft. Above the basalt rock, some areas have $CaCO_3$ (Caliche), with pH ranging between 7.0 and 7.8. Soil P, S and B levels are usually low.

Training Systems

Spindle; row 1 to 4, with 28 blocks of 10 trees. Initially trained by bending branches, which led to blind wood and low productivity. Since 2018, we been slowly transitioning to traditional spindle. This section is notoriously more vigorous than V-Trellis and bi-axis, providing us the opportunity to learn about green spot and vigor management. Since 2021 we been using these blocks for the PGR trial to evaluate Ca related disorders (ongoing project led by Sallato)

V-Trellis; row 5 to 8, with 28 blocks of 22 trees. This block continues to be managed with winter pruning, summer pruning and hedging. Six trees in this section have a root window (rhizotron) to monitor root growth differences between rootstocks (Obj 1).

Bi-axis; row 9 to 11, with 20 blocks of 10 trees. This section was planted a year later (2014). Since 2018 trees have been pruned lightly during the winter to remove undesired branches; redundant,

hanging, and renew wood, followed by summer pruning and hedging. Since 2021, these blocks have been used to evaluated heat monitoring and mitigation practices (ongoing project led by CoPI Khot).

General management:
Disease and pest management is under advice from Jeff Sample (Blehyl Co-op). Mayor challenges have been fire blight (2018-2019), thrips (2021), and mildew (2019-2022). In 2020, the irrigation system was upgraded and divided for each training system, utilizing Wiseconn Engineering monitoring and controls platform. A set of moisture and temperature sensors were installed on each section, and one weather monitoring system for the entire block. A Venturi system was installed for fertigation in 2021. Additional monitoring systems have been installed in the bi-axis section, associated to the heat stress project (for more details review Khot et al, 2021 report)

Research project

1.a. Differences in root growth and nutrient uptake between M9-Nic29 and G41. (Funding source Washington State USDA- Specialty Crop Block Grant. $152,938. Ending 2021). (Sallato)

Root windows (3 x 3 x 3-foot cubes with Plexiglas on one and plywood for other sides) were installed on three random trees per rootstock since 2019. Evaluation of root growth starts prior to bloom and continues every week during spring period when roots are actively growing, and every other week during the summer and fall. Each root window is treated as a replicate unit. Monitoring of root growth is done manually by drawing a quadrant (1.5 x 1.5 ft.) in the middle of the plexiglass and monitoring white roots during the growing season. New growth is recorded and measured on site, then marked with different colors to identify period of growth. We report on cumulative root growth and timing. At the end of the season, each tree is strip harvested to determine yield, crop load and fruit quality. More details can be found project proposal. A detailed explanation of how to develop the root window was shared with the Good Fruit Grower and published in April 2019 (https://www.goodfruit.com/a-window-to-the-roots/)

1.b. Green spot nutrient composition differences, rootstock, and vigor. (Partially funded by Washington State USDA- Specialty Crop Block Grant. $152,938) (Sallato).

From 2018 to 2021, fruit with and without green spot have been collected trees on G41 and M9-Nic 29 rootstock. At harvest fruit from different rootstocks and training systems were collected to determine fruit per tree, crop load and GS incidence. From each experimental unit and rootstock, fruit from six representative trees with (GS+) and without green spot (GS-) symptoms were collected for quality analysis. Then, each individual fruit were separated into peel, flesh, core and seeds to determine fresh and dry matter proportions. Subsequently, each tissue sample was dried, homogenized and sent to a commercial laboratory for nutrient analysis; nitrogen (N), phosphorous (P), potassium (K), calcium (Ca) magnesium (Mg), iron (Fe), zinc (Zn), cupper (Cu), manganese (Mn) and boron (B) analyses following the method recommended for total tissue analyses (Gavlak et al., 2005). First have been published in Sallato et al., 2021. In 2020 and 2021 we added an additional level of GS severity associated to milder symptom (greening), to determine relation with nutrient concentrations.

1.c. Use of AVG (ReTain ®) and artificial pollination to improve fruit set and production (Sallato).

In 2019 to 2021, we studied the effect of an ethylene inhibitor (AVG; ([S]-trans-2-amino-4-(2-aminoethoxy)-3-butenoic acid hydrochloride) (ReTain ®, Valent) and supplemental pollen application on WA 38 fruit set. The trials were conducted in the WA 38 Roza farm and in three commercial orchards; **Buena** 4th and 5th leaf WA 38 trial consisted of five treatments. 1. Pollen, 2.

Pollen + ReTain ® at 80% bloom, 3. Pollen + ReTain ® at petal fall, 4. ReTain ® alone at petal fall and 5. Untreated control. All pollen treatments consisted of two applications (approximately at 30 and 80% open flowers) with 15 g of pollen/acre (70% Red Delicious and 30% Granny smith) each provided as in-kind by Firman Pollen. Treatments were applied with electrostatic sprayer provided as in-kind by OnTarget, USA. **Roza WA 38**, 9th leaf consisted of four treatments: 1. Pollen, 2. Pollen + ReTain®, 4. ReTain ® alone and 4. Untreated control. All treatments consisted of one application at 80% bloom of 30 g/acre equivalent. The application was conducted with battery powered backpack sprayer. ReTain® application were all at 333g/acre rate (1 pouch), provided as in-kind by, Valent Bioscience, USA.

In all trials we determined the percent of open flowers prior to the application, fruit set (July) and percent of single, double or triple at harvest. Results from this and the other commercial sites have been shared in the pre-harvest field day (2021) and 2022 WSTFA annual meeting. A research publication is underway.

1.d. Pruning strategies to promote fruiting wood (Sallato).

During 2021 the entire orchard was pruned before bloom (first week of April) following the advice of WA 38 advisory group (listed as collaborators). During the summer, random sections of the block were selected for summer pruning on different dates: June 26th, July 26th and August 25th. In 2022, during winter pruning a set of seven blocks were left unpruned. Through the year, a different set of three trees within each block (total= 21 trees) were pruned on May 30th, June 16th or September 8th. (Figure 2)

Figure 2. Spring pruning May 30th in WA38 spindle system in three trees, replicated in tree blocks.

Fruit yield, quality and nutrient levels were monitored throughout the season. In 2023, we will continue evaluating regrowth, bloom density, fruit set, and fruit quality.

1.e. Fruit ripening variability between systems and rootstocks (Sallato, Bolivar).
In 2020 and 2021, three trees per training system and rootstocks were selected during harvest, and each fruit was evaluated for starch content utilizing the WA 38 starch index chart (Hanrahan et al, 2019) http://treefruit.wsu.edu/wa38-starch-scale/.

2. Utilize the WA 38 Roza farm as a demonstration block for community engagement and outreach.

Provide a venue to learn together about WA 38 throughout field days, workshops and visits.

RESULTS

Results from our 2021 pre-harvest tour field day, requested us to provide information regarding the history, management, and production over the years. In this report, we focused on providing a detail on management and reporting on production, biennially, fruit size and green spot, and to provide an update on 2022 findings not reported elsewhere.

Evaluate horticultural practices on WA 38 grown on G41 and M9-Nic 29 for better production and fruit quality.

Overall M9-Nic29 had higher productivity (fruit per tree) while equivalent fruit size compared to G41 (Table 1). In the Spindle system, regardless of the rootstock, we have increased the number of fruit per tree and quality since 2019, however, is still highly vigorous (and low crop). In 2022, fruit weight ranged between 230 and 287 g (78 and 73 mm), and green spot incidence were 2% in M9Nic29 and 6% in G.41. On the V-trellis, M9nic29 has also shown more fruit per trees than G.41, and fruit size generally larger on G.41 (Table 1). The trees on bi-axis have been more productive compared to the trees on spindle and V-trellis. In 2021, we overcropped the trees on the V-trellis and bi-axis, which led to small fruit size aprox. 237 g average and between 69 and 85 mm diameter, and reduced green spot incidence (below 4%). Consequently, in 2022 we had reduced biennial bearing and reduced fruit load, specially in the V-trellis (Table 1).

Table 1. Production in fruit per tree, weight and diameter in WA 38 Roza farm from 2019 to 2022.

Rootstock	System	Fruit per tree				Fruit weight (g)			
		2019	2020	2021	2022	2019	2020	2021	2022
M9 Nic29	Spindle	67	58	63	71	290	276	290	287
	V-Trellis	67	66	106	29	221	259	237	289
	bi-axis	65	85	139	76	240	241	237	241
G41	Spindle	53	42	59	76	298	316	298	230
	V-Trellis	46	43	87	39	239	284	298	244
	bi-axis	68	59	113	90	328	257	237	241

Table 2. Green spot incidence and estimated yield in WA 38 Roza farm from 2019 to 2022.

Rootstock	System	Green spot (%)				Bins /acre *				
		2019	2020	2021	2022	trees	2019	2020	2021	2022
M9 Nic29	Spindle	29	7.3	4	2	1210	40	43	52	58
	V-Trellis	14	3.4			2420	73	95	145	48
	bi-axis	1	0.3	0	0	1452	53	71	114	62

	Spindle	45	27.1	13	6	1210	25	28	44	47
G41	**V-Trellis**	56	10.4		2	2420	28	63	149	54
	bi-axis	18	7	4	4	1452	63	49	89	71

*based on fruit without green spot and 925 lb bin. Other defects have not been included.

Based on our results and growing conditions, we estimate that the optimum load on a bi-axis system ranged between 90 to 110 fruit per tree, for both rootstocks. For V-trellis (at 2420 trees per acre), the optimum load ranged between 65 and 80 fruit per tree. In the Spindle (at 1210 trees per acre), we have not reached the optimum load.

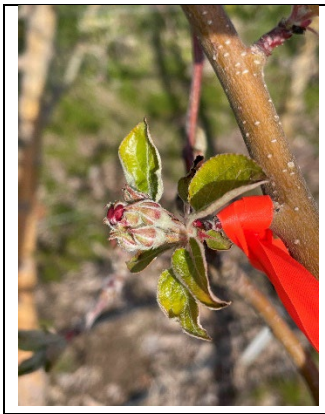
Soil and nutrient management

Initial soil analysis (2019) as recommended for Western soil (Miller et al 2013) indicated mineral deficiencies of phosphorous (10 mg/kg), sulphur (8 mg/kg), zinc (0.50 mg/kg) and boron (0.12 mg/kg) according to recommended levels (http://treefruit.wsu.edu/orchard-management/soils-nutrition/fruit-tree-nutrition/). In 2019, we applied 100 lbs. per acre of mono ammonium phosphate (MAP), 25 lbs of $ZnSO_4$/acre and 2 lbs of B/acre. Since 2019, we have continued with spring ground application of P (MAP) at 150 lbs/acre and foliar B and Zn (fall and spring). In 2022, we added 23 g of Urea per tree (individually) to all replanted trees and pollinizers. Soil chemical analysis in 2022 indicated adequate levels for K (161 mg/kg), Ca (21.6 meq/100g), Mg (2.9 meq/100g), Zn (11.2 mg/kg), Mn (1.47 mg/kg) and Cu (1.9 mg/kg). Soil P and B remains below the recommended level. Thus in 2023 we will increase the dose and method of application.

Leaf chemical analysis were within range for N, (2,1%), P (0.3%), Ca (1.6%), Mg (0.28), S (0.16%) and all micronutrients, while K continues to be above range (2.4%). Regardless, in 2022 nutrient uptake was lower for P, Ca, Zn, Fe, Mn, and B, compared to 2021. In contrast, K uptake was higher in 2022 and N uptake was equivalent both years.

Development during 2022.

First pink was observed April 15th, and king flower starts to around April 25th. A month later, fruit diameter was between 11 to 25 mm, and clusters had between 3 to 4 fruitlets. Around June 16th, fruitlets within the clusters differentiated in size, most continue with three to four fruitlets (Figure 3). Fruit dropped during the month of July. In 2022, the fruit was harvested on October 29th, two weeks later compared to 2021.

		
April 15th	April 22nd	April 25 to 27th (King)

May 27th	June 16th	October 1st

Figure 3. WA 38 fruit development in 2022 Roza Farm.

Research projects;
1.a Differences in root growth and nutrient uptake between M9-Nic29 and G41. (Munguia-Sallato)

Preliminary results showed differences in growth between G41 and M9-Nic 29. In all three years of evaluation (2019 – 2022) root growth starts with temperatures above 59 °F in the soil (data not shown). Consistently G41 has shown higher total root length, root growth rate and longer growth period, compared to M9-nic29. Results from 2019 and 2020 can be seen http://treefruit.wsu.edu/videos/rootstock-differences-in-wa-38/. In 2022, root growth in both rootstock started with temperatures of 52 F (April 4th), at fast rate of 0.8 mm/cm^2/day on M9-nic29 and 1.43 mm/cm^2/day on G.41. The low air and soil temperatures during the month of April, reducing soil temperatures to 44.6 F at 8 inches of soil, impacted root growth rate on both rootstocks (Figure 4). Total root growth during the spring was significantly lower compared to 2020 and 2021.

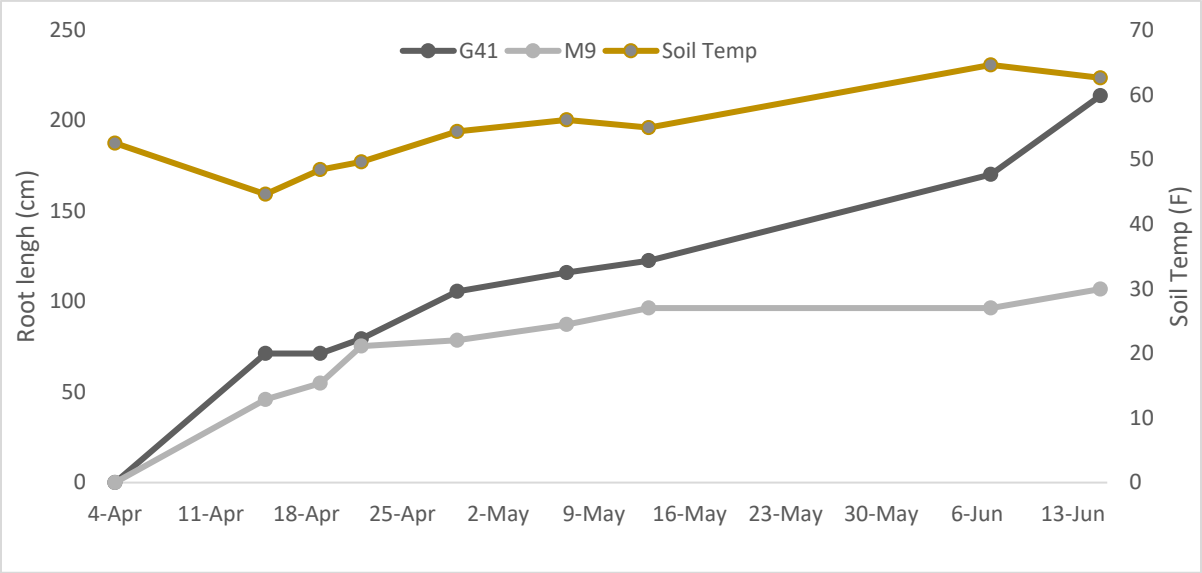


Figure 4. Root growth in WA38 Roza farm in 2022.

Nutrient levels in fruit were significantly different between 2021 and 2022. In 2022, macronutrients N, Ca, Mg, S, and micronutrients Zn, Fe, Mn and B, were lower compared to 2021. While K was

higher in 2022. The reduced nutrient uptake in 2022 can be associated to the impact of environmental conditions on root growth.

Table 3. Fruit nutrient concentration in 2021 and 2022,

	N	K	Ca	Mg	S	Zn	Fe	Mn	B
2021	0.40 a	0.72 b	0.08 a	0.09 a	0.04 a	3.57 a	66.5 a	7.0 a	46.1 a
2022	0.19 b	1.01 a	0.05 b	0.04 b	0.02 b	1.44 b	21.9 b	1.9 b	35.1 b

1.b. Green spot nutrient composition differences, rootstock, and vigor. (Sallato-Munguia-Whiting)

Previous results suggest that there is a strong relation between green spot GS and nutrient balance between calcium (Ca) and nitrogen (N), and that this imbalance is caused by excessive vigor (Sallato et al., 2021). In 2021 we included an intermediate level of GS severity to better understand the symptomatology. In Table 4. We observed a positive correlation between N and B concentration and GS severity (Table 4). This correlation was not observed with Ca and greening, while it was significantly lower in GS ++ compared to the control with no symptoms. Nutrient concentration of P and K were higher in GS fruit, irrespective of the severity.

Table 4. Nutrient concentration in the peel of WA 38 fruit on G41 without green spot (GS -) and two levels of GS: flecking (GS +) and spots (GS ++).

Nutrient	GS -	GS +	GS ++	Pr > F(Model)
N %	0.40 c	0.48 b	0.54 a	<0.0001
P %	0.08 b	0.09 a	0.09 a	0.001
K %	0.83 b	0.95 a	0.94 a	0.007
Ca %	0.09 b	0.10 a	0.08 c	<0.0001
Mg %	0.11 b	0.12 a	0.13 a	<0.0001
B mg/kg	32.5 b	38.1 ab	43.5 a	0.038

Note that Ca related disorders are associated to nutrient imbalances with Ca, not necessarily deficiencies in Ca supply, and is considered a physiological disorder.

1.c. Use of AVG (ReTain ®) and artificial pollination to improve fruit set and production. (Sallato-Whiting)

Results for this objective were reported in detail in last year's continuing report. In addition, results were shared with WA industry at the WSU WA 38 pre harvest field day (October 2021), pollination field days (April 19th and April 21, 2022) in Spanish with infographics "*Mejora de la cuaja en 'WA 38' (*Improving fruit set in 'WA 38'). And at the WSTFA annual meeting Dec 6, 2022 newsflash. A publication including results from commercial orchards is underway.

1.d. Pruning strategies to promote fruiting wood (Sallato)

Summer pruning, as well as winter pruning, will respond differently depending on intensity, diameter, and angle of the wood. Preliminary observations indicates that summer pruning helps control excessive vigor and promotes reproductive bud development. The timing of pruning was positively correlated with the regrowth response, the earlier the pruning timing, the greater the regrowth response. In 2020, summer pruning led to return bloom in the fall, while in 2022, summer pruning in June 16th led to vegetative regrowth (Figure 5)

Figure 6. Difference responses to June pruning between 2020 and 2022. Return bloom during the fall (izq.) after pruning during June in 2020. Vegetative regrowth after summer prune (June 26th) in 2022.

Results related to reproductive bud development, fruit quality and production is under evaluation.

1.c. Fruit ripening variability between systems and rootstocks (Bolivar – Sallato)

Detailed information was shared in previous report and at the Jan 2022, Pom Club meeting (Sallato), and in the Spanish field days led by CoPI Bolivar, "*Perfil de maduración de WA38 en dos portainjertos y tres sistemas de producción- Año 2020. Jenny Bolivar-Medina, Bernardita Sallato. (*WA38 maturation profile on three production systems and 2 rootstock types- 2020). An infographic of the results can be found http://treefruit.wsu.edu/perfil-de-maduracion-de-wa-38-en-dos-portainjertos-y-tres-sistemas-de-produccion-2020/

Utilize the WA 38 Roza farm as a demonstration block for community engagement and outreach.

The WA 38 Roza farm provided a venue for community engagement and outreach. In 2022, the Sallato led a full day workshop for the WSTFA – WSDA and WSU collaborative "Agricultural Leadership Program", where we covered the areas of "plant physiology" (M. Whiting), Crop load management (D. Gleason), Irrigation (A. Moreno), IPM (T. DuPont) and Soil and Nutrient management (B.Sallato), in English and Spanish, for 33 students of the program. Co PI Bolivar hosted 3 field days in Spanish in the areas of pollination, heat stress and nutrient management and maturity assessment, and Sallato hosted a pre-harvest tour in Spanish, with updates on research topics. In 2021 we reached over 60 people during the field days, plus many visitors throughout the season upon request.

A survey conducted after the field days reported 80% increase of knowledge and 50% of the participants, indicated intention to change their management practice for pollination and nutrient management. At the pre harvest WA 38 field day (Spanish), we had 33 attendees and 24 responded to

our survey. Here, most important topics to continue our research and extension work were soil-root-plant interactions, summer pruning, general characteristics, green spot, and pollination. Of the respondents, 83.3% indicated intention to change practices, mentioning summer pruning, vigor management with irrigation, and timing for Ca application.

Project Title: Implementation of a bilingual extension program for Cosmic Crisp®

Report Type: Final Project Report

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Cooperators: Paola Pesantez, AgAID Institute

Project Duration:, 2-Year

Total Project Request for Year 1 Funding: $ 11000
Total Project Request for Year 2 Funding: $ 11000

WTFRC Collaborative Costs: None

Budget 1
Organization Name: Washington State University
Contract Administrator: Hollie Tuttle; Anastasia Mondy
Contract administrator email address: hollie.tuttle@wsu.edu; arcgrants@wsu.edu
Station Manager/Supervisor: Naidu Rayapati
Station manager/supervisor email address: naidu@wsu.edu

Item	2021	2022
Salaries		
Benefits		
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$4,000.00	$4,000.00
Travel	$3,000.00	$3,000.00
Plot Fees		
Miscellaneous	$4,000.00	$4,000.00
Total	$11,000.00	$11,000.00

Footnotes: Supplies includes printing materials ($2000), expenses for the development of graphics and illustration ($2000) Travel section includes Bolivar and Sallato travel expenses to visit orchards, conduct field days and shoot videos (1725 miles @ 0.575 per mile. Miscellaneous covers part of the expenses in the video production and edition costs. Other part of the costs will be covered by our collaboration with the Good Fruit Grower. ($110 per minute of media production, x 10-minute video = $1100. Estimated number of videos/year = 6 x $1100= $6600)

Recap original objectives and significant findings:

1. Translate existing WA 38 and Cosmic Crisp® Extension publications from English to Spanish. Topics include horticultural management, harvest management and post-harvest handling. The information will be accessible in different formats to reach more effectively the Hispanic community (website short articles, pamphlets, infographics, audiovisual resources). *Completed*
    - A survey was designed and distributed to the Spanish speaking community in the WA tree fruit industry. From 37 respondents, 90% were interested in getting access to the information in Spanish, and more than 50% in participating in field days.
    - A section called *Recursos en español* (Resources in Spanish) was created in the WSU tree fruit website. 29 resources for apples, cherries and orchard management have been uploaded.
    - 19 written resources for WA 38 were produced and shared with the Spanish speaking community. From them, 15 were published in *Fruit Matters* newsletter, and uploaded into the *Recursos en español* section. These resources cover different aspects of the WA 38 horticulture, orchard management, pre and post-harvest recommendations.
    - Different formats were used to produce the bilingual (English-Spanish) extension resources and covered the designed and production of flow charts, infographics, starch index cards, a booklet for fruit defects, an a video in Spanish. In addition, summary articles were translated and linked to their corresponding original version in English.
    - The WA 38 Fruit defect guide html document previously published in the WSU Tree Fruit Website, was translated, published and uploaded to the Spanish section. Based on it, a bilingual booklet for fruit defects was designed, printed and distributed to the apple industry across the state.
    - All the resources produced, excepting the summary articles were printed y distributed from July 2021 and December 2022.

2. Develop and deliver interactive WA 38 Extension programs in Spanish to Hispanic workforce to disseminate and demonstrate timely information on pruning, crop load management, irrigation, and maturity/harvest management. Programs will include field trips, workshops, and discussion groups, among others. *Completed*
    - A QR code was created and distributed in the Spanish community participating in field days to facilitate access to the *Recursos en español* section.
    - A total of eight field days were organized across the growing seasons of 2021 and 2022. Three of them were offered in 2021 and five in 2022.
    - 80% of field days attendees expressed an increase of knowledge at the end of each event, and about 50% of the participants in field days related to pollination and nutrition were interested in changing practices
    - 95% of the participants reported that the organization of the field days and the information provided were of high quality.
    - Despite the positive feedback, the participation in field days gradually declined as the growing and harvest season progressed.

3. Establish an Advisory Committee with Spanish native speakers in the apple industry who will help in the identification of priorities in the translation of the information and the delivery of Extension programs. This committee will also identify needs for future programs directed to the Hispanic community. *Completed*

- A Spanish advisory committee was formed by 5 members. The committee met, collaborated and identified priorities and future directions.
- 45 extension resources on WA 38 were compiled, categorized into topics, and prioritized by the advisory group for translation. This process was done once per year.
- 7 topics for field days, and time during the year to offer them, were identified and suggested by the committee.

Results and Discussion

In this final report, the results of the activities carried out from July 1st, 2021 to November 14, 2022 are described.

Objective 1. Translate existing WA 38 and Cosmic Crisp® Extension publications from English to Spanish. Topics include horticultural management, harvest management and post-harvest handling.

Survey

Prior to the translation of documents, and once the project started (July, 2021), we created a community survey to get a better understanding of the priorities for translation of extension resources related to WA 38 that the Spanish speaking community recognizes. The survey was posted in the WSU tree fruit site (http://treefruit.wsu.edu/wa-38-survey-encuesta/). It was also given at the end of field days in 2021, and at presentations in grower meeting sessions in 2021 dedicated to the tree fruit Spanish speaking workforce. To facilitate the prioritization for the translation of documents previously published in the WSU Tree Fruit website, we classified the documents in 6 categories: General WA38 characteristics, horticultural management (crop load, pruning, production systems), nutrition, fruit quality, packing and marketing. In addition, we also asked about the format that they prefer to get the resources (infographics, articles, filed days, videos, etc).

From the responses collected from the survey posted online (via Qualtrics), and in the field days, we found that most of the participants (more than 90%) were interested in getting information of WA 38 in Spanish. In addition, more than 50% of them were interested in participating in field days in Spanish.

Although the number of votes per category varied, and in some cases were scarce, the participants were interested in all the categories, excepting packing and marketing (Figure 1A). The time of the release of the information was based on the season. Spring and summer times had the higher ranking to be preferred to release information related to WA38 characteristics, while winter alone or in combination with spring or summer were preferred for the remaining topics. Fall was the least preferred season to release the information (Figure 1B), which could be related with the busy apple harvesting season, and therefore less time to consult the resources.

Related to the format for delivering the information, most of the participants that answer the question preferred short videos to get information about horticultural management, while they preferred articles for fruit quality topics. For the remaining topics there were no preference related with the format (Figure 1C).

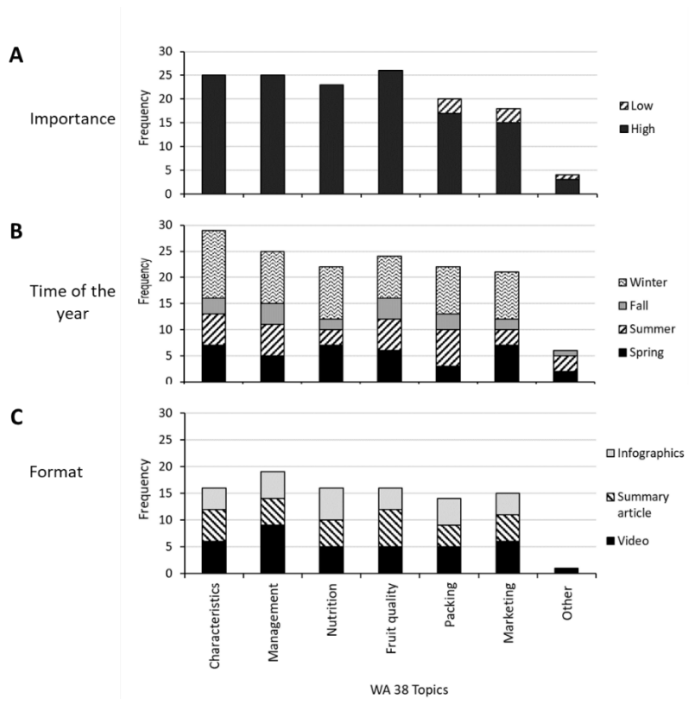

Figure 1. Summary of the results of the 2021 survey to publish extension resources for WA 38. Y axis represent the frequency (votes per category), and X axis WA 38 topics. **A**. Importance of topics for translation (high and low). **B.** Time of the year (Spring, Summer, Fall, Winter) preferred to access the information per topic. **C.** Format preferred to access the information (Video, article, infographics). (n= 51).

Prioritization of resources to be produced in Spanish

The advisory committee classified the WA38 resources already available in English in the WSU tree fruit Website, in three priority categories for translation: high, medium and low (Table 1). The topics related to horticulture, quality standards, harvest criteria, nutrition, pollination, green spot and fruit defects were ranked as high priority. The fruit defects guide that has already been published in the WSU tree fruit website (http://treefruit.wsu.edu/wa-38-defects-guide) was considered important to be translated, and they agreed that the production of a booklet with this information would be a useful resource to have on hand in field conditions. As for the WTFRC continuing reports available at the website, the committee considered them as low priority, and were more interested in the final projects. The optimization of light interception document was considered in between medium priority, and it was suggested to design infographics based on sub-topics within the article.

Table 1. Prioritization for translation to Spanish of WA 38 resources found in the WSU Tree Fruit Website

Priority level (high, medium, low)	Topic	Original Format	Suggested Format in Spanish
High	Quality Standards	Article	Article (1page)
	Harvest criterio	Article	Article (1page)
	WA 38 Fruit Defects guide	Web document	Web document and booklet

	Nutrient differences- green spot	Video (9 min)	Infographics
	Rootstock differences in WA 38	Video (13:23 min)	Infographics
	WA 38 Horticulture: Characteristics	Article	Article
	Pollination, flower biology and fruit development in 'WA38' (2020)	Article	Infographics
	WA 38: understanding green spot origin, timeline, and development (2022)	Article	Summary article or Infographics
	Optimizing harvest time for WA38 (2019)	Article	Infographics
Medium	optimization of light interception, leaf area and yield in "wa38":	Article	Infographics per topic: - Training systems - Rootstocks - Pruning techniques
Low/no needed	Respiration rate and low oxygen limit	Article	Summary article
	Stem punctures in packout (2017-2018)	Article	Summary article
	Marketing and royalty (2020-2021)	8 videos (15min – 1hr:45min)	Summary article
	Fruit quality (2014-2019)	8 videos (1- 32 min)	Summary article
	WA 38 FAQs and information for growers	Article	Article
	WA 38 Size distribution profile in pre-commercial plantings 2020 (box sizes quincy and prosser data 2010- 2016)	Article	Article
	WA38 demonstration trial block (2019)	Article	Article
	WA38 fruit size and dry matter for fruit quality/consumer preference. (2018)	Article	Infographics
	WA 38 rootstock and systems trial (2013)	Article	Article

In addition, the following topics were selected as important to share in field days in Spanish (Table 2).

Table 2. Field day topics identified by the advisory committee

Field day	**When?**	**Approach**
Field day IAREC-la roza- Prosser	Only 2021	- Information about the block. - Research findings.
Field day WTFREC-Sunrise - Wenatchee	Only 2021	- Information about the block. - Research findings.
Evaluation of starch index and harvesting recommendations for WA 38 (Cosmic Crisp®)	Fall 2021 at the Roza. Fall 2022 at the Roza and Sunrise WSU blocks	- Workshop style
Pollination and floral biology	April 2022	In commercial orchards two locations (Central and South areas)

		- Grower experiences - Research findings
Nutrient and Vigor management	June 2022	Research findings- Sunrise and la Roza
Stress management (heat stress)	July 2022	Research findings- Sunrise and la Roza
Green spot	June 2022	Research findings- Sunrise and la Roza

Production, publication and distribution of WA 38 resources in Spanish

The written and audiovisual resources were published in the WSU Tree Fruit Extension team newsletter, *Fruit Matters*. In addition, we created a subsection titled *Recursos en español* (Resources in Spanish) within the WSU Tree fruit website, where all the translated resources have been compiled and are available to consult (Figure 2A). All the written resources created included a link to its respective English version in case the reader wants to compare or learn the terminology in both languages.

In order to make these resources easily accessible to the Spanish speaking community who can have difficulties to navigate the website, we also created a QR code link to this section that bring the user directly to this section (Figure 2B). This QR code was printed and shared with the participants of the field days as well as in presentations of the program. We also explained how to use the code and made sure the participants were able to access and navigate *Recursos en español* section.

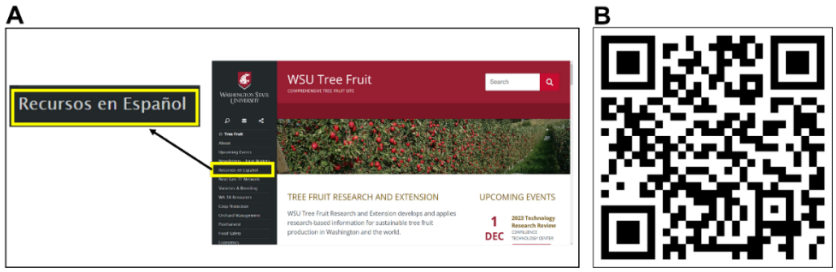

Figure 2. "*Recursos en Español*" (Resources in Spanish) subsection within the WSU Tree Fruit Website where all the WA 38 resources in Spanish are located (A). QR code generated and distributed in the field days to facilitate the access to the subsection (B)

The following documents have produced in Spanish and shared with the Tree fruit industry (Table 3).

Table 3. WA 38 resources in Spanish created in this project.

Title/ Author	Format	Published/shared
Decisiones a tener en cuenta durante la cosecha de WA 38. / Jenny Bolivar-Medina, Carolina Torres, Ines Hanrahan (Harvest decisions)	Flow chart	- Shared on Starch index field days. - To be published on December, 2022. http://treefruit.wsu.edu/recursos-en-espanol/decisiones-a-tener-en-cuenta-durante-la-cosecha-de-wa-38/
Aspectos a considerar durante la cosecha de WA 38. / Jenny Bolivar-Medina, Carolina Torres, Ines Hanrahan (Things to consider when harvesting WA 38 fruit)	Flow chart	- Shared on Starch index field days. - To be published on December, 2022. http://treefruit.wsu.edu/recursos-en-espanol/aspectos-a-considerar-durante-la-cosecha-de-wa-38/
Guía de defectos en WA 38. Translation, / Jenny Bolivar-Medina, Carolina Torres, Ines Hanrahan (WA	Article	Published on July 11, 2022 http://treefruit.wsu.edu/guia-de-defectos-en-wa-38/
	Booklet	Designed ended on September, 2022.

38 Common Defects and Unique Characteristics Near Harvest and During Storage)		Printing and distribution October-December, 2022
Escala de almidón para WA 38. / Jenny Bolivar-Medina, Good Fruit Grower Magazine (WA 38 starch scale).	Video	(https://www.youtube.com/watch?v=t2KWuSY3DYs. August, 2022
*Información actualizada del sistema de apoyo en la toman de decisiones de AWN para el manejo de estrés. / Lav Khot (*AWN updates on decision-tool for heat stress management)	Infographics	Shared in Nutrition, vigor and heat stress field day. Roza, IAREC. July 05, 2022.
Características y Horticultura de WA 38. Translation / Jenny Bolivar-Medina, (WA 38 Characteristics and Horticulture)	Article	Published on April 11, 2022 http://treefruit.wsu.edu/caracteristicas-y-horticultura-de-wa-38/
Mejora de la cuaja en 'WA 38'./ Bernardita Sallato, Juan Munguia, Poliana Francescatto, Matthew Whitting. (Improving fruit set in 'WA 38').	Infographics	Shared in Pollination and fruit set field days. April 19 and 21, 2022.
El desarrollo de green spot en WA 38 se ve afectado por desequilibrio nutricional y portainjerto. / Bernardita Sallato, Matt Whiting, Juan Munguia (Rootstock and Nutrient Imbalance Leads to ''Green Spot'' Development in 'WA 38' Apples)	Summary Article	Published on December, 2021 http://treefruit.wsu.edu/article/el-desarrollo-de-green-spot-en-wa-38-se-ve-afectado-por-desequilibrio-nutricional-y-portainjerto/
Recomendaciones de cosecha y almacenamiento de WA38- 202./ Jenny Bolivar-Medina, Carolina Torres, Ines Hanrahan (Commercial harvest and storage criteria for WA 38- 2021)	Article	Published on October 11, 2021 http://treefruit.wsu.edu/recursos-en-espanol/http-s3-us-west-2-amazonaws-com-treefruit-wsu-edu-wp-content-uploads-2021-10-05173433-commercial_wa38_storage_harvest-espanol-1-docx/ Shared in WA 38 field day in Spanish-Starch Index. September 22, 2021.
Escala de almidón para Cosmic Crisp® cv. WA38. Translation / Jenny Bolivar-Medina, Carolina Torres, Ines Hanrahan (Cosmic Crisp® cv. WA38 starch scale)	Starch scale cards uploaded and printed	Published on October 11, 2021 http://treefruit.wsu.edu/recursos-en-espanol/escala-de-almidon-para-cosmic-crisp-cv-wa-38/ Shared in Starch Index field days. September 22, 2021; September 20 and 22, 2022.
*Perfil de maduración de WA38 en dos portainjertos y tres sistemas de producción- Año 2020./ Jenny Bolivar-Medina, Bernardita Sallato. (*WA38 maturation profile on three production systems and 2 rootstock types- Ayear 2020)	Infographics	Shared in WA38 Field Day in Spanish – Roza, IAREC. September 22, 2021.
Recomendaciones para la cosecha de WA 38- 2021. / Carolina Torres, (Recommendation for WA 38 harvest)	Infographics	Published on September 7, 2021 http://treefruit.wsu.edu/recursos-en-espanol/recomendaciones-para-la-cosecha-de-wa-38/ Shared in field days August 4 and September 22, 2021; September 20 and 22, 2022.

Presencia de grasitud en plantaciones de pre-comercialización de WA 38. Translation. / Jenny Bolivar-Medina, (WA 38 Greasiness incidence in pre-commercialization plantings)	Article	Published on September 7, 2021 http://treefruit.wsu.edu/article/presencia-de-grasitud-en-plantaciones-de-pre-comercializacion-de-wa-38/
Evaluación de portainjertos para manzano 'WA38'. / Erica Casagrande Biasuz, Victor Blanco, Lee Kalcsits ('WA38' evaluation on 9 different rootstocks)	Infographics	Shared in WA38 Field Day in Spanish – Sunrise, TFREC. August 4, 2021.
Cultivos de WA38 en las huertas experimentales de WSU Sunrise y Roza. / Tom Auvil, and Jenny Bolivar-Medina, (WA 38 plots at Sunrise and Roza WSU farms)	Summary article and Infographics	Published on July 22, 2021 http://treefruit.wsu.edu/cultivos-de-wa38-en-las-huertas-experimentales-de-wsu-sunrise-y-roza/ Shared in WA38 Field Day in Spanish – La Roza, IAREC. July 22, 2021.
Manejo de estrés por calor en Manzana./ Lav Khot (Apple hear stress management)	Infographics	Shared in WA38 Field Day in Spanish – La Roza, IAREC. July 22, 2021, and July 05, 2022
Puntos clave acerca de la biología floral, polinización y cuaja de fruta en WA 38. Translation / Jenny Bolivar-Medina (Floral biology and pollination in WA 38)	Infographics	Published on June 4, 2021 http://treefruit.wsu.edu/wa-38-resources/puntos-claves-acerca-de-la-biologia-floral-polinizacion-y-cuaja-de-fruta-en-wa38/ Shared in Pollination and fruit set field days. April 19 and 21, 2022.

Objective 2: Develop and deliver interactive WA 38 Extension programs in Spanish to Hispanic workforce to disseminate and demonstrate timely information on pruning, crop load management, irrigation, and maturity/harvest management. Programs will include field trips, workshops, and discussion groups, among others

Field days and workshops

Our target audience were Spanish speaking growers and workforce in the Washington apple industry that were interested in learning about WA38. A total of eight field days were organized, three of them in 2021 and the remaining five were given in 2022 (Figure 6). In all the field days we provided folders that included the agenda of the day as well as the summaries of the presentations in infographics format in English and Spanish. We also distributed an evaluation form for the field day, and a sticker with the QR presented in Objective 1 (Figure 3A).

Figure 3. Examples of written bilingual resources produced and shared with the tree fruit industry. **A.** Folder showing the materials provided in field days. **B.** Starch Index translated, re-designed and printed. **C.** Fruit defects booklet. Left: front page in Spanish. Right: Content in English- Spanish presented in the gray and white areas of the page, respectively.

The field days in 2021 were given in WSU experimental orchards, Roza at Prosser, and Sunrise at Rock Island. The goals of the first two field days, held on mid-July and early august, respectively, was to provide an overview of the WA38 demo and research blocks that WSU has established and an update of the current studies that WSU research teams in IAREC and TFREC were doing. In the field day at the Roza, topics related to guidelines to the management of WA 38, growth of root systems based on rootstocks, advances in heat stress management were presented. Meanwhile, advances in the evaluation of rootstocks, and horticultural management, as well as harvesting recommendations for WA38 were discussed at the Sunrise orchard. Bilingual infographics of these presentations were shared with participants and are listed in Table 3. Fifteen participants attended the field day at the Roza, and eight were at the Sunrise field day.

The third field day was organized at the Roza and focused on WA38 harvesting recommendations, and maturation analysis via starch index. Starch indexes cards previously created by WTFRC were translated, re-designed, printed, and shared with the attendees who learned and practiced how to use them (Figure 3B). Ten participants attended this event.

In order to increase the participation of the Spanish speaking community in the field days, in 2022 we advertised the events in the WSU Tree Fruit website, Fruit Matters, and sent reminders via mailchimp and texts. We also shared the information through the Good Fruit Grower Magazine, WSTFA news flash, Evenbrite, and personal invitations.

The first two field days in 2022, covered topics related to pollination and fruit set. In these two events participants learned basic floral biology and pollination by a hands-on activity, followed by experiences of growers, information about precision pollination and updates of studies related to this topic. The first field day on April 19 was given at the Roza WA 38 block, with 13 participants attending the event.

During the second event, on April 21, we visited two commercial orchard, Mountain Hill and Columbia Reach, and 25 participants attended the event.

During the field day on July 5 at the Roza, topics related to nutrition, vigor and heat stress management for WA 38 were covered. 17 participants from the industry attended the event. Research teams from IAREC presented their findings. In addition, we invited Pablo Palmandez, UW Agricultural Research and Safety Extensionist who gave an overview of how heat stress affects the persons and how to prevent it.

The last two field days held on September 20 at the Roza and Sept. 22 at the Sunrise WA 38 blocks, with 6 participants in total, covered the most common fruit defects found in Cosmic Crisp®, recommendations for harvesting, and how to properly use the starch index cards in Spanish were provided by representatives from WTFRC, WSU and PVM. At the end of all the field days, attendees evaluated the quality of the events and the presentations, as well as level of knowledge before and after the field days and their intention of changing practices.

In general terms, all the field days in 2021 and 2022, had a positive response from the audience, who found that the organization and quality of the field days in both years were between excellent and good. Likewise, the participants considered that the information presented in these events were excellent. They also reported gained of knowledge at the end of the events in 2021 and 2022 (Figures 4 and 5, respectively). For example, for most of the field days, the level of knowledge perceived by the participants before the event was low for field days related to overview of the WSU blocks in 2021 (Figure 4 A and B), and in 2022, for topics related to floral biology and Starch index field days (Figure 5 A and C). However, this pattern changed as the participants felt that their level of knowledge highly increased at the end of the events.

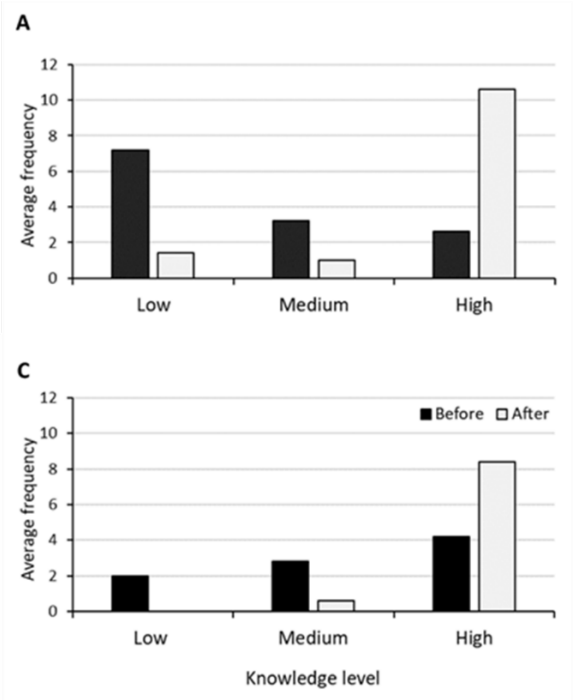

Figure 4. Average knowledge level before and after field days in 2021. **A.** At theRoza (n=13). **B.** Sunrise (n=7). **C,** Starch index evaluation (n=9) Dark bars represent knowledge level before field day, and clear bars after field day

In addition, in field days like the one related to floral biology and pollination, 50% of the participants reported high interest in implement changes in their practices, mainly to explore in more depth the use of assisted pollination and retain to guarantee an optimal pollination of their WA 38 trees. At the end

of all the field days the attendees provided positive no requested feedback. They enjoyed the hands-on activities organized for the field days about floral biology and the starch index and harvest recommendations for CosmicCrisp®. They also appreciated that the information was given in Spanish as they felt more comfortable asking questions and participating in the events.

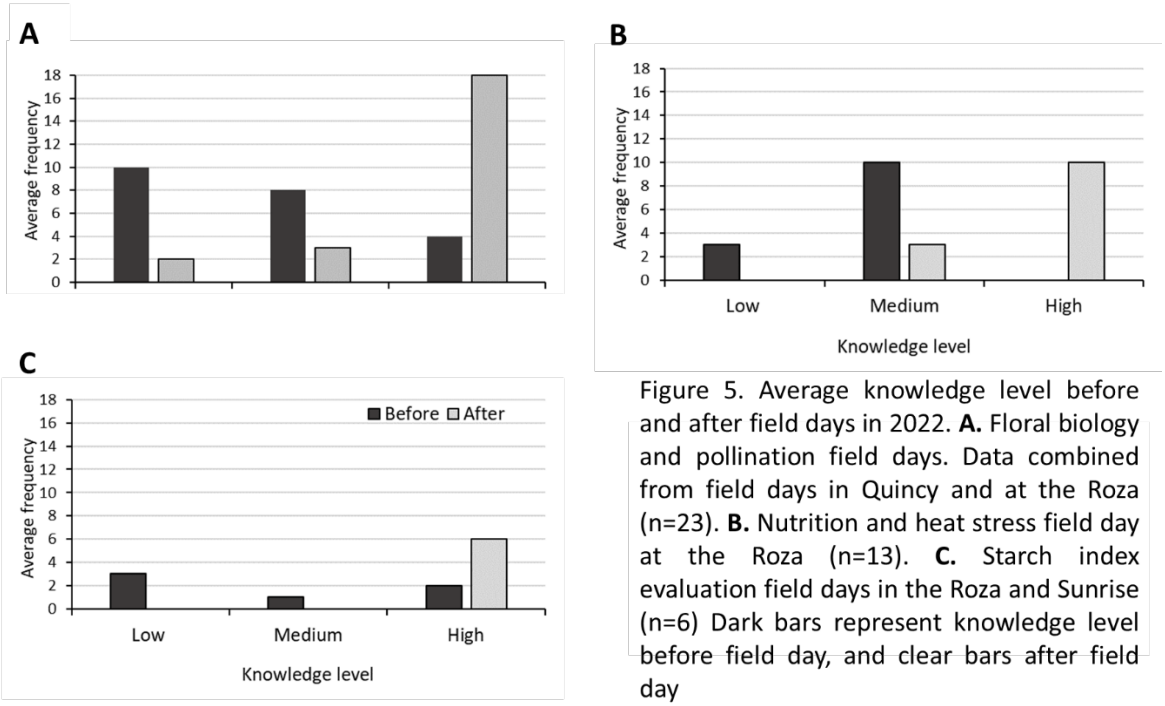

Figure 5. Average knowledge level before and after field days in 2022. **A.** Floral biology and pollination field days. Data combined from field days in Quincy and at the Roza (n=23). **B.** Nutrition and heat stress field day at the Roza (n=13). **C.** Starch index evaluation field days in the Roza and Sunrise (n=6) Dark bars represent knowledge level before field day, and clear bars after field day

Even though the field days have been well received (Figure 6), we noticed that the number of participants in the events got lower as the growing season progressed. This pattern was drastically observed in the starch index field days when only 6 participants in total attended both events. The low attendance confirms the results of the survey in 2021, where fall season was the least preferred time to attend field days. Based on informal conversations with some participants, the low participation could be due to lack of communication within the workforce about the importance of these events, and that are not scheduled as part of their work activities or are perceived as not higher in their priorities specially at the harvesting season. However, prior discussion sessions with WTFRC, PVM and WSU, concluded that it is important to communicate information related to WA 38 in a timely manner, especially the harvest recommendations to guarantee the collection of high-quality fruit. For example, we noticed that prior to the last field days, some of the attendants were not familiar with the quality standards and harvesting recommendations, which often are provided only in English (such as fruit size, background color, stem removal, etc). For that reason, it is imperative to continue expanding the efforts that this project has started by creating more opportunities to transfer the information related to WA 38 in a bilingual format.

Finally, the booklet of the fruit defects guide (Figure 3C) was designed by the end of September 2022. 500 copies were printed and have been distributed to the industry across the state with the help of the advisory committee, WSTFA, Okanagan Horticulture Association, WSU and WTFRC.

Figure 6. Examples of WA 38 Field days in Spanish organized in 2021 and 2022. **A.** At the Roza, 2021. Tom Auvil presented in English an overview of WA 38 demo block, and was translated simultaneously by Jenny Bolivar-Medina. **B.** Floral biology and Pollination Field day, 2022. Participants worked in groups, assembled floral models, and discussed the importance of the floral structures in the pollination process. **C.** Starch Index field day, 2022. Participants prepared their Cosmic Crisp ® samples, applied the iodine solution and assigned the corresponding starch index values to the samples.

Objective 3. Establish an Advisory Committee with Spanish native speakers in the apple industry who will help in the identification of priorities in the translation of the information and the delivery of Extension programs. This committee will also identify needs for future programs directed to the Hispanic community.

Advisory Committee

In July 2021, we invited 20 Spanish speaking members of the tree fruit industry to be part of a Spanish advisory group. Although only 2 persons (Eladio Gonzalez from GSLong, and Victor Bueno from Washington Fruit) answered the call, both contributed with feedback to prioritize the resources to be translated. As recommended by the WTFRC Evaluation Committee, in 2022 we extended the invitation to the community and five persons in total agreed to participate in the committee: Diana Sanchez. Stemilt. Brewster; Leticia Tejo. Fieldin, Inc (previously working in McDouglas, Wenatchee); Eladio Gonzales- GSLong, Naches; Lauren Gonzalez- GSLong; Aylin Moreno- Washington Fruit, Yakima.

As described in the objective 1, the committee contributed to the organization of topics to be translated and provided topic ideas for field days. They also participated in most of the field days organized, proofread some translated documents and provided useful feedback to improve and communicate WA 38 extension resources.

In addition, the committee identified topics of importance to be shared in the near future, not only related to WA 38 but for apples in general such as:

- New rootstocks and WA 38 yield.
- Deficit irrigation to improve fruit size, (especially in Honeycrisp)
- Crop load management
- Soil fertility
- Nutrients availability: how and when to apply nutrients.
- Pruning and tree training systems
- How to manage the vigor in other apple varieties
- Pruning
- More information about the types of pollination that the industry has available.

Executive Summary

Project Title: Implementation of a bilingual extension program for Cosmic Crisp®

Key words: WA 38, bilingual program, resources in Spanish, *Recursos en espanol.*

Abstract

WA 38 is an apple variety released by WSU and its fruit is commercially known as Cosmic Crisp ®. Since the release of WA 38, WSU research teams and the Tree Fruit extension team have provided information focused on improving its cultivation and the production of high-quality fruit. Most of those resources has been only published in English. In Washington tree fruit industry, most of the workforce speaks Spanish, therefore, the WA 38 resources are often out of their reach, especially workers whose knowledge in the English language is limited. The goals of this project were to translate existing information as well as to develop and deliver bilingual (English-Spanish) extension resources related to WA 38. At the beginning of the project, a survey was given to the Spanish speaking workforce. Most of them were interested in getting access to the information in Spanish, and more than 50% in field days. An advisory committee was formed and consulted to prioritize the translation of resources previously published in the WSU tree fruit website, and to provide ideas for field days. A section called *Recursos en español* (Resources in Spanish), and a QR code were created in the WSU tree fruit website, where the written resources in different formats (flow charts, summary articles and infographics) were uploaded. These resources are available to consult and cover different aspects of the WA 38 horticulture, orchard management, pre- and post-harvest recommendations. A total of eight field days, three in 2021 and five in 2022 were given in Spanish and with topics including pollination, fruit set, nutrition, vigor and heat stress management, and harvesting recommendations for WA 38, among others. All the events were well received, and participants evaluated them as the high quality related to their organization and the information provided. Despite the positive feedback, the participation in field days gradually declined as the growing and harvest season progressed. It is important to continue providing resources and events in Spanish or in a bilingual format to create more opportunities to transfer the information related to WA 38 to all the tree fruit industry in Washington.

Project Title: Improving Apple Fruit Quality and Postharvest Performance

Report Type: Final Project Report

PI: Manoella Mendoza
Organization: Washington Tree Fruit Research Commission
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Cooperators:

- WTFRC internal program: Mackenzie Perrault, Ines Hanrahan, Marcella Galeni, Federico Grignaffini, Francisco Sarmiento-Torres, Gerardo Garcia, Gabriela Bolaños, Sadat Amankona.
- Stemilt: Rob Blakey, Hannah Walters, Enrique Garcia
- WA 38 folder distribution: WSU Tree Fruit Extension Team, Agrofresh, GS Long, Storage Control Systems
- WA 38 industry discussion group: Chris Hedges (WSU, organizer after the first meeting), Carolina Torres (WSU), Matt Miles (Allan Bros.) AHP chair, Ines Hanrahan (WTFRC), Manoella Mendoza (WTFRC), Jenny Bolivar-Medina (WSU), Tyler Brandt (PVM), Chris Hargraves (Yakima Fruit), Garrett Grubbs (Chelan Fruit), Lauren Gonzalez (GS Long), Jordan Walker (Roche Fruit), Tom Butler (former member of apple maturity program, Washington Fruit), Craig Anderson (Gilbert Fruit), Jon Onstad (Sage Marketing), Mark Hanrahan (Knight Hill Orchards), others by invitation

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 24,639
Total Project Request for Year 2 Funding: $ 25,327
Total Project Request for Year 3 Funding: $ 26,056

Other related/associated funding sources: in-kind contributions.
Most supplies and fruit are 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.

Primary PI: Manoella Mendoza
Organization Name: WA Tree Fruit Research Commission
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Item	2020	2021	2022
Salaries			
Benefits			
Wages	$15,450.00	$15,900.00	$16,377.00
Benefits	$8,189.00	$8,427.00	$8,679.00
RCA Room Rental	$0.00	$0.00	$0.00
Shipping	$500.00	$500.00	$500.00
Supplies	$500.00	$500.00	$500.00
Travel			
Plot Fees			
Miscellaneous			
Total	$24,639.00	$25,327.00	$26,056.00

Footnotes: Wages and benefits calculated at a yearly increase rate of 3%

The internal program of the WTFRC provides support to scientists and industry. Staff frequently collaborates with a wide variety of programs. The high-priority industry needs not covered elsewhere are tackled by the WTFRC program.

Objectives

1. WA 38 Outreach Material
   a. Distribution of WA 38 starch scale (1-6)
   b. Development of a WA 38 apple defect guide (2020)
   c. Develop harvest criteria information for commercial WA 38 storage (2020 to 2022)
   d. Greasiness incidence summary (published in the Fruit Matters Newsletter 2021 and 2022)

2. WA 38 Collaborative Efforts
   a. Participation in WSU meetings and field days (2020 and 2021)
   b. Lead scientific input to PVM (Marketing & Quality Standard, 2020 to 2022)
   c. Assisting WSU researchers with WA 38 projects
   d. Coordination of WA 38 fruit sampling for Decco, Pace, and Crunch Pak (2020)
   e. WA 38 Industry discussion group
      i. Development of wax protocol (2021)

3. WA 38 Research Projects
   a. DPA phytotoxicity assessment (published in the Fruit Matters Newsletter 2022)
   b. Influence of 1-MCP treatment on fruit flavor considering the starch level at harvest (published in the Fruit Matters Newsletter 2022 and presented at the 2022 WSTFA Research News Flash)
   c. Influence of 1-MCP treatment on starch clearing during RA storage

Significant Findings

<u>Objective 1: WA 38 Outreach Material</u>

a. The WA 38 starch scale was finalized in 2019 and distributed at no cost to the industry. In 2020, 2021, and 2022 industry training was continued via extension events and the distribution of printed copies.
b. A variety-specific defect guide was developed for WA 38. It is available at the WSU Tre Fruit Extension website (http://treefruit.wsu.edu/wa-38-defects-guide).
c. The recommended harvest criteria for commercial WA 38 storage document was developed for the 2021 storage season and updated for 2022. It is available at the WSU Tree Fruit Extension website (http://treefruit.wsu.edu/) under WA 38 resources.
d. Greasiness incidence on WA 38 apples from pre-commercial plantings (ABP phase 3) was summarized. It is influenced by tree age, most prevalent in fruit from young trees (2-3 years old).

<u>Objective 2: WA 38 Collaborative Efforts</u>

a. The in-person meetings held by the WSU extension team had a wide range of participants, including growers, packers, retailers, and researchers (Engl./Span.).

b. The WA 38 Marketing and Quality Standards document was developed for the 2021 season and updated in 2022. It is available at the WSU Tree Fruit Extension webpage (http://treefruit.wsu.edu/) under WA 38 resources. WTFRC facilitated annual scientific input to PVM.
c. Assisted Bernardita Sallato, Karen Lewis, and Meijun Zhu with WA 38 harvest, quality analysis, storage, and transportation.
d. In 2020, several bins of WA 38 were supplied to Decco, Pace, and Crunch Pak to accelerate work on wax and greasiness issues
e. The industry discussion group, under the leadership of Dr. Hanrahan, developed a WA 38 generic waxing protocol is available at the WSU Tree Fruit Extension webpage (http://treefruit.wsu.edu/wa-38-resources/2022-generic-cosmic-crisp-waxing-protocol/)

Objective 3: WA 38 Research Projects

a. DPA phytotoxicity assessment
    i. Diphenylamine (DPA) at 2100 ppm did not cause phytotoxicity on WA 38 apples
b. Influence of 1-MCP treatment on fruit flavor considering the starch level at harvest
    i. The lowest incidence of good flavor in WA 38 was from apples harvested at starch 1.5 (45%) and stored in RA for up to six months. Under the same conditions, fruit harvested at starch levels from 2.0 to 4.5 had better flavor (83 to 100% of good flavor).
    ii. WA 38 apples had a higher percentage of good flavor when not treated with 1-MCP and stored in CA, except at ten months of storage.
    iii. For WA 38 stored in CA, the average fruit firmness was above 17.0 lb., regardless of treatment and storage length.
c. Influence of 1-MCP treatment on starch clearing during RA storage
    i. There was a higher variance between and within treatments in the first few weeks, but the variability decreases over time
    ii. In the first year of the study, the starch degradation in short-term RA storage develops similarly for fruit with and without 1-MCP treatment

Methods

Objective 3: WA 38 Research Projects

In 2021 the WA 38 discussion group met weekly from August to mid-November, via ZOOM and three times in person. During the discussion, the industry members expressed interest in the issues that were investigated in the following projects:

a. DPA phytotoxicity assessment

Diphenylamine (DPA) is an antioxidant compound used for postharvest control of superficial scald on apples. DPA application is very common on Granny Smith apples due to their high superficial scald susceptibility. DPA is also known to cause phytotoxicity on apples, making them unmarketable. The treatment is often applied by drenching with a mix of DPA and a postharvest fungicide but can also be applied via aerosol or fogging within the storage room.

Since no evidence of superficial scald has been seen on WA 38 apple, DPA application is not recommended, but a postharvest fungicide application is advised to decrease postharvest losses from decay. Because Granny Smith and WA 38 harvest timing overlap, a warehouse may face logistics challenges in applying a fungicide alone while avoiding DPA treatment on WA 38.

To assess DPA phytotoxicity on WA 38, apples were harvested from an orchard near Rock Island and another near Quincy in 2021. The apples were drenched with DPA at 2100 ppm mixed with the postharvest fungicide Academy (fludioxonil and difenoconazole). The apples were stored in refrigerated air (RA, 33°F) and evaluated every other week for four months. Phytotoxicity was assessed visually and recorded as absent or present. The sample size for Rock Island and Quincy were 165 and 183 apples, respectively. In 2022, two bins of WA 38 were drenched with a mix of DPA and the fungicide Penbotec (pyrimethanil), at a rate of 1900 ppm.

b. Influence of 1-MCP treatment on fruit flavor considering the starch level at harvest

The compound 1-methylcyclopropene (1-MCP) has been used as a postharvest storage treatment to slow fruit ripening. The 1-MCP binds to ethylene receptors and hinders ethylene-depended reactions, such as fruit maturation (Lee et al., 2012). It can improve firmness retention and reduce the incidence of storage disorders like superficial scald. However, 1-MCP efficacy is highly related to maturity at harvest and time of application, and in some instances, it may inhibit flavor development.

Data was collected from the WSU apple breeding program phase 3 (P3) orchards in Quincy and Prosser from 2010 to 2016. The trees were planted in 2008 on M9 337 rootstock in both locations. After harvest, a sample of 20 to 40 apples was collected and transported to the WTFRC laboratory in Wenatchee. At harvest, quality analysis, starch degradation and external evaluations were performed within 24 hours.

The storage samples, we harvested in 30lb. crates, drenched with a postharvest fungicide at one of the Stemilt facilities and stored in their research storage unit in Wenatchee. Half of each batch was treated with SmartFresh (1-MCP, 1000ppb) within one week of harvest, and samples were stored in refrigerated air (RA, 33°F) and controlled atmosphere (CA, 34°F, 1% CO2, 2% O2) to mimic standard commercial storage conditions. WA 38 was stored in RA for 2 to 6 months and in CA for 4 to 10 months.

Quality analysis of storage samples occurred at the WTFRC laboratory after seven days at room temperature(72°F) to closely mimic fruit quality as sampled by the consumer after transport and handling. Flavor assessment was conducted on 20 apples for each storage sampling combination during quality analysis. The flavor was classified as good, bland (no flavor), or off-flavor. We evaluated a total of 4,230 apples combining locations and years.

c. Influence of 1-MCP treatment on starch clearing during RA storage

Starch degradation is one of the most used procedures to determine apple harvest time. For WA 38 apples, the recommendation is that fruit should be harvested at a minimum starch of 2.0 (WA 38 starch scale: 1 to 6) (Hanrahan & Torres, 2022). For packing and shipping, 90% of the apples must reach or surpass 5.0. Previous studies have reported that fruit picked at 2.0 starch clearance will take an average of six weeks in refrigerated air (RA) storage to reach the required clearance (Musacchi et al., 2019). However, no data is available regarding the effect of 1-MCP treatment on the starch-clearing rate.

In 2021, WA 38 apples were harvested from an orchard near Rock Island, Washington, and immediately stored in RA (33°F). The apples were divided into three treatments: Control, MCP I, and MCP II. The fruit under MCP I and MCP II treatments were treated with SmartFresh™ (100 ppb) 4 and 8 days after harvest, respectively. The apples in the control treatment were not treated with 1-MCP.

The starch degradation was evaluated visually using the WA 38 starch scale. The apples were removed from storage and sampled at room temperature by cutting through the equator and spraying the iodine solution. Starch was read 30 minutes after spraying. The data was collected every week for 11 consecutive weeks on 15 apples per treatment.

Results and Discussions

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

WA 38 Outreach Material

a. Distribution of WA 38 starch scale (1-6)

A starch scale with detailed instructions was developed for WA 38 in 2019. It was distributed free of charge for the apple industry, and industry-wide training was performed in 2019, 2020, and 2021. It included workshops and field days coordinated by the Tree Fruit Extension Team. Over 1000 folders containing the starch scales and other relevant materials, such as the Marketing and Quality Standards and Recommended Harvest Criteria for commercial WA-38 Storage, were distributed at events.

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

b. Development of a WA 38 apple defect guide

A variety-specific defect guide was developed by Ines Hanrahan (WTFRC) and Carolina Torres (WSU) in 2020. 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 of WA 38. It can be found at the WSU Tree Fruit Extension website (http://treefruit.wsu.edu/wa-38-defects-guide). The guide will be updated regularly**.**

c. Develop harvest criteria information for commercial WA 38 storage in 2020, 2021, and 2022

This effort was led by Ines Hanrahan and completed in collaboration with Carolina Torres. Input from the WSU extension team, PVM, and the WA 38 discussion group are included. The document is reviewed and updated yearly to include the latest research results. The 2022 recommendations are available at the WSU Tree Fruit Extension website (http://treefruit.wsu.edu/) under WA 38 resources.

d. WA 38 greasiness incidence in pre-commercialization plantings

The results of this research were published in the Fruit Matters Newsletter in September 2021 and August 2022. The goal was to assess the greasiness incidence of WA 38 apples from two pre-commercialization plantings (Apple Breeding program Phase 3) across years (2010- to 2016). Greasiness was assessed as absent or present at harvest and after refrigerated air (RA) and controlled atmosphere (CA) storage (up to 10 months). 1-MCP was applied in batches of fruit. A total of 4,960 apples from Quincy and 4,678 from Prosser were evaluated.

Greasiness is more prevalent in fruit from 2- to 3-year-old trees. Starch degradation level by tree age shows that WA 38 overall greasiness incidence is not related to starch levels at harvest (Figure 1). For example, Quincy starch levels at harvest were 1.6 for both 2- and 4-year-old trees, but the greasiness levels were 88.9% and 0.8 %, respectively. Similarly, 3- and 6-year-old trees from Prosser had a 2.2 starch degradation level at harvest, and a greasiness incidence of 53.0% and 12.7%, respectively.

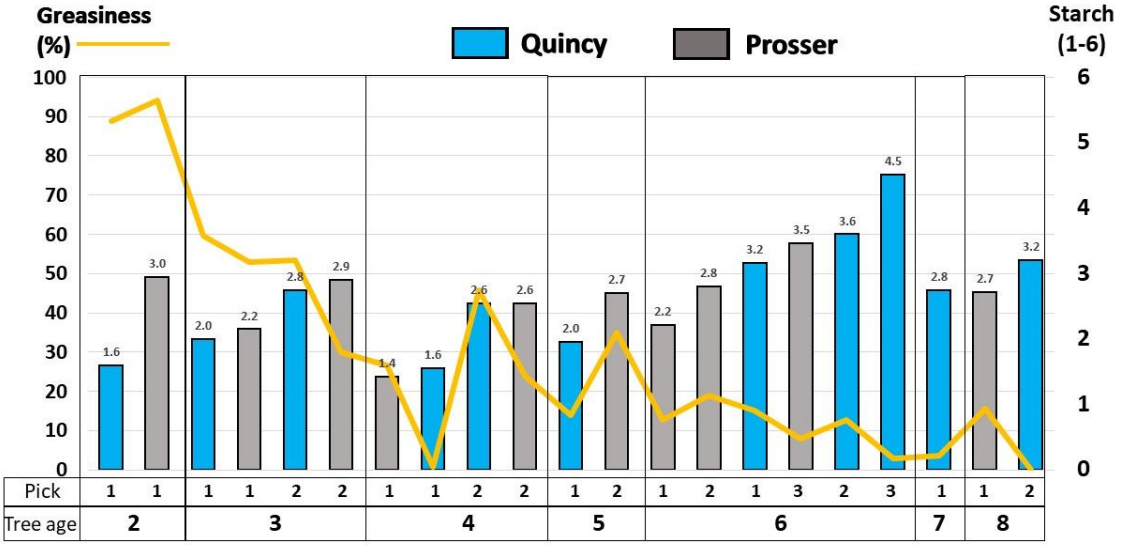

Figure 1. Effect of tree age and starch degradation level at harvest on WA 38 greasiness (%) incidence after storage

Fruit stored in (RA) typically develops more greasiness than fruit stored in CA (data not shown). Treatment with 1-MCP suppresses greasiness development during storage in mature orchards but is less effective during the first few years, when greasiness is high (data not shown). More information can be found at the WSU tree fruit and extension website.
(http://treefruit.wsu.edu/article/wa-38-greasiness-incidence-in-pre-commercialization-plantings/)

WA 38 Collaborative Efforts

a. Participation in WSU meetings and field days

Field days in Spanish were conducted in 2021 and 2022 by Jenny Bolivar (member of the WSU extension team). The WTFRC assisted with printing material, folder organization, and distribution. Ines Hanrahan was a presenter at the Spanish field days, focusing on apple quality, the use of the starch scale, and postharvest issues. The WTFRC staff assisted with event logistics.

b. Led Scientific input to PVM

PVM has published the Marketing & Quality Standard, 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 recommendations, grading criteria for defects and color, and compliance actions. The document is reviewed and updated annually by the Quality Standards Advisory Committee. It can be found on the WSU Tree Fruit and Extension webpage (http://treefruit.wsu.edu/treefruit.wsu.edu%2Farticle%2F2022-commercial-harvest-and-storage-criteria%2F). For more information on industry guidance, refer to https://quality.cosmiccrisp.com/

c. Assisted WSU researchers with WA 38 projects

Helped Bernardita Sallato (WSU) and Karen Lewis (WSU) with the harvest of several bins of WA 38 in Prosser in 2021. WTFRC crew transported and stored the bins at Stemilt RCA in Wenatchee. WTFRC further assisted in conducting titratable acidity analysis for a WA 38 experiment. For Meijun Zhu, three bins of WA 38 were harvested from the Sunrise orchard, in 2020 and 2022. The apples were stored at Stemilt in Wenatchee and transported to Pullman to be used in food safety projects.

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

The 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.

e. WA 38 industry discussion group

A group of industry representatives met in 2021 in 2022 to discuss WA 38 industry issues. In 2021 the group met weekly from August to mid-November, via ZOOM and three times in person. Under the leadership of Dr. Hanrahan, the group developed a WA 38 generic waxing protocol, available on the WSU Tree Fruit Extension webpage (http://treefruit.wsu.edu/wa-38-resources/2022-generic-cosmic-crisp-waxing-protocol/).

Eight hybrid and two in-person meetings were held in 2022. Dr. Hanrahan continued leading this effort joined by Matt Miles (Apple Horticulture and Postharvest Committee Chair). The mailing list currently has 76 members, and everyone interested is welcome to join.

WA 38 Research Projects

a. DPA phytotoxicity assessment (published in the fruit matters newsletter 2022)

In 2021, WA 38 apples treated with a mix of DPA and Academy at 2100 ppm did not develop phytotoxicity symptoms during the four months of observation. This result indicates that WA 38 could be drenched with a fungicide solution containing DPA. We only tested two lots of fruit. Distinct lots may present different levels of sensitivity to a chemical burn. In 2022, 2 bins were drenched with DPA and Penbotec at 1900 ppm. Evaluations are ongoing. It is generally not recommended to use DPA on WA 38 because it does not develop superficial scald.

b. Influence of 1-MCP treatment on fruit flavor considering the starch level at harvest (published in the fruit matters newsletter 2022 and presented in the 2022 WSTFA Research News Flash)

The treatment combinations corresponding to each pick date were grouped based on starch reading at harvest and associated with the flavor classification received during fruit quality analysis after storage. The results below are based on the combined results of fruit harvested at Prosser and Quincy from 2010 to 2016. The RA and CA samples are analyzed separately.

Most fruit stored in RA for up to six months did not receive 1-MCP treatment; thus, it was not possible to determine if 1-MCP had affected fruit flavor in this condition. When differentiating fruit by starch index at harvest, more than 80% of the WA 38 apples were classified as having good flavor, except for fruit harvested at 1.5 starch, of which less than half had good flavor (Figure 3).

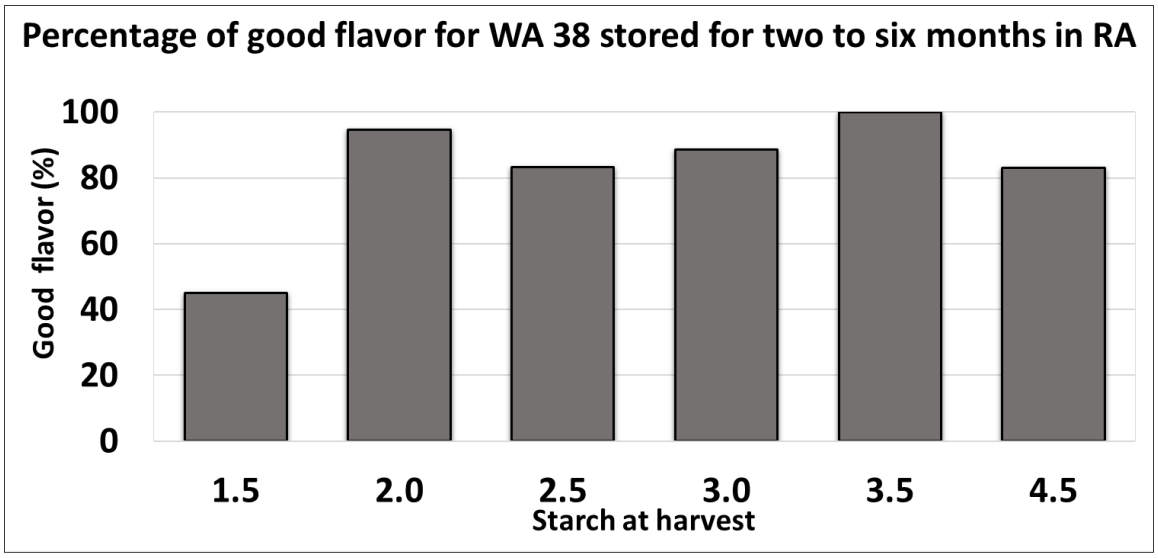

Figure 3. Percentage of good flavor by starch degradation level at harvest of apples stored for up to six months in RA. Total fruit evaluated equal 1040.

At six and eight months of CA storage, at least 90% of the apples harvested at 2.0 to 4.5 starch were classified as having good flavor regardless of 1-MCP treatment (Table 1). However, the fruit treated with 1-MCP scored lower than the untreated fruit at six months and equal to or lower at eight months of storage. Fruit harvested at 1.5 starch and stored in CA for six months had the lowest percentage of good flavor compared with fruit harvested in the 2.0 to 4.5 starch range. The apples treated with 1-MCP scored similarly to untreated fruit but achieved better flavor ratings at ten months in CA.

Table 1. Percentage of good flavor by starch degradation level at harvest for apples stored in CA for six, eight, and ten months with or without 1-MCP treatment. Data summary of fruit harvested at Prosser and Quincy from 2010 to 2016. Total fruit evaluated equal 3060.

	6 months CA		8 months CA		10 months CA	
	no 1-MCP	*1-MCP*	*no 1-MCP*	*1-MCP*	*no 1-MCP*	*1-MCP*
1.5	70	80			80	90
2.0	100		100	100	89	100
2.5	90				60	80
3.0	99	97	97	94	59	68
3.5	100	95	100	100		
4.5	100	97	100	100		
overall	**93**	**92**	**99**	**98**	**72**	**84**

Fruit firmness, titratable acidity and soluble solids were assessed at the same timepoints (data not shown). The average fruit firmness was above 17.0 lb., regardless of treatment and storage length. Apples treated with 1-MCP typically had higher firmness than untreated apples. However, the treatment difference was usually less than 1.0 lb., except for apples harvested at starch level 3.0 and stored in CA for ten months (diff. 1.7lb.). Titratable acidity and soluble solids concentration were comparable between 1-MCP treated and untreated fruit. Greasiness incidence was discussed in the WA 38 outreach material section.

Considering fruit flavor ratings, quality parameters, and greasiness incidence, applying 1-MCP might be beneficial only for the longest-term CA storage if apples are harvested at a 3.0 starch level. For six to eight months of storage, 1-MCP does not appear advantageous or cost-efficient as it may be detrimental to fruit flavor and has no effect on quality parameters.

c. Influence of 1-MCP treatment on starch clearing during RA storage

The starch degradation evolved similarly for the three treatments, increasing by about 1.5 units in 11 weeks. However, rather than a sequential stepwise increase, the starch averages oscillated ± 0.4 units overall between sampling dates (Figure 4). WA 38 apples are ready for packing and shipping when 90% of the apples reach or surpass 5.0 (WA 38 starch scale:1-6). According to the data collected, fruit from control and treated with 1-MCP at four (MCP I) and eight (MCP II) days after harvest would be ready for packing on December 17th,10th, and 23rd, respectively, which is a month later than the sale release date. It is important to mention that the data collected does not indicate the inadequacy of the chosen packing and shipping date, rather, it emphasizes the need for starch assessment for every fruit lot before packing.

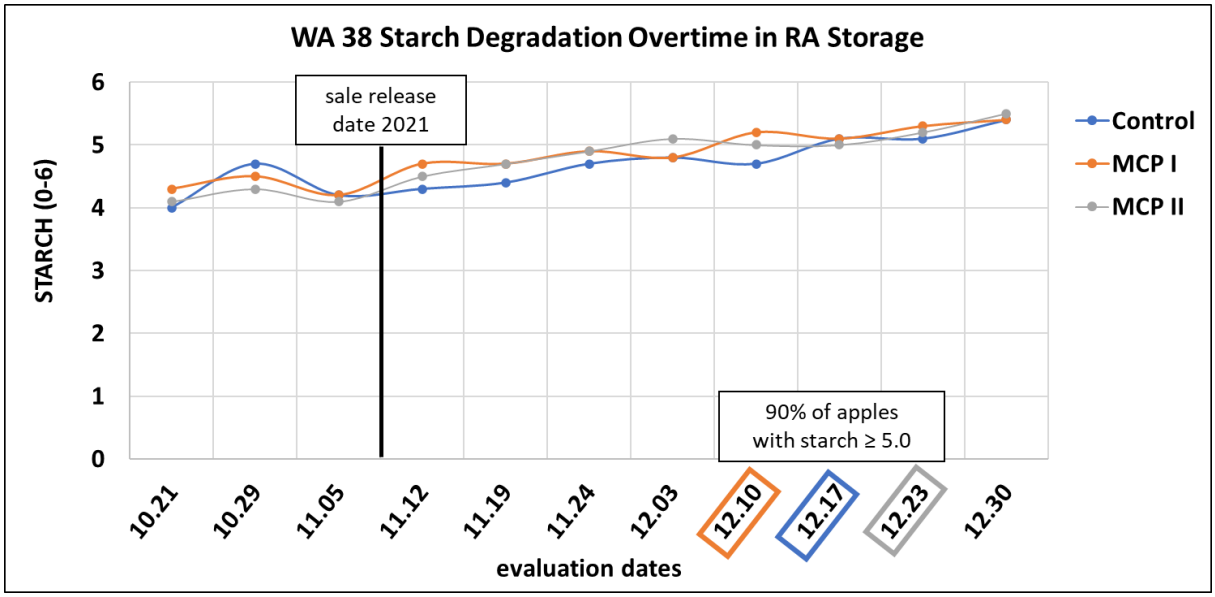

Figure 3. Weekly starch degradation of WA 38 apples stored in RA in 2021. Apples were treated with 1-MCP at four (MCP I) or eight days (MCP II) after harvest. The control treatment did not receive 1-MCP treatment. The date on which 90% of apples reach starch degradation equal to or above 5.0 is December 10th, 17th, and 23rd, for control, MCP I and MCP II, respectively.

There was a slightly higher sample variability on fruit treated with 1-MCP when compared with control. This variation was higher on MCP I, followed by MCP II. The starch deviation within treatment per sampling time decreased over time, showing that apple-to-apple variability declined as fruit matures in storage regardless of treatment (Table 2).

Table 2. Starch degradation variability (standard deviation) of WA 38 apples stored in RA per sampling time and treatment. A darker to lighter yellow shade is used to identify higher to lower variability.

	Standard deviation – variability within treatment										
treatment	**10.21**	**10.29**	**11.05**	**11.12**	**11.19**	**11.24**	**12.03**	**12.10**	**12.17**	**12.23**	**12.30**
Control	± 0.4	± 0.3	± 0.5	± 0.5	± 0.4	± 0.4	± 0.3	± 0.4	± 0.3	± 0.3	± 0.3
MCP I	± 0.6	± 0.4	± 0.6	± 0.6	± 0.3	± 0.6	± 0.3	± 0.4	± 0.3	± 0.3	± 0.3
MCP II	± 0.6	± 0.6	± 0.4	± 0.4	± 0.4	± 0.4	± 0.4	± 0.4	± 0.3	± 0.2	± 0.3

The results suggest a slower starch degradation rate than reported in previous studies. In 2022, tree age will be considered, and the same orchard from 2021 will be sampled to account for year-to-year variability. WA 38 apples will be harvested from one young (2nd or 3rd leaf) and one mature (4th leaf or older) orchard. Half of the fruit will be treated with 1-MCP and stored in either RA or CA.

Project Title: Improving Apple Fruit Quality and Postharvest Performance

Executive summary

Keywords: WA 38 defect guide, WA 38 greasiness, WA 38 starch scale, WA 38 wax protocol

Abstract: The internal program of the WTFRC provides support to scientists and industry. Staff frequently collaborates with a wide variety of programs. The high-priority industry needs not covered elsewhere are tackled by the WTFRC program. This report includes WA 38 outreach material, collaborative efforts, and research results from 2019 to 2021.

Project Outcomes and Significant Findings

Objective 1: WA 38 Outreach Material

a. The WA 38 starch scale was finalized in 2019 and distributed at no cost to the industry. In 2020, 2021, and 2022 industry training was continued via extension events and the distribution of printed copies.
b. A variety-specific defect guide was developed for WA 38 (http://treefruit.wsu.edu/wa-38-defects-guide).
c. The recommended harvest criteria for commercial WA 38 storage document was developed for the 2021 storage season and updated for 2022 (http://treefruit.wsu.edu/).
d. Greasiness incidence on WA 38 apples is influenced by tree age, being most prevalent in fruit from young trees (2-3 years old).

Objective 2: WA 38 Collaborative Efforts

a. The in-person meetings held by the WSU extension team had a wide range of participants, including growers, packers, retailers, and researchers (Engl./Span.).
b. The WA 38 Marketing and Quality Standards document was developed for the 2021 season and updated in 2022 (http://treefruit.wsu.edu/). WTFRC facilitated scientific input to PVM.
c. Assisted Bernardita Salatto, Karen Lewis, and Meijun Zhu with WA 38 harvest, quality analysis, storage, and transportation.
d. Several bins of WA 38 were supplied to Decco, Pace, and Crunch Pak to accelerate work on wax and greasiness issues.
e. The industry discussion group, under the leadership of Dr. Hanrahan, developed a WA 38 generic waxing protocol (http://treefruit.wsu.edu/wa-38-resources/2022-generic-cosmic-crisp-waxing-protocol/).

Objective 3: WA 38 Research Projects

a. DPA phytotoxicity assessment
    i. Diphenylamine (DPA) at 2100 ppm did not cause phytotoxicity on WA 38 apples
b. Influence of 1-MCP treatment on fruit flavor considering the starch level at harvest
    i. The lowest incidence of good flavor in WA 38 was from apples harvested at starch 1.5 (45%) and stored in RA for up to six months.
    ii. WA 38 apples had a higher percentage of good flavor when not treated with 1-MCP and stored in CA, except at ten months of storage
    iii. For WA 38 stored in CA, the average fruit firmness was above 17.0 lb., regardless of treatment and storage length
c. Influence of 1-MCP treatment on starch clearing during RA storage
    i. There was a higher variance between and within treatments in the first few weeks, but the variability decreases over time
    ii. In the first year of the study, the starch degradation in short-term RA storage develops similarly for fruit with and without 1-MCP treatment

Project Title: Reliable Soil Diagnostic Technology for Smart Nutrient Management

Report Type: Final Project Report

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Cooperators: Steve Mantle, Gilbert Plath, Ramon Cuevas, Jenny Bolivar-Medina, SoilTest Lab, Predictive Nutrient Solutions, Inc. in Walla Walla, (PNS), Northwest Agricultural Consultants, Inc. Stuart Goatley (WTFRC).

Project Duration: 2-Year.

Total Project Request for Year 1 Funding: $ 15,670
Total Project Request for Year 2 Funding: $ 14,970

Amount awarded: $152,938
Agency Name: Washington State USDA- Specialty Crop Block Grant
Notes: PI: B. Sallato. Co-PIs: L. Kalcsits, M. Whiting. Costs associated with Objective 1 and wages for hourly support during sample collection incurred in this proposed WTFRC project will be covered by this SCBG Grant.

Amount awarded: ~$35,000
Agency Name: Universidade Federal de Viçosa (UFV), Capes-Print project, Brazil
Notes: Khot's Precision Ag lab has an ongoing collaboration with UFV-Brazil and will host a visiting scholar from UFV, Brazil for a 1-year period (March 2021-February 2022). The UFV-Brazil group has developed a field portable soil nutrient(s) sensing system. In this WTFRC project, we will leverage the expertise of the visiting scholar to test the suitability of this sensing module for mapping the soil attributes from chosen orchard sites. We will then relate those results to other ground-reference methods and other data products.

Budget 1
Primary PI: Bernardita Sallato C.
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Station Manager/Supervisor: Naidu Rayapati
Station manager/supervisor email address: naidu@wsu.edu

Item	2021	2022
Salaries		
Benefits		
Wages		
Benefits		
Equipment		
Supplies[1]	15,670	14,970
Travel		
Miscellaneous		
Plot Fees		
Total	$15,670	$14,970

Footnotes:
[1] Supplies: laboratory analyses of 300 samples @ $35.50/sample for complete soil test including standard and paste extract, and 240 tissue samples (leaves, fruits) @ $18/sample.

INTRODUCTION

A smart orchard project was implemented in Chiawana Orchards, Pasco, in 2020 in collaboration with several industry and university partners. This collaboration initiated a system that enabled the assessment and ground truthing of conventional and new technologies. Under the umbrella of the smart orchard initiative, this project focused on technology for soil chemical management for reliable diagnosis.

Soil physical and chemical testing has been used for more than a century to guide nutrient management practices. Today, new technology could provide opportunities for precision and remote management. Our goal is to develop "smart nutrient management" strategies based on quantifiable needs. For these, our specific objectives were to: a. *Characterize different soil testing methods/technologies and their relationship with plant response, b. Investigate tools to assess and map soil variability and c. Contribute to the "smart orchard initiative" in the area of soil nutrient sensing and management.*

SIGNIFICANT FINDINGS

- Grandview 'Honeycrisp' fruit load was 4.4 and 2.2 times higher in site 3 (S3) compared to site 4 and site 1. While bitter pit incidence was lowest in S3 (2%) and highest in S4 (52%). In 2022, cracking was also significant, with 64% in S3 and no cracking in S4.
- Based on yield, size and culls, S2 was the most productive (or less limited) site.
- Nutrient levels in leaves and soils were adequate for the most part. Leaf N, Ca, Mg, Mn, P and K were good indicators of growth and quality differences among sites.
- Fruit diameter was strongly correlated with leaf Ca and Mn, while BP incidence correlated strongly with P and cracking with B. The ideal timing for leaf tissue sampling were between June 28th and July 28th.
- Soil chemical, physical and biological indicators were significantly different among sites. In general, S3 had higher pH and more Ca, Mg, M.O and microbiological activity. While one of the most limited in terms of fruit quality.
- Soil K and P were excessive in the soil in S4, while adequate in leaves. This suggests uptake issues in the root zone, which could relate to the higher BP incidence in S4. BP incidence correlated negatively with soil pH and positively with P-Olsen. This relation does not imply cause effect, rather provided information regarding limiting conditions at a root level.
- There was a strong correlation between laboratories and soil testing methods.
- Aerial mapping tools provided equivalent maps for vigor distribution, evapotranspiration, and canopy density. While SoilOptix® provided with a more precise variability map for soil texture, Ca, Mg, CEC and B. However, SoilOptix® did not correlate well with the absolute values reported. Thus SoilOptix ® should be utilized for mapping relative differences, not for determining nutrient availability or management.
- E.C mapping correlated well electric conductivity of the soil, at one time. The use of E.C mapping should be timed properly, preferable, at the beginning of the season.
- None of the tools were good predictors of tree productivity, health, and fruit quality on their own. However, the integration of tools; mapping, soil test and tissue samples, provided insightful information related to differences in the block and possible causes.
- Here, soil profile analysis was key to understand the cause of high vigor and quality disorders.

METHODS

This project was conducted in two commercial apple orchards: Chiawana 'Gala' orchard and Grandview 'Honeycrisp' apple orchard. The Chiawana orchard was evaluated in 2020 (unfunded) and 2021, complete details on Chiawana methods and report can be reviewed in previous report (Sallato and Khot, 2022). The Grandview orchard was evaluated in 2021 – 2022. We selected four distinct sites, based on the historical vigor and productivity. Sites 1 and 3 (S1 and S3) were low vigor areas, while sites 2 and site 4 (S2 and S4) were high vigor.

Plant productivity and fruit quality

From each area, three consecutive trees were designated for whole tree monitoring. Two of these areas were also utilized for plant base monitoring systems (Kalcsits), and weather sensors (Mantle and Khot). From each tree, five representative shoots and fruiting spurs (n = 60) were measured for length and diameter during the growing season. At harvest, total fruit per tree were counted to assess yield differences and a sub sample of 40 fruit per tree was taken to the laboratory for fruit quality analysis, including defects, weight and size.

a. Characterize different soil testing methods/technologies and their relationship with plant response.

Soil profile analysis
In-situ soil profile analysis were evaluated March 29th, 2022. Soil pits were excavated with a backhoe in seven areas across the orchard, including sites 1 to 4. The same day, soil profiles were described following USDA NRCS soil taxonomy guide, which includes effective depth (or root depth), color, texture, porosity, structure, drainage, reactivity to HCl (effervescence) and presence of limiting factors. From each profile, two samples were collected at 8 and 12 inches deep for physical and chemical analyses.

Soil laboratory tests; physical, chemical, biological
For each site, three soil samples were collected throughout the growing season, totaling seven timings. On each sampling time, three to four soil cores were obtained from around the tree, combined and sub-divided into three homogeneous samples. Each sample was distributed to three different laboratories. Two laboratories L1 and L2 conducted soil standard analysis tests; soil pH (pH), organic matter (OM), electric conductivity (E.C), soluble solids (SS), cation exchange capacity (CEC), nitrate (NO_3), ammonium (NH_4), phosphorous (P-Olsen), extractable potassium (K), calcium (Ca), magnesium (Mg) and sodium (Na), and micronutrients copper (Cu), manganese (Mn), zinc (Zn), iron (Fe), and boron (B). In 2022, we included additional indicators associated to soil health, and validated by USDA NAPT program; Active carbon (POX-C), ACE protein, Soil Respiration, Potential mineralizable N (PMN, 7-day anaerobic nitrogen), total C and total N. The third sample was sent for resin test analysis (Predictive Nutrient Solution, Inc. in Walla Walla (PNS)) for soil pH, OM, E.C, SS, NO_3, NH_4, P, K, Ca, Mg, Na, Cu, Mn, Zn, Fe and B. All three laboratories are certified by the Soil Science Society of America and the North American Proficiency Testing Program's (NAPT) Plant Performance Assessment Program, Soil and Plant Program, and Soil Performance Assessment Program.

Leaf tissue analysis
Leaf tissue sample were collected to determine nutrient uptake from each replicated tree during the summer. In 2022, we added three additional dates to better understand and correlate nutrient levels over time. At each sampling time, we selected the most recently mature leaf from none bearing shoots.

b. Investigate tools to assess and map soil variability.

Soil variability and mapping were assessed through several methods (Table 1). For each method, we ground truth the information provided by the map, by selecting the values associated to the position of our pre-selected sites. The values obtained from each method were correlated to the corresponding ground truth value obtained in situ. For chemical analysis the correlations were conducted against soil standard analysis.

Table 1. Soil mapping methods and frequency evaluated at the smart orchard project.

Method	Detail*	Frequency
1. SO	SoilOptix® – gamma radiation mapping	2 / Spring - Fall
2. E.C	Electric Conductivity mapping Simplot	1 / 2021
3. SW	Web Soil Survey (NRCS, USDA)	1 / Historic
4. GE	Satellite (Google Earth Pro)	1 / Historic
5. UAS	Drone image: 5-band multispectral sensor	7 / Season

*Details of each technology were reported in details in our previous report.

c. Contribute to the "smart orchard initiative" in the area of soil nutrient sensing and management. Results from soil and plant measurements were shared with Mantle et al. (Innov8ag) to incorporate into their platform and visualization scope of work, as well as to the AgAID project to contribute to their modeling, extension, and broadening participation scope of their project. Field days and outreach activities were coordinated among all smart orchard PIs and coordinated by Jenny Bolivar-Medina.

RESULTS AND DISCUSSION

For synthesis, this report will focus on 2022 results and referring to 2021 when appropriate.

a. Characterize different soil testing methods/technologies and their relationship with plant response.

In Grandview 2022, overall fruit size was 5% smaller than in 2021, while harvested approximately 10 days after. At the beginning of fruit development, S1 had the smallest fruitlet, however, as the season progressed, S2 was the largest and S3 ended with the smallest fruit (Figure 1). In contrast, S4 had the largest fruit in 2021. In 2022, shoot growth was maximum between June 28th and July 28th, being higher in S4, while in 2021, maximum shoot growth was observed between August 4th and August 28th, with no differences between sites (data not shown).

In 2022, total fruit count per tree varied tremendously, between 2 and 135 fruit per tree, being 4.4 and 2.2 times higher in S3 when compared with S4 and S1, respectively (Figure 1). Fruit size at harvest was 11% smaller in S3 (67 mm, 150 box size). Although there were statistical differences between the other sites (S1, S2 and S4), box size remained the same (113) (Figure 1). Bitter pit (BP) levels were highly variable in 2021, ranging from 74% and 1%, however with no differences between sites. In 2022, BP levels at harvest were significantly different among sites, being lowest in S3 (2%) and highest in S4 (52%), still with great variability across the orchard (Figure 2). In addition, cracks and splits accounted for 26% of overall fruit damage, being highest in S3 (64%), while not observed in S4 ($p = 0.004$) (Figure 2).

When estimating production per site, based on yield, size and culls, S1 and S3 were the least productive sites, with estimated 7 and 12 bins per acre, respectively and 7 packs per bin. Although in S3, fruit were smaller (below 150 box count). Site 4 had an estimated 12 bins per acre, with 10 packs per bin, and S2 was the most productive (or less limited) with 23 bins per acre and 17 packs per bin (based on 20 bu/bin and 42lb/bu).

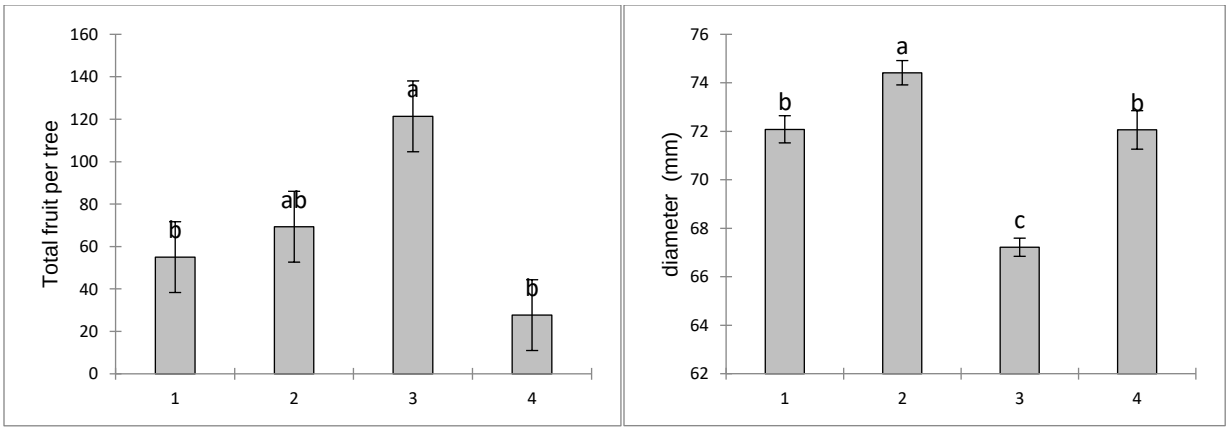

Figure 1. Number of fruits per tree (left) and fruit diameter at harvest (right) between sites in 2022 Grandview orchard. Different letters indicate significant differences between sites (Tukey test, $p < 0.07$)

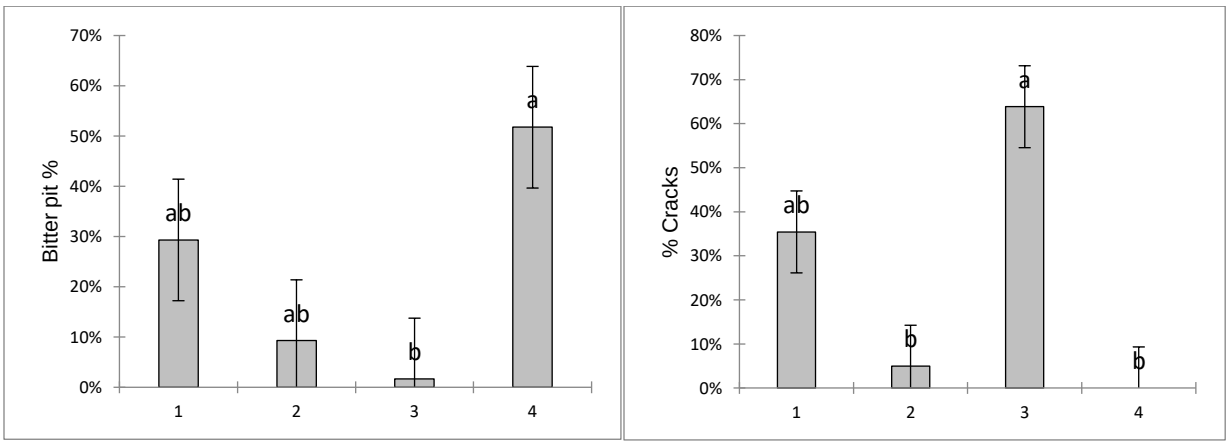

Figure 2. Percent bitter pit (left) and cracking (right) between sites in 2022 Grandview orchard. Different letters indicate significant differences between sites (Tukey test, $p < 0.07$)

Our results agreed with the warehouse pack-outs of Grandview orchard, were out of 93% of total bins processed, 45.5% was packable fruit with estimated 9.6 packs/bin average. Most relevant physiological related culls were undersized fruit, bitter pit and cracking.

Based on the information above, we utilized several tools and technology to identify A) what were the limiting conditions contributing to poor yield and quality, and B) which technologies/or combination of technologies provided us with better information to understand these conditions.

1. *In situ profile analysis*

Soil profile analysis demonstrated great diversity in soil types across the orchard, with distinct stratification (number and depth of soil layers), structure, effective depth, effervescence, porosity, root growth, drainage, among others. While we evaluated seven soil profiles throughout the orchard, in this report I will focus only on those developed at sites 1, 2, 3 and 4.

Site 1, located in the south-east side of the block was associated to Hezel loamy fine sand; with sand in the top 5 inches, followed by loamy fine sand up to 30 inches deep. Roots where scarce in the upper soil, concentrated in the transition between the second and third strata, below 30 inches (Figure 3). The sandy texture and lack of structure leading to excessive drainage and reduce water holding capacity.

Site 2 and 3 were associated to Warden silt loam series. Although S2 had a deeper effective soil depth of 24 inches of sandy loam, lightly alkaline, transitioning smoothly to a highly effervescent silt loam. In S2, roots were more abundant in the upper stratum. While Site 3, was shallower with roots concentrated in the first strata (7 to 10 inches), above a heavier soil (massive), evidencing deficient drainage. In S3 there were fewer roots and signs of anoxia. Also, S3 had a caliche layer at 40 inches. Site 4, was also very different, associated to the Starbuck silt loam soil, with a shallow effective depth of 16 inches over basalt rock. In S4 roots were abundant (Figure 3).

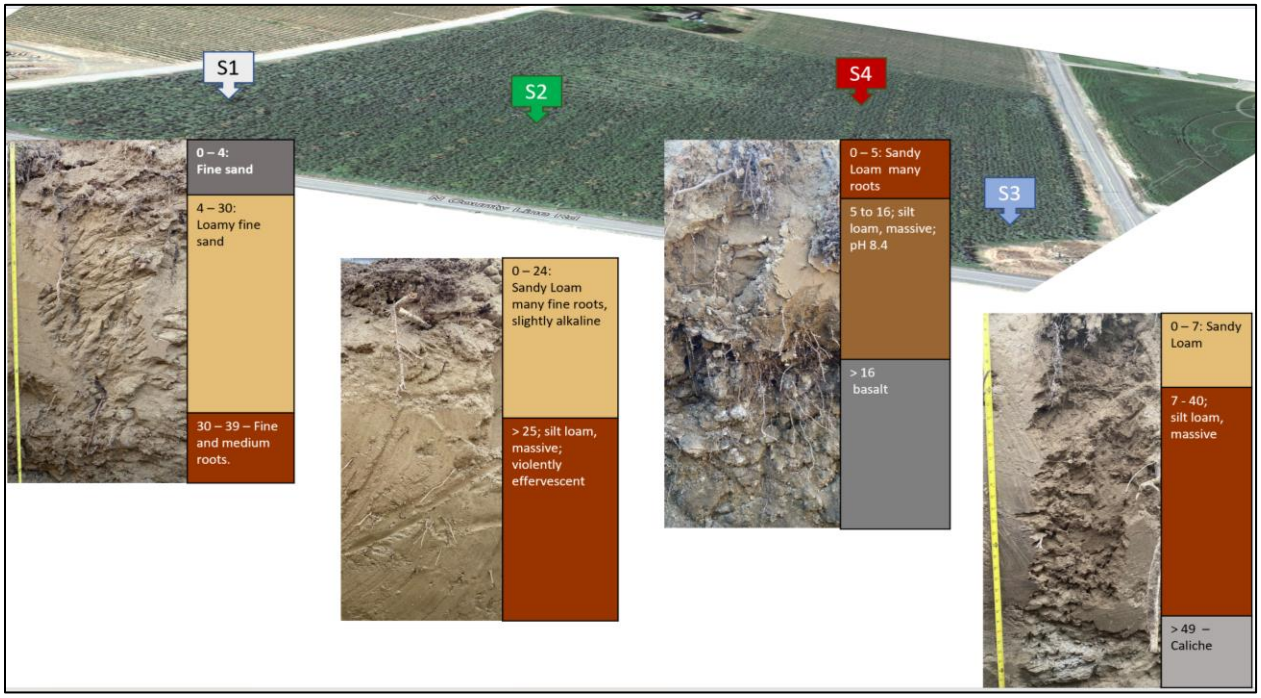

Figure 3. Soil profiles for S1, S2, S3 and S4 sites in Grandview orchard.

Soil profile analysis provided insightful information regarding limiting conditions of the block. Clearly, S1 suffers from excessive drainage, reduced water holding capacity and nutrient leaching. Thus, over time, roots have moved to deeper soil (> 30 inches) in search for moisture, while nonmobile nutrients (such as P) is limited. In contrast, S3 also associated to lower vigor, present opposite limiting conditions; where roots are concentrated in the upper stratum (first foot), due to lack of oxygen, excessive water, and high pH.

Sites S2 and S4, while both were associated to higher vigor, they had distinct fruit quality (S4 had greater BP incidence and reduced crop load). Likewise, S2 has greater root dept compared to S4, while less volume of soil, with higher nutrient accumulation (to be discussed later under soil chemical analysis).

2. *Leaf tissue analysis*

In 2021 and 2022, nutrient tissue concentrations were within adequate range for all nutrients except for Ca in S1, and Zn levels in all sites. Nutrient differences among sites were consistent throughout the season, however samples obtained during June 28th and August 10th, were better correlated with site conditions. Leaf N (2.07 – 2.65), Ca (1.11 – 2.13) and Mg (0.31 – 0.43) were higher in S3, correlating with smaller fruit size and increased fruit cracks. Mn was also higher in S3, which can be associated with excessive water, as Mn becomes more available under anaerobic conditions. In contrast, S2, had more P (0.16 – 0.22) and K (1.03 – 1.74), correlating with higher fruit size and reduced defects (data not shown).

S levels were also within range (0.16 – 0.21) and generally higher in S2 and S3 (heavier soils), but only during the first two sampling times. Micronutrients Zn where below adequate range (< 22 mg/kg) however with no differences between sites, while Fe, Mn, Cu and B were within adequate range and differences were inconsistent (data not shown).

Shoot growth was correlated but weakly with Ca (r = 0.52) and Mn (r = 0.54), while fruit diameter was strongly correlated with Ca (r=0.69) and Mn (r=0.61), but weakly (Figure 4).

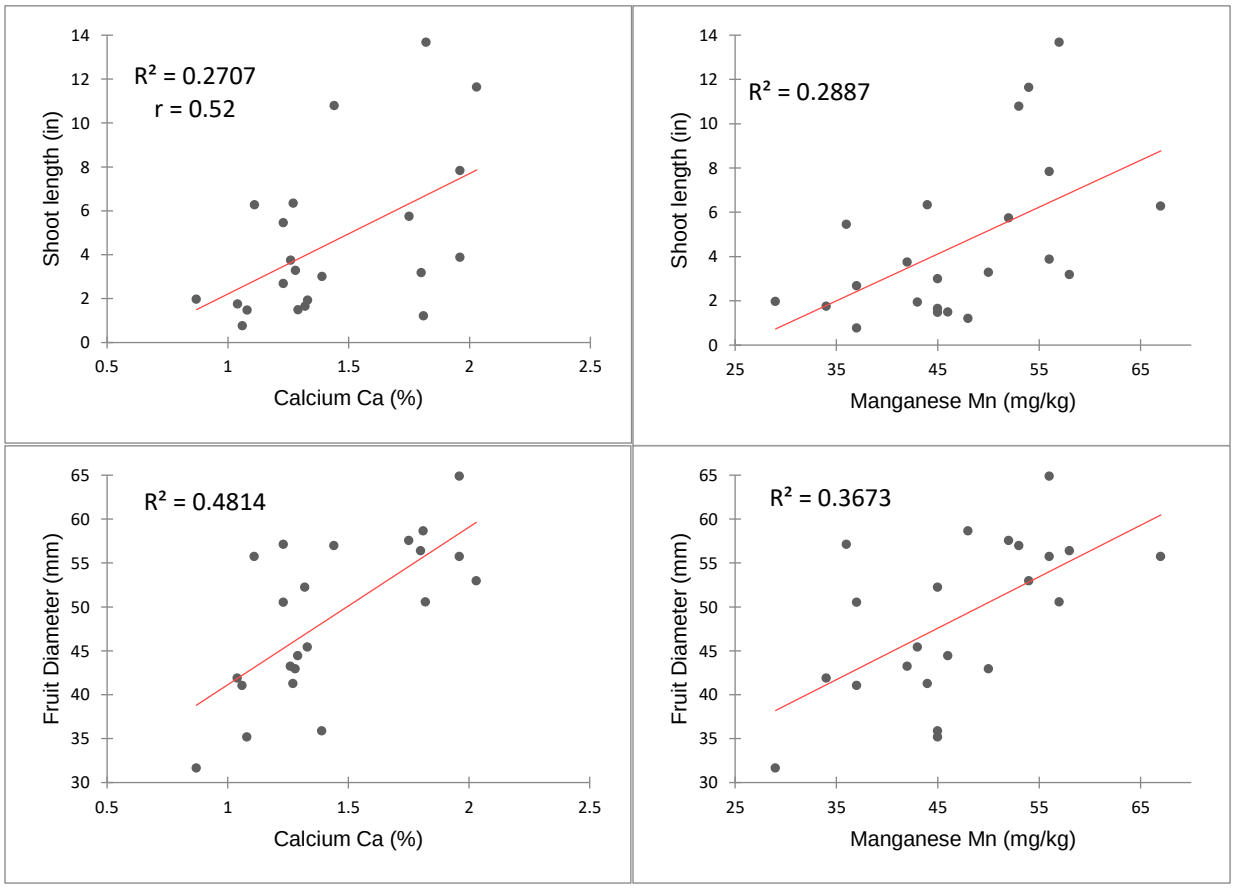

Figure 4. Correlation between shoot growth (top) and fruit diameter (bottom) with Ca (left) and Mn (right) in Grandview 'Honeycrisp' orchard. Where 'R^2' indicates coefficient of determination and 'r' indicates correlation.

Fruit BP incidence was negatively related with N (r = -0.4), P (r = -0.63), S (r = -0.40) and Mn (r = -0.47), thus only strong correlation with P leaf levels. When P levels were above 0.2%, BP incidence was less than 10%. While these relations do not imply cause - effect relationships, they provide insightful information regarding overall conditions where fruit quality was superior. Cracking incidence was weakly correlated with N (r = 0.48), while strongly correlated with B (r = -0.60). Trees with tissue levels above 46 mg/kg, had less than 11% cracking (Figure 5)

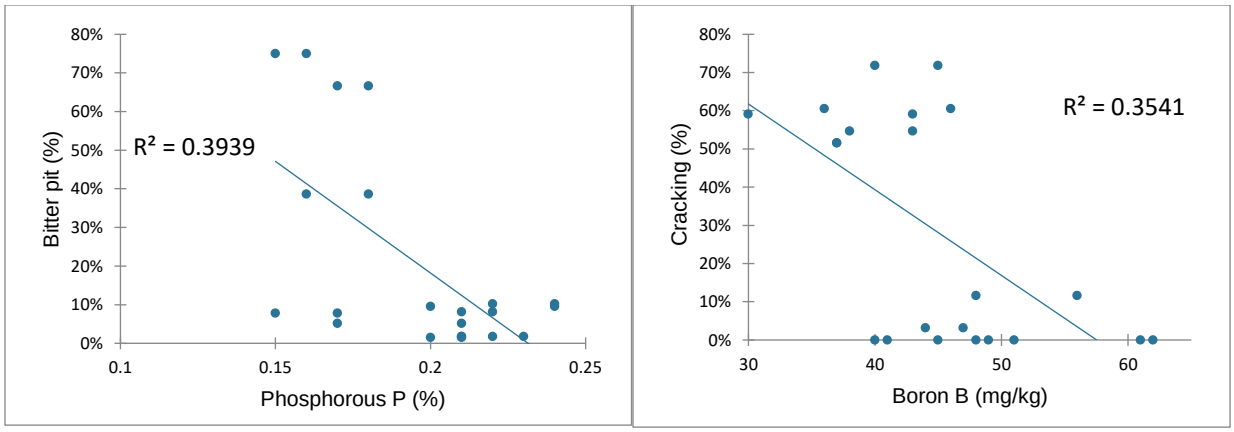

Figure 5. Correlation between bitter pit and leaf P concentration (left) and between cracking and leaf B concentration (right) in Grandview 'Honeycrisp' orchard. R^2 refers to the coefficient of determination, r refers to the correlation.

In agreement with the literature, the most consistent and predictive sampling times were June 28 and July 28th. The later sampling dates were consistent, but only for mobile elements N, P, K. Thus, leaf tissue analysis, collected once per year, from recently mature leaves between June and July, provided useful information to monitor nutrient uptake and deficiencies. However, leaf analyses alone will not inform about causes, nor management.

3. *Soil chemical analysis*

Soil chemical conditions were significantly different among sites, with close association to soil texture, effective depth, and presence of impermeable layers (rock or caliche). Soil pH fluctuated in about 1 point within each site throughout the season averaging 6.4 on S1, 6.7 on S2, 7.7 on S3 and 6.0 on S4. Being consistently higher in S3, with no differences between S1, S2 and S4. Higher pH in S3 is associated to $CaCO_3$ in depth. In agreement, soil available Ca was also significantly higher in S3 across all dates, a condition almost identical to 2021 (Figure 6). Higher available Ca was reflected in higher Ca in leaves (data not shown). Soil available Mg, was stable across the season, ranging from 1.4 and 3.7 meq/100g, being highest in S2 and S3 (data not shown).

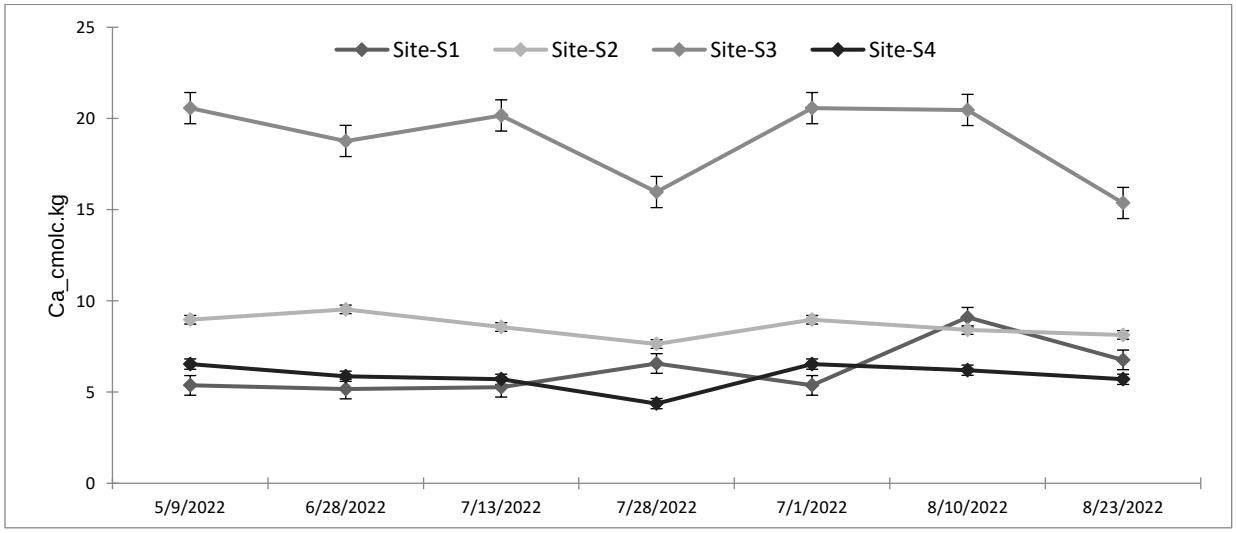

Figure 6. Soil available Ca throughout the growing season in Grandview orchard sites S1, S2, S3 and S4.

Mobile nutrients such as NH4 and NO3, were variable throughout the season, and positively correlated to with irrigation and temperature conditions, with no difference or inconsistent differences between sites (data not shown). Thus, inadequate indicators of N availability, fruit quality or vigor.

Soil K ranged between 222 and 702 mg/kg (Figure 7), considered above adequate range for 'Honeycrisp'. Here S4 had the highest levels for most of the season, followed by S2. However, leaf K was higher only in S2, which could suggest uptake limitations in S4. In both years, K values obtained at the beginning (May 9th) or at the end of the season (Aug 23rd) were better predictors of site conditions and fruit quality differences.

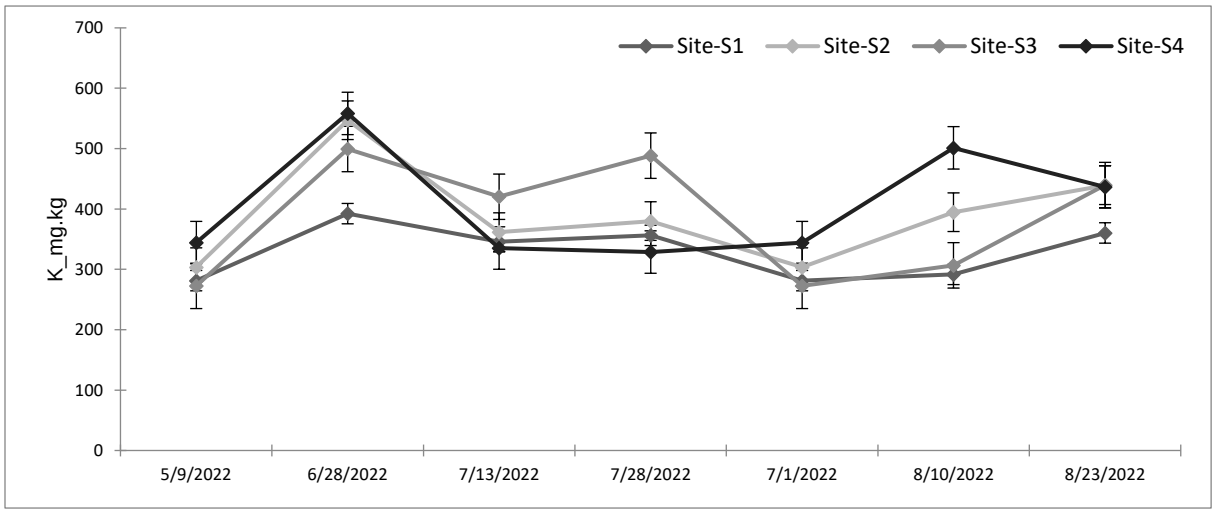

Figure 7. Soil available K throughout the growing season in Grandview orchard sites S1, S2, S3 and S4. Dots corresponding to mean values for site x date. Error bars correspond to standard error.

Similarly, soil available P (Olsen-P) was consistently higher in S4, with levels above recommended (> 40 mg/kg). Surprisingly, it varied throughout the season, however consistent within site (Figure 8). And despite being higher in S4, leaf uptake was higher in S2. Reinforcing possible limitations in uptake.

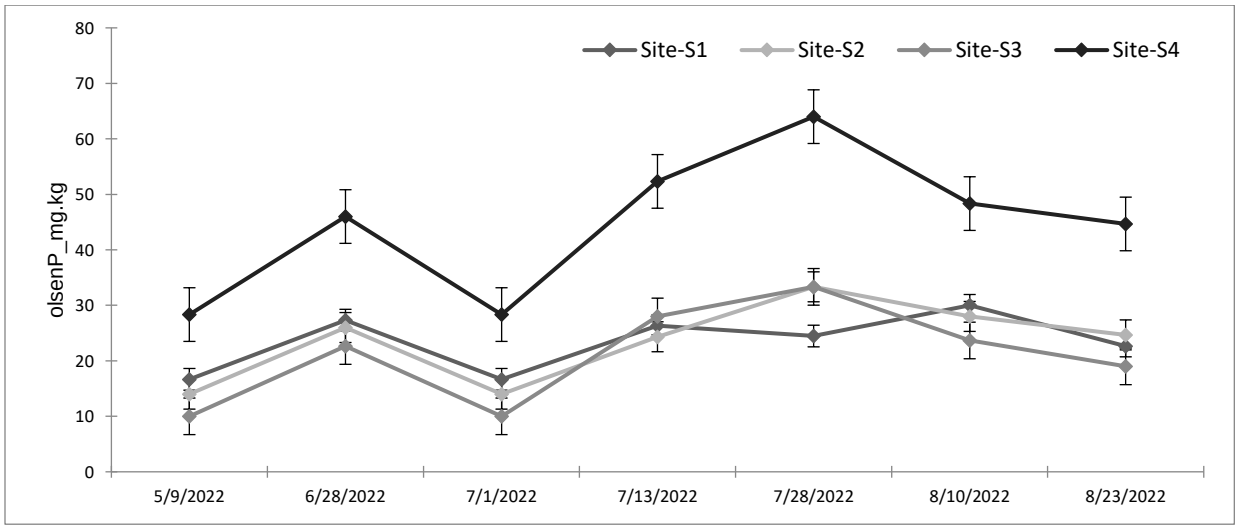

Figure 9. Soil Olsen-P throughout the growing season in Grandview orchard sites S1, S2, S3 and S4. Dots corresponding to mean values for site x date. Error bars correspond to standard error.

Soil Zn, Mn, Cu were adequate and equivalent in all sites for most of the season, while B was generally low (0.09 and 1.3 mg/kg) being higher in S3 and lowest in S1. Soil micronutrients did not correlate with leaf micronutrient uptake (data not shown).

Fruit diameter and weight had weak correlations (-0.65 > r < 0.65) with soil chemical condition. While BP there was a strong negative correlation between BP and soil pH (Figure 10) and Mg (r = -0.73), while strong positive relation with P-Olsen (Figure 10) and Fe (r = 0.76). However, these relations were observed in two or three dates throughout the season, being stronger on June 28th and July 28th.

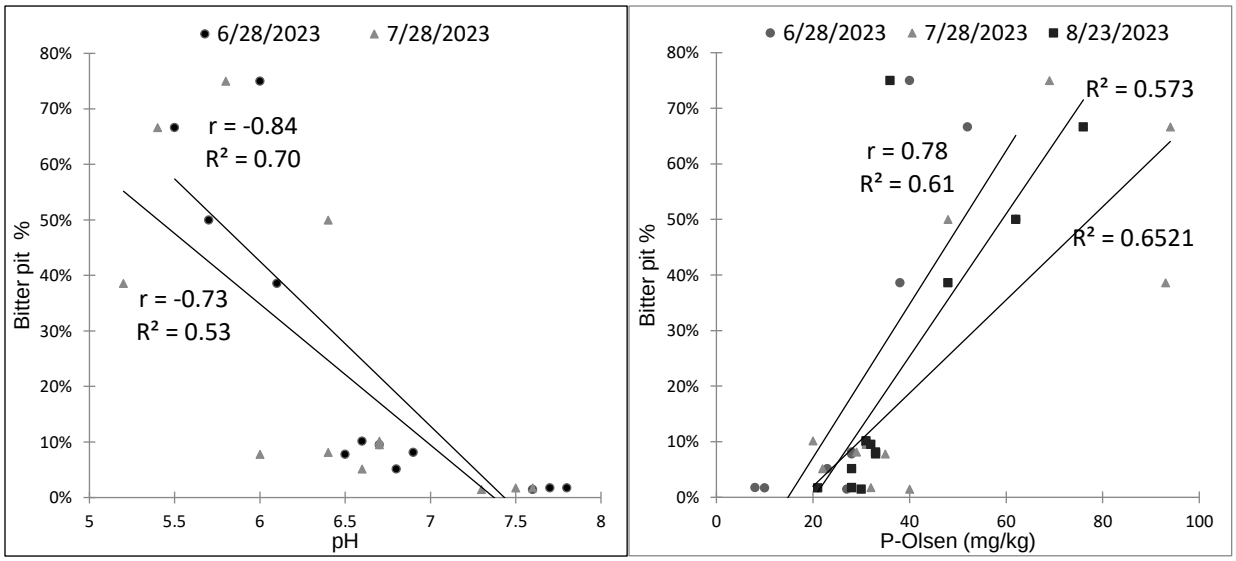

Figure 10. Significant ($p < 0.001$) and strong correlation between bitter pit incidence and (a) soil pH, and (b) available P (P-Olsen) observed on (●) June 28th, (▲) July 28th and (■) August 28th.

Similarly, cracking incidence also correlated significantly and strongly, however in opposite direction with pH (r = 0.79), P-Olsen (r = -0.73), Zn (r = 0.77) and Ca (r = 0.77). But again, not always, with stronger correlations observed when samples were collected on June 28th or end of the season (August 23rd) (data not shown).

4. Soil Health indicators

In 2022 we included additional soil health indicators that relate to biodiversity and habitat capacity of the soil. Soil respiration increased 40% throughout the season, being higher in S3 for most of the season (Figure 11). Interestingly, anaerobic nitrogen was also significantly higher in S3 throughout the growing season (data not shown).

Total N, C, O.M, were strongly correlated throughout the season, and differences between sites were observed only in July and August, where S3 had higher levels compared the other sites. Oddly, ACE protein, another indicator of microbiological activity, was lowest in S3. POX-C and mineralizable C were contradictory and inconsistent throughout the season (data not shown).

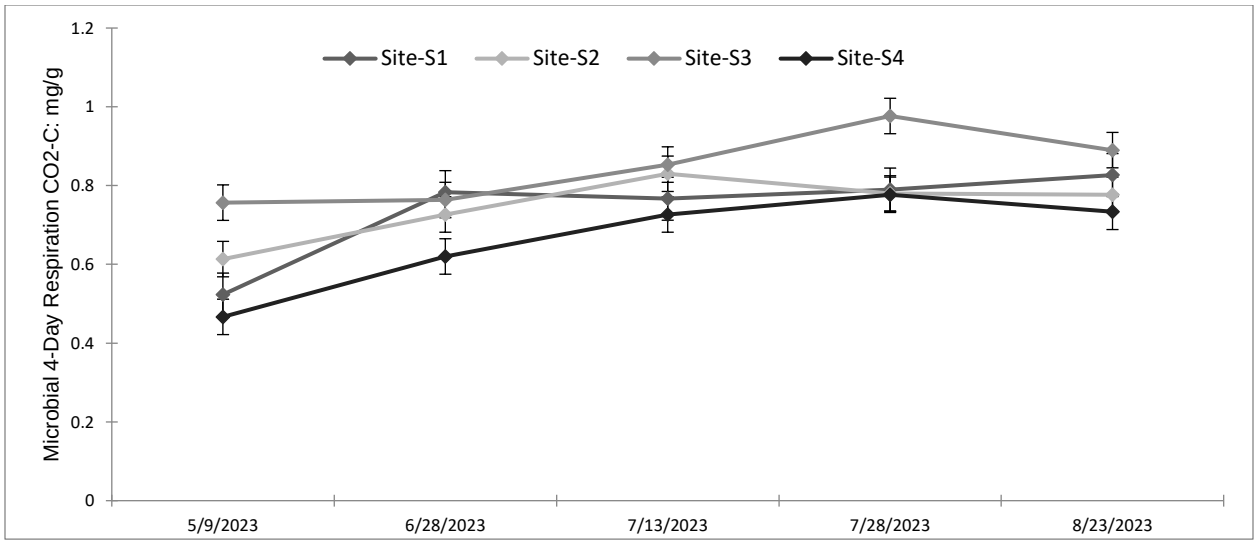

Figure 11. Soil microbial 4-day respiration (CO2-C:mg/g) in S1, S2, S3 and S4, Grandview orchard. Dots corresponding to mean values for site x date. Error bars correspond to standard error.

5. *Correlations between methods and laboratories*

When comparing the two laboratories that conducted the standard test, values were strongly correlated (r > 0.70) for most elements; pH, M.O, NO3, P-Olsen, K, Ca, Mg, Zn, Cu, S, B and POX-C, except for NH4, Na and Mn. (Figure 12). Likewise, the standard test was strongly correlation with the resin test (PNS) (r > 0.56) for NO_3, NH_4, K, Ca, Mg, S, Zn, Fe and Cu under loamy soils, while in sandy soils only NO_3, Ca and K, were correlated (Figure 13).

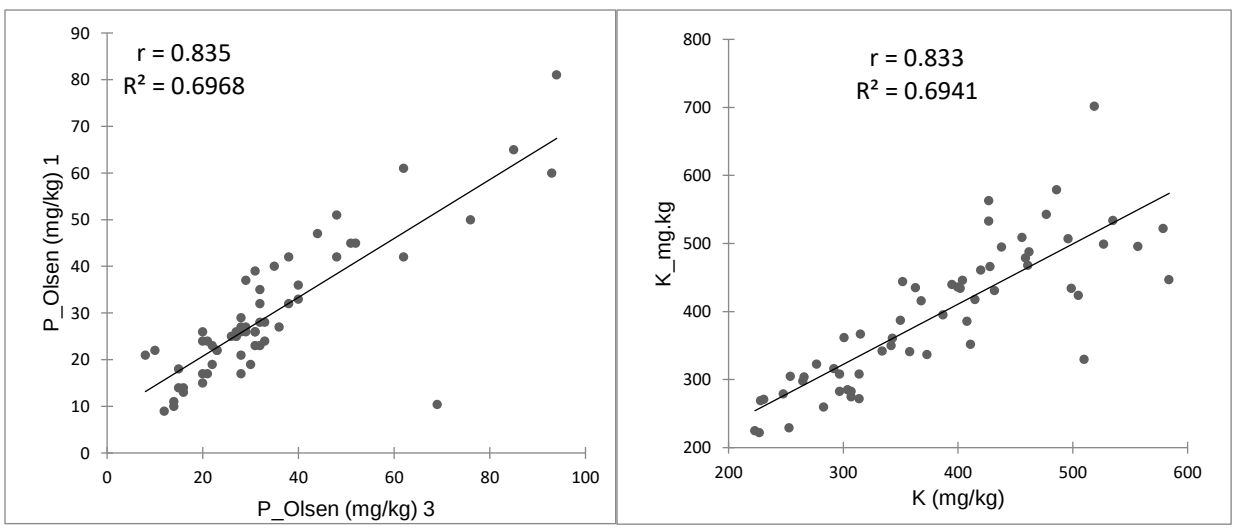

Figure 12. Correlation between laboratories for a) P_Olsen and b) extractable K.

The strong correlation between laboratories utilizing same methodology suggests confidence and accuracy, and values should be comparable. While, although strongly correlated, the resin test uses a different method, thus absolute values are not comparable.

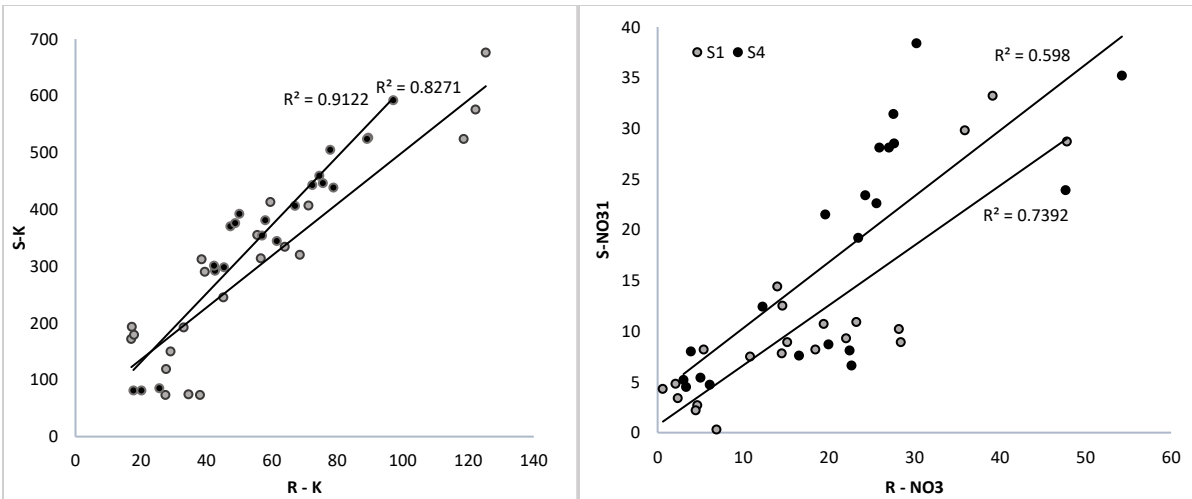

Figure 13. Correlation between a) standard K and resin K and b) standard NO3 and resin NO3, in sandy soil (S1) grey circle and in silt loam soil (S4) black circle.

2. Investigate tools to assess and map soil variability.

SoilOptix® provided 27 variability maps of special information: altitude, physical parameters: Sand, Silt, Clay (Texture), PA water, and chemical parameters; O.M, CEC, pH, NO3, P, K, Ca, Mg, Mn, Fe, Cu, B and salts. When correlating with ground truth values obtained closest to the mapping date, there were positive but weak correlations with pH (r = 0.52), K (r = 0.50) and NO_3 (r = 0.52), and positive strong correlations with O.M. (r = 0.81), Ca (r = 0.95), Mg (r = 0.88) and B (r = 0.85). However, when correlating across for different timings, only Ca (r = 0.95), Mg (r = 0.74), B (r = 0.93) and CEC (r = 0.65) remained significant and strong. The rest of the elements had no correlation or weak with SoilOptix® mapping. In addition, SoilOptix® provided a useful tool to map relative differences, however absolute values were different, thus should not be used for fertilizer recommendations.

The E.C mapping provided three levels of E.C across the orchard (Figure 14), where red is lowest (23.5 – 28.2), yellow intermediate (28.2 – 30.2) and green high (30.2 – 34.8). Here, S1 and S4 were rated low and S2 and S3 were rated intermediate. This relative difference was observed during June 13th, but not the rest of the season. Given that EC is variable and will change in response to irrigation events, the EC should be interpreted accordingly.

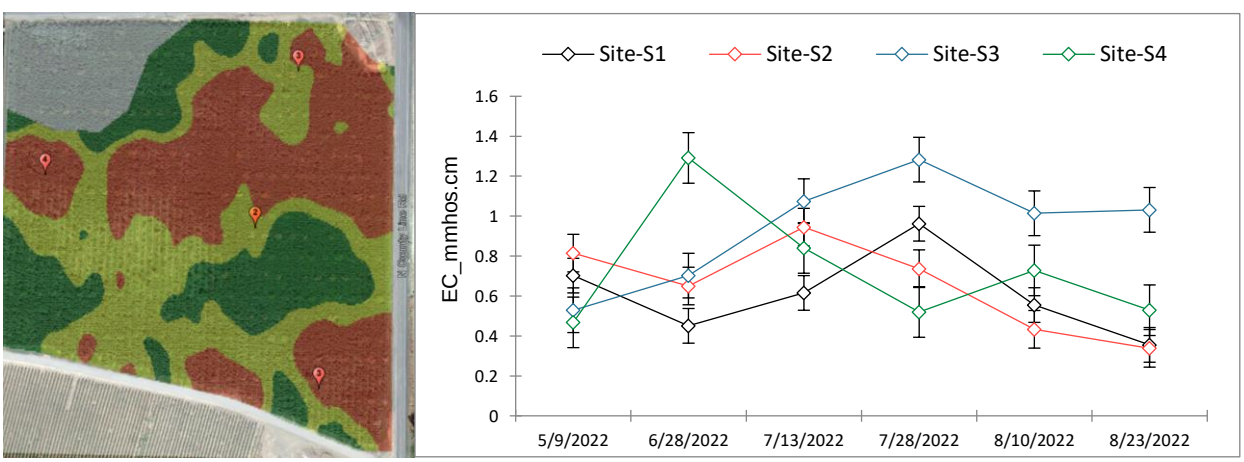

Figure 14. Soil E.C map (left) and E.C readings throughout the season in S1, S2, S3 and S4.

The Soil survey service (SW), provided the greatest amount of information, including elevation, parental materials, ecological, physical, chemical, and biological indicators, water content and availability at field capacity and wilting point, among others. The survey divided the block in five zones; Hezel (1.6%), Quincy (1.4%), Starbuck (12.7%) and Warden (72.4%) (Figure 18). These series were present when evaluating the soil profile, however the area allocated to each unit were inaccurate. Depending on the location, the scale of the information and mapping vary between 1:20,000 to 1:24,000, thus macro scale that needs in situ verification.

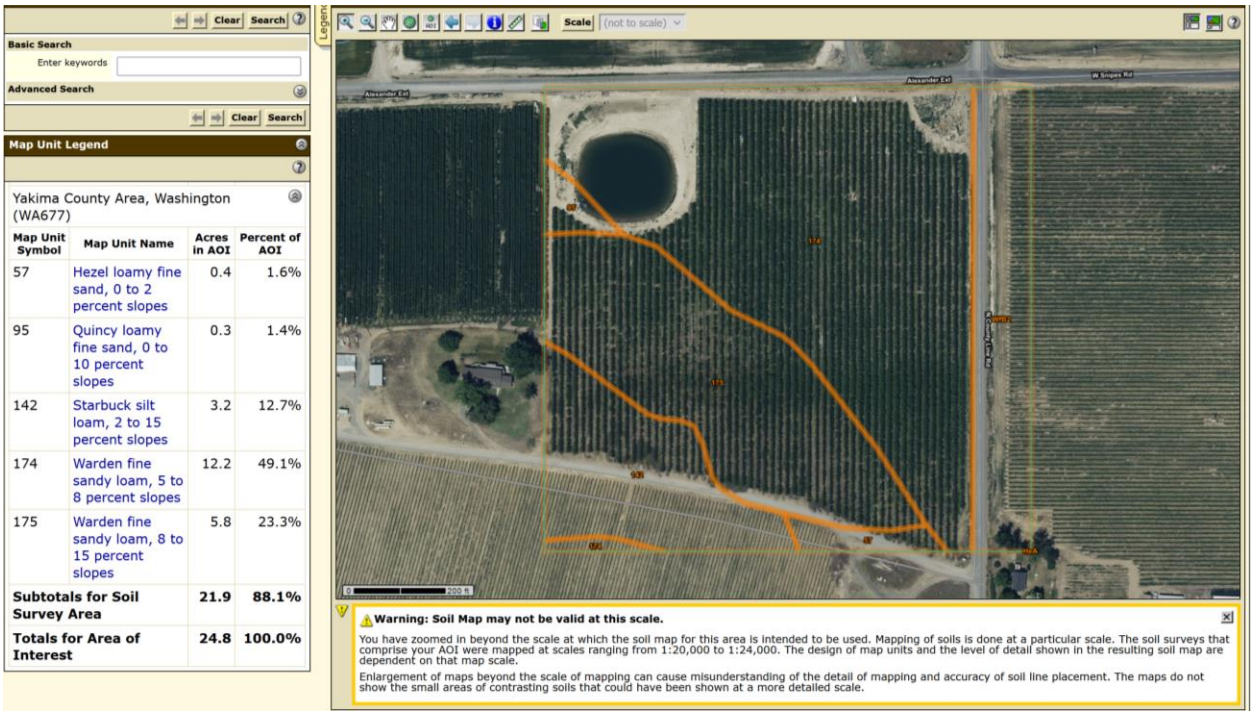

Figure 15. Web soil survey mapping system (USDA) for Grandview site.

Other aerial mapping strategies provided single layers of information associated to tree vigor: water use, evapotranspiration, canopy density, etc. with similar area aggrupation (variability). While all mapping were predictive of vigor differences, no mapping tool could predict fruit size, diameter, or bitter pit incidence, not the rate needed for nutrient application. More detailed comparison between mapping tools were reported in our previous report and it will be summarized in upcoming newsletter article.

3. Contribute to the "smart orchard initiative" in the area of soil nutrient sensing and management.

In 2021 and 2022 we participated in four field days, two in English and two in Spanish. We had over 170 participants, where surveyed individuals indicated they valued the information (95%), and 50% indicated knowledge gain.

EXECUTIVE SUMMARY

Title: Reliable Soil Diagnostic Technology for Smart Nutrient Management

Key words: Soil mapping, soil health, bitter pit.

Abstract:

A smart orchard project was implemented in Chiawana Orchards in 2020 in collaboration with several industry and university partners. This collaboration initiated a system that enabled the assessment and ground truthing of conventional and new technologies. Under the umbrella of the smart orchard initiative, this project focused on technology for soil chemical management for reliable diagnosis.

Soil physical and chemical testing has been used for more than a century to guide nutrient management practices. Today, new technology could provide opportunities for precision and remote management. Our goal is to develop "smart nutrient management" strategies based on quantifiable needs. For these, our specific objectives were to: a. *Characterize different soil testing methods/technologies and their relationship with plant response, b. Investigate tools to assess and map soil variability and c. Contribute to the "smart orchard initiative" in the area of soil nutrient sensing and management.*

This project was conducted in two commercial apple orchards: Chiawana 'Gala' orchard and Grandview 'Honeycrisp' apple orchard. Within each orchard we selected four distinct sites, based on the historical vigor and productivity, two sites were low vigor, and two high vigor. In 2022, Grandview 'Honeycrisp' fruit load was 4.4 and 2.2 times higher in S3 compared to S4 and S1. Bitter pit incidence was lowest in S3 (2%) and highest in S4 (52%). Cracking was also significant in 2022, with 64% in S3 and no cracking in S4. Based on yield, size and culls, S2 was the most productive site, while S3 the least. Leaf N, Ca, Mg, Mn, P and K were good indicators of growth and quality differences among sites. Fruit diameter was strongly correlated with leaf Ca and Mn. BP incidence correlated strongly with P and cracking with B. Soil chemical, physical and biological indicators were significantly different among sites. In general, S3 had higher pH, Ca, Mg, M.O and microbiological activity, while it was also one of the most limited in terms of fruit quality. Soil K and P were excessive in the soil in S4, while adequate in leaves, which suggests uptake issues in the root zone, that could explain BP incidence in S4. BP incidence correlated negatively with soil pH and positively with P-Olsen, however this relation does not imply cause effect, rather provided information regarding limiting conditions at a root level.

When comparing different tools, there was a strong correlation between laboratories and soil testing methods. Aerial mapping tools provided equivalent maps for vigor distribution, evapotranspiration, and canopy density. While SoilOptix® provided with a more precise variability map for soil texture, Ca, Mg, CEC and B. However, SoilOptix® did not correlate well with the absolute values reported. Thus SoilOptix ® should be utilized for mapping relative differences, not for determining nutrient availability or management. E.C mapping correlated well electric conductivity of the soil, at one time, thus should be timed properly, preferable, at the beginning of the season.

None of the tools were good predictors of tree productivity, health, and fruit quality on their own. However, the integration of tools; mapping, soil test and tissue samples, provided insightful information related to differences in the block and possible causes. Here, soil profile analysis was key to understand the cause of high vigor and quality disorders.

Project Title: Managing Ca-related disorders in high vigor conditions

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Cooperators: Poliana Francescatto (Valent), Casey Hubs (Hancock Farmland), Nicolas Newhouse, Matthew Whiting.

Report Type: Continuing Project Report – Year 2

Project Duration: 3 Years

Total Project Request for Year 1 Funding: 13,728
Total Project Request for Year 2 Funding: 14,041
Total Project Request for Year 3 Funding: 14,366

Other funding sources: **Awarded**
Amount: $152,938
Agency Name: 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.

Other funding sources: **Awarded**
Amount: $15,000
Agency Name: Valent BioScience
Notes: Costs associated with product supply and wages for hourly support and data collection will be supported by this project.

Organization Name: Washington State University **Contract Administrator:** Anastasia Mondy
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Station Manager: Naidu Rayapati **Email address:** naidu@wsu.edu

Item	2021	2022	2023
Salaries	5,800	6,032	6,273
Benefits	2,028	2,109	2,193
Wages[1]			
Benefits			
Equipment			
Supplies[2]	5,900	5,900	5,900
Travel			
Miscellaneous			
Plot Fees			
Total	**13,728**	**14,041**	**14,366**

Footnotes: [1] Salary for 50% FTE for 4 month per year for Juan Munguia, Research Assistant at Sallato's laboratory for trial establishment, collecting data, processing fruit in the laboratory and organizing extension activities. [2] Supplies include laboratory analysis and processing of samples for nutrient test (total of 4 soil initial test at 35 USD and 320 leaves and apple nutrient test at 18 USD/sample).

OBJECTIVES

1. Evaluate and manage Ca related disorders utilizing vigor controlling products: Prohexadione-Ca (Apogee ®), ABA (Protone ®) and summer root pruning during fruit enlargement in Honeycrisp and Cosmic Crisp® apples.

Plant growth regulators (PGRs) were evaluated in two Honeycrisp and two WA 38 orchards. PGRs included: Auxin (NAA), Prohexadione-Ca (ProCa), abscisic acid (ABA) and Gibberellic acid (GA_3).

2. Analyze the effect of these products on reducing plant and fruit stresses to effectively control Ca related disorders in WA apple growing regions

We evaluated the effect of the treatments in stem water potential, shoot and fruit growth, final fruit yield, crop load, fruit size, diameter, disorders, and nutrient levels in the flesh.

3. Develop and distribute new strategies to manage Ca related disorder for excessively vigorous conditions.

Preliminary results were shared in grower meetings (Pom Club, Columbia Basin tree fruit Club , WA 38 field day (in Spanish) and at the 118th WSTFA annual meeting news flash.

SIGNIFICANT FINDINGS

- In 2022, HC_1 had four and six times more at harvest and post-harvest BP respectively, compared with 2021. In contrast, HC_2, BP at harvest and after storage were 48% and 89% lower in 2022, with levels about half of those in HC_1.
- In Honeycrisps orchards, post-harvest BP levels where higher in the ABA treatments when compared with ProCa (lowest in BP incidence in HC_1) and with NAA and GA (lowest in BP incidence in HC_2). In contrast, in 2021, BP at harvest and after storage were lower with the ABA treatment (previous report).
- In WA 38, GS incidence ranged between 2.9 and 9.9% in CC_1, being the highest in GA_3 (9.9%) and lowest in ProCa (2.9%) treatments. In CC_2, GS incidence varied between 3.2 and 16.9%, being highest in the NAA treatment, when compared with the control, the ABA and GA_3, with no difference with ProCa.
- WA 38 orchards had higher splits incidence in 2022, when compared to 2021, ranging between 19 and 37%. In CC_1, split incidence was 73% higher in the ProCa treatment. In CC_2, split incidence was 44% higher in the control, compared to the PGR treatments.
- Fruit firmness in HC_1 was slightly higher in GA_3, while in HC_2 firmness was higher in the ABA treatment. Similarly, in CC_1 (WA 38), fruit firmness was 1.3 lbs. higher in the ABA treatment, while unaffected in CC_2.
- In HC_2, crop load was reduced in the NAA when compared with the other PGR treatments, but not different from the control (8.8 fruit/TCSA). In CC_1, crop load varied between 2.4 and 5.1 fruit per cm^2 (TCSA), being lowest in the control and highest in the ProCa treatment.
- In CC_2, crop load ranged between 1.5 and 2.7 fruit per cm^2 (TCSA), being lowest in the NAA treatment, compared with the ABA and GA_3, with no differences with the control.
- In 2022, fruit nutrient levels were significantly lower in N, Ca, Mg, S and micronutrients, and higher in P and K concentration, compared to 2021. Leaf Ca concentration was consistently lower in 2022 compared with 2021, regardless of cultivar, site or treatment.
- In HC_1, higher BP treatment (ABA) had reduced Ca and Zn concentration compared with ProCa (lower BP incidence). Interestingly, Ca concentration in leaves were lowest in the GA_3 treatment when compared with ProCa and the Control.

- In HC_2, only Ca concentration was related to BP incidence, being higher the ABA (high BP) and lowest in the ProCa, GA_3 and NAA treatment.
- It is apparent that weekly treatment with ABA had a stabilizing effect on tree water status, though the benefits were not manifest as any reduction in green spot incidence, but fruit firmness was improved.

METHODS

The project is being conducted in two vigorous Honeycrisp orchards and two vigorous WA 38 orchards, selected due to high incidence of Ca-related disorders during 2020 and above average shoot growth. The 'Honeycrisp' trials (HC) were a 'Honeycrisp' grafted over Fuji/M26 orchard located near Grandview (HC_1), trained with four leaders per graft on a V trellis. And a 'Honeycrisp' on M9-337 located near Prosser (HC_2), planted in a 5 x 10 ft. spacing, with three vertical leaders spaced 20 inches apart on a vertical wall. The WA 38 trials (CC) were conducted in a commercial WA 38 on G41 orchard (CC_1) planted in 2019 located near Prosser, on a V trellis at 2.5 x10 ft (5 ft each side of the trellis) and trained with three vertical leaders, 20 inches apart. And in the WSU research orchard Roza; a WA 38 on G41 (CC_2) planted in 2013 located near Prosser, at 3 x 12 ft on a vertical spindle system. All the orchards are on a silt loam soil, associated to the Warden soil series, characterized by alkaline soils (pH above 7), with the presence of $CaCO_3$ (Caliche) at variable depth, and high water holding capacity.

1. *Evaluate and manage Ca related disorders utilizing vigor controlling products.*

Plant regulators were applied to 10 randomly selected replicate trees (n=50) per site and cultivar according to Table 1. The treatments were applied to the tree foliage, utilizing a powered backpack sprayer (controlled flow) to full coverage. Control trees were sprayed with the same water utilized in the solution.

Table 1. Plant regulator treatments.

Treatments	Concentration	Timing	Reference
NAA (*ProMaxa,3.5% Valent Bioscience*)	5 ppm a.i. 1.42 ml/10L	Honeycrisp: Every week, starting five weeks prior to harvest. (approx. 30-45-60 DAFB)	Banguert, 1979
		WA 38; Every week, starting ten weeks prior to harvest. (30-45-60 DAFB)	
ABA *(Protone ®, 20% Valent BioScience)*	400 mg ai/L 20 g/10L	Honeycrisp: Every week, starting five weeks prior to harvest. (approx. 125 DAFB)	Falchi, et.al, 2017.
		WA 38: Every week, starting ten weeks prior to harvest. (approx. 160 DAFB)	
Prohexadione-Ca *(Apogee ®, 27.5%)*	300 mg ai/L 10.9 g/10L	Honeycrisp: Every week, starting five weeks prior to harvest. (approx. 125 DAFB)	Amarante et.al, 2020
		WA 38: Every week, starting ten weeks prior to harvest. (approx. 160 DAFB)	

GA_3 (*ProGibb®, Valent Bioscience)*	300 mg a.i. /L 7.50 g/10L	Honeycrisp: Every week, starting five weeks prior to harvest. (approx. 125 DAFB)	Amarante et.al, 2020
		WA 38 : Every week, starting ten weeks prior to harvest. (approx. 160 DAFB)	
Control	Water	Same dates as above	-

Evaluation
At harvest, each replicated unit (n=10) were individually assessed for total fruit per tree, total fruit weight, trunk diameter to calculate crop load as the total number of fruit per trunk cross sectional area (TCSA), and at harvest for bitter pit (H_BP%), or green spot (H_GS%). The fruit were taken to the laboratory to determine fruit weight, diameter and other defects, splits, cracks, sunburn, etc. For Honeycrisp trials, fruit were stored at 39 F, for six weeks. After storage, fruit were evaluated for post-harvest BP incidence (PH_BP%).

From each sample unit, a subsample of representative healthy (not pitted fruit) were selected for dry matter and chemical analysis: N, P, K, Ca, Mg, Cu, Mn, Fe, Zn, B.

2. *Analyze the effect of these products on reducing plant and fruit stresses.*

To assess stress, trees were monitored throughout the season including fruit and shoot growth, leaf tissues analysis, stem water potential, stomatal conductance, and leaf temperature. Stem water potential was measured in two to four sun-exposed leaves per tree. Leaves were enclosed before detaching from the tree in aluminum ziploc bags for 30 min to reach equilibrium. Leaves were collected and stem water potential was determined immediately with a pressure chamber (Scholander et al., 1965). Measurements were collected the day before the treatment application, then two and three days after the treatment application.

The experimental data were analyzed using analysis of variance (ANOVA) followed by a Tukey's multiple range test, for mean separation of significant treatment effects. All the analyses were done using XLSTAT software.

3. *Develop and distribute new strategies to manage Ca related disorder for excessively vigorous conditions.*

New findings have been shared with WA growers in two events in 2022; Roza WA 38 field day Spanish (total 32 attendees), and at the WSTFA annual meeting news flash by Juan Munguia de la Cruz, Research Assistant in Sallato's program and M.S. student in Whiting's program.

RESULTS

1.1 Evaluate and manage Ca related disorders utilizing vigor controlling products.

Honeycrisp trials

In 2022, HC_1 had four times more at-harvest BP compared with 2021, averaging 41%. In 2022 there were no differences in BP among treatments. Post-harvest BP was also higher in 2022, ranging between 19 and 59%, six times higher than in 2021. This season, post-harvest BP levels where 40%

higher in the ABA treatment, when compared with ProCa (lowest in BP incidence), however not different from the control (Table 3). Note that in both years the variability of BP at harvest was very high, ranging 0% to 65% for individual replicate trees within a treatment (data not shown), similar to 2021, which might explain the lack of significance in BP levels at harvest.

In contrast, HC_2, BP at harvest and after storage were 48% and 89% lower in 2022, compared to 2021 (data not shown), with levels about half of those in HC_1. Similar to HC_1, there was no effect of PGR treatments on BP at harvest. Oddly, PH_BP was lower for each treatment and highest in trees treated with ABA while NAA and GA_3 treated trees had the lowest BP incidence (Table 3). In contrast, in 2021, BP at harvest and after storage were lower with the ABA treatment (previous report).

Table 3. Fruit quality parameters in two 'Honeycrisp' apple orchards treated with plant growth regulators. Different letters indicate significant differences ($p < 0.1$) among treatments within an orchard. Statistical probability model determined with Tukey (XSLTAT, Andisoft).

Orchard / Treatment		Fruit Firmness (Lb)	Fruit Weight (g)	Fruit Size (mm)	H_BP[a] (%)	PH_BP[b] (%)	Splits (%)
HC_1	Control	19.2 b	173	74	47%	59% ab	7%
	GA_3	20.0 a	174	74	41%	53% ab	9%
	ABA	19.2 b	180	75	38%	69% a	10%
	ProCa	19.8 b	172	73	38%	49% b	12%
Pr > F(Model)		0.075	0.809	0.300	0.487	0.102	0.310
HC_2	Control	16.5 b	204	78	23%	6% ab	9%
	NAA	16.2 b	208	77	17%	3% b	18%
	ABA	17.6 a	201	77	15%	14% a	18%
	GA_3	16.7 b	192	75	14%	3% b	17%
	ProCa	16.6 b	203	77	20%	10% ab	13%
Pr > F(Model)		0.001	0.648	0.478	0.309	0.047	0.149

a Percent bitter pit at harvest out of total fruit harvested.
b Percent bitter pit after six months of storage (39 F), out of all stored fruit.

Fruit firmness in HC_1 ranged between 19.2 and 20.0 lbs., being slightly higher in GA_3, while fruit weight, size and other defects were not affected by the treatments. In contrast, in HC_2 firmness ranged between 16.2 and 17.6 lbs., being higher in the ABA treatment (Table 3). In HC_2, fruit weight, size and splits were also unaffected by the treatments. Regardless of the treatments, cracks or splits were 3 times higher in 2022 compared with 2021 (data not shown).

Regarding tree growth indicators, overall shoot growth was higher in 2022 than in 2021 (data not shown). In 2022, the control in HC_1 had 18% greater shoot length (23 cm) compared to all other treatments which were similar (19 cm). While in HC_2, there were no differences among treatments in shoot growth (data not shown). Surprisingly, in none of the orchards did treatment with GA_3 increase shoot growth. Regardless of the treatments, both orchards had consistently higher shoot length compared to 13 'Honeycrisp' orchards we monitored in South Central WA (Kalcsits and Sallato, nutrient management project). For example, In HC_1 shoot length was 14.5 cm and 19.6 cm in 2021 and 2022, respectively, being 39% and 21% larger than the regional average. In HC_2, shoot length averaged 13 cm and 25 cm in 2021 and 2022, respectively, being 25% and 54% larger than the regional average.

Crop load in HC_1 was highly variable, ranging between 1.1 and 14.8 fruit per cm^2 TCSA, and only weakly correlated (r = -0.39) with BP incidence. In HC_2, crop load varied between 6 and 11.9 fruit per cm^2 TCSA, being lowest in the NAA when compared with the other PGR treatments, but not different from the control (8.8 fruit/TCSA) (data not shown).

Fruit nutrient levels (data not shown)
Fruit nutrient concentration was overall higher in HC_1 (with higher BP incidence), compared with HC_2, except for Zn (data not shown). The greatest differences were observed in the N, P, K, Ca, and B, with 16%, 19%, 31%, 18% and 19% higher concentration, respectively. We observed several differences in fruit nutrient concentration among treatments, in this report I focus on those that were related to BP differences. In HC_1, fruit with highest post-harvest BP incidence (ABA) had reduced P (0.07 %) and reduced B (21.4 mg/kg) concentration, compared with low BP incidence (ProCa). In contrast, in HC_2, high BP incidence (ABA) treatment had lower N concentration (0.29%) compared with NAA (lowest BP incidence), but not different from GA_3, also with lowest BP incidence. Fruit Ca levels were unrelated to BP incidence, however in both sites, fruit Ca concentration were lowest (below 0.04%) in the GA_3 treatments, compared with the control. Fruit nutrient ratios (N:Ca, K:Ca and K+Mg/Ca) were unrelated to BP incidence.

WA 38 trials
In CC_1, GS incidence was fairly low and ranged between 2.9 and 9.9%, being the highest in GA_3 (9.9%) and lowest in ProCa (2.9%) treatments. In CC_2, GS incidence varied between 3.2 and 16.9%, being highest in the NAA treatment, when compared with the control, the ABA and GA_3, with no difference with ProCa (Figure 1).

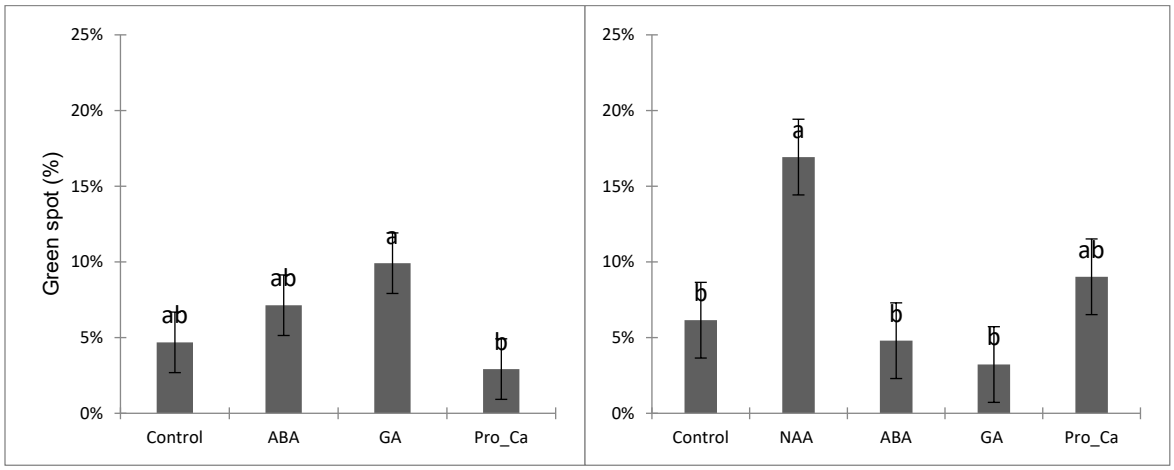

Figure 1. Green spot incidence in WA 38 orchards CC_1 (left) and CC_2 (right) in 2022 treated with PGRs. Different letters above the bars indicate significant differences $p < 0.05$ (Tukey test) within a site. Error bars correspond to the standard error.

Splits and cracks were widely observed in 2022 WA 38 orchards. Here, both WA 38 orchards had higher splits incidence compared to 2021 (data not shown). In CC_1, splits ranged between 19 and 33.3%, being 73% higher in the ProCa treatment, compared with the control (Figure 2). In CC_2, splits incidence ranged between 20 and 37%, with 44% higher incidence in the control, compared to the PGR treatments (Figure 2).

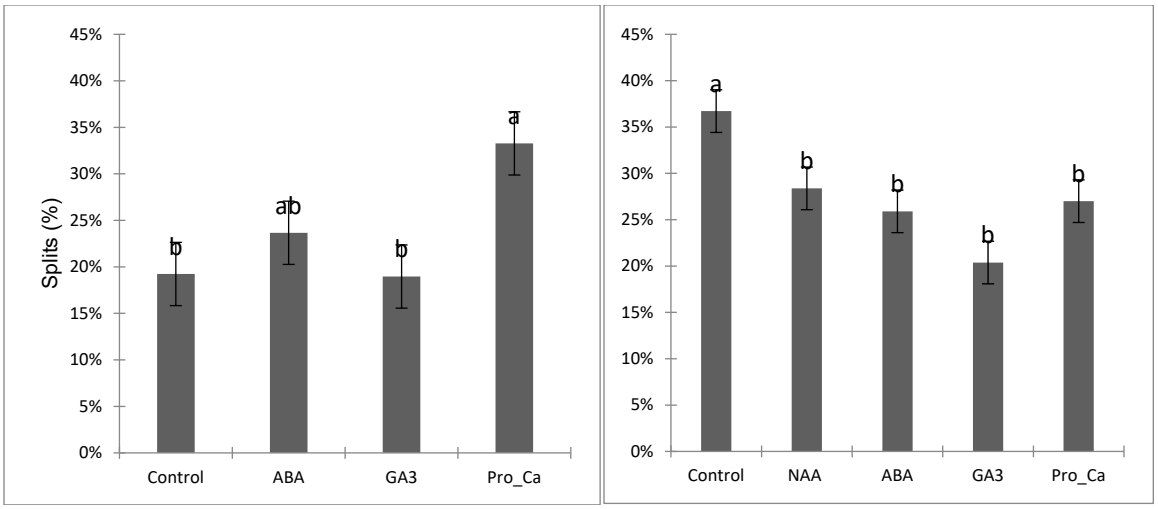

Figure 2. Incidence of splits or cracks in WA 38 orchards CC_1 (left) and CC_2 (right) in 2022, treated with PGRs. Different letters above the bars indicate significant differences $p < 0.05$ (Tukey test). Error bars correspond to the standard error.

In CC_1, fruit weight ranged between 272 and 291 g and of 79 mm of diameter, with no difference between treatments. While fruit firmness (between 20 and 22 lbs.) was 1.3 lbs. higher in the ABA treatment (similar to HC_2). In contrast, in CC_2, firmness and size were unaffected by the treatments, while fruit weight was 19% higher in the NAA treatment when compared with the ABA, however with no difference with the control.

In CC_1, crop load varied between 2.4 and 5.1 fruit per cm^2 (TCSA), being lowest in the control and highest in the ProCa treatment. In CC_2, crop load ranged between 1.5 and 2.7 fruit per cm^2 (TCSA), being lowest in the NAA treatment, compared with the ABA and GA_3, with no differences with the control (data not shown). Regardless of the treatments, crop load was only weakly related to GS % (r -0.27) or other fruit quality parameters.

Similar to 'Honeycrisp', shoot growth in 'WA 38' was higher in 2022, varying between 30 and 39 cm. In CC_1, shoot length in the GA_3 and in the control treatments were 26% larger than in the ProCa. In CC_2 there were no differences between treatments. Regardless of the treatments, shoot growth in 'WA 38' were 58 and 70% higher in CC_1 and CC_2 respectively, when compared with fifteen 'WA 38' orchards being monitored in South-Central WA (Kalcsits and Sallato, nutrient management project) reinforcing that these were relatively high vigor orchards.

Fruit nutrient levels (data not shown)
In 2022, fruit nutrient levels were significantly lower in N, Ca, Mg, S and micronutrients, and higher in P and K concentration, compared to 2021. Similar to 'Honeycrisp', fruit nutrient concentrations in 'WA 38' were different among treatments, however unrelated to GS differences. In this report I will focus on differences associated to fruit quality. In CC_1, the fruit with highest GS incidence (GA_3) had lower K, Ca and Mg concentration, compared with ProCa (lowest in GS). The opposite relation was observed for cracking incidence, where higher concentration of K, Ca, and Mg were observed in the fruit with highest split incidence (ProCa). In CC_2, fruit with higher GS (ProCa) had lower Ca and Mg concentration, while splits were unrelated to nutrient levels.

Leaf nutrient levels (data not shown)
Leaf Ca concentration was consistently lower in 2022 compared with 2021, regardless of cultivar, site or treatment. For example, Ca levels in 'Honeycrips' were 30 and 38% lower in HC_1 and HC_2,

respectively, and 50 and 72% lower in CC_1 and CC_2 respectively. In contrast, Zn levels were consistently higher in 2022 (data not shown). In HC_1, higher BP treatment (ABA) had reduced Ca and Zn concentration compared with ProCa (lower BP incidence). Interestingly, Ca concentration in leaves were lowest in the GA_3 treatment when compared with ProCa and the Control. In HC_2, only Ca concentration was related to BP incidence, being higher the ABA (high BP) and lowest in the ProCa, GA_3 and NAA treatment. In contrast, in WA 38 CC_1, high GS (GA_3) was associated to higher Zn and lower B concentration. In CC_2, nutrient levels in leaves were unrelated to GS or split differences.

2. *Analyze the effect of these products on reducing plant and fruit stresses.*

In CC_1, we measured stem water potential prior to several ABA treatments and again, 48 and 72 hours after those treatments. ABA treatment apparently reduced stress because on several sample dates stem water potential was significantly lower in untreated trees (Figure 3). This effect was particularly evident following treatments on 8 August and 23 August where stem water potential was low in untreated trees (ca. 20 bars, equivalent to -2.0 MPa) but unaltered (or slightly improved) in trees treated with ABA. This effect may be due to the role ABA plays in stomatal closure and maintaining tree water status. Overall, it is apparent that weekly treatment with ABA had a stabilizing effect on tree water status though the benefits of this were not manifest as any reduction in green spot incidence though fruit firmness was improved.

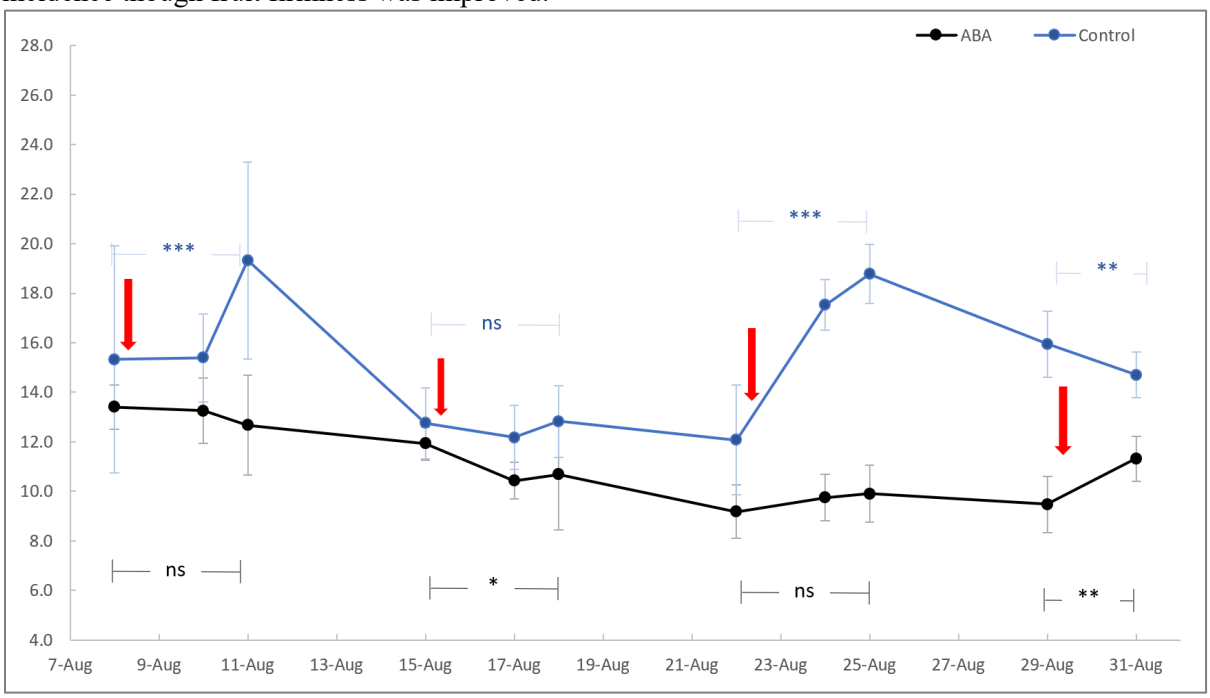

Figure 3. Trends in stem water potential for WA38 trees treated with ABA and untreated. Each arrow indicates the timing of ABA applications. Data are presented in bars (higher readings indicative of greater stress).

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

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City/State/Zip: Prosser/WA/99350

Cooperators: Many Washington State Honeycrisp and WA 38 producers

Report Type: Continuing Project Report

Project Duration: 3-Year

Total Project Request for Year 1 Funding: $ 81,270
Total Project Request for Year 2 Funding: $ 84,015
Total Project Request for Year 3 Funding: $ 86,871

Other related/associated funding sources: none

WTFRC Collaborative Costs: none

Budget 1
Primary PI: Lee Kalcsits
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Station Manager/Supervisor: Chad Kruger
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Item	2020	2021	2022
Salaries	43,200	44,928	46,726
Benefits	15,895	16,530	17,192
Wages	7,800	8,112	8,436
Benefits	1,735	1,805	1,877
Equipment			
Supplies	6,900	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

This project had three objectives aimed at improving fertilizer management and establishing thresholds on fertilizer applications for Honeycrisp and WA 38.

1. **Test how varying rates of N, K, and Mg affects fruit quality traits, disorder incidence, return bloom and tree vigor in Honeycrisp and WA 38 orchards.**
2. **Identify the relation between shoot growth, crop load, and nutrient concentration with disorder incidence for commercial orchards in WA State.**
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.**

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 decreased in incidence from 2020 to 2021 going from almost 12% in 2020 to 3.7% in 2021. This may be a result of trees aging or from the year. 2022 Green Spot evaluations have not been completed and will be included in the presentation of the continuing report
3. N applications increased tree vigor and green spot incidence in 'WA 38' apple and increase bitter pit in Honeycrisp apple.
4. Rootstock heavily contributed to green spot and bitter pit incidence through its effect on vigor.
5. For commercial orchards, overcropping in one year led to a lower than optimum crop load the following year and an elevated risk of bitter pit.

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. 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 controlled experiments.

lbs/acre applied	Nitrogen (N)	Potassium (K)	Magnesium (Mg)
Low	12	50	25
Medium	25	100	50
High	50	200	100

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. Then, 48 fruit was randomly selected from each tree. 16 fruit were used for fruit quality at harvest and the other fruit was stored in regular atmosphere for three months at 1° C and used for disorder evaluation after storage. Elemental analysis was performed using a pooled sample consisting of a peel sample collected from the calyx end of eight fruit from each replicate. Samples were dried, ground, and acid digested then analyzed using an Agilent 4200 MP-AES elemental analyzer. N was analyzed separately with a 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. In 2021, there were 56 orchards sampled. 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. We added a component using fruitlet sap analysis in collaboration with Dr. Lailiang Cheng from Cornell University and are working to compile the results from that study. 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 were 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 was 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. Elemental analysis was completed for 2020 and is in the process of being completed for 2021. Data for elemental composition will be presented for 2020 and disorder incidence, yield, etc. will be presented for both year.

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 started in 2021 and will 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 AND DISCUSSION

For all WA 38 orchards that were sampled in all three years, 88%, 82%, and 82% of target crop load was achieved in 2020, 2021, and 2022, respectively. These targets were based on 5 fruit cm^{-2} TCSA in 2020 and 6 fruit cm^{-2} TCSA in 2021 and 2022. For Honeycrisp, crop load targets were 5 fruit cm^{-2} TCSA for all three years. Honeycrisp orchards achieved a higher % target in 2020 at 95% then fell lower again at 82% and 81% of target crop load for 2021 and 2022, respectively. Lower harvested yields compared to optimum is indicative of the hot period and wind events that led to fruit loss in 2021 and then the cold spring in 2022 that may have caused freezing damage and/or poor pollination. For Honeycrisp, orchards that had higher than optimum crop loads in 2020 averaged 7.6 fruit cm^{-2} TCSA and as a result, yields averaged 40% lower the following year in 2021. These same orchards had only 13.5% bitter pit in 2020 compared to an increase to 33.3% in 2021. Off years drive bitter pit risk. Orchards that were able to maintain crop load within 1 fruit cm^{-2} TCSA target in 2020, were able to maintain consistent crop in 2021 averaging 5.24 fruit cm^{-2} TCSA and then 4.56 fruit cm^{-2} TCSA in 2022. Bitter pit in those orchards with an optimum crop load averaged 5.4% and 17.8% in 2020 and 2021, respectively.

Bitter pit incidence was not different between the two years reported here (2020 and 2021) and averaged approximately 15% across both years (Tables 2 and 3). Fruit weight was significantly higher for WA 38 than Honeycrisp most likely because they were younger trees than Honeycrisp. There were significant differences in fruit peel nutrient concentrations for WA 38 and Honeycrisp. Fruit potassium concentrations were higher for Honeycrisp (Tables 4 and 5). Magnesium concentrations were not different between the two cultivars. Calcium and nitrogen concentrations were higher for WA 38 than Honeycrisp. When a statistical clustering approach was used to cluster outcomes for groups of orchards into five different categories, there were significant differences in bitter pit and green spot among the clusters (Tables 6 and 7). Orchard years that clustered low for bitter pit in Honeycrisp had low vigor and optimum crop load. Although vigor was higher for WA 38 in general, vigor didn't cluster with green spot for commercial orchards. However, crop load clustered closely to green spot where orchard years with low crop load had clear elevated incidence of green spot. Rapid fruit growth associated with high carbohydrate loading during fruit expansion may be responsible for cracks and green spot developing on the peel of WA 38.

There have been significant discussions about the use of fruitlet and leaf testing for predicting bitter pit at harvest. Our results show that there are significant relationships between fruitlet and leaf concentrations in June compared to fruit peel concentrations at harvest. However, fruitlet (K+Mg)/Ca ratios were related to bitter pit incidence in Honeycrisp. The variability around these ratios limits the predictive power. There are many factors that happen following June sampling that can affect final bitter pit risk. Examples of this include rapid fruit growth, post sampling thinning, summer pruning, irrigation management. All of these can change the nutrient ratios and limit the usefulness of those

June fruitlet samples. These samples might indicate if there are some potential problems emerging, but crop load and vigor assessments will probably catch the same issues without needing the nutrient analysis unless the grower is trying a new fertility program or product. Fruitlet and leaf N and K concentrations clustered with bitter pit and green spot risk in Honeycrisp and WA 38 (Tables 8 and 9). Both nutrients are associated with rapid fruit growth and larger fruit sizes. These appear to be targets for early season monitoring and have the potential to be remobilized and accumulate later in the season in developing fruit. N and K also were the most closely correlated with final fruit nutrient concentrations (Figures 1 and 2) and N/Ca and K/Ca ratios in June were somewhat related to the same ratios in fruit peels at harvest (Figure 3). We also had the opportunity to test the peel sap method with traditional fruitlet sampling. Ratios in sap were significantly positively related to bulk nutrient ratios in the fruitlets. (K+Mg)/Ca ratios for both methods were significantly correlated with bitter pit risk for Honeycrisp for commercial orchards (Figure 4). However the predictive power of these ratios in fruitlets was relatively low compared to near harvest. Fruit peel N/Ca ratios remain a good indicator of green spot and bitter pit. These results were supported by findings from our controlled experiments presented in 2021 where elevated N and K applications contributed to elevated green spot risk in WA 38.

Table 1. Commercial orchard sampling for WA 38 and Honeycrisp

	'WA 38'	**'Honeycrisp'**	
2020	23	28	
2021	19	22	
2022	17	22	Total
Total 'Orchard Years'	59	72	**131**

Table 2. Descriptive statistics and range in agronomic variables among commercial orchards for 'Honeycrisp'. * Data still to be collected

	Bitter pit (%)	Shoot length (inches)	Crop load (fruit cm^{-2} TCSA)	Fruit weight (g)
	2020			
Average	16.6	6.5	5.4	231
Minimum	0	1.0	1.1	156
Maximum	94.6	13.3	14.2	325
	2021			
Average	14.4	4.5	4.0	214
Minimum	0	1.8	0.95	111
Maximum	71.9	7.6	9.5	317
	2022			
Average	*	6.6	4.2	253
Minimum	*	3.8	0.85	205
Maximum	*	18.1	11.9	275

Table 3. Descriptive statistics and range in agronomic variables among commercial orchards for 'WA 38'. * Data still to be collected

	Green spot (%)	Shoot length (inches)	Crop load (fruit cm^{-2} TCSA)	Fruit weight (g)
	2020			
Average	13.47	7.9	4.4	286
Minimum	0	3.0	0.8	186
Maximum	72.2	14.4	11.4	385
	2021			
Average	3.91	8.5	5.3	272
Minimum	0	2.7	1.1	184
Maximum	18.75	13.6	10.9	327
	2022			
Average	*	8.0	5.5	277
Minimum	*	5.0	1.8	225
Maximum	*	12.6	12.1	306

Table 4. Descriptive statistics and ranges in fruit nutrient concentrations among commercial orchards for 'Honeycrisp'. * Data still to be collected

	Calcium (mg g^{-1} dw)	Potassium (mg g^{-1} dw)	Magnesium (mg g^{-1} dw)	Nitrogen (mg g^{-1} dw)
	2020			
Average	0.6	10.9	1.1	3.7
Minimum	0.1	6.7	0.7	2.5
Maximum	1.7	15.6	1.6	5.3
	2021			
Average	1.0	8.2	1.0	4.1
Minimum	0.4	6.2	0.8	2.8
Maximum	2.7	11.5	1.3	5.9
	2022			
Average	*	*	*	*
Minimum	*	*	*	*
Maximum	*	*	*	*

Table 5. Descriptive statistics and ranges in fruit nutrient concentrations among commercial orchards for 'WA 38'. * Data still to be collected

	Calcium (mg g^{-1} dw)	Potassium (mg g^{-1} dw)	Magnesium (mg g^{-1} dw)	Nitrogen (mg g^{-1} dw)
	2020			
Average	0.7	7.7	1.0	4.4
Minimum	0.2	5.9	0.7	2.7
Maximum	1.6	13.8	1.9	5.7
	2021			
Average	1.0	6.8	1.0	4.5
Minimum	0.4	5.5	0.8	3.4
Maximum	1.9	8.7	1.3	5.8
	2022			
Average	*	*	*	*
Minimum	*	*	*	*
Maximum	*	*	*	*

Table 6. Clustering of variability in bitter pit among 72 commercial orchard years for 'Honeycrisp'. These are statistically clustered orchards with centered values for each variable.

Risk	Bitter pit (%)	Shoot length (inches)	Crop load (fruit cm^{-2} TCSA)	Fruit weight (g)	Fruit Ca (mg g^{-1} dw)	Fruit K (mg g^{-1} dw)	Fruit Mg (mg g^{-1} dw)	Fruit N (mg g^{-1} dw
Low	8.2	6.5	4.9	226	0.72	9.8	1.02	3.8
Low	11.6	4.9	4.9	179	0.74	9.1	0.98	3.8
Moderate	20.0	8.8	4.6	284	0.64	9.2	1.03	3.8
High	69.2	11.2	3.7	247	0.52	9.2	1.07	3.7
Very High	83.5	17.1	2.8	339	0.27	7.5	1.03	4.0

Table 7. Table 6. Clustering of variability in green spot among 59 commercial orchard years for 'WA 38'. These are statistically clustered orchards with centered values for each variable.

Risk	Bitter pit (%)	Shoot length (inches)	Crop load (fruit cm^{-2} TCSA)	Fruit weight (g)	Fruit Ca (mg g^{-1} dw)	Fruit K (mg g^{-1} dw)	Fruit Mg (mg g^{-1} dw)	Fruit N (mg g^{-1} dw
Low	3.1	8.1	6.0	258	0.97	7.1	0.97	4.4
Low	4.1	9.6	7.5	210	1.09	6.5	0.94	4.6
Moderate	8.5	8.3	4.7	301	0.81	7.4	1.00	4.4
Mod-High	17.7	7.3	3.0	356	0.65	8.5	1.11	4.6
Very High	51.9	8.1	1.7	314	0.32	8.0	0.99	4.9

Table 8. Clustering of variability in bitter pit associated with fruitlet and leaf nutrient concentrations that were sampled in late June. These are statistically clustered orchards with centered values for each variable.

Risk	Bitter pit (%)	Fruitlet Ca (mg g^{-1} dw)	Fruitlet K (mg g^{-1} dw)	Fruitlet Mg (mg g^{-1} dw)	Fruitlet N (mg g^{-1} dw	Leaf Ca (mg g^{-1} dw)	Leaf K (mg g^{-1} dw)	Leaf Mg (mg g^{-1} dw)	Leaf N (mg g^{-1} dw
Low	0.8	0.87	11.9	0.78	6.3	21.6	15.5	4.6	26.1
Moderate	10.6	0.81	13.8	0.88	10.7	21.6	16.5	4.1	27.8
Mod-High	23.0	0.83	13.1	0.73	7.1	25.7	16.8	5.2	28.8
High	57.1	0.95	13.7	0.81	10.1	29.5	15.5	5.9	27.5
Very High	77.3	1.05	15.5	1.04	11.5	21.9	17.5	5.4	30.1

Table 9. Clustering of variability in green spot associate with fruitlet and leaf nutrient concentrations that were sampled in late June. These are statistically clustered orchards with centered values for each variable.

Risk	Green spot (%)	Fruitlet Ca (mg g^{-1} dw)	Fruitlet K (mg g^{-1} dw)	Fruitlet Mg (mg g^{-1} dw)	Fruitlet N (mg g^{-1} dw	Leaf Ca (mg g^{-1} dw)	Leaf K (mg g^{-1} dw)	Leaf Mg (mg g^{-1} dw)	Leaf N (mg g^{-1} dw

Low	3.6	1.86	17.4	1.31	10.2	20.4	22.3	4.3	25.8
Moderate	2.5	1.83	17.9	1.36	11.2	21.2	22.9	3.9	28.0
Mod-High	30.5	1.47	19.0	1.26	11.5	15.3	19.7	3.4	24.9
High	38.9	1.92	19.3	1.60	17.6	23.6	21.8	4.6	29.4
Very High	59.3	1.89	20.5	1.64	15.4	24.4	28.9	4.2	29.8

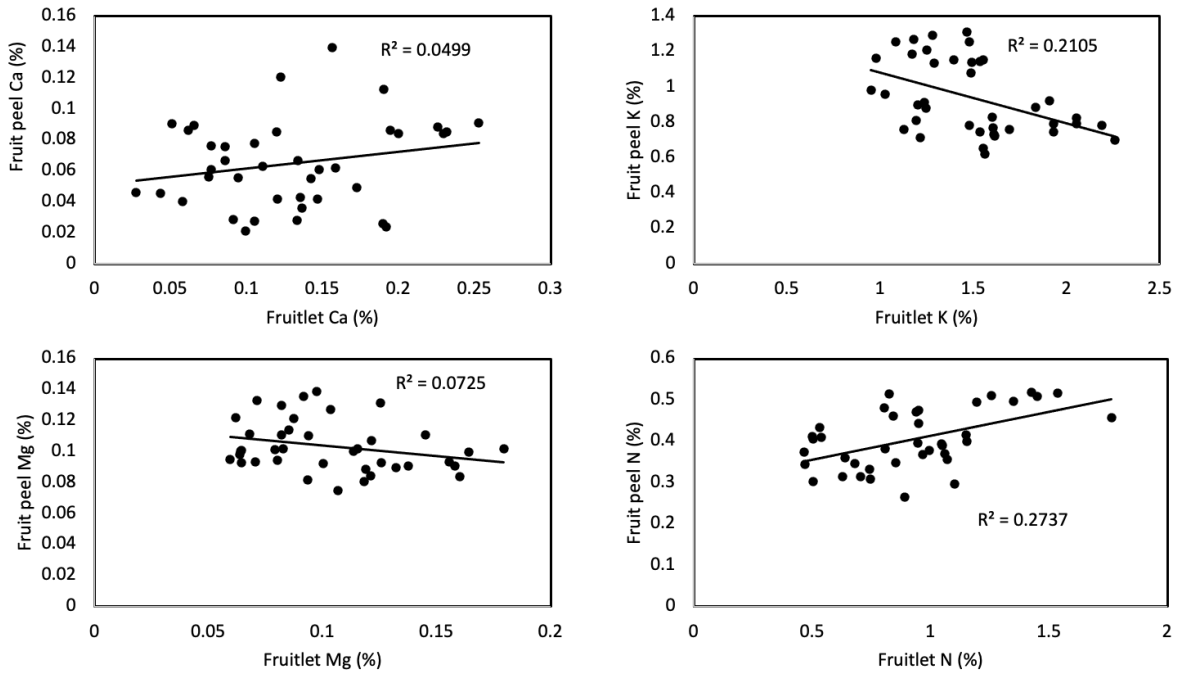

Figure 1. Relationships between fruitlet and leaf and fruit nutrient concentrations for WA 38 and Honeycrisp.

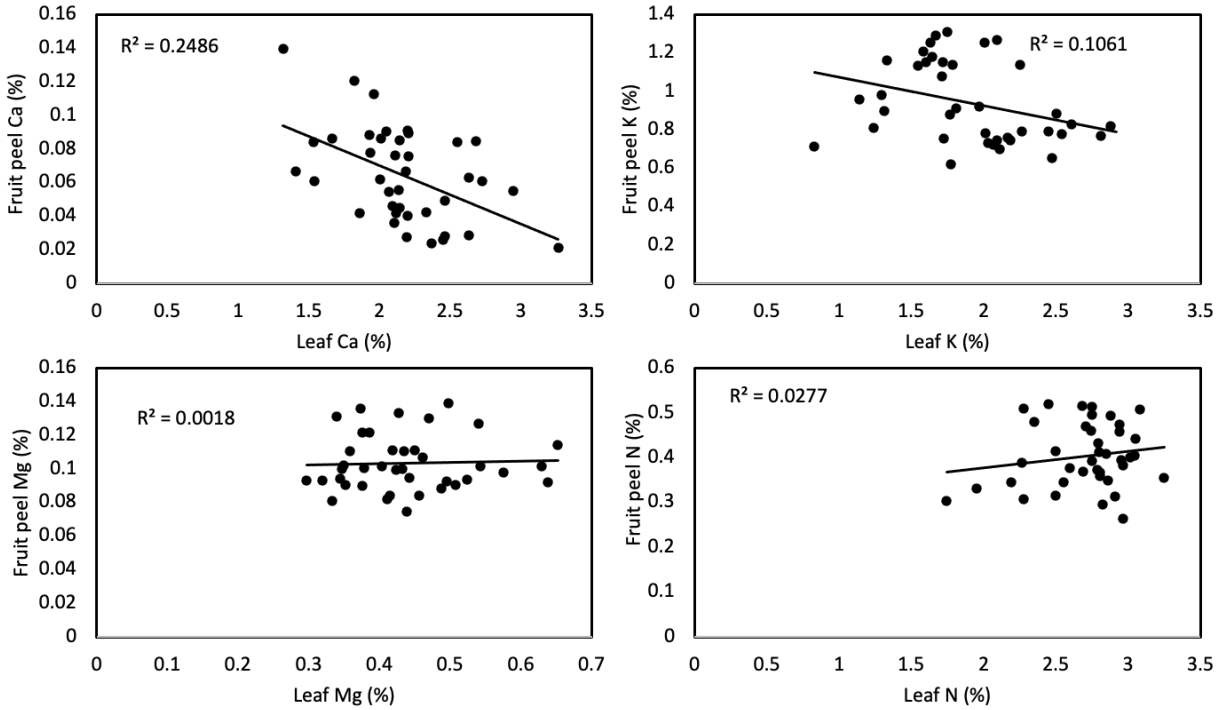

Figure 2. Relationships between fruitlet and leaf and fruit nutrient concentrations for WA 38 and Honeycrisp.

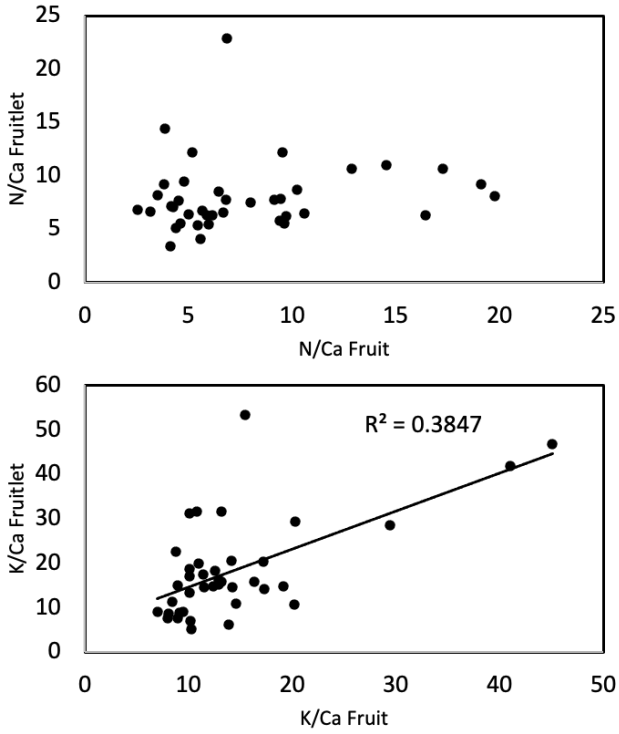

Figure 3. Relationship between N/Ca and K/Ca ratio in fruitlets versus fruit peels at harvest

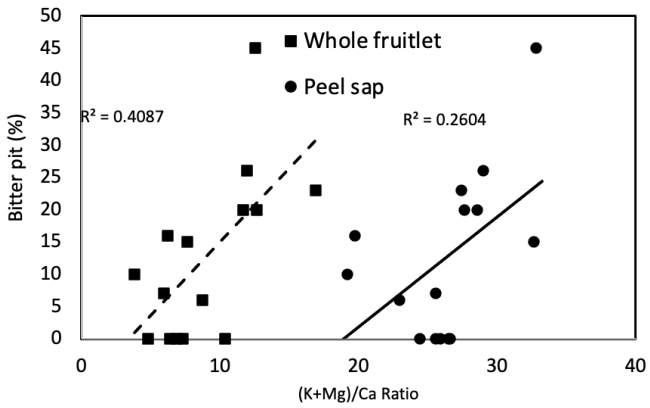

Figure 4. Relationship between peel sap and traditional whole fruitlet analysis and bitter pit in Honeycrisp

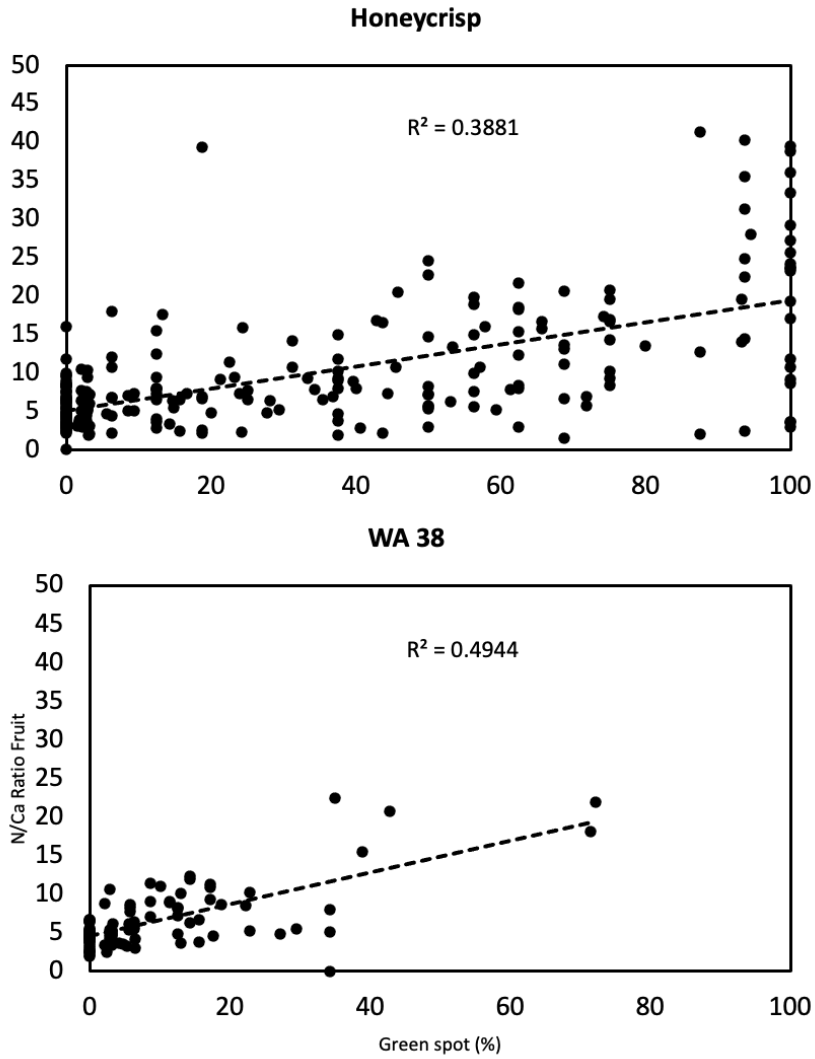

Figure 5. Relationships between N/Ca ratios and bitter pit in Honeycrisp and N/Ca ratios and green spot in WA 38

CONTINUING PLANS

We are working on the final threshold recommendations for leaves, fruitlets, and fruit for K, Mg, N, and Ca concentrations. Once we have the 2022 nutrient concentrations, we can finalize these. Many of the tests determine deficiency but rarely consider excess unless it is a major problem. However, small amounts of excess can still lead to significant losses. We are working on compiling anonymized final soil fertility characteristics, fertilizer management, irrigation management, and orchard training practices to identify key factors leading to successful management of both bitter pit and green spot. The no-cost extension year will be focused on grower outreach and developing material useful for the grower in managing green spot and bitter pit.