2026 Apple Horticulture and Postharvest Research Review

Researchers and industry members from around the world met for the Controlled Atmosphere and Modified Atmosphere conference (CAMA) in Wenatchee. Manoella Mendoza led a tour of local warehouses including a visit to Stemilt.

Photo Source: Torres Lab at TFREC

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Hybrid Format
Yakima, WA

Project Title: Phase 3 Evaluation of Apple Breeding Selections

Report Type: Final Project Report

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Cooperators: Growers: Stemilt Inc., Allan Brothers and Douglas Fruit. Apple Breeding Program Advisory Committee: Aylin Moreno (Taggares Fruit), Oscar Garcia (Taggares Truit), Garrett Henry (Douglas Fruit), Mark Stennes (Agrimacs), Jonathan Cox (Double Diamond), Paul Cathcart (Columbia Reach), Dale Goldy (Gold Crown), Dave Gleason (Superfresh), Jeff Cleveringa (Columbia Fruit), Anne Morrell (Columbia Fruit), Jeff LaPorte (Chelan Fruit), Lauren Gonzalez (GS Long), Sarah Franco (Allan Bros.), Suzanne Bishop (Allan Bros.), Rob Blakey (Stemilt), Bernardo Reyes (Stemilt), Craig Anderson (Gilbert Orchards), Dena Ybarra (Perleberg orchards), Matt Miles (WTFRC commissioner). Technical consultants: Stefano Musacchi, Carolina Torres, Bernardita Sallato, Dave Rudell. Industry partners: AgroFresh and Storage Control Systems.

Project Duration: 3 Years

Total Project Request for Year 1 Funding: $53,478
Total Project Request for Year 2 Funding: $56,127
Total Project Request for Year 3 Funding: $59,791

Other related/associated funding sources: Stemilt and Allan Brothers provide farm crew assistance for pruning, thinning, and field maintenance, Agrofresh donates Smartfresh, and Stemilt assists with SmartFresh and postharvest fungicide application. Columbia Fruit and Columbia Reach assisted with packing line assessment. Washington Fruit donated apple boxes and trays.

Agency: WSU apple breeding program royalties
Amount awarded: ~$500,000 per year (2023-2026)
Notes: Funding supports all other aspects of the apple breeding program (Phase 0 to Phase 2), including all program staff, a full-time farmworker position at WSU Columbia View orchard, and graduate student assistantships. Funds to supplement Phase 3 evaluations are provided as necessary for consumer tastings, equipment, and consumables.
Funding Duration: 2021-2024

Agency Name: WSDA Specialty Crop Block Grant Program
Amount awarded: $220,045
Notes: Establishing rootstock and production system recommendations for new Washington apple selection (WSU 'L') Evans, Musacchi, Sallato. This project will collect complementary information for an elite P3 selection that will be released.
Funding Duration: 2021-2024

Agency Name: Washington Research Foundation
Amount awarded: $99,932
Notes: Rootstock and systems trial for WA 64 apple. Evans, Musacchi, Sallato. This project continues the development of production recommendations for WA 64.
Funding Duration: 2024-2025

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Item	2023	2024	2025
Salaries			
Benefits			
Wages	$28,523.00	$30,079.00	$32,259.00
Benefits	$11,409.00	$12,120.00	$13,204.00
RCA Room Rental	$12,746.00	$13,128.00	$13,528.00
Shipping			
Supplies	$500.00	$500.00	$500.00
Travel	$300.00	$300.00	$300.00
Plot Fees			
Miscellaneous			
Total	$53,478.00	$56,127.00	$59,791.00

Footnotes: Wages/Benefits: calculated based on expected staff wage adjustments. RCA room rentals: 2 rooms, including room operation costs and warehouse fees, adjusted yearly. Supplies: consumables for fruit quality analysis (KOH, distilled water, iodine, etc.). Travel: in-state travel

Justification

New and improved apple varieties are essential for 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. Developing improved apple varieties leads to *sustainable production and enhanced postharvest efficiency*, thereby promoting *sustainability and long-term economic viability by increasing apple packouts*.

P3 selections were planted in three grower-collaborator sites. The advantage of this arrangement is the ability to observe the growth habits and characteristics of advanced selections in a commercial production setting. Having the WTFRC manage P3 provides an independent, industry-oriented evaluation that, with input from industry representatives on the apple breeding program advisory committee (BPAC), ensures that the data collected and information provided align with stakeholders' interests. The project results, including single pick potential, harvest window, storability, and resistance to biotic and abiotic stress, are presented to the BPAC annually. Field visit opportunities are included throughout each season.

Objectives

1. Evaluate and determine the commercial potential of advanced selections of the WABP

Significant Findings

1. Although selection P has good shelf-life potential, it was *discontinued* due to its consistent bi-annual bearing and small fruit size.
2. Although selection R had a lower incidence of storage disorders, it was *discontinued* due to its inconsistent flavor and lower firmness retention.
3. Q and S grew to reach the top wire within the first year on both sites.
4. Selection Q has good firmness retention, losing only about 2 lb. after long-term storage, but bruises relatively easily.
5. Selection S, a yellow-colored apple, has good storage potential, maintaining flavor and texture long term.
6. Selection S presents low storage disorder overall, but internal browning incidence can be high due to advanced maturity at harvest.
7. The clusters of WA 64, also known as selection L, are mostly singles and doubles, but there are differences between sites.
8. WA 64 performed well in packing line assessments, achieving high packouts (71% to 92%).
9. WA 64 is not sensitive to high CO_2 concentration (0.5% O_2/ 5.0% CO_2) and performs well under low oxygen storage (0.6% O_2 and 0.5% CO_2).

Methods

Bud and Bloom observation: Field observations began as the trees started to bloom, occurring at least twice a week, considering the weather patterns and their influence on blooming. The full bloom date is determined for each Phase 3 (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.) are conducted and discussed with field managers. Pest and disease incidence and monitoring are documented during the entire season.

Fruitlet development and pre-harvest: Field activities for this stage start after June drop. Orchard visits occur at least every other week until a month before the predicted harvest. Observations on fruit sets and self-thinning were documented. The orchard crew performed hand-thinning and summer pruning when appropriate, as if the selections were being produced commercially. A specific pruning recommendation plan was put forward following consultation with the grower, BPAC members, and other specialists (e.g., Stefano Musacchi and Bernardita Sallato).

Harvest: Starch degradation, color, background color, and flavor development were assessed during pre-harvest visits. Once the harvest date was established, the harvest was conducted in one to three picks, depending on selection and crop load. The 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 bird peck were 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 the data were 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 postharvest fungicide at a Stemilt drencher location and stored at the Research CA rooms at Stemilt. Stemilt administered the 1-MCP treatment within one week of harvest.

Quality at harvest was assessed within 48 hours. Evaluations included starch degradation (Cornell 1-8), firmness (lb.), soluble solids (% Brix), titratable acidity (% m.a.), color (% of red coverage), background color, size (in.), weight (gr.), DA index, and presence/absence of internal and external defects/disorders.

Post-harvest: Quality assessment was conducted at 3 and 6 months of storage for apples in RA and at 6 and 9 months for apples in CA. Apples with and without 1-MCP treatment were evaluated at the same time points. Quality analysis was conducted after 7 days at room temperature to determine the potential quality for consumers after shipping, handling, and purchase. Per BPAC recommendation, apple quality was also evaluated after 2 weeks at room temperature for fruit stored at 6 months in RA and 9 months in CA, starting with fruit harvested in 2024. This analysis will assess the potential quality for consumers in a scenario where the time from fruit handling to consumption exceeds one week. Box size distribution data was generated from individual fruit weights. Fruit was distributed at meetings and events as available.

Advanced Phase 3

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

- commercial packing line handling: glossiness, bruising, stem puncture, cullage, size and packout data is collected. Fruit is evaluated in the laboratory after the packing line run on the same day, after 7 days in RA storage, and 7 days in RA + 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).

WA 64 (selection L)

In addition to packing line handling and a formal taste panel, this selection was tested for CO_2 sensitivity and Low oxygen storage. The CO_2 sensitivity test was performed using the USDA CA chambers (0.5% O2/ 5.0% CO2, 37°F), and low oxygen storage experiment was performed in two SafePods (control: 2.0% O_2 and 1.0 % CO_2, treatment: 0.6% O_2 and 0.5% CO_2), both set at 37°F.

Results and Discussion

During this reporting period, we evaluated five selections: L, P, Q, R, and S. Selection L (WA 64) has been released, while selections P and R have been discontinued (refer to previous reports for details). Selections Q (Cripps Pink × Honeycrisp) and S (Honeycrisp × WA 2) remain active within the program. This report will provide detailed information on selections Q, S, and WA 64.

Selections Q and S:

These two selections were topworked in Quincy in 2020 and in Sagemoor in 2021. Most of the trees reached the top wire within one year. Tree growth is similar on both sites, with some blind wood in the middle section and heavily cropped treetops. Both locations were defruited in the first year and hand-thinned in the following years.

At the Quincy site in 2023 and 2024, the farm crew performed hedging and summer pruning in accordance with Stefano Musacchi's recommendations. In Sagemoor, winter pruning was performed following selection-specific recommendations by Bernadita Sallato.

Fruit was harvested in 2022 from Quincy and from both sites in the following years. In 2022, all ABP selections and apple varieties harvested by the WTFRC crew generally had less color and higher bruising incidence. Also, we observed a period of stagnation in starch degradation for a few weeks, followed by rapid depletion, which might have led to fruit being picked at an advanced maturity stage. In 2023, the harvest timing was adjusted, and the fruit had better color and lower bruise incidence. The information in the following sections summarizes all the data collected until 2024. Quality analysis for 2025 is ongoing.

Selection Q

A bicolor apple with medium to large fruit, with box size peaking at 72 (range 72-88). Fruit typically has a short stem and is considered easy to pick. This selection was strip-picked in two picks, except in the first year of production in Quincy, when it was harvested in three picks. Starch degradation at harvest ranged from 2.4 to 5.6 (Cornell, 1-8). It has a good texture and flavor, but the percentage of bland and off-flavor increased with storage duration and days at room temperature (7 to 14 days).

Selection Q retains firmness well. At harvest, it ranged between 15.3 and 17.0 lb. in Quincy and 16 to 20 lb. in Sagemoor, with a maximum loss averaging 2 lb. during storage. SSC increases by about 2.0 (% Brix) from harvest to 9 months CA. Titratable acidity varied, with an average of 0.55, but in some instances, it decreased to 0.25.

There was a low incidence of preharvest and storage disorders. The incidence of bitter pit, soft scald, superficial scald, and split was below 1% per pick. Internal browning was most often observed in fruit stored in CA for 9 months, with a few instances in fruit stored in RA for 6 months. Incidence for each time point was higher in Quincy (up to 20%) than in Sagemoor (up to 7%). This selection bruises relatively easily compared with apples that are more resistant to bruising, like WA 64 or WA 38.

Action plan: In addition to the standard evaluations, this selection will be analyzed on the AWETA research packing line at TFREC to collect data on standard packout grading (color and size) and bruise sensitivity.

Selection S

This is the only single-colored apple selection in P3 currently in production. At harvest, the color at both sites ranged from pale yellow to golden, with a darker color and a blush on the sun-exposed side of the apple. At Sagemoor, the trees are planted on a spindle system on G.969 rootstock at 4 x 12 ft spacing. In Quincy, the trees were planted in a biaxis system on M9 Nic.29 and G.41 at 3 x 12 ft spacing. Two additional plots were planted near Pasco, at Douglas Fruit orchards in 2025, one with and one without netting.

Biannual bearing was observed on both sites, but more prominently in Quincy, where 2023 was a low-production year. 2024 was heavily cropped and not thinned properly, resulting in small, under-colored apples and no crop in 2025. Fruit in P3 is typically hand-thinned to observe inherent tree bearing characteristics. Due to the highly variable crop load scenario, selection S will be chemically thinned in 2026.

In Quincy a wide variation in starch degradation at harvest (Cornell 2 to 7) was observed on the first pick in 2022 and 2024 (heavy crop years). The second and third picks had advanced starch degradation at harvest (7.3 ± 0.6). In 2023 (low crop), the fruit in Quincy was harvested in a single pick, about 2 weeks earlier than in 2022, and starch degradation was 2.4 ± 1.1. In Sagemoor, starch degradation was less advanced at harvest in 2023 (1.8 ± 0.7) than in 2024 (4.8 ± 0.4).

No bitter pit or pre-harvest drop was observed. However, overall limb rub incidence was considerable, ranging from 11.2% to 16.5% across all years and locations. Sunburn development is more prominent in Sagemoor than in Quincy, resulting in 13% cullage due to sunburn (Figure 1). In Sagemoor, Parka is used to mitigate sunburn, while overhead cooling is used in Quincy. The latter has no fruit loss due to sunburn cullage. Blush was also more prominent in Sagemoor (56%) than in Quincy (13%) (data not shown).

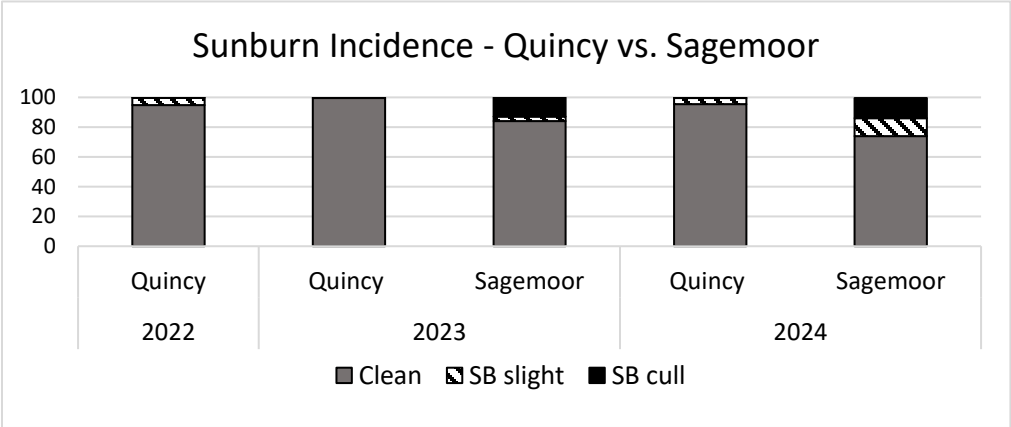


Figure 1. Total annual incidence of sunburn for selection S by location and sunburn type: no sunburn (clean), slight, and severe (cull)

Overall, greasiness was low, with a higher incidence in fruit stored for 6 months in RA. Stem puncture and soft scald were below 2%, and superficial scald was below 1%. Split incidence is typically higher in Quincy (2.8%) than in Sagemoor (1.9%). Average per pick can range from 0.8% to 6%, with a higher incidence on the last pick. The highest incidence of bruising was observed in the first year, when fruit was harvested at advanced maturity. Flavor and texture are consistently highly rated.

Senescent internal browning was observed in all years and locations, ranging from 0% to 80% (Table 1). In 2022, fruit from Quincy developed internal browning in most of the timepoint/treatment combinations, starting at 3 months in RA and increasing by pick and time in storage. In 2023 and 2024, internal browning at a much lower rate and was first observed after 6 months in storage. In Sagemoor, the incidence of internal browning was very low in 2023 and increased in 2024, when apples were harvested at a more advanced stage of starch degradation. These results indicate that the high incidence of this disorder is due to advanced maturity at harvest and might also be influenced by crop load.

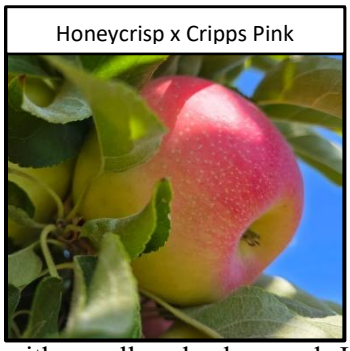
Table 1. Internal (senescent) browning incidence for selection S harvested from Quincy and Sagemoor, by year and storage timing evaluation. Years of heavy bearing are indicated with an asterisk (*).

	Quincy						**Sagemoor**	
Year		2022*		2023	2024*		2023*	2024
Har. Date	9.28	10.6	10.12	9.11	9.12	9.18	9.5	9.1
Starch (1-8)	5.8	7.3	7.4	2.4	4.6	-	1.8	4.8
	Internal Browning incidence (%)							
Harvest	0	0	0	0	0	0	0	0
3 mo RA	0	40	43	0	0	0	0	3
3 mo RA+MCP	18	38	40	-	0	0	-	-
6 mo RA	8	33	33	18	0	33	10	8
6 mo RA+MCP	0	80	35	-	0	3	-	-
6 mo CA	25	58	65	25	15	48	10	53
6 mo CA+MCP	38	58	40	-	-	13	-	-
9 mo CA	10	55	70	23	13	30	0	0
9 mo CA+MCP	23	80	75	-	13	38	-	-

Action plan:

- To mitigate internal browning, some batches of selection S were conditioned at 50°F for a week and stored in RA (33°F) or CA (0.5% O_2/ 5.0% CO_2, 37°F) in 2025, following Rudell's recommendation.
- To address annual bearing, the selection will be chemically treated on all sites in 2026, including the new plantings at Douglas fruit.
- For sunburn mitigation, adjustments to spray applications are needed at Sagemoor, and the addition of netting at Douglas fruit will provide insight into the efficacy of different sunburn mitigation techniques, including the overhead cooler in Quincy.

WA 64 (Selection L) – Sunflare™



WA 64 is the new release from the WSU apple breeding program and has received the commercial name of Sunflare™. Commercial planting availability and the first commercial harvest are predicted for 2026 and 2029, respectively.

This selection was grafted in 2015 on both Prosser and Quincy locations, on M9.337 and G.41 rootstocks, respectively. Tree structure (type III) is comparable to cv. Braeburn, and harvest timing is similar to cv. Golden Delicious, with bloom time similar to cv. Gala in Quincy.

WA 64 is a bicolored symmetrical apple that colors well when exposed to sunlight, typically achieving 50% to 70% red/pink blush with a yellow background. It is slow to brown, easy to pick, and pre-harvest drops 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.

Self-thinning was observed and recorded as qualitative data in the P3 sites for a few years of production. In 2023, sections of 30 trees in each location were marked with ribbons during bloom, and the number of clusters was recorded. The number of fruitlet clusters and cluster classification (singles, doubles, triples, and quadruples or higher) were recorded after the June drop but before hand-thinning. The tree sections were selected that had 50 to 60 bloom clusters.

In Quincy, fruitlet distribution was similar across years, with more than 60% of clusters set as singles, and triples and quadruples combined equaling 8 to 10% (Figure 2). In Prosser, the crop load was more evenly distributed between categories, with an increase in quadruples from 2023 (12%) to 2024 (21%). A higher percentage of single fruitlets and a lower percentage of quadruples were observed in Prosser in 2025 in comparison with previous years. The clusters in Prosser were hand-thinned to singles and doubles, and production levels were similar to previous years (data not shown).

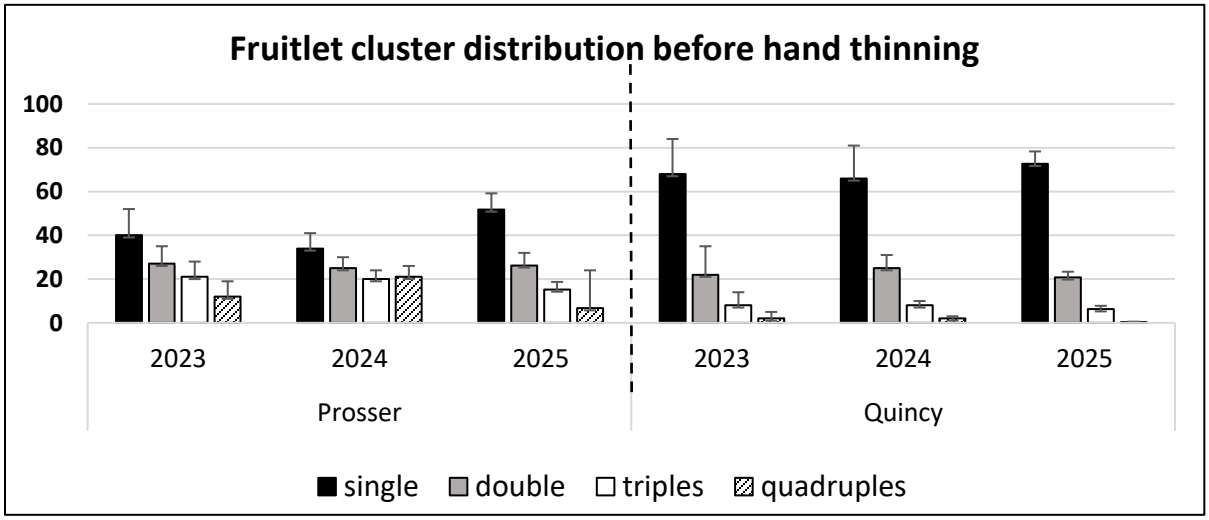


Figure 2. Distribution (%) and standard deviation of WA 64 fruitlet cluster by category (singles, doubles, triples, and quadruples or higher) in 2023, 2024, and 2025 for Prosser and Quincy.

Fruit flavor after harvest

This data was collected to determine whether WA 64 apples can be eaten immediately after harvest, meaning no delays in packing and shipping. WA 64 harvested from Prosser and not treated with 1-MCP was stored in RA for one week. The apples were tasted at room temperature during four events, 10 to 18 days after harvest. A total of 97 participants evaluated the appearance, flavor, and texture of WA 64 apples on a scale from 1 (poor) to 5 (outstanding). The combined scores for categories 4 and 5 were 93% for appearance, 94% for flavor, and 96% for texture. No starchy or poor flavor was detected. These results indicate that WA 64 can be immediately packed after harvest.

Packing line assessments

Packing line handling evaluations, including glossiness, bruising, stem puncture, decay, storage disorders, and fruit flavor, were conducted in 2022, 2023, and 2024. In 2022, two bins of WA 64 were harvested from Quincy, and the evaluations occurred in March and August of 2023 using fruit stored in RA and CA, respectively (Figure 3A). In 2023, 2 bins of apples were harvested from each site, and one bin per site was stem clipped. One set (stem clip vs. non-stem clipped) was stored in RA and evaluated in May; the other in CA was evaluated in August 2024 (Figure 3B and C). In 2024, 4 bins of apples from Quincy were evaluated in August 2025. The Cripps Pink grading program was used for all packing line assessments.

Packed fruit (≥ 25 % red color + small or no defect) was above 70% for all packing line assessments, achieving as high as 92% for fruit from Prosser. Quincy is a challenging site for color development, and the variability is reflected in the increase of the "25 to 49% + no defect" category in 2023. In 2024 fruit was classified in only three packout grades, and total packable fruit was 91% (Figure 3D). WA 64 from Prosser typically has better color and size than Quincy, thus delivering higher packouts. Stem clipping provided slightly higher packouts in fruit from Quincy (Figure 3B), with less fruit allocated for processing and lower cullage. Fruit from Prosser that was not stem clipped had higher packouts but slightly higher cullage.

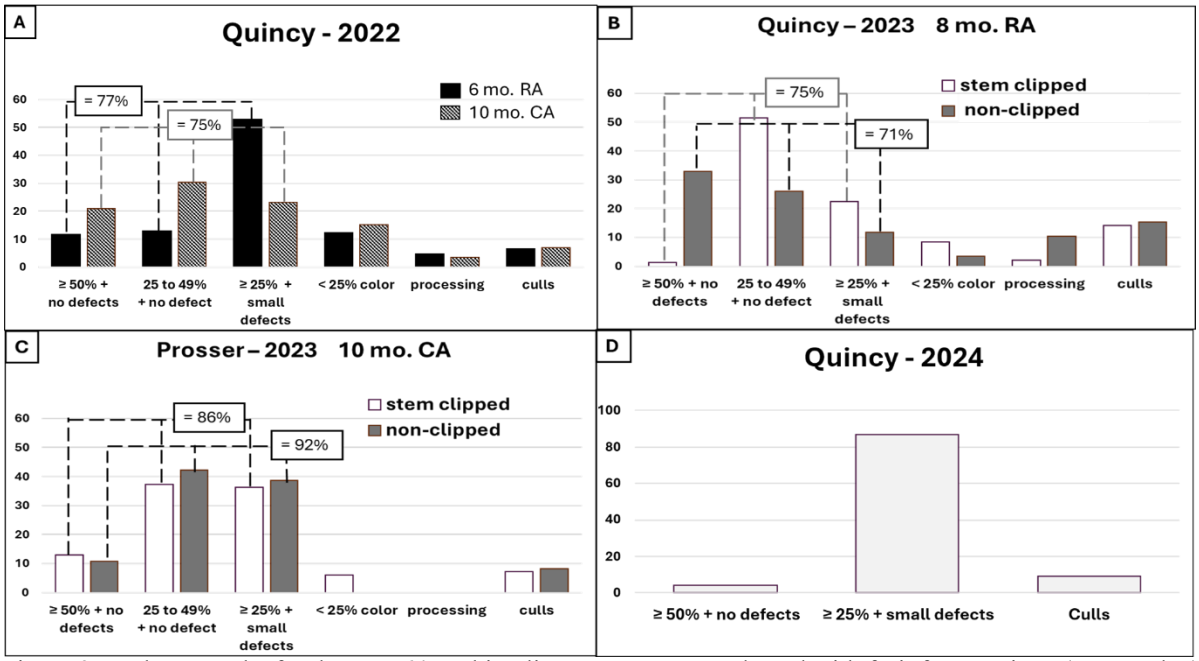


Figure 3. Packout results for the WA 64 packing line assessment conducted with fruit from Quincy (A, B and D) or Prosser 2023 (C). In 2022 and 2024, the fruit was not stem clipped. The sample size for 2022 and 2023 is one bin per treatment. In 2024, sample size is four bins.

Storage trials

CO_2 sensitivity: WA 64 apples were stored for 6 months in CA chambers at the USDA with high CO_2 concentration. Evaluations occurred monthly for external symptoms, and fruit was kept at room temperature for two weeks after the end of the experiment. No internal or external CO_2 injury was found. This evaluating as scheduled to be repeated in 2025, but it was not executed due to the government shutdown.

Dynamic controlled atmosphere: Fruit from Quincy and Prosser were transported to Union Gap and stored for 6 months at the Storage Control Systems cold room (37°F) in two SafePods (control: 2.0% O_2 and 1.0 % CO_2, treatment: 0.6% O_2 and 0.5% CO_2). In summary, fruit quality parameters were equivalent between treatments and similar over time. Better ratings for flavor were observed on fruit with lower oxygen levels after 6 months in storage and a week at room temperature.

WSDA experiment samples: WA 64 samples were collected from Sunrise in 2024 and 2025, and Roza in 2025 and stored in RA and CA, without 1-MCP. Apples were separated by rootstock and tree training system to evaluate the impact of these variables on fruit quality.

Outreach

- The article WA 64 – Tree Characteristics and Horticulture (authors: Bernardita Sallato, Sara Serra, Manoella Mendoza, Kate Evans, and Stefano Musacchi) is available at the WSU Tree Fruit website
- Legacy Grower Meeting in 2024
- WA 64 Session in the WA State Tree Fruit Association 2024 Annual Meeting
- Total of 6 WA 64 field days from 2023 to 2025. Both P3 plantings will continue to host the WA 64 field days in conjunction with the WSDA sites (Sunrise and Roza)
- WA 64 News and Tasting events held in Yakima and Wenatchee in 2025
- Two Fruit Matters articles about fruit quality and storability will be written in 2026, based on the data collected by WTFRC in P3

Project Title: Phase 3 Evaluation of Apple Breeding Selections

Keywords: apple breeding, cultivar performance, postharvest storage, field evaluation

Executive summary

The Washington State University Apple Breeding Program (WABP) seeks to deliver new apple varieties that enhance the long-term sustainability, profitability, and competitiveness of the Washington apple industry. Phase 3 (P3) evaluations play a critical role in this effort by assessing advanced breeding selections under commercial orchard and postharvest conditions. The Phase 3 (P3), managed by the Washington Tree Fruit Research Commission (WTFRC), provides an independent, industry-focused evaluation framework informed by direct collaboration with growers and the Apple Breeding Program Advisory Committee (BPAC).

Advanced apple selections were planted and evaluated at multiple grower-collaborator sites to capture performance across commercial production environments. This approach enabled detailed assessment of tree growth habits, cropping behavior, harvest timing, fruit quality, storability, and susceptibility to biotic and abiotic stress. Results were shared annually with BPAC members and the industry through data summaries and field visits, ensuring alignment with industry priorities.

During the reporting period, five selections (L, P, Q, R, and S) were evaluated. Based on cumulative field and postharvest performance, selections P and R were discontinued due to unfavorable horticultural and quality traits, including biennial bearing, small fruit size, inconsistent flavor, and reduced firmness retention. Selections Q and S remain active and continue to be evaluated. Selection Q demonstrated strong tree vigor, good firmness retention, and low disorder incidence, but exhibited higher bruise susceptibility and increased bland or off-flavor development with extended storage. Selection S showed excellent long-term storability and consumer-relevant texture and flavor, but performance was influenced by crop load variability, sunburn incidence at certain sites, and a risk of senescent internal browning associated with advanced maturity at harvest.

WA 64 (selection L), now commercially released as Sunflare™, consistently demonstrated high packout potential, strong firmness retention, low disorder incidence, and favorable consumer acceptance. The selection performed well in commercial packing line assessments, with packouts ranging from 71% to 92%, and showed tolerance to elevated CO_2 and low-oxygen storage conditions. Consumer taste panel results indicated excellent appearance, flavor, and texture shortly after harvest, supporting immediate packing and marketing opportunities.

Overall, this Phase 3 evaluation program successfully supported data-driven advancement, discontinuation, or commercialization decisions for elite apple selections. The outcomes strengthen the WABP pipeline, reduce industry risk associated with new cultivar adoption, and contribute directly to sustainable production systems and improved postharvest efficiency for Washington growers.

Project Title: Life Cycle Assessment for Apple Production in the Pacific Northwest.

Report Type: Final Project Report

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Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 161,531
Total Project Request for Year 2 Funding: $ 192,195
Total Project Request for Year 3 Funding: $ 174,900

Other Funding Sources: None
WTFRC Collaborative Costs: None

Budget 1
Primary PI: Greg Thoma
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	2023	**2024**	**2025**
Salaries (fully burdened) Thoma & Matlock	$32,500	$48,500	$55,000
Benefits			
Wages			
Benefits			
RCA Room Rental			
Shipping			
ISO Review Panel		$15,000	
Travel	$4,800		
Plot Fees			
Miscellaneous			
Total	$37,300	$63,500	$55,000

2025 expenses to be invoiced:
Thoma salary: $55,000
ISO review ($15,000) paid in 2025 following completion of the review.
Travel: $0

Budget 2

Co PI 2: Janjoris van Diepen
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Contract Administrator: Janjoris van Diepen
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	2023	2024	2025
Salaries	$65,200	$92,400	$92,400 **+$10,000 = $102,400**
Benefits			
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies			
Travel	$2,500	$2,500	$2,500
Survey Dissemination			
Miscellaneous			
Total	$67,700	$94,900	$94,900 **+ $10,000 = $104,900**

Actual costs 2025

Salaries: $102,400

Perpetual EcoInvent data license $2500

Travel: $0

Budget 3

Co-PI 3: Georgine Yorgey (WSU lead Co-PI), Suzette Galinato (WSU Co-PI)
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Contract administrator email address: arcgrants@wsu.edu
Station Manager/Supervisor: Chad Kruger (Tree Fruit Research and Education Center Director, Center for Sustaining Agriculture & Natural Resources Director)
Station manager/supervisor email address: cekruger@wsu.edu

	2023	2024	2025
Salaries (1)	$14,901	$22,962	
Benefits	$4,972	$10,064	
Wages (2)	$28,800	$9,318	
Benefits	$2,938	$951	
RCA Room Rental			
Shipping			
Supplies (3)	$1,920		
Travel (4)	$3,000	$500	
Plot Fees			
Miscellaneous		$5000	
Total	$56,531	$48,795	$ 0

Life Cycle Assessment for Apple Production in the Pacific Northwest.

Objectives

We conducted a lifecycle assessment to evaluate the environmental impacts of apple production from orchard establishment through harvest and cold storage (or alternate supply chain stage where the apples are ready for delivery to the consumption stage). The analyses included upstream (e.g., suppliers) and downstream (e.g., waste management) processes associated with apple orchard and warehouse operations (e.g., production of raw, auxiliary, and operating materials), including all relevant inputs, emissions into the air, water, and soil, and disposal of all elements of production (e.g., pruning wood and end-of-life trees). This enables the apple industry to respond with cost-effective adaptive strategies to sustain production and profitability into the future, address buyer concerns, take advantage of government programs, and prepare for potential federal regulatory oversight (e.g., reduction in GHG emissions) being developed. The results of this LCA offer insights into the entire production system from which the sector could construct a public policy or public relations narrative regarding the impacts of tree fruit production on climate change and other environmental impacts.

The primary project goals were to provide a baseline assessment of the environmental sustainability of Northwest apple production and to ***develop a scenario analysis tool*** that will support the evaluation of management decisions over the orchard life cycle and provide the standard against which future improvements can be documented. These objectives were achieved through stakeholder-engaged efforts to define the sector's most relevant data and sustainability metrics.
At scale, we envision a continuum of orchard stages. As new practices and technologies emerge, the scenario tool can inform decisions regarding the next establishment phase's management. Since environmental sustainability metrics are vital components of the scenario analysis tool, baseline life cycle impact assessment results are a key deliverable from this project and implemented in the tool.
Specific objectives of this project are:

- Design and test a comprehensive life cycle data collection survey to provide data for a baseline sustainability assessment [complete] (e.g., Carbon and water footprint, energy consumption, eutrophication, etc.) and the development of a scenario tool for the evaluation of alternate management scenarios (e.g., biomass to energy versus composting of end-of-life trees).
- Provide an evaluation of current sustainability metrics of a range of management alternatives of NW apple production – that is, ***a baseline suite of metrics against which future progress can be evaluated***.
- Develop an LCA model for environmental impact assessment and scenario testing.
- Engage stakeholders in the development of a scenario analysis tool with which producers can simulate alternate management practice effects on environmental sustainability metrics that can be used to identify strengths and weaknesses of alternate management systems to identify environmental hotspots as opportunities for improvement.

Significant Findings

Workflow 1: Survey implementation

- An extensive list of over 100 questions for apple orchard growers was created based on a literature review, expert judgment and previous LCA experience. This list formed the basis for focused, in-depth interviews. Subsequently a shortened survey was deployed via Qualtrics and available for most of 2024. A total of 62 surveys (including focus group interviews) were used to generate the inventory model in the Simapro LCA software platform.

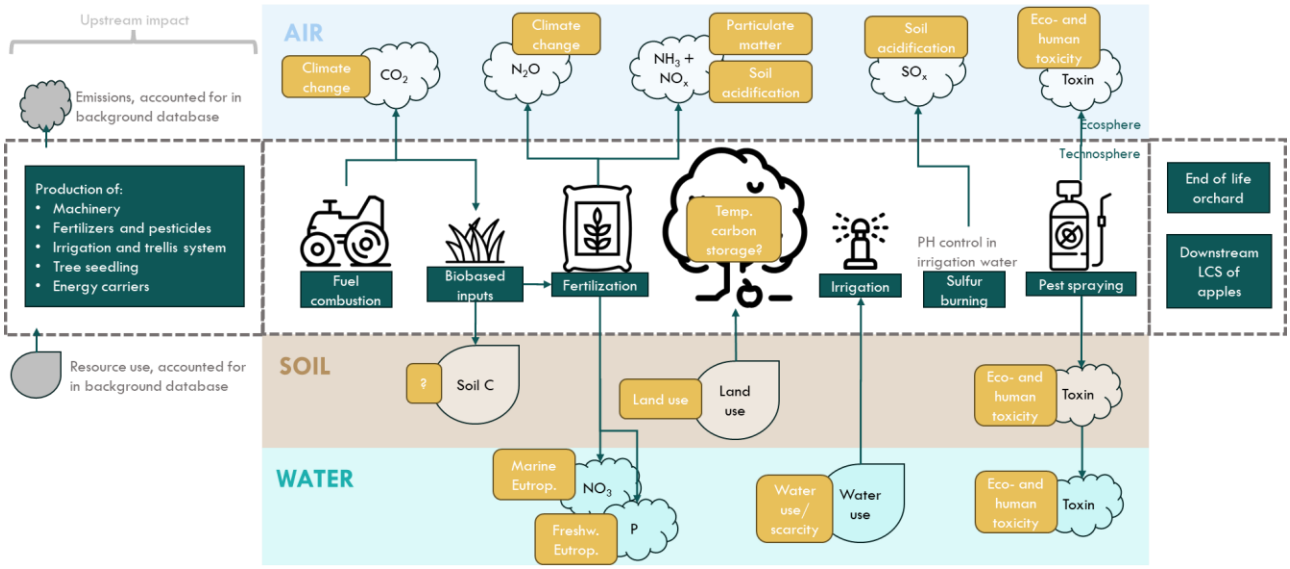

Figure 1. Schematic overview of processes considered in the LCI and associated environmental impacts.

Workflow 2: Life cycle inventory and report

- A parameterized lifecycle inventory (LCI) model has been created in the SimaPro software platform. The model includes an accounting of inputs (including upstream processes), outputs, and emissions of the establishment and production phases of apple cultivation. The model formed the basis for the full lifecycle impact assessment and sensitivity which was delivered in early 2025. A schematic overview of the production activities within the scope of the model, and the associated environmental impacts, are depicted in Figure 1.
- The full LCA report has undergone an ISO conformance review and the panel report has been submitted along with the full report which describes the LCA process and methodologies employed in detail.

Workflow 3: LCA tool development

- The learnings regarding management practices and LCA calculations form the basis for the scenario analysis tool. It was developed with stakeholder engagement and delivered separately from this report with a user's manual.

Quantifying the impacts of current Pacific Northwest apple production practices on the environment is important to understand the environmental impacts associated with apple production and supply chains in the region, to position the apple industry to be in compliance with buyer demands, and to engage with the USDA Climate-Smart Commodities Program. The LCA from this project has provided a baseline environmental profile and can assist in identifying opportunities for greenhouse gas mitigation and other sustainability efforts, in turn allowing the apple industry to make impactful, data-driven decisions. Further, the effort can support communication to educate the Pacific Northwest apple industry, retail partners, and consumers about the sustainability characteristics of apple production. Finally, the research provides the industry with a credible, science-based narrative showcasing its efforts as good caretakers of the land and resources.

Broadly, this project has relied on stakeholder-engaged life cycle inventory data collection, which was used in standard LCA software to calculate carbon and water footprints and other sustainability

indicators. A lifecycle inventory model is constructed as a set of linked unit processes. Each unit process accounts for a specific activity in the supply chain (e.g., drip or other irrigation systems, or application of crop protection chemicals) and captures the full production chain of the system under study.

The overarching structure of this project was highly integrated from the outset. Close coordination between the three workflow activities has been achieved through weekly or biweekly team meetings. Our efforts in year 3 were focused on completing the scenario tool and finalizing the ISO review of the full LCA report.

Scenario assessment tool

The spreadsheet tool and user's guide have been separately submitted to the WTFRC and not reproduced in this report.

The tool was constructed to include baseline life cycle impact assessment results and to support simulation of management practices on environmental sustainability metrics. It will allow users to identify strengths and weaknesses of alternate management systems to identify environmental hotspots as opportunities for improvement.

We met with stakeholders in 2025 to discuss the tool requirements and development process (Figure 1). The goal is to provide utility for a range of operators. While the vertically integrated operations (i.e., through to the retail receiving gate) are likely to have someone who can be assigned to use the tool, we know from many other situations that small, independent farms and ranches generally struggle to use complicated tools / models – both from a technical skillset perspective and from a "too many competing priorities" perspective. To lower the barrier to adoption, we have included several ready-to-run templates for users to modify to match their operations.

Figures 2 and 3 present examples of the output generated from the scenario tool, here comparing the benchmark from the ISO reviewed report against an alternative high density/high yield scenario.

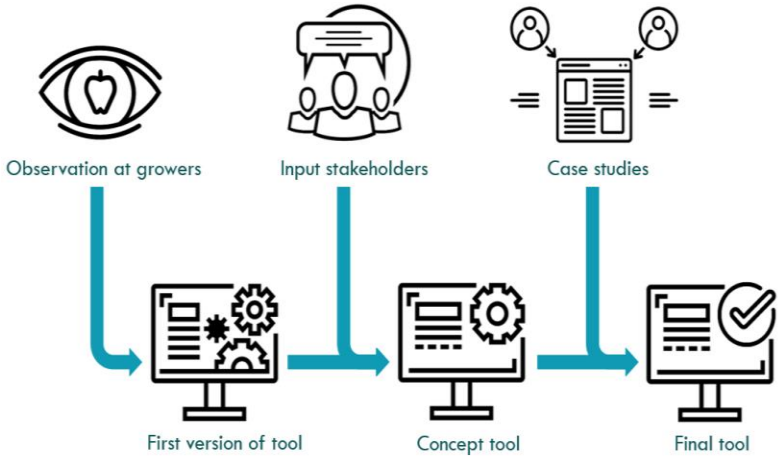

Figure 1. Schematic of the stakeholder-engaged process for creating the scenario analysis tool.

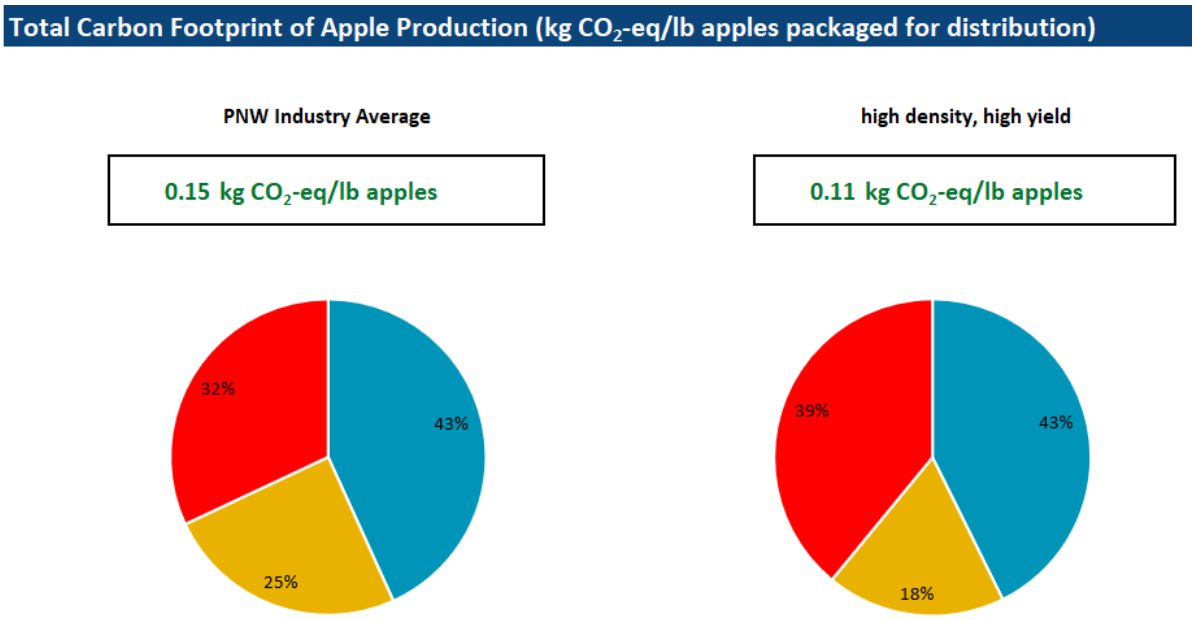

Figure 2. Example of graphical output from the scenario tool, providing a comparison of the benchmark LCA from the ISO report to one of the pre-populated scenarios.

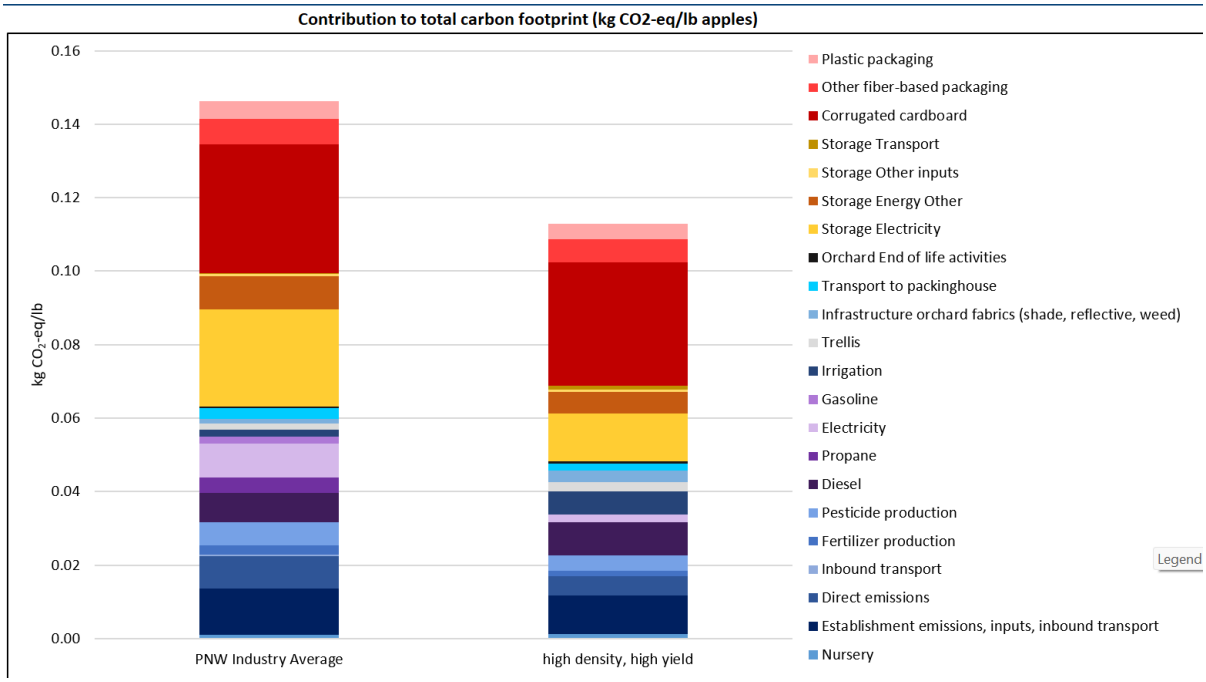

Figure 3. Example contribution analysis output from the scenario tool

Executive Summary: The Environmental Footprint of PNW Apples

The Life Cycle Assessment (LCA) establishes a clear environmental benchmark for Pacific Northwest (PNW) apples, which constitute **over 76% of the U.S. fresh apple crop**. The study confirms that PNW production methods are **highly efficient** but critically reveals that over half of the environmental burden occurs **after the apple leaves the orchard**. This analysis provides the necessary data to accurately quantify our regional advantages, defend our production standards, and focus investment for the highest possible return.

Baseline Findings: Pinpointing the Footprint (The "Whys and Hows")

The baseline footprint for **one pound** of packaged PNW fresh apples is **0.15 kg CO_2-equivalents** (climate change impact) – about the same as 0.4 kWh of electricity. The system boundary spans nursery → establishment → full production/harvest → transport to storage → climate-controlled storage → packaging for distribution. Indicators: climate change, fossil resource scarcity, water consumption, land occupation. A key finding is the split in the footprint contribution across the supply chain, demonstrating significant post-orchard contributions to consumer-ready apples:

Footprint Breakdown

The LCA shows that the **post-harvest system dominates** the overall carbon and fossil energy footprints, contributing **57%** and 62% of the total impact, respectively and contributes 47% to land occupation. For climate change, the breakdown is: **Packaging Materials: 32%; Storage-Processing: 25%**, and **Orchard Production: 43%.** Of the 43% attributable to the orchard: **Energy use** (diesel, electricity for pumping, etc.) is the single largest factor at **37%** of the production footprint.

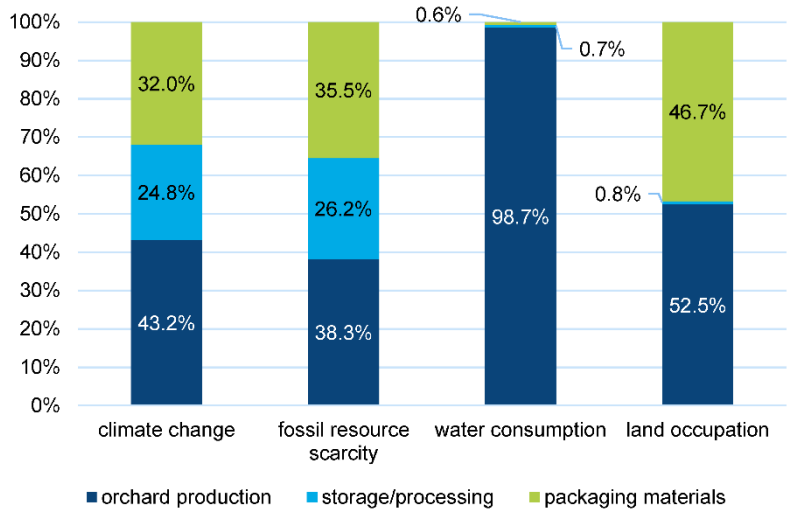

Figure 4. Contribution analysis for primary impact categories.

Critical Context: Why PNW Apples are Climate-Friendly

- **Hydroelectricity Advantage:** The regional reliance on hydroelectric power is a major competitive advantage. A sensitivity analysis demonstrated that utilizing hydro-generation (the predominant central Washington source) in place of the average Western U.S. grid mix results in a **27% reduction in the total carbon footprint**. This highlights how the region's clean energy infrastructure significantly cuts the post-harvest footprint from cold storage.
- **Water Consumption:** Water consumption impact is **dominated by the apple production phase**. This confirms that continuous focus on irrigation efficiency is paramount for regional water stewardship.
    - Water conservation practices are common in **high density orchards**. Growers already use sprinkler and drip irrigation systems, and they're working towards implementing sensor technology that only irrigates when and where water is most needed.
- **Perennial Crop:** Pacific Northwest tree fruit production can be climate friendly by its nature as a perennial cropping system. In addition to carbon stocks in the trees and soil, growers use **reduced**

or no tillage practices in the orchard and plant vegetation in between tree rows that helps to improve **soil organic matter** and **prevent soil erosion and pesticide runoff**.

Actionable Recommendations: Maximizing Investment

The LCA demonstrates that the highest-impact areas for improvement are in energy use and materials. The sector's strategy should focus on implementation of the following strategic actions:

Track	Strategic Focus	Recommendation	Easiest/Hardest
I. Post-Harvest Efficiency	Cold Storage (25% impact)	Focus on optimizing refrigeration and warehouse energy use and purchasing **Renewable Energy Certificates (RECs)** to formally claim the 27% hydroelectric benefit across the supply chain.	**Easiest Win:** Clear ROI on energy upgrades and a quantifiable environmental claim.
II. Supply Chain Innovation	Packaging Materials (32% impact)	Prioritize collaboration with suppliers to innovate packaging (e.g., lower-carbon substrates, increased recycled content) to drive the largest absolute reduction in the total footprint.	**Strategic Challenge:** Requires significant, collective industry R&D investment.
III a. Orchard Optimization	Energy Use (37% of production impact)	Invest in precision agriculture and electrification of on-farm equipment to reduce diesel consumption and maximize the benefit of PNW's low-carbon grid for powering irrigation and field work.	**Continuous Effort:** Direct control by growers with mid-level investment needed.
III b. Orchard Optimization	Improve data quality and granularity	Implement training and adoption incentives for the scenario analysis tool under development.	**Continuous Effort:** Direct action at sector level with moderate time investment needed.

The Value Proposition

This LCA proves that the investment was essential. It arms industry leaders with the specific data needed to **market the PNW apple as a verifiable, low carbon commodity** and provides the roadmap to focus resources on the efforts with the greatest future sustainability gains.

Over half of the 0.15 kg CO_2e per lb footprint occurs after harvest: packaging (32%) and storage & processing (25%). Thus, interventions like **lower-impact packaging**, **storage energy efficiency**, and **refrigerant management** can move the needle materially, alongside orchard energy efficiency.

- A 10% reduction in packaging impacts lowers total footprint by ~3.2%.
- A 10% reduction in storage/processing impacts lowers total by ~2.5%.
- A 10% reduction in orchard energy lowers total by ~1.6%.
- A 10% increase in yield could lower the total by ~4.6% (e.g., yield increase through genetic improvement enabling fixed inputs).

Life Cycle Assessment for Apple Production in the Pacific Northwest.

Keywords: Sustainability, Lifecycle Assessment, Carbon Footprint, Scenario Analysis

Abstract:
The Life Cycle Assessment (LCA) establishes a clear environmental benchmark for Pacific Northwest (PNW) apples, which constitute over 76% of the U.S. fresh apple crop. The study confirms that PNW production methods are highly efficient but critically reveals that over half of the environmental burden occurs after the apple leaves the orchard. The baseline footprint for one pound of packaged PNW fresh apples is 0.15 kg CO_2-equivalents (climate change impact) – about the same as 0.4 kWh of electricity. The system boundary spans nursery → establishment → full production/harvest → transport to storage → climate-controlled storage → packaging for distribution. A key finding is the split in the footprint contribution across the supply chain, demonstrating significant post-orchard contributions to consumer-ready apples.
This analysis provides the necessary data to accurately quantify regional advantages, defend production standards, and focus investment for the highest possible return. This enables the apple industry to respond with cost-effective adaptive strategies to sustain production and profitability into the future, address buyer concerns, take advantage of government programs, and prepare for potential federal regulatory oversight (e.g., reduction in GHG emissions) being developed.

Project Title: Towards next generation maturity indices: apple biomarker discovery; AP-22-101A

Report Type: Continuing Project Report - Year 3

Primary PI: Dr. Loren Honaas
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City/State/Zip: Wenatchee, WA 98801

Cooperators: AllanBrothers Inc., Stemilt LLC.

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $ 266,267
Total Project Request for Year 2 Funding: $ 155,303
Total Project Request for Year 3 Funding: $ 160,510

Other related/associated funding sources:
Funding Duration: annual congressional appropriation
Amount: $85,000
Agency Name: USDA ARS
Notes: 3-year total = $255,000: Personnel $180,000, Consumables/Supplies $30,000, Equipment (including computational resources): $45,000

Funding Duration: The funding source has expired but resources are still available.
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 provide the computing power necessary for model development.

Funding Duration: 2017-2022
Agency Name: US National Science Foundation (NSF) Award #1659300
Amount: $150,000
Notes: A portion of this award was used to fund 600 Terabytes of storage for execution of scientific workflows and storage of results. We will use that infrastructure for this project.

Funding Duration: 2020-2025.
Amount: $100,000
Agency Name: Auburn Harkess Start-Up Funds
Notes: These funds are being used to purchase molecular genomics reagents and equipment for apple DNA and RNA isolation and sequencing.

Budget 1
Primary PI: Dr. Loren Honaas
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Contract administrator email address: chuck.myers@ars.usda.gov, sharon.blanchard@ars.usda.gov
Station Manager/Supervisor: N/A
Station manager/supervisor email address: N/A

Item	2022	2023	2024
Salaries	44,775	45,894	47,042
Benefits	18,806	19,276	19,758
Wages			
Benefits			
Equipment			
Supplies			
Travel			
Miscellaneous			
Plot Fees	5,500	5,700	5,900
Total	69,081	71,410	72,700

Footnotes: Plot fees for WSU SRO blocks that provide validation samples.

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
Station Manager/Supervisor: N/A
Station manager/supervisor email address: N/A

Item	2022	2023	2024
Salaries	61,509	63,969	66,528
Benefits	19,677	20,464	21,282
Wages			
Benefits			
Equipment	46,000		
Supplies			
Travel			
Miscellaneous			
Plot Fees			
Total	127,186	84,433	87,810

Footnotes: Postdoc will be co-advised by all project PIs. Salaries and benefits are estimated to be inflated by 4% per year per WSU guidelines.

Budget 3
Co-PI 3: Dr. Alex Harkess
Organization Name: Auburn University and HudsonAlpha Institute for Biotechnology
Contract Administrator: Mercedes McKoy
Telephone: 334-844-3951
Contract administrator email address: MLF0015@auburn.edu
Station Manager/Supervisor: Optional
Station manager/supervisor email address: Optional

Item	2022	2023	2024
Salaries			
Benefits			
Wages			
Benefits			
Equipment			
Supplies			
Travel			
Miscellaneous	70,000		
Plot Fees			
Total	70,000	0	0

Footnotes: Miscellaneous funds are for RNA-Seq: this is global gene activity analysis of a majority of the total validation samples, estimate analysis of 350 total samples from 45 cultivar/years.

Budget 4 N/A
Co-PI 4: Dr. James Mattheis
Co-PI requests no funding.

Objective:
Develop and improve methods for biomarker discovery.

A. Use novel analytics and modeling approaches to strengthen biomarker discovery approach
B. Generate new global-scale gene activity data from current and new multi-year samples for rapid validation
C. Investigate disagreement between technologies for gene activity estimates to enhance translation to NGMIs

Next year goals:

1. Formal invention report/manuscript describing NGMIs
2. Prototype kit protocol and cost summary
3. Compile all new data (including related projects) for model updates

Significant findings/results from 2025:

1. Additional data for the NGMI project
    a. ~150 new tissue samples collected in 2025, including 2 new cultivars
    b. ~400 new RNA-Seq samples processed, with ~350 samples in queue at HA
2. NGMI test on at-harvest samples provides actionable storability info in commercial lots
3. Modeling paper that reveals data requirements: updated and submitted
4. NGMI updates - at harvest and future (7 days out) predictions of maturity
5. Prototype test kit deployed

Methods (Significant findings indicated in parentheses)

Analysis of validation samples

(Significant finding #1)

We have been building a catalog of samples that we can use to test and/or improve our pome fruit maturity prediction models for the last 8 years. These include 17 cultivars and 18 accessions (35 genotypes in total) from an original RosBreed apple population at Washington State University's Sunrise Orchard (rosbreed.org). Combined, this is at least 132 unique orchard-year combinations (e.g. orchard a vs. b; orchard a year 1 vs. 2; cultivar a vs. b). Altogether, a grand total of 1,903 unique biological samples have been collected and retained in our sample catalog, over half of which have been sequenced and hundreds more are queued for analysis. Each unique sample has corresponding at-harvest fruit quality data (weight, color, internal ethylene concentration (IEC), defects/disorders, firmness, Brix˚, TA, starch), as well as postharvest outcomes. In the 2025 harvest season, we added an additional 147 unique biological samples to our sample catalog. Most of these samples (111) are from two new cultivars and are sampled across a variety of harvest time courses, including samples from 9 commercial lots where sampling and harvest timing was guided by growers. This project (2023-2025) has added 630 samples in total, comprising ~1/3rd of our total sample catalog.

Raw sequencing data are processed as described in Hadish *et al.* (2022) & Honaas *et al*. (2021). Gene activity profiles are used to predict various fruit physiological traits (IEC, starch, harvest date, disorder incidence, etc.) Model predicted values are plotted against the actual values and Root Mean Square Error (RMSE) and Pearson's R^2 were used to assess model performance.

NGMIs in storage trials

(Significant finding #2)

The USDA ARS Tree Fruit Research Lab has been running storage trials on 'Scilate' apple for several years as part of other projects. The goal of these trials is to find storage parameters that promote long term storability of this cultivar, marketed as Envy™. These trials point to at-harvest fruit maturity as a primary indicator of fruit storability. They also confirm that fruit Internal Ethylene Concentration (IEC) is a superior maturity index compared to starch clearing. In the most recent years of this collaboration, we have taken peel samples of these fruit at harvest (as per Honaas *et al.* 2019). A subset of these tissue

samples (19 lots across 2 years) was used to test our prototype Next Generation Maturity Index (NGMI); this *beta* model interprets the activity of 8 genes and renders an estimate of IEC. This test leverages components our prototype test kit, which is described in more detail below. We compared the actual IEC values for these fruit lots with the NGMI predicted IEC values using standard linear regression techniques.

Evaluation of trait prediction model performance as a function of input data
(Significant finding #3)
As we reported previously, we have been using very large, publicly available data sets to estimate how models that predict plant traits improve as more data are added. Following the same approach as we previously reported, we expanded this experiment to include other plant species and other traits. Submission of an updated manuscript is pending.

NGMI model update
(Significant finding #4)
The NGMI model used to estimate IEC in the storage trial experiments discussed herein renders a prediction based on the activity of 8 genes; apart from running on a pared-down set of genes, the model, and evaluation thereof, is like those previously described (Hadish *et al.* 2024). For 'Scilate' apple we identified 3 IEC categories that were useful in our experiments: <2ppm, 2-10ppm, >10ppm. The utility of these is based largely on the accuracy of models trained on subsets of the IEC values, but also on ranges of IEC that seem to be relevant for predicting disorder profiles in USDA storage trial experiments. These IEC parameters were determined based on models built from the whole data set (all data currently in hand). In parallel we were developing qPCR assays and preparing fruit peel samples for qPCR analysis. The model with 8 genes used across 19 lots represents a special sample set for which we have storage trial outcomes (commercial and USDA), rich fruit quality data that includes our state-of-the-art computer vision data, an abundance of RNA-Seq data, and qPCR data across multiple orchards and years, each with records of production and postharvest practices, plus weather data by virtue of AgWeatherNet.

Our published work (Hadish *et al.* 2024) shows that model performance increases rapidly by doubling the number of genes from 10-20, so we expect improvements as more qPCR data are added to this special set of samples. Another improvement in the current model is the ability to make predictions of *future* IEC values, that is, to forecast the fruit IEC 7 days into the future. These models are conceptually similar to those described above and they are evaluated similarly.

Prototype test kit
(Significant finding #5)
The current prototype kit has proprietary components; therefore the following section is limited in detail. The prototype kit consists of a custom sampling device developed in cooperation with OPS Diagnostics (see below). The apple peel samples from 6-8 fruit (e.g. sun side & shade side; n≈15 peel samples) are collected in a polycarbonate vial and processed in a modified Plant Synergy 2.0 RNA extraction kit from OPS Diagnostics. The purified RNA is analyzed via qPCR (Hadish *et al.* 2024) targeting 8 select genes from the IEC prediction model and 2-3 internal reference genes. The qPCR data are used as input for the model, which rapidly (less than a second per sample) computes an IEC value for the samples.

Materials and Methods work cited
Hadish *et al.* 2024 - https://doi.org/10.1371/journal.pone.0297015
Hadish *et al.* 2022 - https://doi.org/10.1186/s12859-022-04629-7
Honaas *et al*. 2021 - https://doi.org/10.3389/fpls.2021.609684
Honaas *et al.* 2019 - https://doi.org/10.1016/j.postharvbio.2018.09.016

Results and Discussion

NGMI inputs and outputs

(Significant findings #1 & 3)

To illustrate what prototype NGMIs actually measure, we created a plot that shows the activity pattern, over time, for the top 50 genes in a prediction model (Figure 1, reproduced from AP-22-101A annual report 2025). These top 50 genes were selected from ~40,000 possible apple genes based on a sophisticated analysis of our unprecedented apple RNA-Seq postharvest data set that spans 8 years and 17 cultivars (>120 unique orchard/cultivar/years).

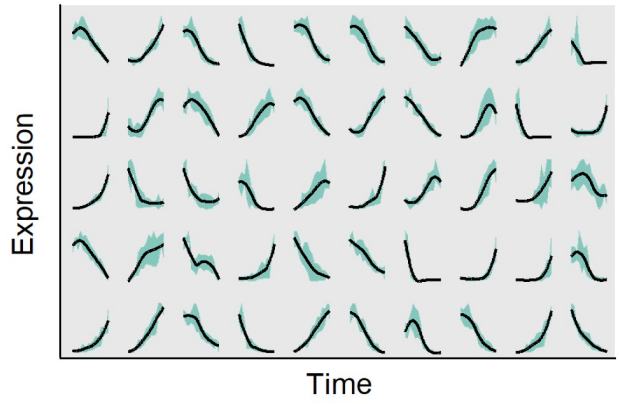

Figure 1. Prototype NGMIs integrate information from many gene activity signatures to make a prediction. Shown are normalized gene expression (i.e. gene activity) profiles in the 10-week apple time course experiment for the 50 genes most predictive of harvest week in our prototype model. Each line represents the change in gene expression of a single gene over time. Time is on the x-axis and normalized gene activity is on the y-axis for each gene. Shaded regions show standard error.

With this model, using only the combined information of these 50 gene activity patterns, we can estimate the level of fruit IEC, and many other fruit attributes with high accuracy. So, the NGMI input is a collection of gene activity signatures, and the output is a prediction of some fruit attribute. For the storage trials of 'Scilate' apple, we trained NGMI models to predict IEC as it is a useful, but impractical, maturity index compared to starch clearing. Furthermore, based on USDA storage trial data IEC is a useful indicator for internal disorder risk.

NGMIs can provide actionable data: proof of concept

(Significant findings #2 & 4)

A key hurdle for using our NMGI is the very high cost and slow turnaround time of generating the input data (RNA-Seq), so a major focus of this current project has been to build a high accuracy model that runs on cheap data that is faster and easier to generate. We tested this model on 'Scilate' apple storage trial samples using qPCR to generate the input values. The model output at-harvest IEC predictions were reassuringly good and comparable to RNA-Seq based predictions (Figure 2). The IEC 1 week forecast model has similar accuracy to the at-harvest one (RMSE: 1.08ppm & R2: 0.85).

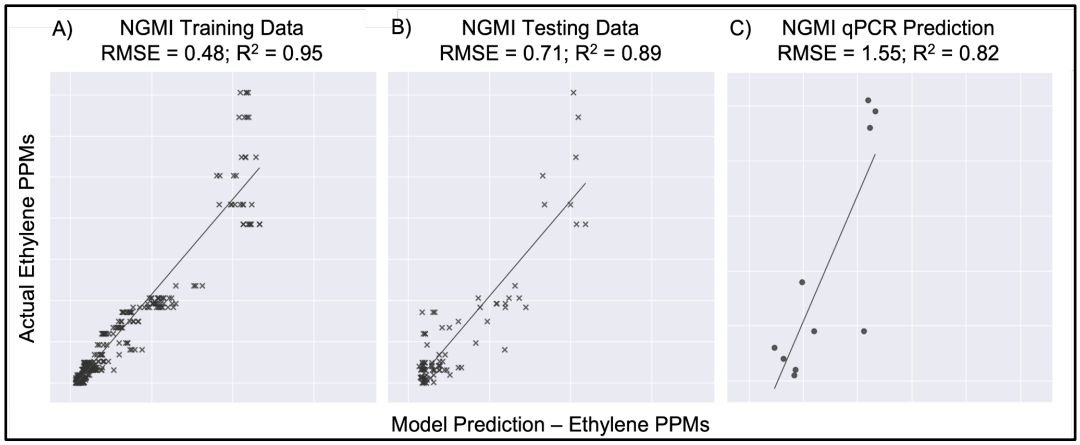

Figure 2. NGMIs estimate IEC with high accuracy. NGMI Trait Prediction Model for IEC apple (A) RNA-Seq training data, (B) RNA-Seq testing data, (C) predictions based on gene expression results from 8 preliminary qPCR biomarkers tested on 19 unique 'Scilate' at-harvest samples.

The prototype NGMI kit

(Significant finding #5)

Producing an IEC estimate using cheaper and faster qPCR data vs RNA-Seq data illustrates the potential for a point of contact test that measures just a few genes. But for a test that can be run to completion on-site with minimal equipment and supplies requires more efficient methods to generate the test samples. We have worked with project partner OPS Diagnostics to develop a sampling device (Figure 3) that seamlessly integrates into their high efficiency Plant Synergy 2.0 plant RNA kit (opsdiagnostics.com). The device captures peel samples into a vial that is pre-loaded with a solid-state extraction matrix and metal beads. The rest of the extraction protocol is based on commercially available products that use relatively inexpensive pieces of equipment (small homogenizers and benchtop centrifuges). Tests of the vial capacity indicate up to 16 samples (8 fruit x 2 faces) can be processed efficiently with the current kit, which is an acceptable size for one of a minimum 3 replicated biological samples needed for measuring gene activity (i.e. 3 replicates of 6 fruit each). Furthermore, we have benchmarked qPCR results generated from the custom Synergy 2.0 kit extracts against our gold standard lab protocol extracts.

While the qPCR step is expensive and somewhat time consuming, we expect that the assays we are developing in another project (AP-24-103 – see below) will be cheaper and faster. Importantly, qPCR provides critical ground truth for the detection technologies we are using in this and related projects.

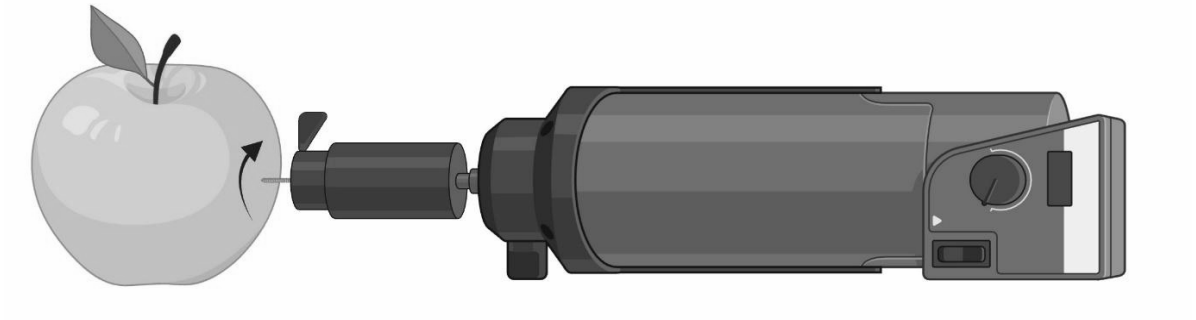

Figure 3. Rendering of OPS Diagnostics Fruit Peel Sampler.

Synergy with other WTFRC-funded projects

TR-24-100A: This project supported the development of a software called Granny that uses computer vision to estimate starch clearing in apples (and other visually scored traits). As detailed in reports for that project, data from Granny was used in place of human ratings of apple fruit to train trait prediction models. These models are trained like those discussed above and perform as well as models trained on human ratings. Advantages to the use of computer vision compared to human ratings include lower variance, higher granularity, and a digital record (by virtue of the pictures) of starch clearing patterns. With sufficient development it may also be faster.

AP-23-103A: This project aims to understand the etiology for postharvest decay using modeling methods as described above - the key difference is that the trait we model on is postharvest decay. In addition to the rates of decay, all other fruit quality parameters (such as those discussed above) have also been recorded. These new RNA-Seq and trait data will roughly double the size of our foundational data set (from ~350 to now ~700) which should enhance model accuracy.

AP-24-103: This project aims to build a biomarker panel that can predict losses in firmness of apple fruit during storage. New data from this project will add 3x more data to the current preliminary data set, which was also funded by the WTFRC. Another project goal is to develop a rapid and sensitive test that will run the biomarker panel on-site. Initial work towards this goal leveraged samples and data from AP-22-101A owing to the more mature trait prediction models and much larger sample catalog. Such tests will be equally useful across all NGMI projects discussed herein, and can be rapidly adapted as current models are improved and new ones are developed.

Executive Summary

We have shown that we can produce a next generation maturity index result using equipment, supplies, and methods which suggest a commercially viable protocol is a possibility. The test results suggest that we can estimate important fruit attributes from gene activity profiles alone. The IEC prediction model is just a proof-of-concept - we have shown previously that we can estimate many different fruit attributes using a similar strategy, and the predictions improve as more data are added. Reassuringly, we have shown that our prototype NGMI can render actionable predictions that rely on just a handful of gene activity profiles. Importantly, qPCR and related technologies can be rapidly tuned to detect new genes, so as models are improved and new ones are developed, the test kits can be rapidly adapted to measure new input gene profiles to predict fruit attributes of choice.

Project Title: Reducing CO_2-related disorders during Honeycrisp rapid CA treatment

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Cooperators: Sarah Gabel, Emmi Klarer, Stemilt Growers LLC., McDougall & Sons Inc.

Report Type: Continuing Project Report

Project Duration: 3 Years

Total Project Request for Year 1 Funding: $22,000
Total Project Request for Year 2 Funding: $85,000
Total Project Request for Year 3 Funding: $85,000

Other related/associated funding sources: Awarded
Funding Duration: 2022 - 2025
Amount: $115,317/3 yrs.
Agency Name: USDA-ARS, In-house project
Notes: In-house project with complimentary objectives. Funds for storage maintenance and costs ($8000/yr), supplies and materials ($3000/yr), travel ($1000/yr), and 0.1 FTE (PI) and 0.1 FTE (technical).

Other related/associated funding sources: Awarded
Funding Duration: 2023 - 2027
Amount: $555,828/4 yrs.
Agency Name: USDA-NIFA
Notes: Project submitted to SCRI program on FY21 was highly scored but not funded. Reviewer concerns will be addressed with stakeholder consultation and the proposal resubmitted for FY22.

Budget
Primary PI: David Rudell
Organization Name: USDA-ARS
Contract Administrator: Sharon Blanchard
Telephone: 509-664-2280 (SB)
Contract administrator email address: Sharon.Blanchard@usda.gov

Item	2022	2023	2024
Salaries (GS-7)*		43,311	45,764
Benefits (40%)		18,124	18,305
Wages	5,000	5,000	5,000
Benefits			
Equipment			
Supplies	5,000	4,565	3,931
Travel			
Miscellaneous**	12,000	12,000	12,000
Plot Fees			
Total	22,000	85,000	85,000

Footnotes: *Estimated 3% salary increase; **22% of instrument service contract

Objectives:

1. Determine influence of CO_2 levels on disorder development during rapid CA treatment.
2. Determine influence of temperature on disorder development during rapid CA treatment.
3. Monitor flesh chemistry to indicate which CO_2 level treatment conditions may elevate risk of developing soft scald/soggy breakdown or CO_2-related/other disorders.

SIGNIFICANT FINDINGS

1. Rapid CA conditioning eliminated soft scald/soggy breakdown.
2. CO_2-related internal browning incidence decreased with increasing conditioning temperature.
3. Rapid CA conditioning does not compromise quality (6 months) regardless of how long it was delayed.
4. Fruit quality was not impacted by conditioning temperature.
5. CO_2-related symptoms developed as a result of elevated CO_2 during rapid CA in all seasons where symptoms developed.
6. Internal browning associated with elevated CO_2 during rapid CA develops after transfer to long-term CA (low CO_2) storage.
7. Differences of CO_2 sensitivity was observed using cortex chemistry monitoring.
8. Bitter pit and leather blotch were more common at orchards where fruit was less mature at harvest.
9. Apples stored in atmospheres comprising higher CO_2 had higher titratable acidity and total soluble solids.

METHODS

Objective 1: Determine influence of CO_2 levels on disorder development during rapid CA treatment.

In year 3, Honeycrisp apples were harvested as close to commercial harvest as possible from 9 orchards near Bridgeport, Mattawa, Quincy, and Royal City, WA. Harvest maturity (internal ethylene concentration, firmness, starch index, titratable acidity, and soluble solids) and external/internal appearance were evaluated, and fruit were imaged. Apples were treated with 1-MCP (about 1 ppm), then stored in 2.5% O_2 and (0.5, 1, 2, 3, 5%) CO_2 for 7 days at 50 °F. Following conditioning, apples were stored for 6 months in 2.5% O_2 and 0.5% CO_2 at 37 °F upon which external and internal disorders, firmness, titratable acidity, and soluble solids were evaluated. All statistical analysis was performed using SAS version 9.4 TS Level 1M8.

Objective 2: Determine impact of initial fruit temperature during conditioning.

To determine the impacts of conditioning temperature during rapid CA, Honeycrisp apples were harvested approximately one week after commercial harvest from an orchard in Mattawa, WA. Harvest maturity (internal ethylene, firmness, starch index, titratable acidity, and soluble solids) and external/internal defects were evaluated, and fruit were imaged. Apples were treated with 1-MCP (about 1 ppm) and immediately placed in CA in 0.5% O_2 and 2.5% CO_2 at (37, 46, 50 °F) for 7 days. Due to previous difficulties in observing disorders in the apples and to establish whether disorders would develop, 2 trays were stored in a room held at 51 °F for 7 days. Following conditioning, apples were stored in 2.5% O_2 and 0.5% CO_2 at 37 °F for 6 months upon which external and internal disorders, firmness, titratable acidity, and soluble solids were evaluated.

Objective 3: Monitor flesh chemistry to indicate which treatment conditions may elevate risk of developing soft scald/soggy breakdown or CO_2-related browning.

Honeycrisp apples were picked from the same 9 orchards in objective 1 at commercial harvest. They were treated with 1-MCP and underwent a 7-day conditioning period at 50 °F, 2.5% O_2, and one of the following five CO_2 levels: 0.5%, 1%, 2%, 3%, and 5%. During the conditioning period, apple cortex was assessed for injury and sampled for metabolic analysis at days 0 and 7 for all orchards, but also at days 2 and 4 for three of the nine orchards. Cortex samples were frozen in liquid nitrogen and ground using an analytical mill (IKA model A 11 B S001) and were stored at -80 ºC prior to targeted analysis for chemicals linked to CO_2-sensitivity (McTavish et al., 2025).

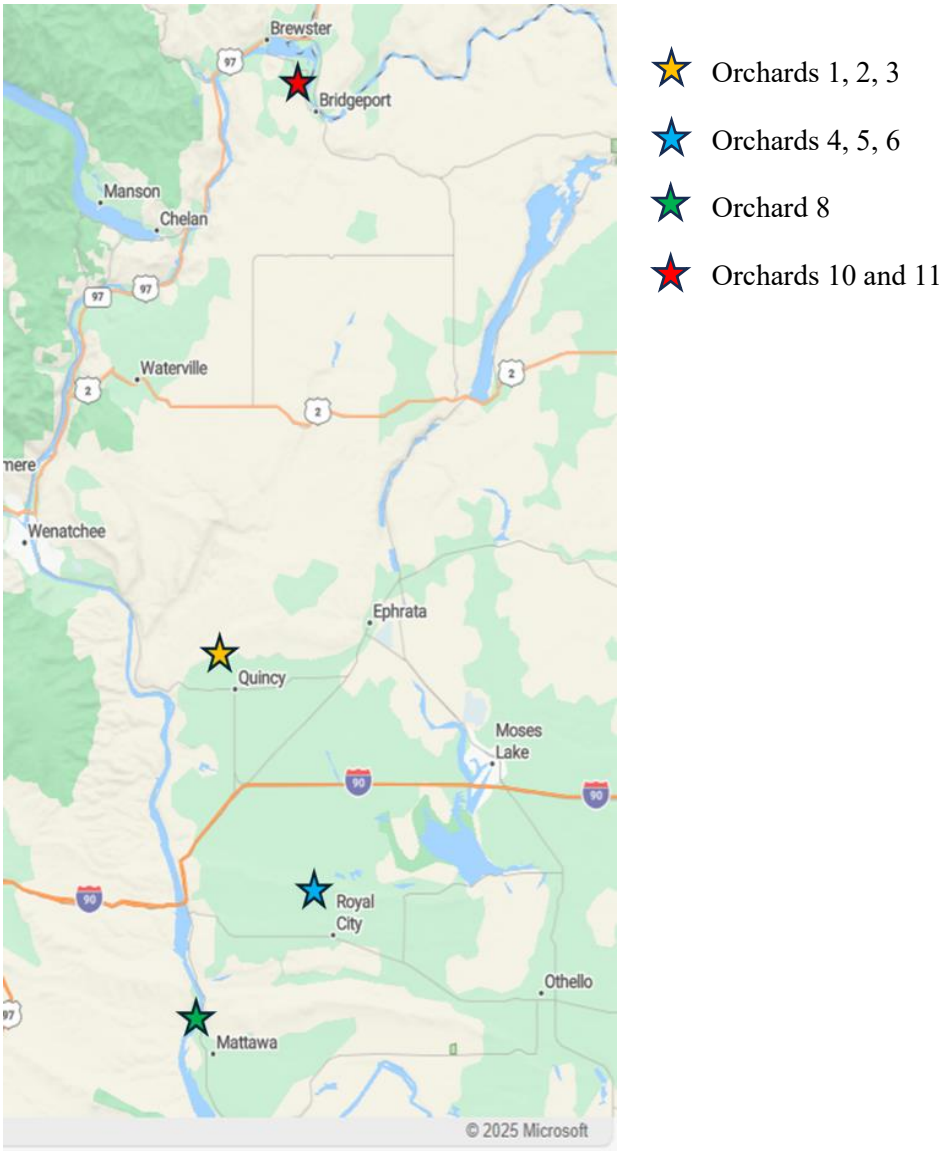

Figure 1. Distribution of orchards near four cities (Bridgeport, Quincy, Royal City, and Mattawa) of Washington State.

RESULTS AND DISCUSSION

Objective 1: Determining influence of CO_2 levels on disorder development during rapid CA treatment

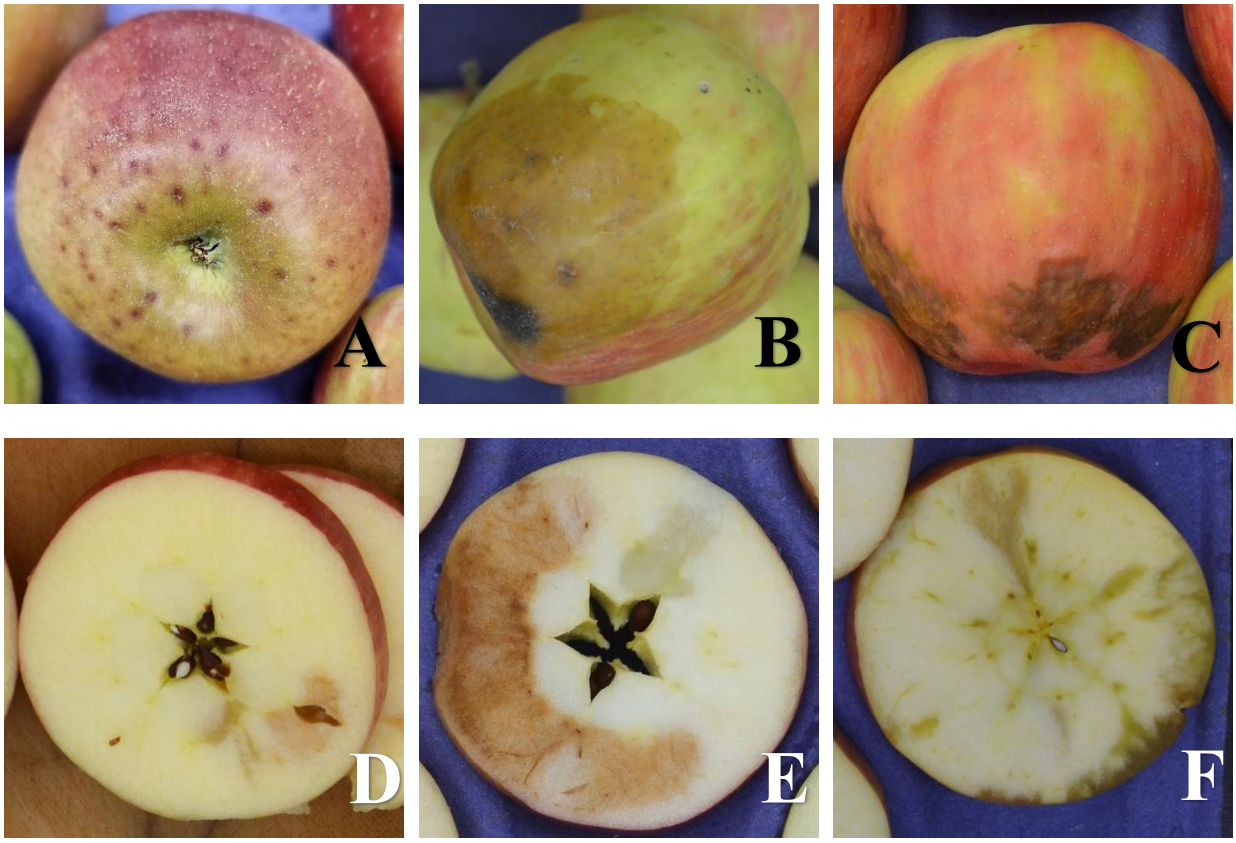

Figure 2. Disorders of Honeycrisp observed in the 2024 sampling season. (A) Bitter pit (B) Soft scald (C) Leather blotch (D) CO_2 browning and lens-shaped cavity (E) Soggy breakdown (F) Watercore. Brightness was enhanced 20% on pictures D-F.

Honeycrisp apples were harvested immediately ahead of commercial harvest from 7 orchards and immediately after harvest for 2 (orchards 10 and 11). Apples were temperature conditioned during the initial pull down to 2.5% O_2 and different CO_2 concentrations to observe response to CO_2 levels as well as susceptibility to bitter pit, soft scald, leather blotch, soggy breakdown, and watercore. Averaged starch index values at harvest were between 4 and 6 at all orchards except orchards 3 and 10 with values of 2.08 and 3.17 respectively. Likewise, orchards 3 and 10 produced the least internal ethylene further indicating fruit from these orchards was less mature than those from the other 7 orchards.

Bitter pit, leather blotch, and watercore incidence remained insignificant on apples harvested from every orchard, but notably fruit from orchard 3 developed high frequencies of leather blotch (up to 84%), regardless of conditioning treatment (Table 1). The relatively less mature apples from orchards 3 and 10 explains the higher incidence of bitter pit in fruit originating

Table 1. Percent disorder incidence after 6 months of storage of Honeycrisp harvested at 9 different orchards and conditioned at 50 °F alongside rapid CA with 2.5% O_2 and varying CO_2 levels to display differences in disorder development among orchards. CO_2-related internal browning and lens-shaped cavities were orchard dependent and, in those orchards with significant incidence, was primarily associated with 5% CO_2 during conditioning. Other disorders were less linked with conditioning variables and were, instead, equally prevalent in all treatments as in the case of leather blotch. Data were analyzed comparing CO_2 conditioning treatments within a single orchard using either a Pearson's Chi-Square table or a Fisher's exact test if the data was sparse (cells with counts <5), followed by pairwise comparisons using Pearson's Chi-Squares (n=36; α=0.05). Letters indicate significant differences of disorder incidence within a single orchard, which were observed at some orchards for soft scald, CO_2 damage, and soggy breakdown.

Orchard #	CO_2 Treatment	Bitter Pit	Soft Scald	Leather Blotch	CO_2-relatedinternal browning and cavities	Soggy Breakdown	Watercore
1	0.50%	0 a	2.86 a	0 a	14.29 ab	0 a	0 a
	1%	2.78 a	0 a	0 a	11.11 a	0 a	0 a
	2%	0 a	0 a	0 a	11.11 a	5.56 a	0 a
	3%	0 a	0 a	0 a	5.56 a	2.78 a	0 a
	5%	0 a	0 a	0 a	30.56 b	0 a	0 a
2	0.50%	0 a	0 a	0 a	5.56 a	0 a	0 a
	1%	0 a	16.67 b	0 a	11.11 a	11.11 b	0 a
	2%	0 a	0 a	0 a	0 a	0 a	0 a
	3%	0 a	0 a	0 a	16.67 a	0 a	0 a
	5%	0 a	8.33 ab	0 a	11.11 a	8.33 ab	0 a
3	0.50%	0 a	0 a	75.00 a	2.78 a	0 a	0 a
	1%	0 a	0 a	72.22 a	2.78 a	0 a	0 a
	2%	0 a	0 a	83.33 a	2.78 a	0 a	0 a
	3%	0 a	0 a	75.00 a	0 a	0 a	0 a
	5%	0 a	0 a	77.78 a	0 a	0 a	0 a
4	0.50%	0 a	8.57 a	2.86 a	2.86 a	0 a	0 a
	1%	0 a	0 a	8.33 a	5.56 a	0 a	0 a
	2%	0 a	0 a	8.57 a	2.86 a	0 a	0 a
	3%	0 a	5.71 a	0 a	0 a	0 a	0 a
	5%	0 a	5.56 a	13.89 a	2.78 a	0 a	0 a
5	0.50%	0 a	0 a	8.82 a	2.94 a	0 a	0 a
	1%	0 a	0 a	8.33 a	0 a	0 a	0 a
	2%	0 a	0 a	3.03 a	3.03 a	0 a	0 a
	3%	0 a	0 a	8.57 a	8.57 ab	0 a	0 a
	5%	0 a	2.86 a	11.43 a	25.71 b	0 a	0 a
6	0.50%	0 a	0 a	0 a	0 a	0 a	0 a
	1%	0 a	0 a	2.78 a	0 a	0 a	0 a
	2%	0 a	0 a	2.86 a	0 a	0 a	0 a
	3%	0 a	0 a	0 a	0 a	0 a	0 a
	5%	0 a	0 a	2.86 a	0 a	0 a	0 a
8	0.50%	0 a	0 a	0 a	0 a	0 a	0 a
	1%	0 a	0 a	2.78 a	8.33 a	0 a	0 a
	2%	0 a	0 a	0 a	5.56 a	0 a	0 a
	3%	0 a	0 a	0 a	5.56 a	0 a	0 a
	5%	0 a	0 a	0 a	2.78 a	0 a	0 a
10	0.50%	2.94 a	0 a	2.94 a	11.76 a	0 a	8.82 a
	1%	11.11 a	0 a	0 a	13.89 a	0 a	2.78 a
	2%	8.82 a	0 a	0 a	17.65 a	0 a	8.82 a
	3%	2.78 a	0 a	0 a	5.56 a	0 a	0 a
	5%	5.71 a	0 a	2.86 a	8.57 a	0 a	0 a
11	0.50%	2.78 a	0 a	2.78 a	8.33 a	0 a	0 a
	1%	0 a	0 a	2.78 a	2.78 a	0 a	0 a
	2%	0 a	0 a	0 a	2.78 a	0 a	2.78 a
	3%	0 a	0 a	0 a	5.56 a	0 a	0 a
	5%	0 a	0 a	2.78 a	0 a	0 a	0 a

from orchard 10 and leather blotch at orchard 3, both disorders associated with immature apples. Soft scald and soggy breakdown incidence were different among conditioning treatments for apples from

orchard 2, where the 1% CO_2 treatment had more instances of these disorders than all other treatments except the 5% CO_2 treatment (Table 1). Incidence on fruit from other orchards was insignificant. Honeycrisp from orchards 1 and 5 developed CO_2-related internal browning, primarily when CO_2 levels were adjusted to 5%.

Table 2. Fruit quality after 6 months of storage of Honeycrisp conditioned at 50 °F alongside rapid CA with 2.5% O_2 and varying CO_2 levels to display differences in CO_2 sensitivity among orchards. Firmness was not impacted by CO_2 % during conditioning. TA was elevated with CO_2% during conditioning. Data were analyzed comparing CO_2 conditioning treatments within a single orchard using ANOVA followed by Tukey's comparison or – if the data were not normally distributed or did not have equal variances – a Kruskal-Wallis test followed by Wilcoxon DSCF pairwise comparisons (n=36 for firmness and 18 for TA and °Brix; α=0.05). Letters indicate significant differences between CO_2 treatments within a single orchard, which were observed at some orchards for TA and °Brix.

Orchard #	CO_2 Treatment	Firmness (lb)	TA (g/L)	°Brix
1	0.50%	14.28 a	3.98 ab	14.48 a
	1%	14.39 a	3.79 a	14.47 a
	2%	14.59 a	3.91 ab	14.81 a
	3%	14.67 a	4.14 ab	14.72 a
	5%	14.55 a	4.39 b	14.57 a
2	0.50%	14.46 a	3.95 a	14.16 a
	1%	15.06 a	4.06 a	14.11 a
	2%	14.85 a	4.12 a	13.99 a
	3%	14.87 a	4.22 a	14.22 a
	5%	14.21 a	4.02 a	13.97 a
3	0.50%	14.42 a	5.42 a	13.19 a
	1%	14.89 a	5.61 a	13.16 a
	2%	14.97 a	5.61 a	12.96 a
	3%	14.86 a	5.56 a	12.98 a
	5%	14.94 a	5.52 a	13.23 a
4	0.50%	13.15 a	3.70 a	12.24 ab
	1%	13.84 a	4.05 ab	12.39 ab
	2%	13.12 a	4.13 b	12.13 a
	3%	13.53 a	4.16 b	12.59 b
	5%	13.45 a	3.93 ab	12.53 b
5	0.50%	14.94 a	4.80 a	13.45 a
	1%	15.44 a	4.52 a	12.91 b
	2%	15.58 a	4.52 a	13.14 ab
	3%	15.12 a	4.77 a	13.22 ab
	5%	15.16 a	4.80 a	13.12 ab
6	0.50%	14.72 a	3.50 a	13.86 ab
	1%	14.97 a	4.07 b	13.75 abc
	2%	14.66 a	3.79 ab	13.56 c
	3%	14.48 a	3.42 a	13.61 bc
	5%	14.84 a	3.54 ab	13.91 a
8	0.50%	15.94 a	5.38 a	13.64 a
	1%	15.70 a	5.46 a	13.74 a
	2%	15.58 a	5.44 a	13.58 a
	3%	15.54 a	5.46 a	13.52 a
	5%	15.66 a	5.54 a	13.51 a
10	0.50%	16.47 a	6.24 ab	17.77 a
	1%	17.16 a	6.31 ab	17.66 ab
	2%	17.77 a	6.33 ab	17.67 ab
	3%	17.52 a	6.22 a	17.88 a
	5%	17.73 a	6.79 b	17.32 b
11	0.50%	14.74 a	4.47 ab	14.09 a
	1%	15.08 a	4.67 abc	14.23 a
	2%	15.23 a	4.40 a	14.16 a
	3%	14.86 a	4.85 bc	14.41 a
	5%	15.01 a	4.90 c	14.43 a

There were no significant differences in fruit firmness among treatments at any orchard after 6 months of storage. Conditioning treatment did impact titratable acidity and soluble solids concentrations (Table 2). Significant differences of titratable acidity among treatments occurred following storage of apples from orchards 1, 4, 6, 10, and 11. The majority of these differences were linked with higher CO_2 % during conditioning, including stepwise increases with CO_2 levels in a few cases rather than only the extremes. CO_2 level during conditioning impacted soluble solids concentrations for fruit from orchards 4, 5, 6, and 10. Most of these differences were between adjacent treatments in CO_2 concentration rather than extremes.

Objective 2: Determining impact of initial fruit temperature during conditioning

Honeycrisp apples were harvested from orchard 8 (objective 1) about 2 weeks after commercial harvest. Apples were conditioned at various temperatures to observe disorder incidence rates response. Average starch values of 5.89 and an IEC of 40.71 ppm at harvest indicate these apples were well into the climacteric stage of ripening. Firmness and titratable acidity were consistent across treatments, but soluble solids varied when comparing the temperature treated apples to the control treated apples (Table 3). All temperature conditioned apples (37, 46, and 50 °F) had significantly more soluble solids compared to the air treated apples. There were no significant differences in apple quality between the temperature treatments.

Table 3. Fruit quality after 6 months of storage of Honeycrisp conditioned at 2.5% CO_2 and 0.5% O_2 and varying temperatures. The air stored, unconditioned comparison is listed at the bottom. Results demonstrate that temperature treatment following 1-MCP treatment had little influence on fruit quality. Data were analyzed comparing treatments using ANOVA followed by Tukey's comparison or – if the data were not normally distributed or did not have equal variances – a Kruskal-Wallis test followed by Wilcoxon DSCF pairwise comparisons (n=36 for firmness and 18 for TA and °Brix; α=0.05). Letters indicate significant differences between treatments, which were observed among the °Brix values.

Treatment	Firmness (lb)	TA (g/L)	°Brix
37 °F	13.61 a	4.04 a	13.14 ab
46 °F	13.92 a	4.09 a	13.20 ab
50 °F	13.92 a	4.17 a	13.42 a
Air	13.64 a	4.03 a	12.50 c

There were no instances of bitter pit, leather blotch, or watercore in any of the apples sampled from orchard 8 at the late harvest (Table 4). Apples conditioned at 37 °F were more sensitive to CO_2 than those conditioned at 46 °F. Also, apples conditioned at any temperature developed more CO_2-related internal browning and lens-shaped cavities than those stored in air without conditioning. There was no significant incidence of soggy breakdown and soft scald.

Table 4. Percent disorder incidence after 6 months of storage of Honeycrisp conditioned at 2.5% CO_2 and 0.5% O_2 and varying temperatures. The air stored, unconditioned comparison is listed at the bottom. Results indicate that lower temperatures during conditioning elevate CO_2-sensitivity. Data were analyzed comparing treatments using either a Pearson's Chi-Square table or a Fisher's exact test if the data was sparse (cells with counts <5), followed by pairwise comparisons using Pearson's Chi-Squares (n=36; α=0.05). Letters indicate significant differences of disorder incidence.

Treatment	Bitter Pit	Soft Scald	Leather Blotch	CO_2-related internal browning and cavities	Soggy Breakdown	Watercore
37 °F	0 a	0 a	0 a	30.56 a	0 a	0 a
46 °F	0 a	0 a	0 a	11.11 b	2.78 a	0 a
50 °F	0 a	0 a	0 a	13.89 ab	0 a	0 a
Air	0 a	5.56 a	0 a	2.78 bc	2.78 a	0 a

Objective 3: Monitoring flesh chemistry during conditioning period

Honeycrisp cortex was collected from the apples from all locations used for objective 1 before and after the conditioning period and, for 3 of the 9 orchards, an additional two times during the conditioning period. The purpose for sampling cortex during the conditioning period was to monitor levels of natural chemicals found to be associated with risk of CO_2-related internal browning in our previous project (McTavish et al., 2025). This was attempted in 2023 at one orchard but disorder incidence was low, hence it was repeated in 2024 using multiple orchards.

CO_2-related browning risk was analyzed during the conditioning period for orchard 5. Cortex was analyzed for changes in levels of risk-associated natural chemicals. These are chemicals related to cell membrane integrity that were also identified earlier by us as associated with superficial scald risk. The relationship of one natural chemical group, the acylated steryl glycosides (ASGs), changes in ratio with its related steryl ester (SE). We are monitoring this ratio during conditioning to determine if risk caused by the environment during conditioning will lead to browning during long-term storage. If CO_2 levels were adjusted to 5%, apples from orchard 5 produced a higher ASG:SE ratio of these compounds than those conditioned in an atmosphere comprising 1% CO_2 (Figure 3). The spike in ASG:SE is potentially correlated with long-term disorder development, as 25.71% of the apples from orchard 5 conditioned in 5% CO_2 exhibited CO_2-related injuries and those conditioned in 1% CO_2 showed none (Table 1). This analysis is ongoing and expected to be completed for all samples from all orchards regardless of storage outcome with the intent of determining if these changes are linked with risky conditions or strictly with eventual symptom development.

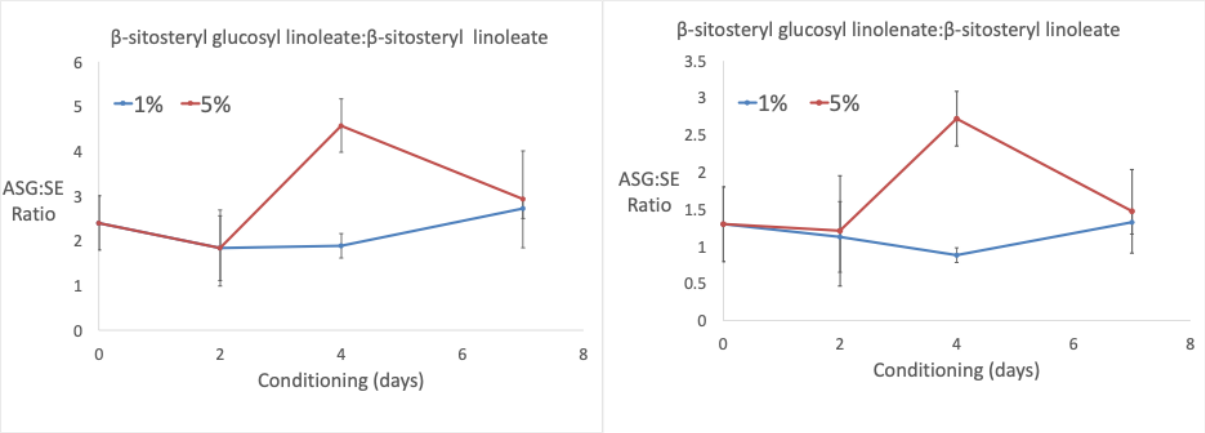

Figure 3. Natural chemicals levels increasing during rapid CA conditioning of Honeycrisp in conditions more likely to cause apples to develop internal browning. Apple cortex chemistry was analyzed during the asymptomatic conditioning period to determine if changes in CO_2-risk related chemistry would reflect disorder outcome. This was true for orchard 5 where there was a spike of the ASG:SE ratio between 2 and 7 days. Analyses are ongoing for the remainder of samples in this and other orchards. Error bars indicate standard error (n=6).

References:

McTavish, C., Klarer, E., Milne, S., Valdez, N., Mattheis, J., Leisso, R. and Rudell, D., 2025. Risk assessment candidates for carbon dioxide-related internal browning of apple during controlled atmosphere storage. Postharvest Biology and Technology, 230:113840.

Project Title: Improving Apple Fruit Quality and Postharvest Performance

Report Type: No-cost extension

Primary PI: Manoella Mendoza
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Project Duration: 3 Years

Total Project Request for Year 1 Funding: $ 36.661
Total Project Request for Year 2 Funding: $ 39,802
Total Project Request for Year 3 Funding: $0

Other related/associated funding sources: in-kind contributions = $30K.
Notes: Stemilt conducts the application of SmartFresh and donates and applies postharvest fungicides. The WA 38 apples are harvested from two Stemilt blocks. Vertigo-Tech provided the microwave sensor prototype. USDA is providing CA storage (chambers). The industry donates other miscellaneous supplies (trays, boxes, bags, etc.). The Dutch government, via Orchard of the Future collaboration: "Developing bilateral innovation projects (BIS) for technology development and application," provides funding for Vertigo Tech (traveling, machine shipping).

Item	2023	2024	2025
Salaries			
Benefits			
Wages	$24,802.00	$27,011.00	
Benefits	$10,629.00	$11,576.00	
RCA Room Rental	$1,180.00	$1,215.00	
Shipping			
Supplies			
Travel			
Miscellaneous			
Total	$36,611.00	$39,802.00	$0.00

Footnotes: Wages/Benefits: calculated based on expected staff wage adjustments.

Justification

One of the WTFRC internal program targets is to tackle high-priority industry needs that are not covered elsewhere. The Commission has used this project structure for several years, which enables us to respond swiftly to significant industry topics related to apple quality and postharvest issues. This includes the development of variety-specific starch scales (e.g., Honeycrisp, WA 38), testing various methodologies (e.g., bitter pit prediction methods for Honeycrisp), products (e.g., NSure sampling kit, Accuvin malic acid test), and equipment (e.g., Felix F750, DA meter).

This project structure is also beneficial for reducing required funding, as harvest and quality analysis for multiple objectives are conducted concurrently. In this funding cycle, the WA 38 was used for the starch degradation and the Fresco testing. Additionally, a new objective was added, and data was collected for the Granny software (WA 38, Honeycrisp, and Gala) at no additional cost, as it was integrated into the Fresco testing protocol. Granny Smith apples were also harvested this season and used for testing the Fresco and the Granny Software. The outcomes of this project lead to straight-forward, directly actionable results benefitting both organic and conventional growers, regardless of the size or scale of their operations.

Objectives

1. Evaluate new technologies to assess fruit quality parameters
    a. Testing Fresco (microwave sensor) to measure apple quality parameters non-destructively
    b. **(New)** Collaborate with Honaas lab to test the Granny software (image-based analysis) for starch degradation
2. Investigate the effect of 1-MCP on WA 38 starch degradation during RA and CA storage
3. Assess the influence of 1-MCP on WA 38 fruit flavor

Significant Findings

Fresco Microwave Sensor

- Wavelengths correlated with firmness, soluble solids, and titratable acidity were identified in the microwave spectra region
- Positive correlation of soluble solids, firmness, and titratable acidity is weak, moderate, and strong, respectively, when predicting quality parameters for a randomly selected set of apples, unseen by the model
- Soluble solids prediction was affected by the narrow distribution range, but the mean absolute percent error (MAPE) and bias are low, indicating that the error between prediction and observed value is low.
- The best-fitting model was for titratable acidity, with a coefficient of determination above 0.7 and low error metrics.

WA 38 Research

- WA 38 starch clearance rate and variability are influenced by tree age, with fruit from mature trees displaying a more homogeneous starch degradation compared to fruit from young trees
- Fruit from young trees (2 and 3 years) might need additional time in storage to achieve 90% of the fruit with a starch clearance of 5.0 required for packing and shipping

- 1-MCP treatment and CA storage did not impact the starch degradation rate
- 1-MCP had an adverse effect on the flavor of fruit harvested from mature trees in 2023, while fruit from mature trees that were not treated with 1-MCP was preferred among all tree age and treatment combinations

NOTE: The WA 38 research was concluded in 2024, and the findings were incorporated into the preceding report. As there are no new developments during this reporting cycle, I will refrain from reiterating the results here. All pertinent information regarding this project will be included in the final report. Progress on the Granny Software will be reported by the PI.

1. Evaluation of new technologies

a. Fresco microwave sensor

Methods

Fresco is a new sensor developed by Vertigo Tech, a startup, spin-off of Delft University of Technology in the Netherlands, and part of the Orchard of the Future Dutch-USA collaboration. The sensor is a hand-held device that uses low-energy microwaves to non-destructively measure fresh fruit quality parameters such as Brix, titratable acidity, firmness, juiciness, and dry matter. One differential is that microwaves penetrate deeper into the fruit flesh in comparison with, for instance, NIR sensing. The sensor is in the pilot stage, with prototypes tested by Vertigo Technologies, the Wageningen University in the Netherlands (part of the Next Fruit 4.0 Cool data), and the WTFRC.

In 2023, one bin each of Gala, Honeycrisp, and WA 38 was harvested at commercial maturity and treated with postharvest fungicide before storage. The WA 38 and Gala were stored in a Stemilt RCA room (RA, 33°F), and Honeycrisp was conditioned and stored in the WTFRC cold room at 37°F. Fruit was not treated with 1-MCP. A total of 700 apples per variety were sampled during four sessions, from January to April. The apples were taken out of storage the day before quality assessment, numbered, placed in trays, and left at room temperature for one day. Each apple was processed individually.

In this first phase, we collected data for three apple quality parameters: firmness (lb.), soluble solids content (% Brix), and titratable acidity (% m.a.). The goal was to use the data to pinpoint the wavelength associated with each quality parameter and test the accuracy of the prediction by comparing the results of the destructive measurements with the values predicted by Fresco. The Fresco measurements were taken on the sun-exposed and shaded sides of each apple, at the same location where firmness was measured with the Fruit Texture Analyzer. The Brix and titratable acidity were measured from the apple juice made with the sun-exposed and shaded pieces. The Felix 750 produce quality meter was used to collect non-destructive Brix values, and the results will be compared with the Brix predictions provided by Fresco. Peter Balk at Wageningen University also evaluated the same quality parameters as the WTFRC for two apple varieties (Gala and Elstar), with the addition of dry matter assessment.

To analyze the 2023 data, a partial least square (PLS) analysis was conducted to appraise the correlation between prediction (non-destructive) and destructive methods and to assess the accuracy of the non-destructive method. The regression model was validated using a K-fold and a Train/Test split. A 10-fold cross-validation performs the fitting procedure ten times, with each fit being performed on a training set consisting of 90% of the total training set selected at random, with the remaining 10% used as a "hold-out" set for validation. When using Train/Test split, part of the data is not used in training but only as a test set, unseen by the model.

UPDATE

In 2024, *Granny Smith* apples were incorporated into the experiment to expand the data range, with particular emphasis on soluble solids content (°Brix) and titratable acidity (TA). Pre-harvest samples were collected from all apple varieties to assess the potential of the Fresco device as a pre-harvest evaluation tool. In 2025, dry matter content was added as an additional quality parameter and evaluated during storage (January to March) alongside the other fruit quality measurements.

The dataset was analyzed using a neural network approach, with 80% of the data allocated for model training, 10% for validation, and 10% for testing. However, a substantial portion of the 2024 data could not be used because the probe accumulated dirt during continuous use without adequate cleaning, which caused signal drift (see Figure 1). *As a result, a no-cost extension was requested to allow for additional data collection during the 2025 season, enabling the development of a more robust apple quality model that can be validated in the subsequent year.*

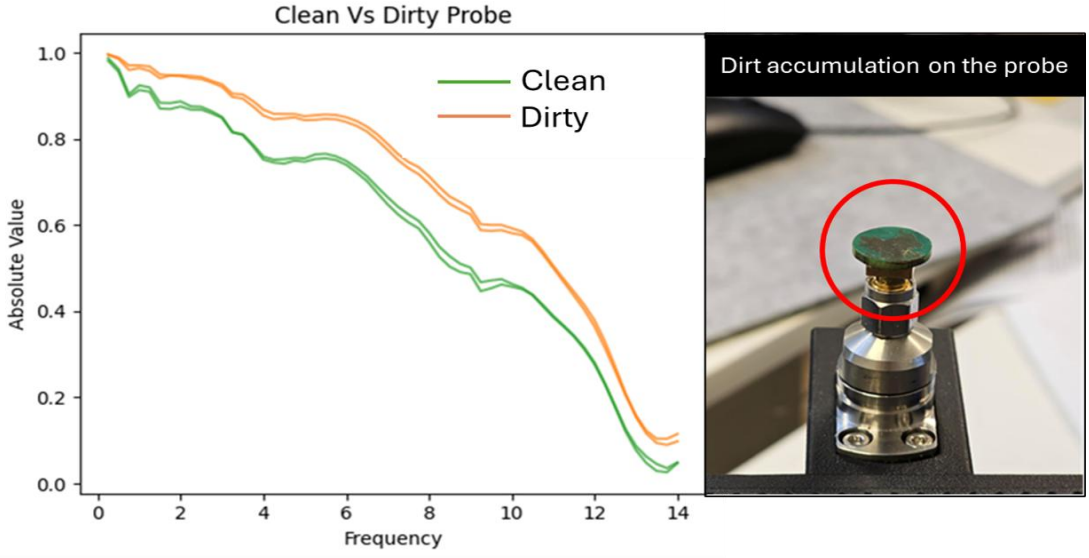

Figure 1. Comparison of the absolute value at different frequency points (GHz) for measurements taken with a clean (green, lower values) and dirty (orange, higher values) probe.

The results and discussion section is based on the updated statistical analysis of the 2023 dataset and a small portion of the 2024 data that was not affected by signal drift.

Results and discussion

Figure 1 shows the data distribution of soluble solids (A), firmness (B), and titratable acidity (C) assessed via destructive methods. The overall range of variability between time points is low, especially for soluble solids. This range restriction can affect the correlation coefficient. Titratable acidity showed the highest variability among apple varieties, but the mean values across sessions were similar, except for WA 38 and Gala at the first-time points.

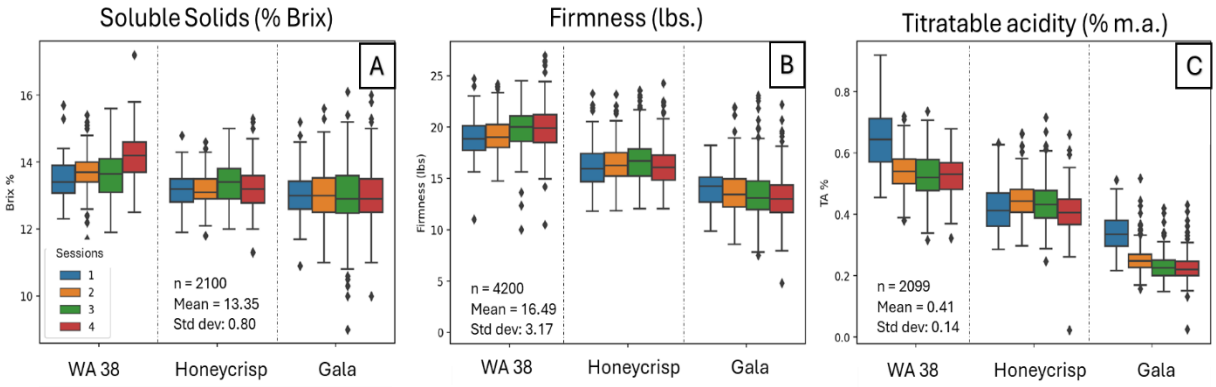

Figure 2. The data distribution of soluble solids (A), firmness (B), and titratable acidity (C) was measured using destructive methods for WA 38, Gala, and Honeycrisp apple varieties. Quality parameters were evaluated at four different times during the storage period. The graph shows the total number of data points (n) and the mean and standard deviation (Std dev) for each quality parameter.

The data from the three cultivars were combined to train, validate, and test the model for each quality parameter. Model training was conducted on 80% of the dataset, and validation and testing were conducted on the remaining 20%, split equally. Validation and testing were conducted on a portion of the dataset unseen by the model (Figure 3). The model training and testing scatterplots (Figure 3) and the correlation coefficient and error (Table 1) show variability in prediction accuracy between quality parameters.

Positive correlations for soluble solids, firmness, and titratable acidity are weak, moderate, and strong, respectively, when predicting quality parameters for a randomly selected set of apples unseen by the model. Among the three tested quality parameters, the best-fitting model was for titratable acidity. The predictions for the training set were equivalent to those for the testing set, with a coefficient of determination (R^2) above 0.7 and low error metrics (RMSE, MAE, and Bias) for both sets. For firmness, R^2 was lower and error metrics were higher for the testing set than for the model (Table 1), indicating that predictions were less accurate for the independent set of values.

Soluble solids have the lowest R^2 values for both the training and testing datasets. This can be attributed to the narrow Brix distribution in the dataset, which creates a notable data artifact known as range restriction. However, the MAPE and Bias are low, indicating that the model's estimator's expected value is close to the true underlying population parameter it aims to estimate. The MAE indicates that, on average, the difference between predicted and measured values is less than 0.5% Brix (Table 1). Increasing the range of soluble solids values might yield a higher coefficient of determination.

In 2023, the prediction of soluble solids was assessed using the Felix F-750 instrument based on a generic apple model (data not shown). The correlation coefficient for the Felix prediction was lower (R^2 = 0.194) than the Fresco prediction (R^2 = 0.465). It's important to emphasize that high correlation coefficients (above 0.7) can be achieved with the Felix instrument when developing a variety-specific model or conducting a Partial Least Squares (PLS) analysis using the data generated by Felix. However, this experiment evaluated the instrument's ability to deliver quality parameter results promptly without requiring further data manipulation by the user. Additional data analysis will be conducted throughout this project to better compare the predictive power of both instruments.

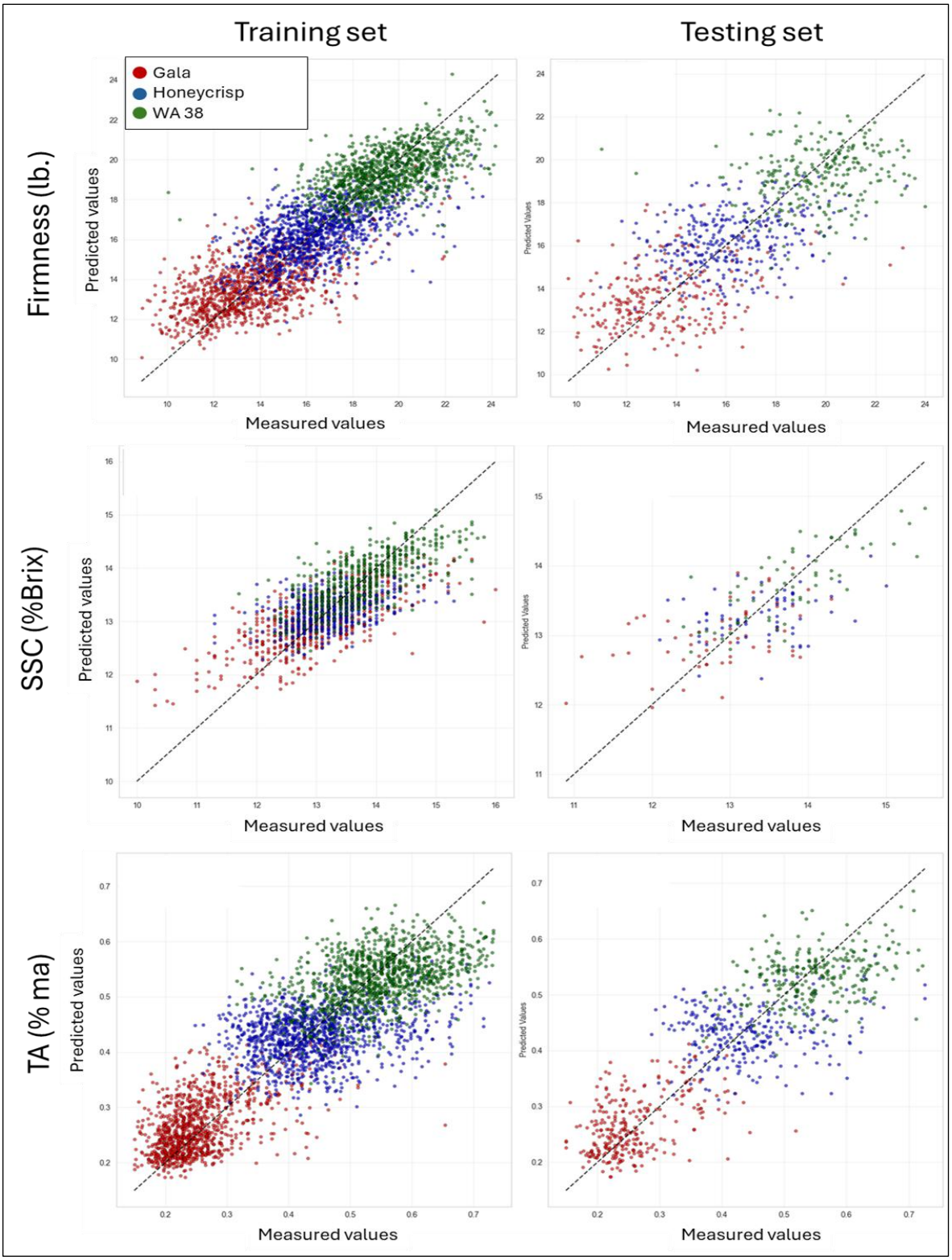

Figure 3. Soluble solids (%Brix), firmness (lbs.), and titratable acidity (% ma) correlation between non-destructive predicted values calculated by Fresco and destructive measurements (measured values) for model training and testing using Neural Network modeling.

Table 1. Coefficient of determination (R^2), root mean square error (RMSE), maximum absolute error (MAE), mean absolute percentage error (MAPE), and Bias for the sample sets used by Fresco in the model training and testing to predict soluble solids, firmness, and titratable acidity values.

Parameter	sample set	R^2	RMSE	MAE	MAPE	Bias
soluble solids (%Brix)	Training set	0.525	0.548	0.421	3.2%	0.015
	Testing set	0.465	0.575	0.450	3.4%	0.020
Firmness (lb.)	Training set	0.722	1.615	1.260	7.9%	0.070
	Testing set	0.563	2.066	1.623	10.3%	0.114
Titratable acidity (% malic acid)	Training set	0.738	0.072	0.056	14.3%	0.005
	Testing set	0.724	0.074	0.057	14.7%	0.006

Conclusion

The wavelengths associated with firmness, soluble solids, and titratable acidity were identified in the microwave spectra region. A positive correlation was achieved for the three quality parameters with a varying prediction power range. The strongest correlation was found for titratable acidity, followed by firmness and soluble solids concentration. The narrow distribution range affected the prediction of soluble solids, but the MAE and Bias are low, indicating a low error between the prediction and the observed value. Overall, the results are promising, but more data is needed to evaluate the instrument's accuracy.

Microwave sensor functionality updates

The Fresco sensor has been updated based on feedback from the WTFRC and other beta testers. Improved functions include reduced waiting time to reach optimal operating temperature, a cell phone connection to facilitate data entry, and a repositioned on/off switch.

In 2023, the system was limited to collecting and displaying spectral data as the sole measurement output. However, in 2024, we were able to select the measurement parameters, with results presented post-assessment. Data can now be uploaded directly to OneDrive using a Wi-Fi connection.

Upcoming work

In 2025, we conducted additional data collection for Honeycrisp, Gala, and Cosmic Crisp varieties. Granny Smith apples were incorporated into the experimental framework to enhance the diversity of data distribution. Pre-harvest samples were collected, and the potential for using Fresco as a pre-harvest assessment tool will be evaluated. In addition to the three quality parameters, dry matter will be evaluated during storage in 2026.

Project Title: Risk Assessment For Loss of Firmness During Storage in Gala

Report Type: AP-24-103; Continuing

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Cooperators: None

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $118,762
Total Project Request for Year 2 Funding: $96,035

Other related/associated funding sources:

Requested

Awarded

Funding Duration: 2024-2026
Amount: $248,000
Agency Name: FPPC PRSC
Notes: Funded. This proposal aims to use preliminary data gathered for Next Generation Maturity Indices for pear to develop prototype ripening capacity tools. Similar methods are being used and are therefore transferable. Some resources (e.g. apple genomes, computers) are useful to both projects.

Funding Duration: 2022-2025
Amount: $582,080
Agency Name: WTFRC
Notes: The work funded by this project has relevance to the work proposed here. Similar methods as are used for Next Generation Maturity Index development are proposed here and are therefore transferable. Some resources (e.g. apple genomes, computers) are useful to both projects.

Funding Duration: 2023 - 2027
Amount: $60,000
Agency Name: T&G Global
Notes: Materials Transfer/Research Agreement for Scilate storage trials aimed at managing internal disorders.

WTFRC Collaborative Costs:

Primary PI: Alex Harkess
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Contract Administrator: LaShawn Jackson
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Station Manager/Supervisor: N/A
Station manager/supervisor email address: N/A
Email Address: N/A

Item	2024	2025
Salaries	**$61,000**	**$62,830**
Benefits	**$14,762**	**$15,205**
Wages		
RCA room rental		
Shipping		
Supplies[1]	**$10,000**	**$15,000**
Travel[2]	**$3,000**	**$3,000**
Plot fees		

Miscellaneous[3]	**$30,000**	
Total	**$118,762**	**$96,035**

Footnotes:
[1]A hi-spec computer and reagents for LAMP assay development and testing
[2]Project partners in AL to visit PNW, meet with stakeholders, see orchards and packing houses, receive training in YR 1 on apple sampling methods, train Honaas lab personnel in LAMP assay protocol YR 2
[3]RNA-Seq of 150 samples

Budget 2
Co-PI 2: Alan Yocca requests no funding

Budget 3
Co-PI 3: Loren Honaas requests no funding

Budget 4
Co PI 4: Stephen Ficklin requests no funding

Objectives

1. Add additional cultivar years of storage trial RNA-Seq data to improve upon existing firmness models.

2. Establish a multi-cultivar genetic framework to study apple fruit quality changes (loss of firmness) during storage.

3. Begin development of LAMP assays for a short list of firmness model genes

Year 3 NCE report

Significant Findings/Results

- In-person visit to orchards and packing houses created new opportunities for genomics collaborations
- All proposed RNA sequencing is complete
- RNA-seq costs were reduced 30% with new technology collaborations
- Colorimetric CRISPR-Cas12a assay development shows viable promise for low-cost in-field testing of RNA biomarkers for firmness (or any other measurable trait with a marker)

Methods

This project ultimately expands upon the foundational work used to generate predictive models for firmness loss in (Hadish et al. 2024) (https://doi.org/10.1371/journal.pone.0306187). While the postharvest storage protocols remained consistent with this 2018 study, a 2019 cohort was harvested from a new location: SRO Block 5 (Gala apples on M9 rootstock).

Experimental Design and Treatment Conditions The study evaluated fruit stored under two temperature regimes (1°C and 10°C) combined with varying atmospheric conditions and chemical treatments. The specific parameters were defined as follows:

- **Source Material:** Gala apples, SRO Block 5 (M9 rootstock).
- **Atmospheres:** Air vs. Controlled Atmosphere (CA: 2% O2, 1% CO2).
- **Treatments:** Control vs. 1-MCP application.

Sampling Time Points Sampling frequency was stratified based on storage stability and temperature conditions:

- **1°C Air:** Weekly (Weeks 1–5), then Monthly (Months 2, 3, 4, 5, 7, 9).
- **1°C CA & 1°C 1-MCP (Air/CA):** Bi-monthly to Quarterly (Months 1, 3, 5, 7, 9).
- **10°C Air:** Weekly (Weeks 1–8) to capture rapid physiological changes.

RNA sequencing completion

All proposed sampling and RNA sequencing for this project is complete (Objective 1). All samples were shipped to HudsonAlpha for library preparation and RNA-Seq. The majority of samples met the target input of ~1000ng total RNA, with a subset of 32 samples provided at ~600ng. Our ability to use reduced amounts of total RNA as input has greatly decreased the project costs as we detail below.

Postdoctoral Associate Nicole Szeluga in Harkess' lab has led sequencing efforts, as Dr. Alan Yocca (a co-PI on this proposal) has since moved on to a position in pangenomics at Bayer. Illumina TruSeq or Twist Biosciences libraries were prepared for 183 RNA samples and sequenced on an Illumina NovaSeq X with paired-end 150 nt reads. High quality sequencing data was produced for every proposed sample, at an average of 25 million read pairs per sample. These data are currently being analyzed for the model improvement portion of this proposal (Objective 2).

Building deep genomics industry collaborations to support WTFRC-funded research

During the course of this project our RNA-seq workflow underwent a major transformation that ultimately allowed us to reduce the cost of apple RNA-seq by roughly 30%, thanks to strategic partnerships with two genomics companies: Twist Biosciences (San Francisco, CA) and n6 Tec (Pleasanton, CA). A significant bottleneck in the field of genomics is 1) the preparation of high quality nucleic acid, 2) the preparation of DNA/RNA sequencing libraries, 3) the actual sequencing, 4) re-do'ing failed sequencing runs or increasing data amount for specific samples that were undersequenced.

Our first major improvement is that we have replaced legacy RNA-seq library preparation protocols (Illumina TruSeq, as performed above) with Twist's rapid, cost-effective library preparation system, significantly increasing our throughput while lowering reagent costs. This alone reduces the time to prepare a sequencing library from 2 days to less than 1 day. Next, the integration of the n6 iconPCR box has transformed our downstream processing by automating the normalization of libraries before sequencing (Figure 1). The n6 icon system utilizes an innovative method to normalize library concentrations during the PCR amplification step itself, ensuring that every sample reaches a consistent molarity regardless of the starting input. Additionally, reducing the number of PCR cycles increases the quality of data produced by reducing the number of artificial duplicate sequencing reads, which often can consume 5-20% of sequencing data that needs to be thrown out during analysis. This "self-normalizing" capability allows us to bypass expensive and time-consuming individual Quality Control (QC) and quantification steps prior to pooling. For example, this will eliminate the need to check every individual RNA tube for concentration before beginning the sequencing library prep, a step which often takes days of person-time and has a per-sample cost of reagents. Furthermore, because the resulting pools are perfectly balanced, we have effectively eliminated the need for costly re-sequencing runs often caused by uneven coverage or sample dropouts. These sequencing re-runs take additional time as well, since it is now (perhaps counter-intuitively) difficult to sequence a small number of samples in the growing world of genomics, given the growing size of genome sequencing machines. This collaboration with n6 and the overarching fruit biomarker project is being featured in a poster presentation by n6 at the Plant and Animal Genome (PAG) conference in January, as well as in a talk by Dr. Szeluga in the Postharvest Ripening, Senescence, and Technology workshop. N6 is taking a particular interest in highlighting this partnership for improved fruit biomarker development, and additional white papers will be released this year as well as social media.

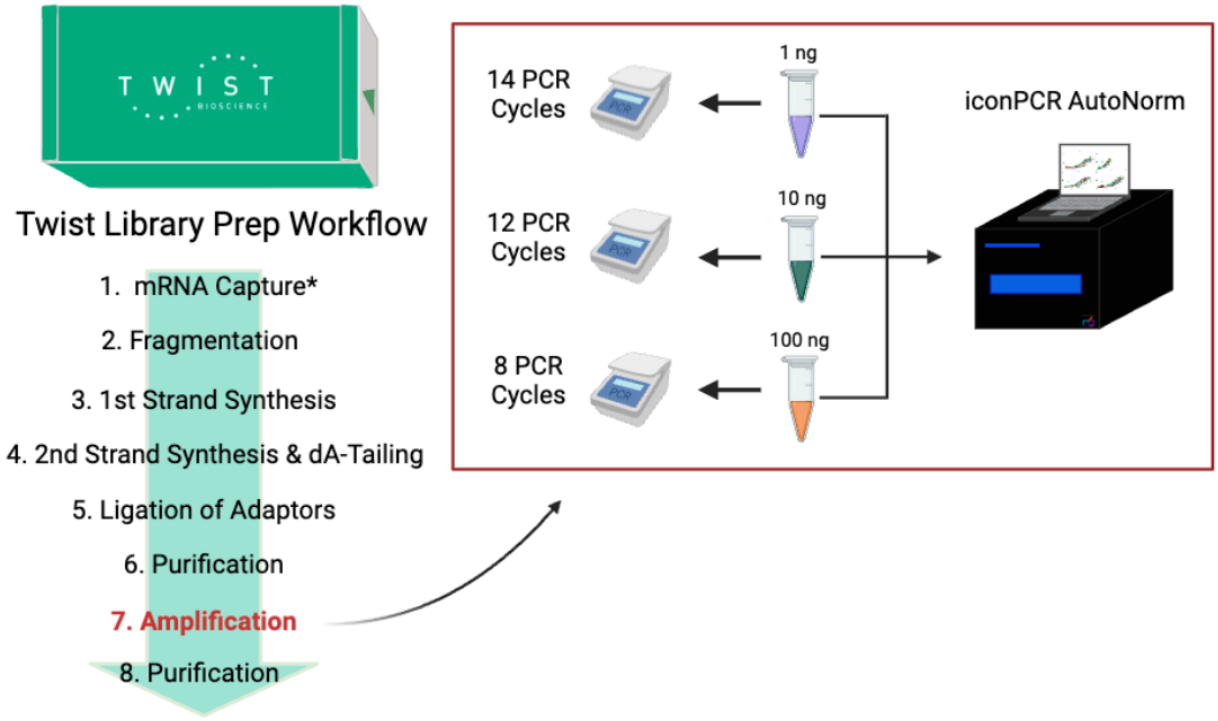

Figure 1: N6 Tec's iconPCR normalizes cDNA libraries across a 96-well plate during the amplification step of a library prep workflow regardless of initial input.

Development of qPCR and LAMP assays for top genes

This year began the development of an assay to measure the expression of the biomarkers identified from the gene model for loss of fruit quality. qPCR analysis of samples is necessary to confirm the expression of our top biomarkers prior to the colorimetric assay. First, RNA is converted to cDNA using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher, Waltham, MA), amplified using GoTaq® qPCR Master Mix (Promega, Madison, WI), and quantified on the QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher, Waltham, MA).

In our assay design, we amplify the cDNA biomarkers using a LAMP assay because it is a faster and cheaper alternative to qPCR or PCR amplification. The LAMP assay is combined with a Reverse Transcription step (RT-LAMP) (ThermoFisher Scientific, Waltham, MA, USA) step that converts RNA to cDNA to simplify and hasten the process after RNA extraction (Figure 2B). After cDNA is amplified using RT-LAMP, it can be quantified with a Cas12a-Gold Nanoparticle colorimetric assay that we are actively developing, visible to the naked eye without any additional or expensive equipment.

The use of Gold Nanoparticles (AuNPs) for a common field test may sound expensive, but when used in a colorimetric assay, is a highly cost-effective tool to visualize biomarker expression. When dispersed, 15 nm-sized gold nanospheres reflect a deep red coloring and when aggregated, reflect a deep purple color. Utilizing the color change for our colorimetric assay, we ordered two batches of 15 nm AuNPs labelled with single stranded DNA (ssDNA) from Luna Nanotech (Markham, ON, Canada). The ssDNA sequences conjugated onto AuNP-A and AuNP-B were based on the work of the Li lab at Sichuan University in Chengdu, CN (Li et al. 2019). When AuNP-A and AuNP-B are brought close in proximity by a ssDNA linker, their color shifts from red to purple (Figure 2C). This ssDNA

linker is a short 30 nucleotide sequence with 15 nucleotides complement to the ssDNA arm on AuNP-A and the other 15 nucleotides complement to the ssDNA arm on AuNP-B (Table 1).

In our colorimetric assay, we propose to use Cas12a (also known as Cpf1) to cut the ssDNA linker and prevent the aggregation of AuNP-A and AuNP-B, resulting in a red color (Figure 2C). Cas12a is used because it features a RuvC active site that indiscriminately cleaves ssDNA such as our linker. RuvC is allosterically activated upon the binding of DNA to the ribonucleoprotein complex (Cas12a + crRNA) (Paul and Montoya 2020; Wörle et al. 2022). In our assay, our LAMP product binds to the Cas12a-crRNA complex to activate RuvC nuclease activity. This color change is sensitive to the amount of amplified DNA marker present in the reaction. We can measure the absorbance at 550 nm wavelength using a spectrophotometer to quantify the color change based on a pre-existing standard curve (Figure 2D). With multiple quantified biomarkers, we can now rank the apple lots based on maturity and estimate when the apples will reach peak maturity or how close the fruit was to maturity when harvested to predict storage outcomes.

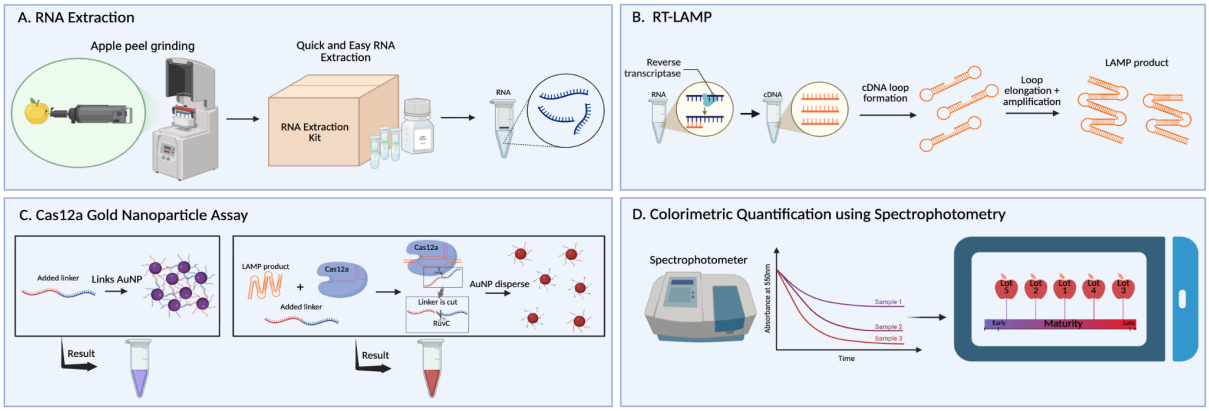

Figure 2: Cas12a-based colorimetric assay reduces cost, time, and difficulty of evaluating firmness biomarkers. Our partnership with OPS Diagnostics has produced a tissue collection tool and easy extraction method to collect and extract RNA more efficiently than full RNA extraction protocols (A). RNA is amplified using RT-LAMP assay (B). After amplification, the LAMP product binds to Cas12a, activating ssDNA cutting activity. This ribonucleoprotein cleaves the ssDNA linker and disperses gold nanoparticles (C). The color change can be quantified using spectrophotometry (D).

Results and Discussion

Improved firmness biomarker model outcomes with additional RNA-seq data

We have just finished all RNA sequencing for this project in the last month, so we are now just beginning to evaluate model performance improvement and softening biomarker development. This will be the primary focus of the NCE request that has been granted, as all other goals of the proposal have been completed.

Genomics industry partnerships are increasing visibility of WTFRC and decreasing biomarker discovery costs

This project highlights a strategic evolution in how the Washington Tree Fruit Research Commission approaches genomics research to improve storage outcomes. Historically, agricultural genomics has relied on standard, off-the-shelf legacy protocols that are typically tuned for human genomics. However, by actively forging early-stage partnerships with biotech innovators like Twist Biosciences and n6 tec, we are moving beyond simply using technology to optimizing it specifically for tree fruit applications.

The primary beneficiaries of these technical advancements are the growers and packing houses that fund and rely on this research. By integrating Twist's rapid library preparation and the n6 iconPCR AutoNorm system during the life of this project (Figure 3), **we have achieved a 30% reduction in RNA-seq costs**. This cost reduction demonstrates our commitment to responsible stewardship of Commission funds, allowing us to stretch research dollars further, screening more samples and gathering more data for the same investment. These methods will be applied to several active WTFRC-funded proposals that use the Harkess Lab at HudsonAlpha for sequencing (e.g. NGMI biomarkers from Co-PIs Honaas and Ficklin). Moving forward, the elimination of time-consuming individual Quality Control (QC) steps and the reduction of sequencing re-runs means we can deliver data faster for biomarker development across any trait. For a grower or breeder, this acceleration reduces the "time-to-insight," enabling faster decisions regarding selection and harvest management. Pragmatically this means we are now discovering and applying new fruit trait biomarkers faster, and with less money. We are quickly moving beyond basic science and into translational field applications with these technological advancements funded by this proposal.

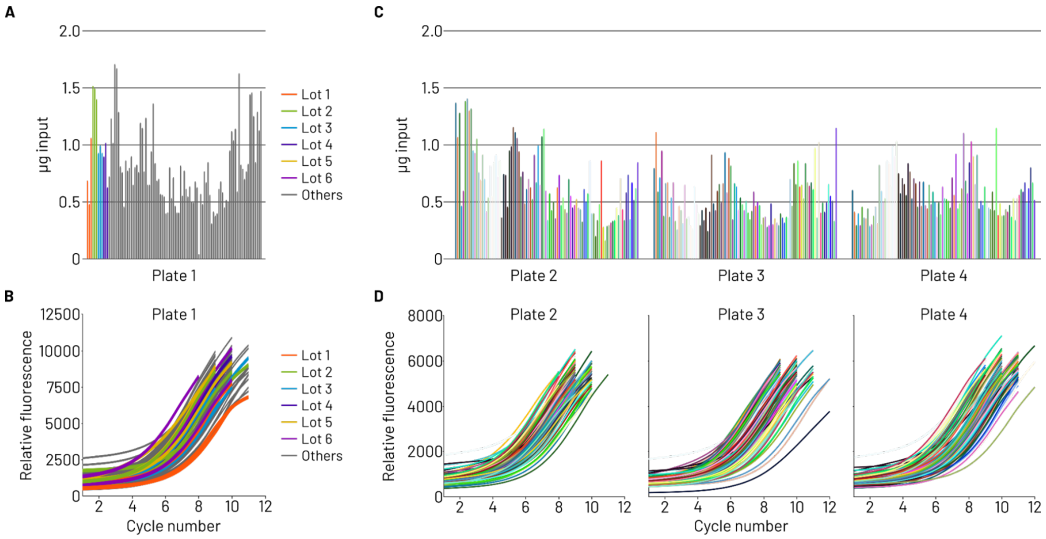

Figure 3: RNA-seq library autonormalization with n6. With highly variable input RNA amounts (a,c) across 4 plates of libraries, PCR cycle number can be precisely controlled to yield normalized library pools and reduce sequencing costs (b,d).

Significant progress towards inexpensive in-field biomarker testing

In-field biomarker testing, meaning in the orchard or packing house, has been hampered by two main problems. First, the isolation and QC of RNA is often only feasible in a controlled laboratory setting. Second, the physical test itself (e.g. RNA sequencing, qPCR) typically requires expensive capital equipment, reagents, and expertise. As we continue to develop better models and biomarkers based on our RNA-seq data for a variety of traits, our goal is ultimately to provide industry tools to assay these biomarkers quickly and inexpensively in the field.

To solve the first problem of efficient RNA isolation without expensive equipment, the development of an efficient tissue collector was the creation of another partnership between our team and OPS Diagnostics. This tool quickly collects evenly sized peels and places them in an RNA-stable buffer for downstream extraction. This buffer is compatible with OPS Diagnostics' fast and easy RNA extraction buffer. The extraction produces excellent quality RNA suitable for downstream application. This work has largely been performed in Co-PI Honaas' laboratory and overlaps with several other WTFRC-funded projects on next-generation maturity indices.

To solve the second problem of the physical biomarker test/assay, we have successfully used RT-LAMP to amplify biomarkers of interest. We partnered with Luna Biotech (Markham, Ontario, Canada) to conjugate ssDNA to 15nm gold nanospheres. The second AuNP design contained a 1 kDA backfill necessary to stabilize the nanospheres and prevent the passive aggregation observed in the first batch. After adjusting the salt concentration in our reaction, we observed a visible and quantifiable color change with the addition of our ssDNA linker (Figure 4). The presence of the salt was necessary to destabilize the backfill and allow for aggregation to occur.

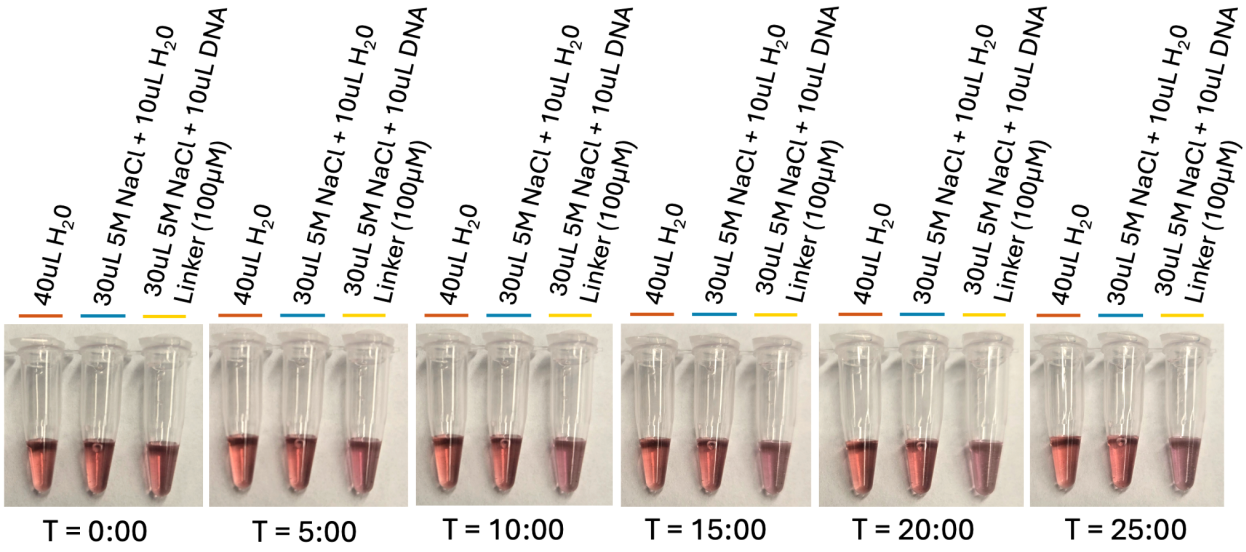

Figure 4: Gold nanoparticles change color in the presence of a ssDNA linker. A solution with equal parts AuNP-A and AuNP-B does not aggregate with the addition of water or salt. With

the addition of a ssDNA linker complement to the ssDNA arms attached to the 15-nm gold nanospheres, the AuNPs aggregate and change color over a period of 25 minutes.

Using spectrophotometry, we quantified the absorbance of the AuNPs every five minutes over the course of 35 minutes and saw the amplitude of absorbance shift from 520 nm to 550nm in the samples containing the ssDNA linker (Figure 5). **Based on both of these experiments, we estimate that it will only take 15 minutes to assay a biomarker panel**.

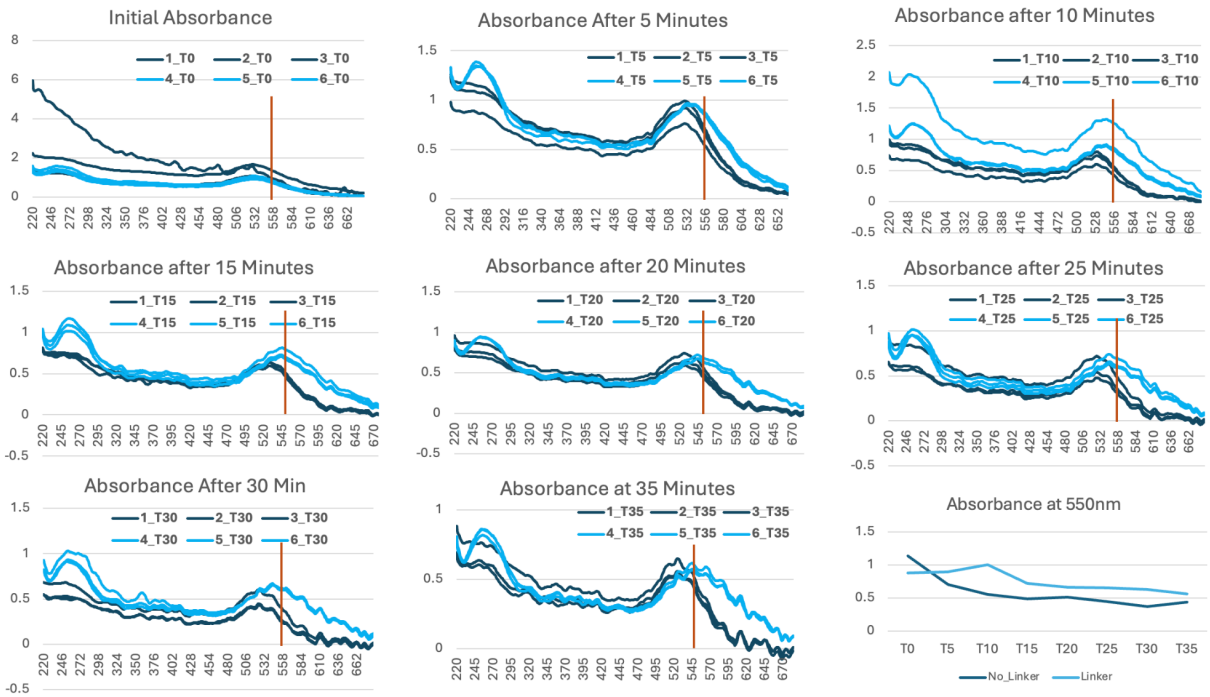

Figure 5: The absorbance value at 550nm is greater in the sample containing a linker than the samples without a ssDNA linker post 5 minutes. The graphs track the absorbance values of six samples over the course of 35 minutes using a spectrophotometer (DeNovix). The final graph tracks the average absorbance value at 550nm over the course of 35 minutes.

The pilot development of our Cas12a-based colorimetric assay represents the beginning of a pivotal shift in agricultural diagnostics. Historically, the assessment of RNA biomarkers for traits like apple softening has been tethered to centralized laboratories, relying on expensive and time-consuming methods such as quantitative PCR (qPCR) or RNA sequencing (RNA-seq). By successfully engineering a highly sensitive, field-deployable assay, we will have effectively decentralized this capability, moving precision molecular diagnostics from the benchtop to the orchard and packing house. Crucially, the accessibility of this test does not come at the expense of performance. Leveraging the enzymatic precision of Cas12a, our designed colorimetric assay could demonstrate sensitivity thresholds comparable to qPCR, capable of detecting femtomolar concentrations of target RNA (Guo et al. 2023). This high sensitivity is vital for detecting low-abundance transcripts associated with early-stage softening before visible degradation occurs. The visual readout, eliminating the need for fluorescence readers or thermal cyclers, ensures that sophisticated molecular data is accessible without capital-intensive infrastructure.

Table 1: Comparison of industry gold standard techniques to pilot colorimetric assay

Feature	**Current Standard (qPCR/RNA-seq)**	**New Cas12a Colorimetric Assay**
Location	Centralized Lab	In-Field / Orchard / Packing House
Time to Result	Days (shipping + processing)	Minutes/Hours
Equipment	Thermal cyclers, Fluorometers	Heat block (or ambient), Visual
Cost	High ($$$)	Ultra-Low ($)
Sensitivity	High	High (Comparable)
Expertise Required	Ph.D. / Lab Tech	Minimal Training

While RNA-seq and qPCR remain essential for discovery and validation, they are over-engineered for routine screening applications of known biomarker genes or gene panels. Our findings suggest that Cas12a-enabled diagnostics are poised to replace these legacy methods as the new industry standard for in-field trait verification. By providing a binary or semi-quantitative visual result, we strip away the complexity of data analysis required by sequencing, offering actionable intelligence that empowers decision-making on the ground. Beyond apple softening, this platform establishes a modular framework for agricultural diagnostics. The programmable nature of the Cas12a guide RNA allows us to rapidly pivot this technology to detect other critical biomarkers, such as maturity markers, disease resistance genes or pathogen presence, with minimal redevelopment. As we move toward potential commercialization, this technology promises not only to reduce waste in the apple supply chain but to democratize access to molecular breeding tools across the agricultural sector.

Citations

Guo, Hangyu, Yaqin Zhang, Fange Kong, et al. 2023. “A Cas12a-Based Platform Combined with Gold Nanoparticles for Sensitive and Visual Detection of Alternaria Solani.” *Ecotoxicology and Environmental Safety* 263 (115220): 115220.

Hadish, John A., Heidi L. Hargarten, Huiting Zhang, James P. Mattheis, Loren A. Honaas, and Stephen P. Ficklin. 2024. “Towards Identification of Postharvest Fruit Quality Transcriptomic Markers in Malus Domestica.” *PloS One* 19 (3): e0297015.

Li, Yongya, Hayam Mansour, Tony Wang, Sudarsana Poojari, and Feng Li. 2019. “Naked-Eye Detection of Grapevine Red-Blotch Viral Infection Using a Plasmonic CRISPR Cas12a Assay.” *Analytical Chemistry* 91 (18): 11510–11513.

Project Title: Mitigating WA 38 greasiness and related quality defects

Report Type: Final Project Report

Primary PI: Carolina Torres
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Cooperators: Anne Plotto, USDA-ARS, USHRL

Project Duration: 3 Years
Total Project Request for Year 1 Funding: $ 84,050.00
Total Project Request for Year 2 Funding: $ 84,012.00
Total Project Request for Year 3 Funding: $ 86,092.00
Other related/associated funding sources: None
WTFRC Collaborative Costs: None

Budget 1
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Item	2022	2023	2024
Salaries	$54,000.00	$56,160.00	$58,406.00
Benefits	$20,050.00	$20,852.00	$21,686.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$4,000.00	$1,000.00	
Travel			
Plot Fees			
Miscellaneous			
Total	$78,050.00	$78,012.00	$80,092.00

Footnotes: Salaries: Research personnel to carry out field and laboratory work, fruit evaluations and data analyses in years 1, 2, and 3. Benefits: $20,050, $20,852, and $21,686 are requested for benefits tied to the research personnel.
Supplies: Supply costs of $4,000 in year 1 and $1,000 in year 2 are requested to pay for supplies for fruit quality evaluation

Budget 2
Co-PI: David Rudell
Organization Name: USDA-ARS
Contract Administrator: Chuck Myers and Sharon Blanchard
Telephone: (510) 559-5769 (CM, 509-664-2280 (SB)
Contract administrator email address: Chuck.Myers@usda.gov, Sharon.Blanchard@usda.gov

Item	(Type year of project start date here)	(Type year start date of year 2 here if relevant)	(Type year start date of year 3 here if relevant)
Salaries			
Benefits			
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$3,000.00	$3,000.00	$3,000.00
Travel	$3,000.00	$3,000.00	$3,000.00
Plot Fees			
Miscellaneous			
Total	$6,000.00	$6,000.00	$6,000.00

Footnotes: Supplies: Supply costs of $4,000 in year 1 and $1,000 in year 2 are requested to pay for supplies for fruit quality evaluation. Travel: $3,000 is requested in years 1, 2, and 3, respectively, for associated travel for Dr. Anne Plotto.

Project Title: Mitigating WA 38 greasiness and related quality defects

Peel greasiness has been the most prominent issue during the first years following the commercial launch of WA 38 (Torres & Gomez, 2020; Hedges and Torres, 2021). This is a common phenomenon on apples and can develop while the fruit is on the tree or during the cold chain. Some of the factors involved in skin greasiness are genetic differences between cultivars, tree age, growing environment (seasonal variation), storage environment (temperature, atmosphere, relative humidity), length of storage, and fruit maturity (Yang et al., 2017). The objectives of this project were:

1. Further, define harvest maturity guidelines limiting greasiness in the cold chain.
2. Establish ethylene mitigation protocols that reduce greasiness for both conventional and organic production.
3. Determine the limitations of wax/detergent for mitigating greasiness in the post-storage cold chain.
4. Identify and determine protocols for mitigating off-flavors associated with greasiness.

Significant Findings:

1. Maturity progression varied between growing sites and seasons. The starch degradation rate was temperature-dependent during the growing season. Warmer weather led to higher rates of starch degradation and greater dispersion of starch indexes at harvest.
2. Overall, skin greasiness was more related to fruit maturity and cooler weather during the last part of the growing season than just tree age.
3. There was a higher incidence (and severity) of greasiness in fruit from air storage compared to that stored in controlled atmosphere (CA; 2.5% O_2, 1.5% CO_2), but more consistently on that from H2 (1 week after commercial harvest). In all cases, greasiness increased once in the simulated shelf-life period (7 days at 68^{o}F).
4. Retain® reduced greasiness incidence and severity postharvest up to 4 months, but not during 'shelf-life' once fruit was removed from cold storage. In general, the earlier the applications (14 and 21 days before harvest), the more effective control of skin greasiness was achieved.
5. Although 1-MCP formulations were able to delayed ripening, they had inconsistent effects over skin greasiness.
6. All detergents tested were able to remove skin greasiness effectively, and all coatings continued to control greasiness during the cold chain.
7. The off-flavor, described as bitterness, was mostly detected in the flesh of the unexposed section of the fruit, and linked with a higher ratio of aroma compounds typical of unripe apples over the ripe ones. Following the harvest recommendations and avoiding the harvest of immature fruit should be able to minimize it.

Results

Objective 1: Further define harvest maturity guidelines, limiting greasiness in the cold chain. Maturity progression was evaluated weekly starting at 4 weeks before commercial harvest. Following each harvest, fruit was stored 33°F in air or in a controlled atmosphere (2.5% O_2, 1.5% CO_2). Fruit quality (ripeness, skin greasiness, and physiological disorders) was evaluated monthly for 6 months. Skin greasiness was rated using a 4-point subjective scale, rubbing the fruit against the hand, and rated as (0) no greasiness to (3) severe greasiness.

Maturity was different in fruit from different locations and growing seasons, with ethylene production, starch index, and chlorophyll degradation (DA meter) reflecting these differences. Figure 1 shows the frequency of SI values in fruit harvested commercially and one week later. In 2022, the warmest year, there was a greater dispersion of SI values with no differences between the two harvests in fruit from both locations (Mattawa and Quincy), compared to the other seasons.
Skin greasiness was site-dependent; the warmest site (Mattawa) had, in general, less greasy fruit at harvest than the cooler site (Quincy) (Fig. 2). In both orchards, there was a higher incidence of greasiness in the later harvest, emphasizing the role of harvest maturity in this defect.
As in the previous seasons, in 2024-2025, CA storage reduced fruit softening (~0.8 lb), ethylene production, and background color change (I_{AD} values) compared to air storage (Table 1). Overall, throughout all growing seasons, skin greasiness incidence and severity were lower in fruit stored in CA compared to those in air. In addition, greasiness increased during the 'shelf-life' period (7 days at 68ºF). Fig. 3 shows the results from the 2024/2025 storage season.

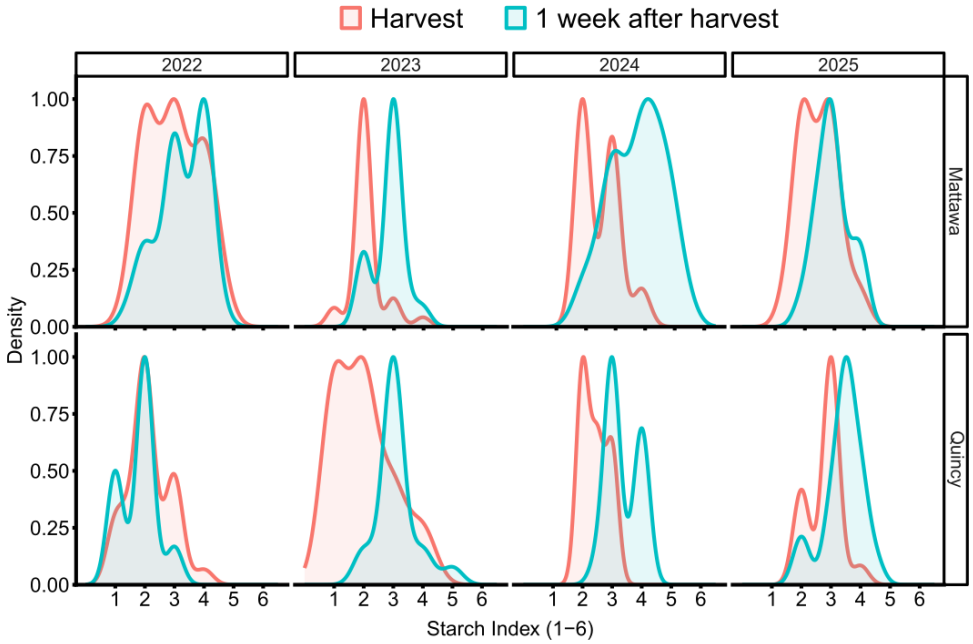

Figure 1. Density plots from the starch index of fruit from Mattawa and Quincy in 2022, 2023, 2024, and 2025 at commercial harvest and one week later (1 week after harvest, wah) or up to three weeks later (2024). Overlap shapes show no differences in the starch degradation population in fruit sampled at each timepoint.

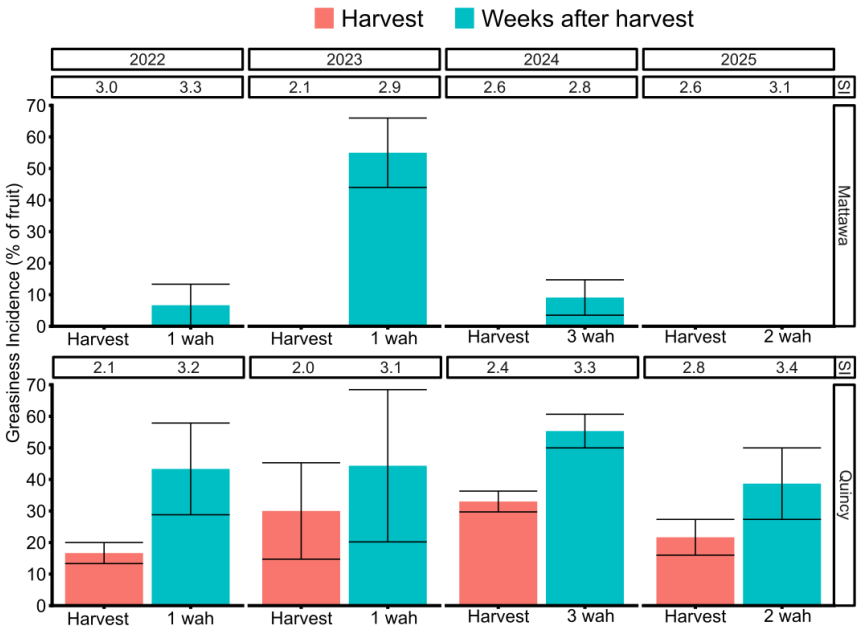

Figure 2. Bar plots of greasiness incidence (average) from Mattawa and Quincy in 2022, 2023, 2024, and 2025 at commercial harvest and up to 3 weeks later (week after harvest). Bars present the standard error. The average starch indexes at each harvest point are shown on top of each column.

Table 1. Relative ripeness and greasiness (average values) during air and CA storage at 33°F during the 2024-2025 season.

	Harvest	Storage Eval.	Firmness (lb)		Backg. Color (1-4)		I_{AD} (0-2.2)		SI (1-6)		Ethylene (ppm)	
			RA	CA	RA	CA	RA	CA	RA	CA	RA	CA
Mattawa	H1	1mo+1d	18.7	19	3.4	3.4	0.4	0.3	4.2	3.9	17.8	15.3
		2mo+1d	17.7	18.1	4	4	0.3	0.3	5	4.8	38.7	18.8*
		3mo+1d	17.1*	19	3.9	3.9	0.2*	0.3	5.8	5.7	55.1	12.6*
		4mo+1d	18.2	17.0*	4	4	0.2*	0.3	6	6	54.9	14.1*
		5mo+1d	16.7	16.5	4	4	0.2*	0.3	6	6	48.9	11.9*
		6mo+1d	16.7*	17.9	4	3.8	0.2*	0.3	6	6	15.7	7.1*
	H2	1mo+1d	17.2	16.0*	4	4	0.3	0.3	4.8*	5.8	22.5	23.7
		2mo+1d	16.6	16.5	4	4	0.2	0.3	5.9	5.7*	31.3	21.1*
		3mo+1d	16.7	16.9	4	4	0.2*	0.3	5.9	5.8*	44.5	24.8*
		4mo+1d	16.2	16	4	4	0.7*	0.9	6	6	54.6	15.7*
		5mo+1d	16.5*	17.5	4	3.9	0.2*	1.1	6	6	39.8	12.9*
		6mo+1d	15.9	16.5	4	4	0.2	0.2	6	6	29.1	14.3*
Quincy	H1	1mo+1d	19.2	19	2.2	2.2	0.9	0.8	4.2	4	16	0.3*
		2mo+1d	19.2	19.5	2.9	2.7	1	1	5.1	5.1	47	19.0*
		3mo+1d	18.2*	19.5	3.3	3.2	0.7*	0.9	5.6	5.7	70.3	8.0*
		4mo+1d	17.5*	19.4	3.2*	3.6	0.8	0.9	5.9	5.9	80.7	10.3*
		5mo+1d	17.2*	18.4	3.3	3.5	0.8	0.7	6	6	63.7	10.7*
		6mo+1d	16.7*	19.6	3.6	3.5	0.8	0.8	6	6	71.1	12.3*
	H2	1mo+1d	18.4	18	3.6	3.8	0.8	0.7	3.9	4.2	48	48.8
		2mo+1d	18.2	18.6	3.3	3.6	0.8	0.8	5.3	5.0*	54.7	15.4*

3mo+1d	18.1*	19.1	3.9	3.7	0.5*	0.8	5.8	5.8	72.7	11.6*
4mo+1d	17.2*	18.5	3.9	3.6*	0.2*	0.3	6	6	77.3	9.0*
5mo+1d	17.9*	20.9	3.4	3.1*	0.6	0.3*	6	6	42.3	11.2*
6mo+1d	17.1*	18.4	3.9	4	0.7	0.8	6	6	44.2	9.6*

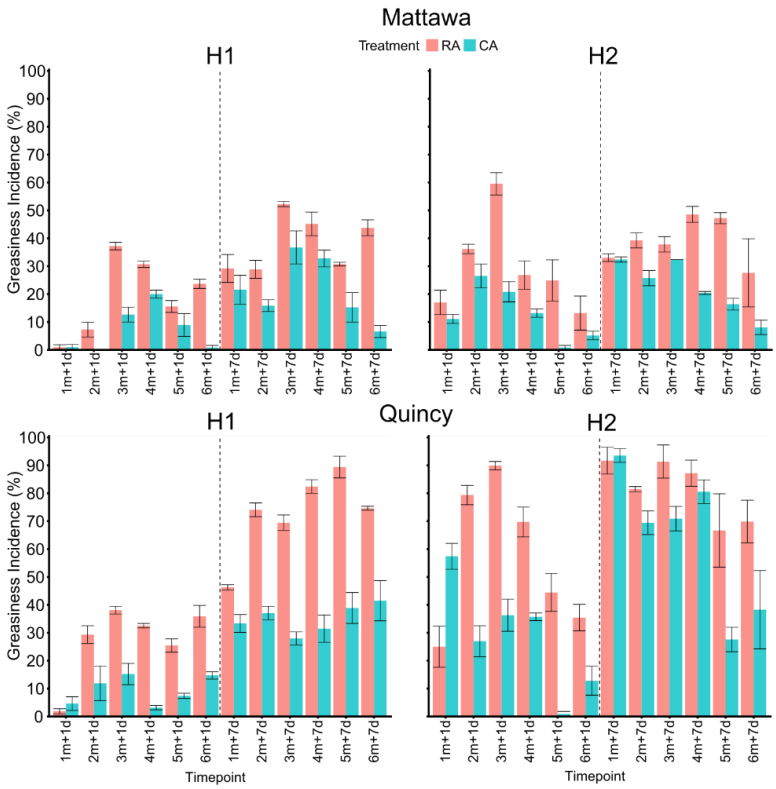

Figure 3. Bar plots of greasiness incidence (average) from Mattawa and Quincy in the 2024 season up to 6 months in storage (RA or CA) plus 1 or 7 days at room temperature (20 ºC). Bars present the standard error.

<u>Objective 2</u>: Establish ethylene mitigation protocols that reduce greasiness for both conventional and organic production.

Table 2 shows treatments applied in 2022, 2023, 2024 and 2025. Preharvest application of Retain®, either at half or full rate, suppressed ethylene production and delayed the starch degradation at harvest (Table 3 shows results for 2024/2025). Fruit greasiness was present at the time of the first application (2 weeks before harvest) in 2023. In 2024, it appeared in the UTC one week after commercial harvest. In 2025, no greasiness at the time of harvest was detected in any of the treatments.

There were no differences between treatments in ethylene production or starch degradation postharvest (Table 3 shows results for 2024/2025). All Retain® reduced greasiness incidence and severity after 1 day at 68ºF. The effect disappeared after 7 days at 68ºF and 4 months of storage (Table 3 shows results for 2024/2025).

As in 2022, in 2023, Harvista™, either applied at a single (1x) or double (2x) rate, was not able to reduce ethylene production nor affect the starch degradation pattern on the fruit compared to the UTC. Nevertheless, in 2023 Harvista™ 2x was able to better retain firmness after 4 months of air storage (Table 4). Harvista™ 1x plus SF was able to retain flesh firmness for up to 12 months of

storage (+3.0 lb than UTC and Harvista™ 1x). No differences in skin greasiness between treatments were observed at harvest. Ethylene production was suppressed using Harvista™ 1x plus SF (T5) and UTC plus SF (T4) for up to 8 months plus 7 days at 68ºF. Similar results were found in 2024. Skin greasiness severity was reduced by Harvista™ 1x plus SF treatment in 2023. This did not occur in the 2024 season (Table 3).

Table 2. PGR treatments (rate and time of application) during the 2023, 2024, and 2025 seasons.

Year	Treat.	Material/a.i.	Rate	Timing	Others
2022	T1	Untreated control (UTC)	NA	NA	• Orchard location: Zillah, WA • Sylcoat (0.1% OSi) added to Retain® applications. • Two harvests: At the time of UTC (commercial), and 1 week later.
	T2	AVG (ReTain®)	Full rate (24 oz/acre)	7 DBH	
	T3	ReTain®	Half-rate (12 oz/acre)	7 DBH	
2023	T1	Untreated control (UTC)	NA	NA	
	T2	ReTain®	Full rate (24 oz/acre)	21 DBH	
	T3	ReTain®	Full rate (24 oz/acre)	14 DBH	
	T4	ReTain®	Full rate (24 oz/acre)	7 DBH	
2024 & 2025	T1	Untreated control (UTC)	NA	NA	
	T2	ReTain®	Full rate (12 oz/acre)	14 DBH	
	T3	ReTain®	Half rate (24 oz/acre)	14 DBH	
	T4	ReTain®	Full rate (24 oz/acre)	7 DBH	
2022 & 2023	T1	UTC			• Orchard location: Royal City, WA • Two harvests: At the time of UTC (commercial), and 1 week later. • Only harvest 1 for SF treats.
	T2	1-MCP (Harvista™ 1.3 SC) (1x)	Full dose	14 DBH	
	T3	Harvista™ (2x)	Full dose	14 & 7 DBH	
	T4	UTC plus 1-MCP (SmartFresh™; SF)	100 ppm	At harvest	
	T5	Harvista™ 1x plus SF	T2 plus SF (100 ppm)	At harvest	
2024 & 2025	T1	UTC			
	T2	UTC plus 1-MCP (SmartFresh™; SF)	100 ppm	At harvest	

AVG: Aminoethoxyvinylglicine; 1-MCP: 1-methylcyclopropene; DBH: Days before harvest

Table 3. Fruit maturity (starch index, SI, flesh firmness, internal ethylene concentration, IEC, titratable acidity) and greasiness incidence and severity (low) during storage at 33ºF of WA 38 apples treated with AVG (Retain®). Season 2024/2025.

	Eval.	Treat.	SI (1-6)	Firmness (lb)		IEC (ppm)		Titratable Acidity (% malic acid)		Greasiness Incidence (% fruit)		Greasiness Low Greasiness (% fruit)	
				+1d	+7d	+1d	+7d	+1d	+7d	+1d	+7d	+1d	+7d
H1	Harv.	UTC[Y]	2.2	18.6	-	0.9 b[z]	-	0.6	-	0.0	-	0.0	-
		T2	2.3	18.1	-	1.4 c	-	0.6	-	0.0	-	0.0	-
		T3	2.4	18.1	-	0.6 a	-	0.6	-	0.0	-	0.0	-
		T4	2.5	18.0	-	0.4 a	-	0.5	-	0.0	-	0.0	-
	4 m	UTC	6.0	16.8 c	15.4 a	43.7 b	159.3 c	0.4	0.4	46.0	61.1 b[x]	42.2 b	40.2
		T2	6.0	16.4 a	15.2 a	56.5 c	148.2 b	0.4	0.4	38.8	66.7 a	33.0 b	39.8
		T3	6.0	16.6 b	16.1 b	35.2 a	94.0 a	0.4	0.3	35.5	54.8 b	34.8 c	45.2
		T4	6.0	17.0 c	15.8 b	37.3 a	150.4 b	0.4	0.4	42.9	73.7 a	36.8 a	32.6
	8 m	UTC	6.0	15.6 a	15.1	15.9 b	201.9 c	0.3	0.2	29.4	62.0 a	25.4	50.9

		T2	6.0	16.3 b	14.9	11.8 a	167.9 b	0.2	0.2	29.4	64.8 a	28.1	47.9
		T3	6.0	15.8 a	15.4	10.1 a	145.9 a	0.3	0.2	14.3	30.6 b	12.2	26.3
		T4	6.0	15.5 a	15.9	15.1 b	151.3 a	0.3	0.2	23.8	39.8 b	20.2	30.4
	Harv.	UTC	3.8 b	17.4	-	2.2 b	-	0.5		0.0	-	0.0	-
		T2	2.8 ab	17.4	-	0.8 a	-	0.6		0.0	-	0.0	-
		T3	2.6 a	18.6	-	0.8 a	-	0.6		0.0	-	0.0	-
		T4	3.3 b	17.7	-	1.6 b	-	0.6		0.0	-	0.0	-
H2	4 m	UTC	6.0	16.3 b	15.3	46.8 b	181.9 c	0.3	0.3	25.6 a	55.6 a	24.0	41.1
		T2	6.0	15.7 a	15.2	45.5 b	153.2 b	0.4	0.3	26.9 a	51.8 a	24.2	40.1
		T3	6.0	15.4 a	15.2	36.0 a	136.3 a	0.3	0.3	19.0 b	34.4 b	18.4	29.8
		T4	6.0	16.3 b	15.1	38.8 a	182.9 c	0.3	0.3	17.6 b	27.9 b	17.1	25.6
	8 m	UTC	6.0	16.0 b	14.8	10.2 b	155.6 b	0.2	0.2	14.6	21.6	14.1	19.3
		T2	6.0	15.2 a	14.5	5.8 a	160.6 c	0.3	0.2	19.0	27.1	18.6	24.2
		T3	6.0	15.6 ab	14.7	8.2 ab	131.3 a	0.3	0.2	7.7	27.7	7.7	26.5
		T4	6.0	15.3 a	14.6	6.7 a	160.0 c	0.2	0.2	10.3	34.1	10.3	29.1

[Y] UTC= Untreated Control

[Z] ANOVA (P≤0.05). Different letters indicate significant differences between treatments within timepoints (Tukey, P≤0.05).

[x] Kruskal-Wallis (P≤0.05). Different letters indicate significant differences between treatments within timepoints (Dunn Test, P≤0.05).

Table 4. Fruit maturity (starch index, SI, flesh firmness, internal ethylene concentration, IEC, titratable acidity) and greasiness incidence and severity (low) during storage at 33ºF of WA 38 apples treated with 1-MCP (SmartFresh (SF)). 2024/2025 season.

	Eval	Treat.	SI (1-6)	Firmness (lb)		IEC (ppm)		Titratable Acidity (% malic acid)		Greasiness			
										Incidence (% fruit)		Low Greasiness (% fruit)	
				+1d	+7d	+1d	+7d	+1d	+7d	+1d	+7d	+1d	+7d
H1	Harvest		2.8	18.8	-	1.2	-	0.6	-	0.0	-	0.0	-
	4 m	T1	6.0	18.8 *[z]	18.2*	48.6	119.8	0.3*	0.3	23.0	40.5*[x]	68.2	37.050
		T2	6.0	20.5	20.5	18.4*	99.5	0.5	0.4	21.4	24.6	68.6	0.5
	8 m	T1	6.0	18.0	17.3	8.0	106.8	0.2	0.2	13.9	26.8	86.1	67.721
		T2	6.0	18.4	18.2	11.1	100.4	0.2	0.2	35.4*	59.0*	29.5*	1.4*
	12 m	T1	6.0	16.7*	16.2*	15.6	86.7	0.1*	0.2	10.2	18.4	60.3*	67.461
		T2	6.0	17.7	17.2	8.7*	38.3*	0.2	0.2	9.7	18.1	90.3	1.9
H2	Harvest		2.6	19.3	-	2.4	-	0.6	-	0.0	-	0.0	-
	4 m	T1	6.0	19.5	17.1*	29.6	95.1	0.4	0.3*	25.9*	47.2*	57.9*	30.7*61.4
		T2	6.0	19.8	19.5	4.5*	9.7*	0.4	0.4	13.2	33.3	86.8	
	8 m	T1	6.0	17.4*	16.0*	7.9*	94.6	0.2	0.2*	0.0	25	0.0*	58.8

	T2	6.0	19.3	18.5	16.1	60.1	0.2	0.3	18.6*	29.8	77.9	59.5
12 m	T1	6.0	16.2*	14.4*	3.6*	106.3	0.2	0.1*	14.7	21.2	77.1	73.96
	T2	6.0	19.8	19.1	17.1	101.6	0.2	0.2	18.3	24.7	70.9	65.2

[Z] ANOVA (P≤0.05). Asterisks indicate significant differences between treatments within timepoint.
[x] Kruskal-Wallis (P≤0.05). Different letters indicate significant differences between treatments within timepoints (Dunn Test, P≤0.05).

Objective 3: Determine the limitations of wax/detergent for mitigating greasiness in the post-storage cold chain.
Experiments done in 2022 and 2023 are shown in Table 5. For the coating trials, fruit was cleaned with Epi-Clean (60 s), dried in air, and coated with the different formulations (Table 5).
In general, all detergent treatments were able to remove the greasiness on the fruit for up to 7 days at 68ºF regardless of the initial amount of greasiness in 2022, and up to 1 day in 2023 (Fig. 4). All coatings tested in all trials were able to maintain grease-free fruit for up to 21 days at RT plus 30 days in cold storage plus 21 additional days at RT (Fig. 5).

Table 5. Detergent and coating treatments in the 2022 and 2023 seasons.

Year	Fruit condition	Product	Treat.	Material	Rate	Application Time
2022	Low greasiness High greasiness	Detergents	1	UTC (Water)	NA	30 s
			2	Acidex Duo	25 ml / L	30 & 60 s
			3	Epi-Clean	25 ml / L	30 & 60 s
	Low greasiness	Coatings	1	UTC	NA	-
			2	PrimaFresh 360 HS	0.4 g /fruit	30 s
			3	Shield-Brite AP-450	0.4 g /fruit	30 s
			4	Xedasol	0.4 g /fruit	30 s
2023	High greasiness	Detergents	1	UTC (Water)	NA	30 s
			2	Acidex Duo	25 ml / L	30 & 60 s
			3	Epi-Clean	25 ml / L	30 & 60 s
		Coatings	1	UTC	NA	-
			2	PrimaFresh 360 HS	0.4 g /fruit	30 s
			3	Shield-Brite AP-450	0.4 g /fruit	30 s
			4	Xedasol	0.4 g /fruit	30 s

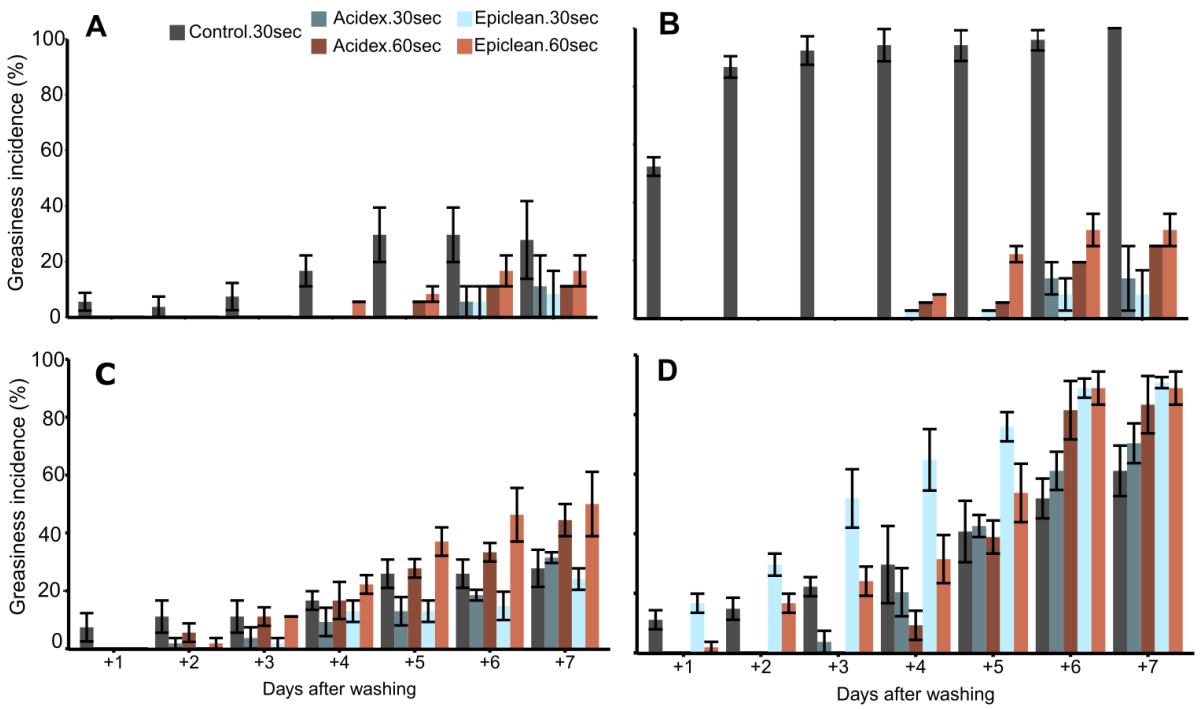

Fig. 4. Greasiness incidence (% fruit) in WA 38 apples in 2022 (A: Low greasiness lot; B: High greasiness lot) and 2023 (C: Trial 1; D: Trial 2). All fruits were stored at 33 ºF and evaluated for up to 7 days at 68 ºF after washing with different detergent treatments (Mean ± Standard Error).

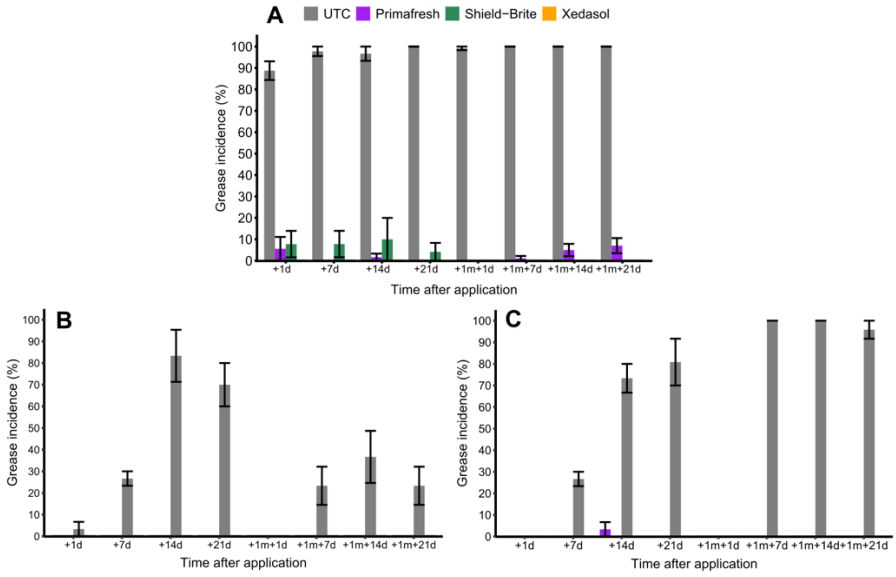

Fig 5. Greasiness incidence (% fruit) in WA 38 apples in 2022 (A) in fruit with low greasiness and evaluated up to 21 days at 68ºF after coating treatments, and after 1 month in cold storage (33ºF) and up to 21 days at 68ºF; and in 2023 stored for 6 months (B) or 10 months (C) for up to 21 days at 68ºF with or without 1 month of air storage (33 ºF) after the coating treatment.

<u>Objective 4</u>: Identify and determine protocols for mitigating off-flavors associated with greasiness.

In 2022, fruit sampled from long-term CA lots where the off-flavor was identified were used for an *ad hoc* taste panel of individuals accustomed to apples to identify the "bitter" off-flavor as well as hypothetical off-flavor components. Apples (n=160) were sampled in sections representing quarters of the fruit (stem/calyx end; sun-facing/shaded) (n=320 in total). Apple samples (peel separated from the flesh) were rated for eating quality, and the adjacent peel/cortex flash was frozen in LN_2 and stored at -80 ºC until processing and instrumental analysis. Samples were rated as "bitter", "musty", "chemical", "medicinal", "metallic", "tingly", "astringent", or an "after taste", all descriptors of the off-flavor determined by panelists during training. The aroma profile was analyzed using gas chromatography/mass spectrometry to allow for a snapshot of the aroma, approaching a direct comparison of the profile and the panelists' experience. Data were analyzed using statistical approaches that identified the presence, nature, and associated aroma components linked with those samples identified as imparting an off-flavor.

Off-flavor was identified in at-least one sample given to each panelist. Bitter samples were often detected in the same fruit as samples that were not considered bitter. While peel typically produces the highest levels of aroma, bitterness was more commonly associated with cortex tissue (Fig. 3), the shade side of the apple (>60%), and the shoulder and calix-end sections.

The aroma profile was compared with off-flavor detected by the sensory panel. Off-flavor was determined as a positive response to any of the following descriptors as they could all be considered descriptors of the off-flavor according to the sensory panel survey: "Bitter", "chemical", "medicinal", "metallic", "tingly", "astringent", or "after taste" (Fig. 6). The aroma profile (35 natural aroma chemicals) was consistent among samples from the same apple, but links with off-flavor were not obvious until whole apples were categorized according to presence of off-flavor. An apple was considered if off-flavor was detected in 50% or more of the comprising cortex or peel samples. This more clearly revealed associations with aroma chemicals linked with unripe apples, especially more "cut grass" and less ripe apple aroma chemicals (Fig. 7). This classification was even more apparent when projected using the ratio of 2-methylpropyl acetate, an aroma note associated with apples that were considered "good" overall, to one that was associated with "bad" flavored fruit, 2-hexenyl acetate. Consequently, once these associations were confirmed using this binary categorization, we could follow levels of these and related aroma compounds back to compare them with sensory classifications of individual samples, finding that links between the limited set of aroma compounds and sensory classification still held true.

Subsequent work should focus on determining any associations among phenotype, unripe aroma notes, and harvest maturity to resolve whether harvesting maturity impacts consumers' experience of off-flavors. Rather than any single chemical causing the off-flavor, it is possible that another significant factor, such as maturity, is impacting flavor consumer perception of bitterness such as a pre-association between that attribute and green, grassy aroma notes.

No bitterness or off flavor was detected in fruit from any of the previous experiments (Objectives 1, 2, and 3) performed during 2023, 2024, and 2025.

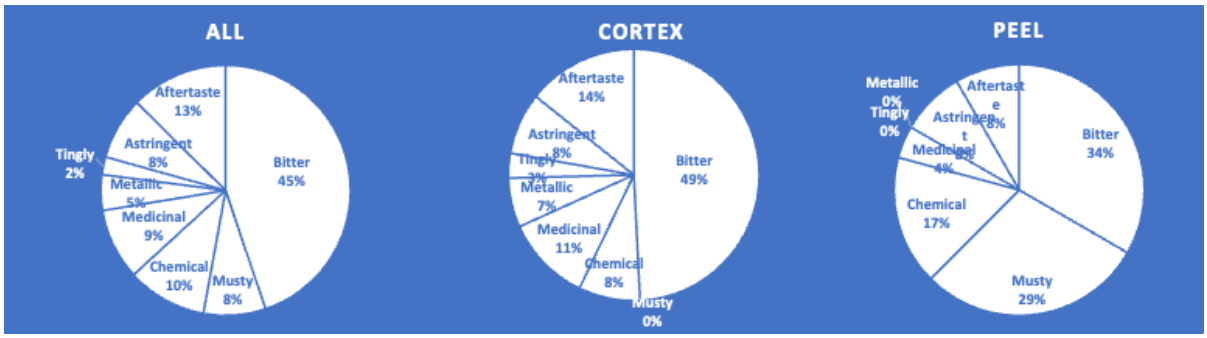

Fig. 6. Descriptors of taste found by the trained panel. Values indicate % of samples (n=320) in each descriptor.

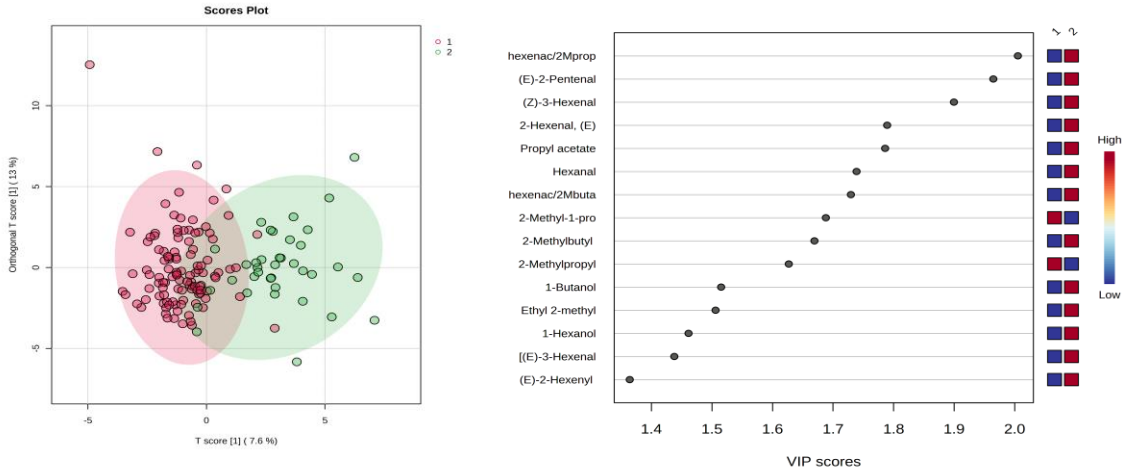

Fig. 7. Flesh tissue samples (left) from WA 38 apples were identified by a sensory panel as having off-flavor (green) or not (red). This indicates that the aroma profile, as a whole, is different between apples considered to have an off-flavor and those with no issue. It also reveals that there are one or more aroma components related to this difference. Natural aroma chemicals (right) that are most associated with off-flavored samples include those typically most abundant in unripe or immature apples, while those prominent in samples considered good are typically more prominent in ripe apple aroma profile. The ratio of unripe to ripe aroma components are also high in this list, further highlighting the relationship between unripe aroma and perceived off-flavor.

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Yang, Y., Zhou, B., Zhang,J., Wang, C., Liu, C., Liu, Y., Zhu, X., Re, X. 2017. Relationships between cuticular waxes and skin greasiness of apples during storage. Postharvest Biol. Technol. 131, 55-67.

EXECUTIVE SUMMARY

Project Title: Mitigating WA 38 greasiness and related quality defects

Keywords: apple, cosmic crip, fruit quality, Retain, Harvista, Smartfresh, coating, maturity indexes

Abstract:

Skin greasiness remains a key postharvest challenge for WA 38 apples, particularly during long-term storage and marketing. This project evaluated how harvest maturity, growing conditions, and postharvest practices influence greasiness development and related quality defects, including off-flavors, with the goal of identifying practical strategies growers can apply throughout the cold chain. Results showed that fruit maturity at harvest is a major driver of greasiness risk. Starch breakdown

progressed differently among sites and seasons, largely due to temperature differences during the growing season. Warmer conditions led to faster starch degradation and greater variability in maturity at harvest, increasing the risk of greasiness. In contrast, cooler temperatures late in the season were associated with higher greasiness development.

Storage conditions also influenced greasiness development. Fruit stored in regular air developed more greasiness than fruit stored under controlled atmosphere (CA). In all storage regimes, greasiness increased once fruit were removed from cold storage and placed into shelf-life conditions (7 days at 68°F).

Ethylene management before and after harvest played an important role too. Retain® applications reduced greasiness at harvest and during cold storage for up to four months, with earlier applications (14–21 days before harvest) provided a better control. However, Retain® did not prevent greasiness development once fruit were removed from cold storage. Among postharvest treatments, a single application of Harvista™ combined with SmartFresh (SF) or SF alone reduced greasiness incidence during storage.

During packaging, detergent washes were able to eliminate greasiness for 1–7 days before the reappearance of greasiness, while coatings helped maintain control during the cold chain, but neither approach fully prevented greasiness during shelf life.

An off-flavor described as “bitterness” was most often detected in immature fruit and was linked to aroma profiles typical of unripe apples. Following recommended harvest maturity guidelines and avoiding immature fruit is the most effective way to reduce this off-flavor risk.

PROJECT OUTCOMES

- **Refined harvest maturity guidelines** confirmed that fruit maturity at harvest is the primary factor influencing WA 38 greasiness and off-flavor risk. Cooler late-season conditions increased greasiness, reinforcing the need for careful, block-specific harvest timing.
- **Validated ethylene-management tools** showed that early preharvest Retain® applications (14–21 days before harvest) reduced greasiness during cold storage, while only Harvista™ (1x) combined with SmartFresh consistently reduced greasiness severity postharvest across storage conditions.
- **Defined storage and handling impacts** demonstrated higher greasiness incidence in regular air storage compared to CA, with greasiness increasing during shelf-life in all cases.
- **Established practical limits of post-storage interventions**, with detergents providing only short-term greasiness removal (1–3 days) and coatings offering extended but incomplete control during the cold chain.
- **Identified the source of off-flavors**, linking bitterness primarily to immature fruit and unripe aroma profiles, and confirming that proper harvest maturity is the most effective mitigation strategy.

Project Title: Genomic approaches to understand the etiology of postharvest decays

Report Type: Continuing Project Report

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Cooperators: N/A

Project Duration: 2-Year

Total Project Request for Year 1 Funding: $71,285
Total Project Request for Year 2 Funding: $109,025

Other related/associated funding sources: None

WTFRC Collaborative Costs: None

Budget 1
Primary PI: Dr. Achour Amiri
Organization Name: Washington State University-TFREC
Contract Administrator: Stacy Mondy
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Contract administrator email address: anastasia.mondy@wsu.edu
Station Manager/Supervisor: Chad Kruger

Item	2023	2024	
Salaries1	$43,680.00	$0.00	
Benefits1	$15,492.00	$0.00	
Salaries2	$0.00	$31,500.00	
Benefits2	$0.00	$10,205.00	
RCA Room Rental	$0.00	$0.00	
Shipping	$0.00	$0.00	
Supplies3	$11,300.00	$0.00	
Travel4	$813.00	$0.00	
Plot Fees	$0.00	$0.00	
Miscellaneous	$0.00	$0.00	
Total	$71,285.00	$41,705.00	$0.00

Footnotes:

1: Salaries are for a Scientific Assistant in Amiri Lab to conduct work related to fruit inoculation, sampling, and RNA extraction in Year 1 (2023) at monthly salary of $5,200 for 12 months at 0.7 FTE. Benefits are calculated based on 35.5% rate with a 4% inflation each year.

2: Salaries are for a Scientific Assistant in Ficklin Lab to conduct work related to RNAseq analyses in Year 2 (2024) from samples collected in Year 1, at monthly salary of $5,200 for 12 months at 0.5 FTE. Benefits are calculated based on 35.5% rate with a 4% inflation each year.

3. Supplies include $5,000 for microbiological media, Petri dishes, pipette tips, filters, bottles and other miscellaneous to be used to grow and maintain inocula of fungi for inoculation. Supplies also include $6,300 for RNA extraction estimated at $35/sample and estimated 180 total samples.

4. Travel to experimental sites in Washington States, to meet collaborators in Pullman to discuss data, and to attend extension event and apple review days. Estimated 1,300 miles a year at $0.625/mile.

Budget 2
Co PI 2: Alex Harkess
Organization Name: Hudson Alpha Institute for Biotechnology
Contract Administrator: Nancy Westfall
Telephone: 256-327-0400
Contract administrator email address: nwestfall@hudsonalpha.org

Item	2023	2024	
Salaries	$0.00	$0.00	
Benefits	$0.00	$0.00	
Wages	$0.00	$0.00	
Benefits	$0.00	$0.00	
RCA Room Rental	$0.00	$0.00	
Shipping	$0.00	$0.00	
Supplies	$0.00	$0.00	
Travel	$0.00	$0.00	
Plot Fees	$0.00	$0.00	
Miscellaneous1	$0.00	$67,320.00	
Total	$0.00	$67,320.00	$0.00

Footnotes:
1: Funds are for RNAseq work and analyses to be conducted at the Hudson Alpha Institute for Biotechnology. We plan to collect 3 samples at 10 different sampling times from 3 different cultivars for a total of 200 samples and two pathogens (*P. expansum* and *B. cinerea*). RNAseq analysis estimated at $170/sample.

Objectives

1) Understand gene activity in three major cultivars in relation to infection by *P. expansum* and *B. cinerea* during several preharvest phenological stages and during storage using RNA-seq analyses. Here, we focus on these two major pathogens with a possibility to extend the research to other pathogens in future studies.

2) Compare three major apple cultivars, i.e., Honeycrisp (highly susceptible), Gala (moderately susceptible), and Granny Smith (less susceptible) in terms of gene expression to better understand differences seen between cultivars in WA commercial warehouses.

3) Use the new knowledge to identify gene activity that could potentially predict risk for decay development early enough to allow a timely deployment of appropriate strategies to reduce fruit loss.

Significant Findings

1) Fruit of three cultivars, namely Honeycrisp, Gala, and Granny Smith, were inoculated weekly with spore suspensions of *Penicillium expansum* and *Botrytis cinerea* in the orchard 5, 4, 3, 2, 1 week preharvest, at commercial maturity (harvest = 0 week), and 1, 2, 4, 8, and 24 weeks postharvest.

2) When wounded and inoculated, the three cultivars expressed decay symptoms differentially after 2 and 5 weeks in regular atmosphere storage at 34°F for Gala and Granny Smith and at 37°F for Honeycrisp.

3) *B. cinerea* showed higher early infection rates compared to *P. expansum*.

4) After two weeks of storage, Granny Smith apples did not exhibit any symptoms of blue mold, irrespective of the inoculation stage. In contrast, Gala and Honeycrisp apples expressed the highest blue mold incidence at four weeks preharvest and one week postharvest, respectively. Afterward, the incidence of blue mold exceeded 70% after five weeks of storage, regardless of the cultivar and inoculation stage.

5) Internal ethylene production was significantly induced by inoculations with *P. expansum* and *B. cinerea* in comparison to the control across nearly all inoculation stages.

6) Overall, *Botrytis cinerea* induced higher ethylene production compared to *P. expansum*.

7) The cultivar Honeycrisp produced more ethylene than the two other cultivars when inoculated with either pathogen.

8) As anticipated, fruit firmness decreased over time and did not show significant differences between cultivars at comparable inoculation times.

9) 260 RNA samples were extracted from the cultivars treated with water or inoculated with the pathogens were submitted for sequencing at Hudson Alpha Institute for Biotechnology. Results are expected early 2026.

Methods

Activity 1. **Fruit inoculation and sampling** [2023, Amiri]

To assess gene activity changes as result from infections by *P. expansum* (blue mold) or *B. cinerea* (gray mold), fruit from Honeycrisp, Gala, and Granny Smith will be used. These three cultivars have been selected for this study for two main reasons: 1) because they have a range of susceptibility to postharvest decay, allowing comparison of gene activity that may relate to this trait, and 2) because high quality genomes of Gala (Sun et al. 2020), Honeycrisp (Kahn et al. 2022), Granny Smith (Honaas' AP-19-103 final report) are available, facilitating deeper insights into the genetics of decay susceptibility. Fuji, another important cultivar, was suggested by a reviewer, however, because there is not currently a Fuji genome, we decided to include the aforementioned cultivars owing to the expanded possibilities offered by their new genome resources.

Activity 1.1. Isolates growth and inoculum preparation. Three isolates of *P. expansum* and three isolates of *B. cinerea* will be grown on potato dextrose agar until profuse sporulation is observed and will be used to prepare spore suspensions by mixing equal volumes form each isolate, and the final concentration will be adjusted to 10^5 spore/ml. Fresh inoculum will be prepared for each inoculation time.

Activity 1.2. Fruit inoculation and fruit sampling. Trials will be conducted in research blocks at the WSU Sunrise experimental orchard in East Wenatchee, WA. In 2023, fruit from each of the abovementioned cultivars will be inoculated with spore suspensions of *P. expansum* and *B. cinerea* 5, 4, 3, 2, 1 and 0 weeks before estimated commercial maturity (Table 1). At each sampling time, three fruits on trees, will be tagged, and punctured with a sterile syringe (2 mm x 3 mm) at four equidistant points of equatorial zone of each fruit. Fruit will be inoculated with a 20-µl aliquot of the spore suspension of each pathogen, separately. At each inoculation time, three wounded apples, from the same trees, will be inoculated with 20 µl of sterile water and will be used a mock (control) fruit. To avoid cross-contaminations and additional external inoculum, fruit will be covered with fruit protect bags until sampling is conducted. To account for potentially confounding circadian rhythm bias, inoculation and sampling will be conducted at 1-3 pm in the afternoon for each inoculation and sampling time. Inoculated and mock apples will be harvested in labeled Ziplock bags 48 hours post-inoculation and immediately transported in cooler to WSU-TFREC. Tissue samples will be taken as described below and will be frozen at -80°C (-112°F) until used for RNA extraction.

Table 1. Cultivars, number of fruit, and sampling times expected in this study

	Weeks preharvest												Weeks postharvest									
	5		4		3		2		1		0		1		2		4		8		Total	samples x 2
Cultivar	Cont.	Inoc.	Cont.	Inoc.	Cont.	Inoc.	Cont.	Inoc.	Cont.	Inoc.	Cont.	Inoc.	Cont.	Inoc.	Cont.	Inoc.	Cont.	Inoc.	Cont.	Inoc.	samples	pathogens
Honeycrisp	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	60	120
Gala	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	60	120
Granny Smith	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	60	120
																					180	**360**

Cont. = control (non-inoculated fruit), Inoc. = Fruit inoculated with either *P. expansum* or *B. cinerea*

For fruit to be sampled and tested during storage, 24 additional apples from each cultivar, will be harvested at commercial maturity, similar to sampling done at 0-week preharvest (Table 1), and will be immediately transported to WSU-TFREC and stored at 35°F (for Gala and Granny Smith) and at 55°F for 10 days then at 37°F hereafter for Honeycrisp to mimic the preconditioning step commonly done at commercial warehouses for this cultivar. Six apples will be sampled at 1, 2, 4 and 8 weeks postharvest (Table 1) and three will be inoculated with spore suspensions of *P. expansum* or *B. cinerea* as described above for preharvest fruit and three apples will be inoculated with water and used as a control.

Experimental postharvest fruit will be stored in similar conditions used for preharvest fruit before proceeding with RNA extraction. For both pre and postharvest fruit, quality assessment of starch, firmness, weight, soluble solids, and titratable acidity will be conducted on a fruit subsample from the same tree at each sampling time.

Activity 2. **Tissue sampling, RNA extraction, and quality analyses** [2023-24, Amiri]

Sampling will be performed by removing 8 mm diameter plugs, including peel tissues surrounding the wound and 4 mm of mesocarp (flesh) tissue using a disposable biopsy punch, centered on the wound site, which will be immediately sectioned into two small disks with a sterile scalpel blade. Each biological replicate will contain 24 discs (3 fruits and 4 wounds per fruit). All sectioned discs will be frozen in liquid nitrogen and stored at −121°F. RNA will be extracted using protocols described previously (Honaas and Kahn 2017, Mellidou et al. 2014) and evaluated for quality and purity before storing at −121°F.

Activity 3. **Sequencing and data analyses** [Harkess & Honaas 2024]

Following methods developed by Honaas in the scope of WTFRC project AP-19-103, RNA samples of sufficient high-quality (RIN≥8, $A_{260/280}$ ≥1.8, $A_{260/230}$ ≥2.0) will be analyzed with RNA-Seq at the HudsonAlpha Institute for Biotechnology core facility. Libraries will be constructed using Illumina's TruSeq RNA Library Prep Kit V2 (https://www.illumina.com/) and sequenced to a target sample depth of ≥35 million reads (a higher-than-normal target because the RNA samples are a mixture of fruit and fungal RNA – essentially a double sample). RNA Seq data will be mapped and quantified as described in Honaas et al. (2020), following additional recommendations for analyzing a mixed sample transcriptome as outlined in Honaas et al. (2016).

Activity 4. **Use comparative genomics to discover genes and potential markers in Honeycrisp, Gala, and Granny Smith related to infections and decay development** [Ficklin, Honaas, Amiri, 2024**]**

Recent work by Honaas' team has explored methods to select genes that are related to postharvest fruit quality traits. This has led to the identification of genes that may be useful someday as risk assessment tools, but also that help us learn about the genetics that control important fruit quality traits. Honaas' current project (AP-22-101A) takes large gene activity tables called "gene expression matrices" or GEMs and uses a mix of conventional methods with machine learning techniques (Honaas et al. 2021) to iterate through the data to select genes associated with various traits, like at-harvest fruit maturity or susceptibility to postharvest disorders. The project described here will add gene activity data from three additional cultivars and orchards to Honaas' data but will also add a new synergistic dimension – fruit susceptibility to postharvest decays. Using similar approaches described in AP-19-103 and AP-22-101A, we will identify genes that are relatable specifically to susceptibility to postharvest decays as a function of maturity (sampling done over time) and cultivar and expand the scope of the larger biomarker project by leveraging additional expertise and genome-scale data related to postharvest pathology.

A major benefit will be a first glimpse into how apple fruit gene activity changes in response to postharvest pathogens as a function of maturity (Table 1), and across cultivars that show a range of susceptibility to postharvest decay. This will help us learn about targets for future investigations, as well as potential molecular mechanisms that may explain differences in susceptibility to postharvest pathogens across cultivars. All-together, this project will enhance our understanding of how at-harvest fruit maturity impacts susceptibility to postharvest decays, and why there might be differences in risk among cultivars.

Expected outcomes. This study will generate extended across-cultivar genomic knowledge related to pathogen infections. The data generated could help discover Honeycrisp, Gala, and Granny Smith polymorphisms potentially linked to decay development or resistance in a dynamic way pre and postharvest. One hoped long-term objective will rely on comparing data from major cultivars to gather new knowledge (genes or markers) to informs us if similar or different pathways are triggered by the two most important pome fruit pathogens, *P. expansum* and *B. cinerea*. We will also learn about how the time of infections matters for decay occurring later in storage.

Pitfalls and limitations. Because seasonal variability and weather conditions may affect genomic response of fruit to pathogen infections, large and complex data expected form this study will have to be analyzed carefully and accurately. Samples from different cultivars and sampling times following fungal inoculations will be useful to acquire a new cross-cultivars knowledge about the genomics of pathogen infection pre and postharvest that on the long term will be very useful to identify genes and markers that may help predict decay outbreak.

Results

Decay incidence and severity

All main effects, including cultivar, pathogen, inoculation time, and most interactions had a significant effect on the incidence of blue mold and gray mold. During the preharvest season, disease incidence and severity generally increased as the inoculation time approaches harvest and differences among cultivars are most pronounced during this period. The pathogen *Botrytis cinerea* exhibited higher early infection rates in comparison to *Penicillium expansum* (Figure 1). The time point at 0 WPr (harvest = commercial maturity) emerges as a crucial transition point. Disease development patterns undergo significant changes with both pathogens demonstrating increased aggressiveness (Figure 1). At this stage, differences in cultivar resistance become less pronounced.

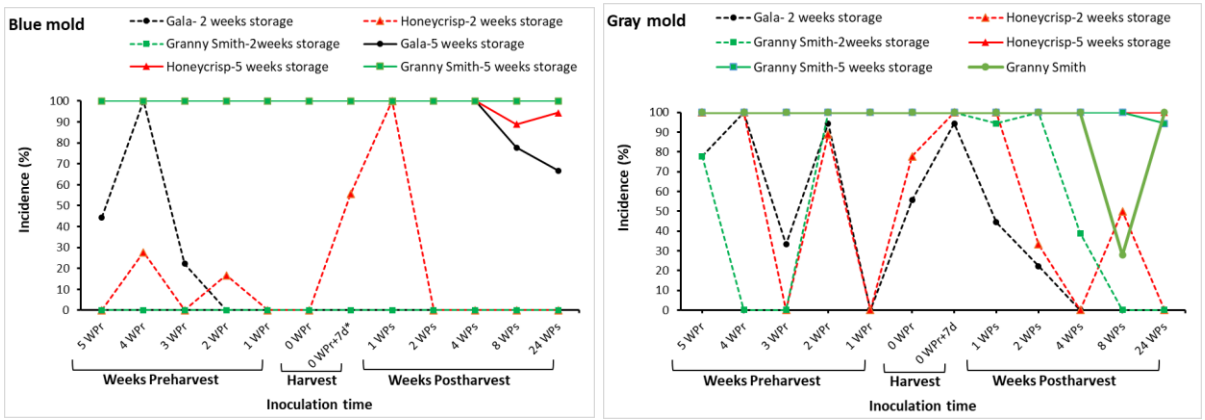

Figure 1. Incidence of blue mold (right) and gray mold (left) after two weeks (dashed lines) and 5 weeks (solid lines) in a regular atmosphere at on three apple cultivars inoculated with *Penicillium expansum* and *Botrytis cinerea* at different preharvest (WPr) and postharvest (WPs) times (weeks). For the 0 WPr + 7d, fruit were inoculated on the tree after one week passed commercial maturity to simulate over-maturity conditions.

Gala showed higher blue mold severity than Honeycrisp during the preharvest period 5 weeks preharvest to 1 week preharvest, than an inverse situation was observed in storage with higher severity

observed on Honeycrisp (Figure 2, left). Granny Smith was resistant to blue mold infection after 2 weeks in storage and exhibited the lowest blue mold virulence at almost all pre and postharvest inoculation stages when storage was extended to 5 weeks.

In terms of gray mold susceptibility, both Gala and Honeycrisp exhibited significantly greater vulnerability 5 and 4 weeks prior to harvest when compared to Granny Smith (Figure 2, right). As the harvest period approached, the susceptibility of Gala to gray mold decreased, whereas that of Granny Smith increased, ultimately aligning with the susceptibility levels observed in Honeycrisp (Figure 2, right).

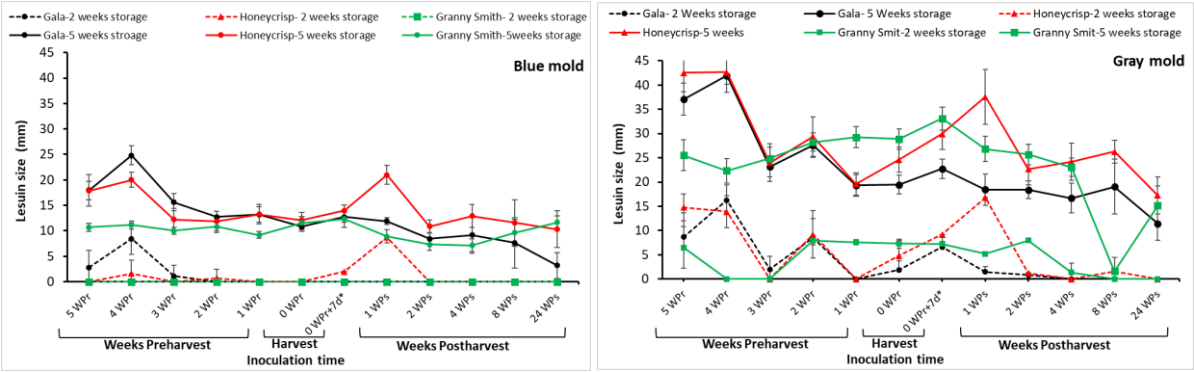

Figure 2. Severity of blue mold (left) and gray mold (right) after two weeks (dashed lines) and 5 weeks (solid lines) in a regular atmosphere at on three apple cultivars inoculated with *Penicillium expansum* and *Botrytis cinerea* at different preharvest (WPr) and postharvest (WPs) times (weeks). For the 0 WPr + 7d, fruit were inoculated on the tree after one week passed commercial maturity to simulate over-maturity conditions.

Fruit Quality Parameters

Seven fruit quality parameters were assessed at each weekly inoculation, including weight, firmness, Brix, titratable acidity (TA), internal ethylene production, color, and red over color. No significant differences were observed in weight, Brix, or titratable acidity across cultivars and pathogens. The firmness of the apples decreased over time yet exhibited similar trends among cultivars at the same sampling intervals (Figure 3).

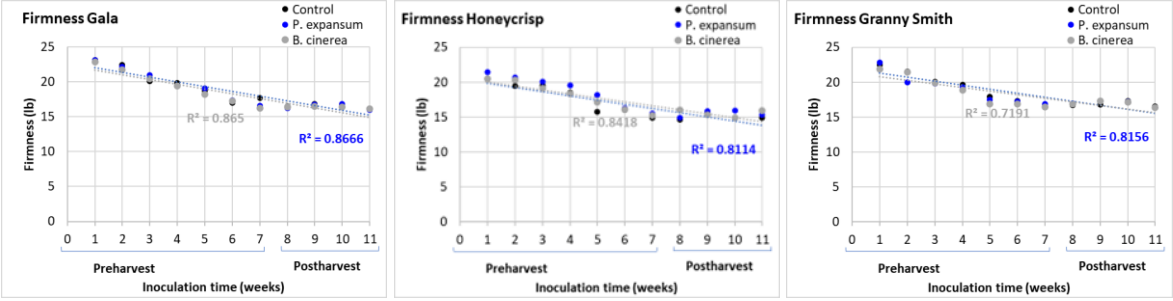

Figure 3. Firmness (lb) of Gala, Honeycrisp, and Granny Smith apples inoculated weekly at different maturity stages with *Penicillium expansum* and *Botrytis cinerea* at different preharvest (WPr) and postharvest (WPs) times (weeks). For the 0 WPr + 7d, fruit were inoculated on the tree after one week passed commercial maturity to simulate over-maturity conditions.

Interestingly, internal ethylene production was significantly induced by inoculations with *P. expansum* and *B. cinerea* in comparison to the control at nearly all inoculation stages. Overall, *Botrytis cinerea* elicited a greater production of ethylene compared to *P. expansum*. Notably, the cultivar Honeycrisp produced more ethylene than the other two cultivars when inoculated with either pathogen (Figure 4).

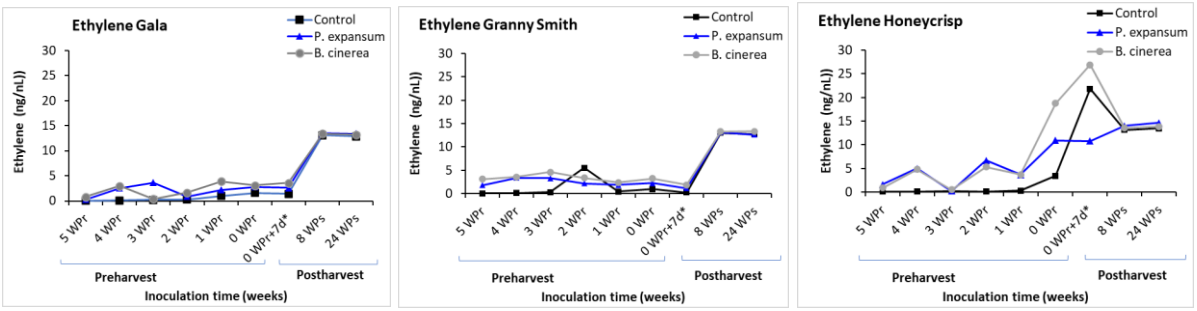

Figure 4. Firmness (lb) of Gala, Honeycrisp, and Granny Smith apples inoculated weekly at different maturity stages with *Penicillium expansum* and *Botrytis cinerea* at different preharvest (WPr) and postharvest (WPs) times (weeks). For the 0 WPr + 7d, fruit were inoculated on the tree after one week passed commercial maturity to simulate over-maturity conditions.

Significance to the industry

The objective of this study was to understand gene activity in three major cultivars in relation to infection by *P. expansum* and *B. cinerea* during several preharvest phenological stages and during storage using RNA-seq analyses. The ultimate goal will be to use the new knowledge to identify gene activity that could potentially predict risk for decay development early enough to allow a timely deployment of appropriate strategies to reduce fruit loss.

Although the ongoing genomic analysis will determine whether such differences are governed by specific genes, the variability in response to elicitation between the two pathogens and the three cultivars observed at the phenotypic level may provide opportunities to identify genetic markers that can assist in predicting the risks of gray and blue mold infections. If confirmed and validated, such tools will enhance the development of informed management strategies aimed at reducing decay and increasing packout.

Ongoing and future activities

- RNA samples are being sequenced at Hudson Alpha Institute for Biotechnology. Expected first quarter of 2026.
- Sequences will be cleaned and analyzed using bioinformatic tools to look for potential markers (3rd quarter of 2026)
- Final Report to be submitted: January 2027

Project Title: UV-C and antimicrobial spray to control decay and food safety risks

Report Type: Continuing Project Report **Year**: 2 of 3

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Cooperators: Columbia Fruit Packers

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $71,136
Total Project Request for Year 2 Funding: $99,250
Total Project Request for Year 3 Funding: $80,519

Other related/associated funding sources: Awarded
Funding Duration: 2024 - 2027
Amount: $249,956
Agency Name: Washington State Specialty Crop Block Grant Program
Notes: N/A

WTFRC Collaborative Costs: None

Budget 1
Primary PI & CO-PI 2: Qingyang Wang & Joy Waite-Cusic*
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Item	2024	2025	2026
Salaries	$28,924.00	$30,081.00	$15,642.00
Benefits	$7,466.00	$8,063.00	$4,354.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$9,000.00	$9,000.00	$7,000.00
Travel	$582.00	$582.00	$582.00
Plot Fees			
Miscellaneous			
Total	$45,972.00	$47,726.00	$27,578.00

Footnotes:
Salaries and Benefits: Salaries are requested for a Graduate Research Assistant (GRA) at 0.49 FTE for 2.5 years at 25.8% benefit rate. Salaries are calculated with an increase of 4% per year, and benefits are calculated with an inflation rate of 8%.
Supplies: Funds are requested for purchasing laboratory consumables (such as gloves, centrifuge tubes, and pipette tips), microbiology experiments (such as growth media, Petri dishes, inoculation loops), and chemical analysis (such as antimicrobial agents and assay solutions for phenolic content measurements).
Travel: Funds are requested to cover the mileage for transportation to WSU/cooperator facility for sample collection and facility visiting once a year adhering to university policies, delineated as $0.67 per mile × 868 miles = $581.56
*PI Wang and Co-PI Waite-Cusic will co-advise one GRA and share supplies throughout this project.

Budget 2
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Item	2024	2025	2026
Salaries	$12,309.00	$12,678.00	$13,059.00
Benefits	$9,355.00	$9,635.00	$9,925.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$2,000.00	$2,060.00	$2,122.00
Travel			
Plot Fees	$1,500.00	$1,500.00	$1,500.00
Miscellaneous			
Total	$25,164.00	$25,873.00	$26,606.00

Footnotes:

Salaries and Benefits: Salaries are requested for a Faculty Research Assistant (FRA) at $49,236/year for 3 months, and 76% benefit rate. Salaries are calculated with an increase of 3% per year, and benefits are calculated with an inflation rate of 3%.

Supplies: Funds are requested for purchasing laboratory consumables and microbiological supplies such as growth media, Petri dishes, inoculation loops, and pipette tips.

Plot Fees: Annual plot fee at the Southern Oregon Research and Extension Center is $1500.

Budget 3

Co-PI 4: Claire Murphy

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Station Manager/Supervisor: Naidu Rayapati

Station manager/supervisor email address: 509-786-9215

Item	2024	2025	2026
Salaries		$15,562.00	$16,184.00
Benefits		$1,559.00	$1,621.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies		$8,000.00	$8,000.00
Travel		$530.00	$530.00
Plot Fees			
Miscellaneous			
Total	$0.00	$25,651.00	$26,335.00

Footnotes:

Salaries and Benefits: Salaries are requested in years 2 and 3, respectively, for a graduate student's summer hourly salary and a part-time hourly employee. Benefits are requested in years 2 and 3, respectively, which are tied to the graduate student's summer hourly salary and a part-time hourly employee.

Supplies: Funds are requested for purchasing laboratory consumables and microbiological supplies such as growth media, Petri dishes, inoculation loops, and pipette tips.

Travel: Funds are requested to cover the mileage for transportation to cooperator facility for sample collection and facility visiting adhering to university policies, delineated as $0.655 per mile × 808 miles = $529.24

OBJECTIVES

The overall goal is to is to combine a short-time UV-C light treatment with an antimicrobial spray (sanitizers approved for organic use and plant-based agents) to create a practical and cost-effective strategy for reducing postharvest apple losses due to decay-causing fungi (*Penicillium expansum,* blue mold) and foodborne pathogens (*Listeria monocytogenes*).

1. Define and optimize UV-C treatment to inactivate decay-causing fungi and foodborne pathogens.

2. Identify and evaluate plant-based antimicrobials and organic sanitizers to prevent or delay the growth of decay-causing fungi and foodborne pathogens.

3. Optimize integrated lab-scale UV-C and antimicrobial treatment to inactivate and delay the growth of decay-causing fungi and foodborne pathogens on the surface of apples while preserving product quality.

4. Assess the optimized UV-C and antimicrobial integrated treatment using a pilot-scale system followed by simulated bulk cold storage.

Based on feedback from the 2025 annual stakeholder review, the project scope was expanded to address additional, industry-driven research questions, including:

- Evaluate the effect of drying with UV-C treatment
- Assessment of whether combining UV-C with high temperatures enhances efficacy

The current project report (Year 2) summarizes progress and findings related to Objectives 1-4, as well as results from the additional research questions identified during the prior annual review.

SIGNIFICANT FINDINGS

- Short-duration UV-C treatments consistently reduced *Penicillium expansum* spores and *Listeria* on apple surfaces.
- UV-C was most effective on exposed or wound-like surfaces (e.g., equatorial region), while liquid antimicrobial treatments (e.g., hydrogen peroxide) were critical for controlling contamination in anatomically complex regions such as the stem and calyx.
- Integrated treatments outperform single interventions, but treatment sequence matters.
- Incorporating a drying step significantly enhanced the efficacy of UV-C and antimicrobial treatments, improving outcomes for both decay control and food safety under packing-line–relevant conditions.
- Validation studies using conveyor UV-C system support scalable implementation.

METHODS

PART I: Studies on Decay-Causing Fungi (*Penicillium Expansum*)

<u>*Defining UV-C Inactivation Doses*</u>: Spores of *P. expansum* were exposed to UV-C under controlled laboratory conditions using both continuous and single-dose applications. Initial evaluations were conducted in spore suspension, followed by validation on apple surfaces at three anatomically relevant infection sites: stem, calyx, and equatorial region. Key parameters evaluated included: Exposure time (seconds), UV-C dose (kJ/m^2), and initial spore concentration (10^5–10^7 CFU).

Antimicrobial Screening and Selection: A range of organic sanitizers and antimicrobial agents were screened using spore suspension assays and disk diffusion assays. Hydrogen peroxide, lactic acid, and acetic acid were evaluated across multiple concentrations and contact times, with hydrogen peroxide selected for further integration studies based on consistent sporicidal performance.

Integrated Treatments on Apples: Surface-sanitized apples were inoculated with *P. expansum* spores using a single-drop method at the equatorial region (surface), stem, or calyx. Treatments included: UV-C alone (1.56 kJ/m²), hydrogen peroxide alone (3%, 1 min), sequential UV-C followed by hydrogen peroxide, and non-treated control. Apple samples were stored under high-humidity, room-temperature conditions and monitored for disease incidence and progression for up to 30 days. Experiments include three independent replicates with 45 apples for each replicate.

Optimization and Exploratory Assessments: Additional studies were conducted to evaluate if the "base treatment" can be enhanced in efficacy by: Treatment sequence, reduced hydrogen peroxide concentration with longer contact time, extended UV-C exposure durations, and addition of surface drying (forced air/heat).

PART II: Studies on Foodborne Pathogens (*Listeria Monocytogenes*)

Bacterial Strains and Inoculum Preparation: Listeria innocua was used as the primary surrogate organism for *L. monocytogenes*, based on comparable UV-C sensitivity. For validation, select experiments directly compared *L. innocua* and *L. monocytogenes* under identical UV-C exposure conditions. Cultures were grown to stationary phase and adjusted to target inoculation levels of approximately 6 log CFU/apple for surface inoculation studies.

UV-C Treatment on Apple Surfaces: Whole Gala apples were surface-inoculated with *Listeria* and exposed to UV-C at varying exposure times and doses. UV-C doses ranged from low (≈10 mJ/cm²) to high (>3000 mJ/cm²), achieved by adjusting exposure time and distance. Following treatment, surviving populations were enumerated and reported as log CFU reduction per apple.

Surrogate Validation: To confirm suitability of *L. innocua* as a surrogate, apples inoculated with either *L. innocua* or *L. monocytogenes* were exposed to identical UV-C treatments (5 s; 29.7 mJ/cm²). Log reductions were compared using unpaired statistical tests.

Combination UV-C and Hydrogen Peroxide Treatments: Combination strategies were evaluated in both planktonic systems and on apple surfaces. Treatments included: 1) Sequential application of H_2O_2 followed by UV-C, 2) sequential application of UV-C followed by H_2O_2, and 3) simultaneous exposure to UV-C and H_2O_2. Hydrogen peroxide was applied at 5% concentration with exposure times ranging from 30 s (surface spray) to 6 min (planktonic studies). UV-C doses varied depending on experimental configuration.

Conveyor-System and Drying Integration: A lab-scale conveyor system was used to simulate packing-line conditions. Apples inoculated at approximately 6 log CFU/apple were treated with UV-C alone, H_2O_2 spray alone, or combined UV-C + H_2O_2 treatments. Selected treatments were evaluated with and without a forced-air drying step (42 °C, 1.5 m/s airspeed, ~20% RH, 120 s) to assess the impact of surface moisture reduction on treatment efficacy.

RESULTS AND DISCUSSION

PART I: Studies on Decay-Causing Fungi (*Penicillium Expansum*)

UV-C Inactivation Kinetics and Dose Response: UV-C exposure resulted in a rapid reduction in *P. expansum* spore viability, characterized by a sharp initial decline in surviving spores within the first 10-20 seconds (equal to 0.52-1.04 kJ/m² using our UV system) of exposure. This was followed by a slower inactivation phase, suggesting that a subpopulation of spores exhibits increased tolerance to UV-C stress. No statistically significant differences were observed between continuous and single-dose UV-C applications at equivalent exposure times, indicating that total delivered dose, rather than exposure mode, affect inactivation. (Figure 1A).

UV-C efficacy was strongly dependent on both dose and initial spore concentration. At moderate contamination levels (10^5–10^6 CFU), UV-C doses ≥1.56 kJ/m² achieved complete or near-complete inactivation. In contrast, high spore loads (10^7 CFU) showed substantial survival even at higher doses. These results informed the selection of ≥1.56 kJ/m² as a target dose for whole-apple trials. (Figure 1B).

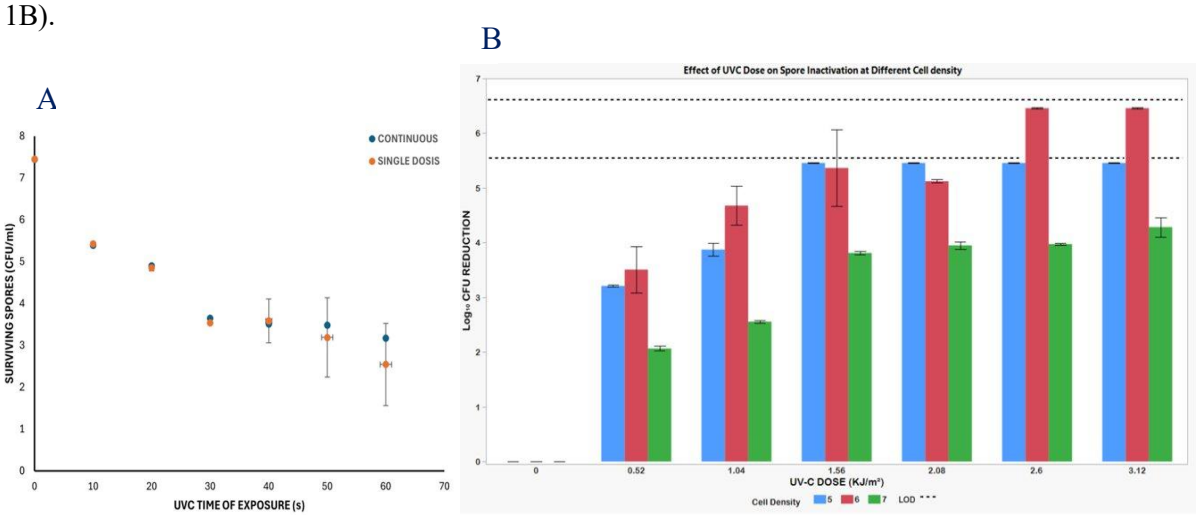

Figure 1. UV-C dose-response and inactivation kinetics of *P. expansum* spores. (A) Survival of *P. expansum* spores following continuous versus single-dose UV-C exposure as a function of exposure time. (B) Effect of UV-C dose (kJ/m²) on spore survival at different initial spore concentrations (10^5–10^7 CFU). Data demonstrate dose dependence and increased resistance at higher spore loads.

Antimicrobial Screening Results: All antimicrobial agents evaluated demonstrated concentration-dependent reductions in spore survival. Among the agents tested, hydrogen peroxide, lactic acid, and acetic acid consistently reduced spore counts across both suspension and disk diffusion assays. Hydrogen peroxide emerged as the most effective and consistent sporicidal agent and was therefore selected for further optimization. Further evaluation of hydrogen peroxide revealed that contact time had a stronger influence on spore inactivation than concentration alone. Short contact times (1 min) resulted in limited to moderate reductions even at higher concentrations, whereas extending contact time substantially improved efficacy. A hydrogen peroxide concentration of approximately 3% achieved greater than 1-log reduction at short contact times and served as the baseline condition for integrated treatment studies.

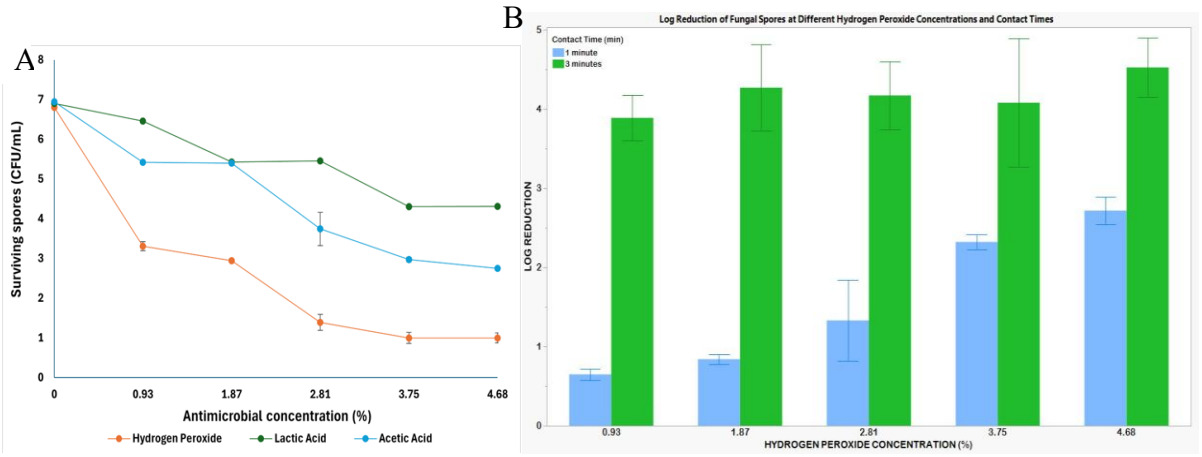

Figure 2. Antimicrobial screening results. (A) Spore suspension assay showing concentration-dependent reductions in spore survival for selected antimicrobials. (B) Effect of hydrogen peroxide concentration and contact time on spore inactivation.

Treatment Effects on Apple Disease Development During Storage (Equatorial Region): To evaluate treatment performance under stringent conditions, disease development studies were conducted using a deliberate "worst-case" inoculation and storage scenario. Apples were manually wounded and wet-inoculated by introducing a spore suspension directly into wounded equatorial tissues, followed by storage at room temperature (20 °C) and ~90% RH. These conditions were intentionally selected to accelerate decay development and challenge treatment efficacy beyond typical commercial storage environments.

Under these aggressive conditions, untreated control apples exhibited rapid disease development, with high disease incidence (100% disease rate) observed within the 20-day storage period. Hydrogen peroxide treatment alone resulted in only a modest reduction in disease incidence (93% disease rate) at the equatorial region and was not statistically different from the untreated control. This limited efficacy likely reflects the difficulty of achieving sufficient antimicrobial contact and persistence within wounded tissues following a single spray application. UV-C treatment alone significantly reduced disease incidence (84% disease rate) compared with the control, demonstrating its effectiveness as an initial, non-chemical intervention for inactivating *P. expansum* at exposed wound sites. The combined UV-C followed by hydrogen peroxide treatment provided the greatest overall reduction in disease incidence at the equatorial region (80% disease rate) and was statistically superior to the control. It is hypothesized that under typical commercial cold-storage conditions, where pathogen growth rates are substantially lower, these effects are expected to translate into even greater relative benefits for decay suppression.

Table 1: Evaluation of Treatment Effects on Disease Incidence of Equatorial Region over 20 days.

TREATMENT	TOTAL DISEASED COUNTS	OVERALL INCIDENCE (%)
CONTROL	45	100.00%
HYDROGEN PEROXIDE 3%	42	93.30%
UV-C TREATMENT	38	84.40%
COMBINED TREATMENT	36	80.00%
WATER	44	97.80%

Treatment Effects on Apple Disease Development During Storage (Stem and Calyx): The stem and calyx regions represent anatomically complex and commercially relevant infection sites, as these natural openings frequently serve as entry points for *P. expansum* during harvest, handling, and storage. Disease development at these sites was evaluated using the same worst-case experimental framework described above, including wet inoculation into the openings and accelerated storage conditions.

At the stem region, treatment effects were pronounced and clearly differentiated. Apples treated with hydrogen peroxide alone or with the combined UV-C + hydrogen peroxide treatment exhibited complete suppression of visible decay throughout the storage period (0% disease rate). In contrast, UV-C treatment alone resulted in little reduction in disease incidence (95% disease rate), while control had 100% disease rate. These findings indicate that liquid-based antimicrobial treatments are particularly effective at covering and disinfecting the stem cavity, where surface irregularities and shadowing limit direct UV-C exposure. Similarly, at the calyx region, hydrogen peroxide demonstrated the strongest reduction in disease incidence (4% disease rate) relative to the control (100% disease rate). UV-C alone and the combined treatment showed minimal additional benefit at this site (93% disease rate). Collectively, these results underscore the marked site-specific differences in treatment efficacy, where liquid-based treatments are particularly effective at penetrating the complex stem cavity structure, where UV-C penetration is limited. An integrated approach that leverages the strengths of both treatments may provide the most consistent disease control across the entire fruit surface, even under worst-case contamination and storage conditions.

Table 2: Evaluation of Treatment Effects on Disease Incidence of Stem (top) and Calyx (bottom).

Treatment (on stem)	Total Diseased Counts	Overall Incidence (%)
Control	45	100%
Hydrogen Peroxide (3%)	0	0%
UV-C Treatment	43	95.5%
Combined Treatment	0	0%

Treatment (on calyx)	Total Diseased Counts	Overall Incidence (%)
Control	45	100%
Hydrogen Peroxide (3%)	2	4.4%
UV-C Treatment	42	93.3%
Combined Treatment	42	93.3%

Influence of Treatment Sequence: Treatment order significantly affected disease outcomes. Applying UV-C prior to hydrogen peroxide resulted in consistently lower disease incidence compared with the reverse sequence (Figure 3). These results suggest that UV-C may be most effective when applied to relatively clean, untreated surfaces, providing rapid initial inactivation before antimicrobial application.

Figure 3: Effect of treatment sequence. Apples on the left were treated with UV-C followed by hydrogen peroxide, whereas apples on the right were treated with reversed order. Apples were stored at room temperature with ~90% RH for 30 days.

Optimization of Treatment Parameters: Reducing hydrogen peroxide concentration to 1% while extending contact time to 3 minutes showed promising reductions in disease incidence, indicating potential opportunities to enhance efficacy by reducing oxidative stress of apples received from higher concentration of hydrogen peroxide, while lowering chemical usage (Figure 4). Increasing UV-C exposure beyond 30 seconds did not further reduce decay, suggesting diminishing returns at higher fluence levels.

Figure 4: Effect of hydrogen peroxide concentration and treatment time on decay development of apple surface. treatment sequence. Apples on the left were treated with 3% hydrogen peroxide for 1 min, whereas apples on the right were treated with 1% hydrogen peroxide for 3 min. Apples were stored at room temperature with ~90% RH for 30 days.

Role of Surface Drying and Heat: Preliminary trials incorporating surface drying using a brief heat/air treatment prior to storage demonstrated enhanced reductions in both spore survival and visible decay, particularly when combined with UV-C and antimicrobial treatments. While these results are preliminary and require increased replication, they suggest that synergistic presence of heat during drying or the lower surface moisture enhance the overall treatment efficacy against disease development. (Figure 5).

Treatment	Log Reduction	Disease Rate (%)
Control	0	90
Drying + No UVC	0.2798	70
Drying + Combine	0.7178	40

Control
90% H – room temperature
Over 20 days.

Drying + No UV-C
90% H – room temperature
Over 20 days.

Drying + Combine
90% H – room temperature
Over 20 days.

Figure 5: Assessment of surface drying combined with UV-C and antimicrobial treatment.

PART II: Studies on Foodborne Pathogens (*Listeria Monocytogenes*)

UV-C Dose-Response for Listeria on Apple Surfaces: UV-C treatment resulted in significant, dose-dependent reductions of *L. innocua* on the surface of Gala apples. Increasing UV-C exposure time and delivered dose led to greater log reductions, confirming UV-C as an effective non-chemical intervention for *Listeria* control on apples. Even short exposure times achieved measurable reductions, supporting the feasibility of UV-C integration into high-throughput packing operations. (Figure 6).

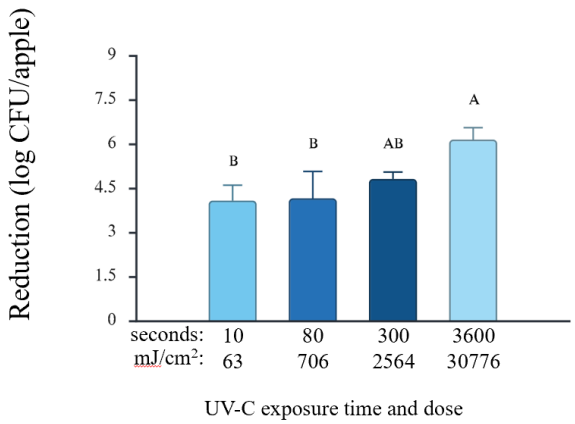

Figure 6: Reduction of L. innocua from the surface of Gala apple due to UV-C

Validation of L. innocua as a Surrogate for L. monocytogenes: Direct comparison of *L. innocua* and *L. monocytogenes* exposed to the same UV-C dose (29.7 mJ/cm²) showed no statistically significant difference in log reduction between the two species. This result supports the use of *L. innocua* as a conservative and appropriate surrogate for UV-C intervention studies, particularly for pilot-scale and conveyor-based experiments where pathogen use is restricted. (Figure 7).

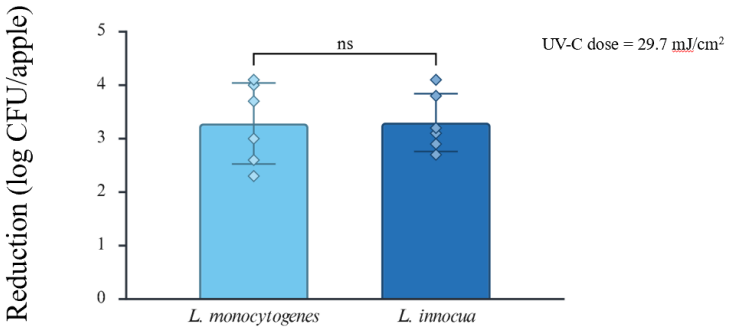

Figure 7: Reduction of *L. innocua* vs. *L. monocytogenes* from the surface of Gala apple due to 5 seconds exposure to UV-C.

Planktonic Combination Treatments: UV-C and Hydrogen Peroxide: In planktonic systems, all combination treatments of UV-C and hydrogen peroxide produced greater reductions than individual treatments alone. Among the combinations evaluated, treatment sequence influenced efficacy. Sequential application of UV-C followed by hydrogen peroxide generally resulted in higher reductions than the reverse sequence, while simultaneous treatments produced intermediate effects. These findings suggest that UV-C–induced cellular damage may increase bacterial susceptibility to subsequent oxidative stress from hydrogen peroxide. (Figure 8).

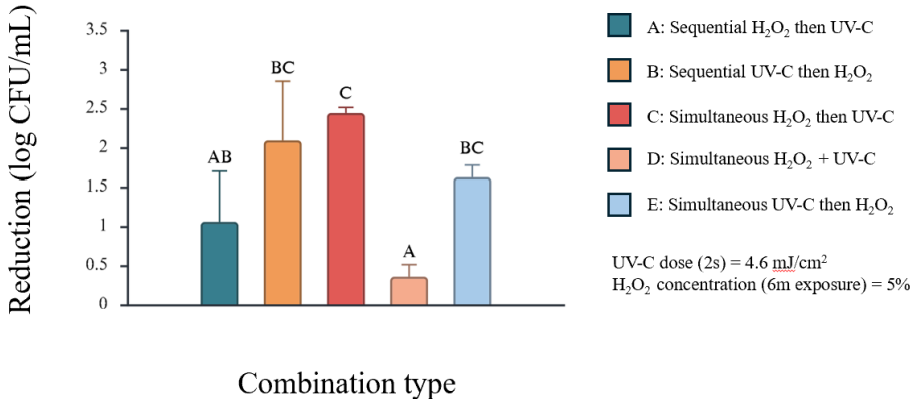

Figure 8: Planktonic reduction of *L. innocua* due to different combinations of UV-C and H_2O_2.

Combination Treatments on Apple Surfaces: Similar to planktonic study, on apple surfaces, combined UV-C and hydrogen peroxide treatments achieved significantly greater reductions of *L. innocua* than either treatment alone. UV-C followed by hydrogen peroxide consistently outperformed hydrogen peroxide followed by UV-C, reinforcing the importance of treatment order under realistic surface conditions. Treatments involving potable water controls confirmed that observed reductions were attributable to antimicrobial effects rather than mechanical removal. (Figure 9).

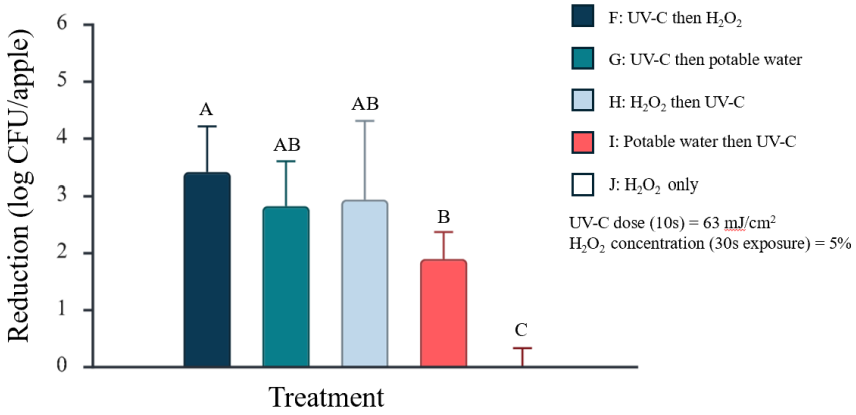

Figure 9: Reduction of *L. innocua* from the surface of Gala apple due to combination UV-C and hydrogen peroxide spray.

Conveyor-System Evaluation and Role of Drying: Under simulated conveyor-system conditions (Figure 11), all antimicrobial treatments reduced *Listeria* populations relative to untreated controls. Incorporation of a forced-air drying step substantially improved treatment outcomes across all interventions. The combination of hydrogen peroxide spray followed by UV-C, when paired with drying, produced the greatest log reductions (Figure 10). These results indicate that the synergistic presence of heat during drying and/or the low surface moisture strongly influences *Listeria* survival and treatment efficacy, and that integrating drying with UV-C and antimicrobial sprays may significantly enhance food safety outcomes in commercial packing lines.

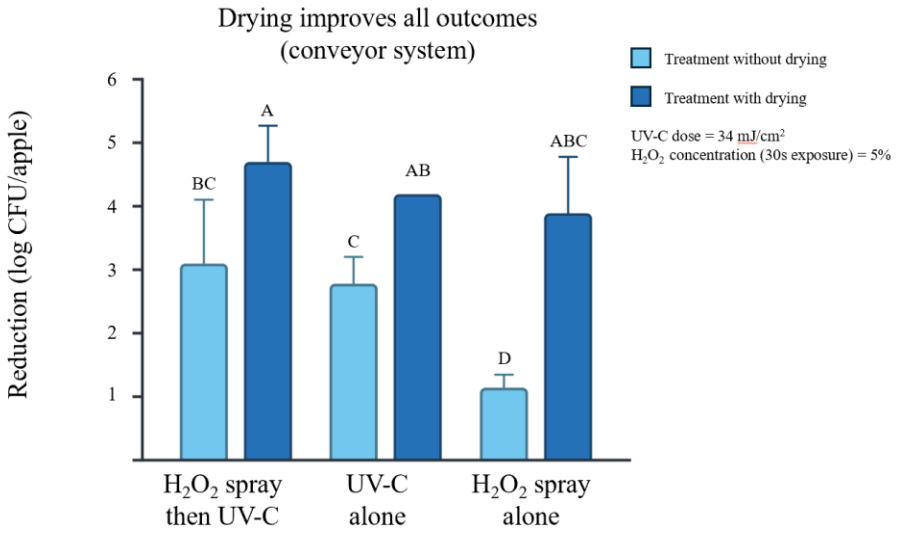

Figure 10: Effect of drying on the overall treatment performance on apples.

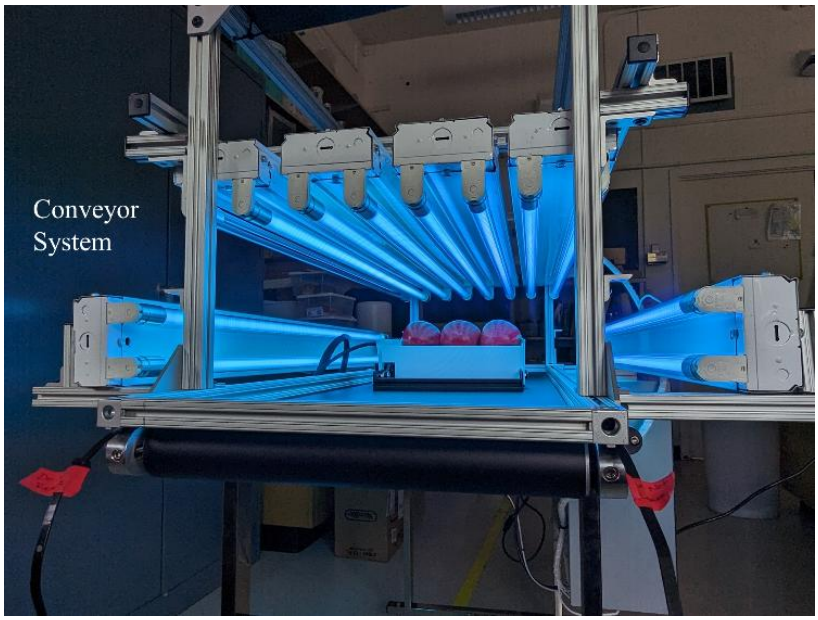

Figure 11: Lab-scale conveyor UV-C system used for this study.

Next Steps:

- Increase replication for combined UV-C + antimicrobial + drying treatments.
- Transition optimized treatments to simulated commercial cold storage and packing line study.
- Integrate fungal and bacterial findings to develop unified, practical recommendations for industry adoption.

Project Title: Food-Grade Antimicrobial Coatings for Storage Bins to Prevent Decay

Report Type: Continuing Project Report

Primary PI: Luyao Ma, PhD
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CO-PI 3: Claire Murphy, PhD
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City/State/Zip: Pullman, WA 99164

Cooperators: Two apple packinghouses in Washington have agreed to be cooperators. The identity of the operations that are willing to serve in a cooperator role will be kept confidential.

Contact information: Luyao Ma, luyao.ma@oregonstate.edu, 541-737-0659

Project Duration: 3 Years

Total Project Request for Year 1 Funding: $69,759
Total Project Request for Year 2 Funding: $79,982
Total Project Request for Year 3 Funding: $82,644

Other related/associated funding sources: Oregon State University will provide an in-kind contribution towards the tuition of one Graduate Research Assistant hired for this project**.**
Funding Duration: 2025 - 2027
Amount: $79,203
Agency Name: Department of Food Science and Technology, Oregon State University

WTFRC Collaborative Costs: None

Budget 1
Primary PI & Co-PI 2: Luyao Ma & Qingyang Wang*
Organization: Oregon State University (main campus)
Contract Administrator: Irem Turmer
Telephone: 541-737-4933
Contract administrator email address: sponsored.programs@oregonstate.edu

Station Manager/Supervisor: Lisbeth Goddik
Station manager/supervisor email address: lisbeth.goddik@oregonstate.edu

Item	2025	2026	2027
Salaries	$40,830	$42,055	$35,961
Benefits	$10,939	$11,711	$10,799
Wages	-	-	-
Benefits	-	-	-
RCA Room Rental	-	-	-
Shipping	-	-	-
Supplies	$15,500	$13,500	$8,000
Travel	$2,490	$2,490	$2,490
Plot Fees	-	-	-
Miscellaneous	-	-	-
Total	$69,759	$69,756	$57,250

Footnotes:
*** Primary PI Ma and Co-PI Wang will co-advise one Graduate Research Assistant (GRA) and share supplies.**
Salaries: Funds are requested to cover the salaries of three key personnel, including (i) one GRA at 100% FTE for Year 1 & 2 and at 80% FTE for Year 3 (base salary at $33,654/year); (ii) PI Ma at 2.5% FTE each year for 3 years (base salary at $122,676/year); (iii) co-PI Wang at 3% FTE each year for 3 years (base salary at $97,308/year). Salaries are calculated with an increase of 3% per year.
Benefits: Funds are requested to cover the fringe benefits of three key personnel, including (i) one GRA at 100% FTE for Year 1 & 2 and at 80% FTE for Year 3); (ii) PI Ma at 2.5% FTE each year for 3 years; (iii) co-PI Wang at 3% FTE each year for 3 years. Benefits are calculated at the rate of 0.546 with an inflation rate of 3%.
Supplies: Funds are requested to purchase supplies for microbiology experiments (microbiological media, petri dishes, inoculation loops, sanitizers, microplates, etc.), antimicrobial agents and coating materials (sanitizers, plant-based antimicrobial chemicals, baking yeast, coating chemicals, spray bottles, etc.), and laboratory consumables (gloves, centrifuge tubes, pipette tips, etc.)
Travel: Funds of $2,490 per year for 3 years are requested to cover travel costs to Washington State University and cooperator facilities for sample collection, field visiting, and project meetings adhering to university policies. Mileage: $0.67 per mile × 900 miles × 2 times per year = $1,206. Vehicle rental: $100 × 2 days × 2 times per year = $400. Lodging: $150 per night × 2 nights × 2 times per year = $600. Per diem: $71 × 2 days × 2 times per year = $284.

Budget 2
Co-PI 3: Claire Murphy
Organization: Washington State University's Irrigated Agriculture Research & Extension Center
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Item	2025	2026	2027
Salaries	-	-	-
Benefits	-	-	-
Wages	-	$6,106	$12,813
Benefits	-	$616	$1,281
Shipping	-	-	-
Supplies	-	$2,250	$8,900
Travel	-	$1,200	$2,400
Miscellaneous	-	-	-

Total	-	$10,226	$25,394

Footnotes:

Wages: $6,106 and $12,813 are requested in Year 2 & 3, respectively, for summer student hourly employees to collect and process samples for Objective 3.

Benefits: $616 and $1,281 are requested in Year 2 & 3, respectively, for benefits tied to the hourly Wages.

Supplies: $2,250 and $8,900 are requested in Year 2 & 3, respectively, to purchase supplies such as swabs, microbiological media, petri dishes, etc.

Travel: $1,200 and $2,400 are requested in Year 2 & 3, respectively, for mileage and travel associated with sample collection, adhering to all university policies.

Objectives

1. Evaluate the antimicrobial efficacy of bio-encapsulated FDA-approved agents against *Penicillium expansum* (blue mold).
2. Develop antimicrobial coatings using the bio-encapsulated agents on wooden and plastic coupons that simulate apple bin materials to inactive blue mold.
3. Assess the efficacy of antimicrobial coatings in reducing cross-contamination of blue mold and natural microflora (e.g., total coliforms, yeasts, and molds) in apple bins in orchards, as well as their cost and impact on apple quality.

Significant Findings

- The disc diffusion assay proved to be a reliable and reproducible method for quantifying the antifungal efficacy of both chemical- and plant-based agents against *Penicillium expansum*.
- Hydrogen peroxide and Sporocide®, both widely used industrial sanitizers, demonstrated significant inhibitory effects against *P. expansum* across the tested concentration range (0.5-5.0%).
- At lower concentrations (0.5% and 1.0%), hydrogen peroxide exhibited significantly greater antifungal efficacy against *P. expansum* compared with Sporocide®.
- Among the three essential oils evaluated, eugenol showed significant antifungal activity at concentrations ranging from 1.0% to 10.0%, whereas thyme oil and tea tree oil did not exhibit measurable inhibition under the disc diffusion assay conditions.
- Hydrogen peroxide, Sporocide®, and eugenol oil all demonstrated clear dose-dependent inhibition trends within their respective tested concentration ranges.
- Heat treatment effectively inactivated yeast cells, establishing a functional foundation for subsequent encapsulation of antimicrobial agents.

Methods

Screening Antimicrobial Efficacy of FDA-Approved Agents Against *P. expansum*

During Year 1 (from September 2025 to December 2025), standardized methodologies and experimental protocols were established to evaluate the effects of FDA-approved sanitizing agents against the common postharvest apple pathogen, *Penicillium expansum.* Antimicrobial agents were selected based on safety, prior industry application, established efficacy, and/or perceived potential efficacy based on literature review. A total of five agents were screened, including two chemical sanitizers and three essential oils. The chemical sanitizers evaluated were hydrogen peroxide and Sporocide®, a commercially available bleach alternative formulated from hydrogen peroxide and peracetic acid. Both chemical sanitizers were tested at concentrations of 5%, 2%, 1%, and 0.5%. The essential oils screened for antifungal activity included eugenol oil, tea tree oil, and thyme oil.

Antifungal efficacy was assessed using a standardized disc diffusion assay. Briefly, *P. expansum* spores were harvested from an established cultures and suspended at 10^6 CFU/ml before being evenly spread onto potato dextrose agar (PDA) plates. After drying, 20 µL of each diluted antimicrobial agent was applied to a 6 mm sterile diffusion disc, which was then placed onto the inoculated PDA surface. Plates were sealed and incubated at 25° Celsius (C) / 77° Fahrenheit (F) for

5 days. Following incubation, fungal lawn growth was visually assessed, and zones of inhibition were quantified as the diameter of complete growth suppression surrounding each disc, with no visible mycelial or spore development. Data were collected from three independent biological replicates with four technical replicates per treatment and compared across agents and concentrations.

Development of a Yeast Particle-Based Encapsulation Approach for Antimicrobial Agents

We have also been developing new yeast particle-based encapsulation methodology to enhance antimicrobial stability, delivery, and duration of activity. The yeast cells required specific heat treatment to induce cellular death and cytoplasmic expulsion to transform the living active dry yeast to yeast capsules. Yeast cells are a promising vehicle for antimicrobial delivery, acting as a natural barrier for the agents. We hypothesize that this allows the agent to be preserved for longer against natural deterioration and slows the release of anti-microbial agents to prolong agent efficacy.

Dry active yeast (*Saccharomyces cerevisiae*) was subjected to heat treatment at 95°C / 203°F to induce complete cell inactivation and cytoplasmic expulsion, thereby transforming viable yeast into hollow yeast capsules. Following heat treatment, yeast cells were repeatedly washed to remove intracellular contents. The inactivated yeast cells were then weighed (% w/w), resuspended in antimicrobial agent solutions, and subjected to vacuum loading (99% vacuum with an approximate lag time of 30 seconds) to facilitate agent uptake into the internal void spaces. After vacuum treatment, yeast cells were washed with water to remove the unencapsulated agents. Encapsulated yeast cells were either used immediately or suspended in deionized water and stored at 4°C / 39.2°F prior to testing.

Evaluation of Antimicrobial Effects of Yeast-encapsulated Antimicrobial Agents

A rapid screening protocol was developed to evaluate the antimicrobial performance of yeast-encapsulated agents. To accelerate the optimization of encapsulation parameters, *Listeria innocua* was selected as the initial model organism due to its rapid growth and established use as a surrogate for *Listeria monocytogenes*. This approach enables efficient screening prior to validation against *P. expansum,* which requires longer culture and incubation times.

L. innocua culture were grown overnight on a shaker at 37°C / 98.6 °F, and then diluted to 10^7 CFU/ml. Aliquots (10 µl) of *L. innocua* were added to 1 ml of antimicrobial solutions, achieving a final *L. innocua* concentration of 10^5 CFU/ml. Treatment groups included yeast-encapsulated agents, empty (non-loaded) yeast cells, free (non-encapsulated) agent solution, and non-treated groups (negative control). The treatments were conducted for 15 minutes, 1 hour, and 24 hours at 25°C / 77°F. Antimicrobial efficacy was quantified using plate count assays, with colony-forming units enumerated following incubation to assess treatment performance.

Statistical Analysis

Results were presented as means ± standard deviations and were based on twelve independent experiments (n = 12). Data were analyzed using one-way analysis of variance (ANOVA). After verifying homogeneity of variances, the Games-Howell post hoc test was applied to identify statistically significant differences among treatments, with significance defined as $P < 0.05$.

Results and Discussions

The project was initiated on September 16, 2025, following the onboarding of a graduate research assistant and master's student, Ms. Kalli Wagon. The research progress summarized in this report reflects activities conducted during the reporting period from September 15, 2025 to December 31, 2025.

Five antimicrobial agents were selected for initial screening to evaluate their efficacy against the common blue mold pathogen *Penicillium expansum*. Two chemical-based sanitizers, hydrogen peroxide and Sporocide®, were included as benchmark treatments. In parallel, essential oil-based antimicrobials were evaluated using disc diffusion assays to assess their potential for mold growth

inhibition. Three essential oils were screened, among which eugenol essential oil demonstrated the most promising antifungal activity. Eugenol is a plant-derived compound commonly extracted from cloves and other botanical sources. Tea tree oil and thyme oil did not exhibit measurable inhibitory effects against *P. expansum* under the tested conditions; therefore, these treatments were not advanced to subsequent dilution or efficacy studies.

The antifungal efficacy of Sporocide® is summarized in **Figure 1**. Sporocide® solutions were evaluated across a concentration range from the labeled use concentration (5.0%) to 0.5%. A clear concentration-dependent response was observed, with increasing Sporocide® concentration corresponding to larger zones of inhibition. Notably, measurable inhibition was maintained even at reduced concentrations, indicating robust antifungal activity across the tested range. At the labeled concentration (5.0%), Sporocide® achieved an inhibition zone of 2.12 ± 0.13 cm against *P. expansum* after 5 days of incubation. These results suggest that Sporocide® has strong potential for postharvest mold control applications, with flexibility for dose optimization to balance efficacy, chemical usage, and cost considerations in commercial settings.

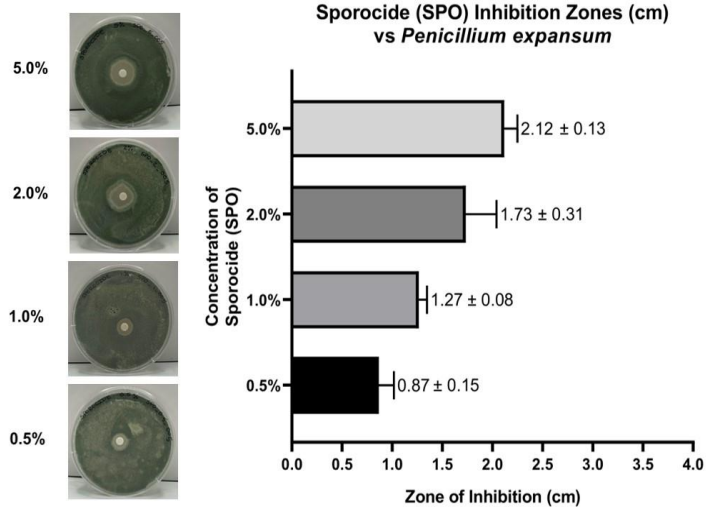

Figure 1. Evaluation of the chemical sanitizer Sporocide® against *Penicillium expansum* using a disc diffusion assay. Zones of inhibition (cm) were quantified to assess antifungal efficacy. Representative images of plated mold lawns with diffusion discs at varying Sporocide® concentrations are shown on the right. Results are based on twelve independent experiments (n = 12).

Similarly, hydrogen peroxide exhibited antifungal activity against *P. expansum* across all tested concentrations (0.5-5.0%). Disc diffusion assay results (**Figure 2**) demonstrated a clear concentration-dependent response, with inhibition zone diameters increasing as hydrogen peroxide concentration increased. Specifically, inhibition zones expanded from 1.10 ± 0.06 cm at 0.5% hydrogen peroxide to 2.10 ± 0.06 cm at 5.0%. In addition to suppressing mycelial growth, hydrogen peroxide treatment visibly reduced spore production, as evidenced by a less intense greenish mold lawn in representative plated samples (**Figure 2**, right panel). These findings support the potential of hydrogen peroxide as an effective postharvest sanitation option, offering scalable antifungal performance and compatibility with existing sanitation systems when applied at appropriate concentrations.

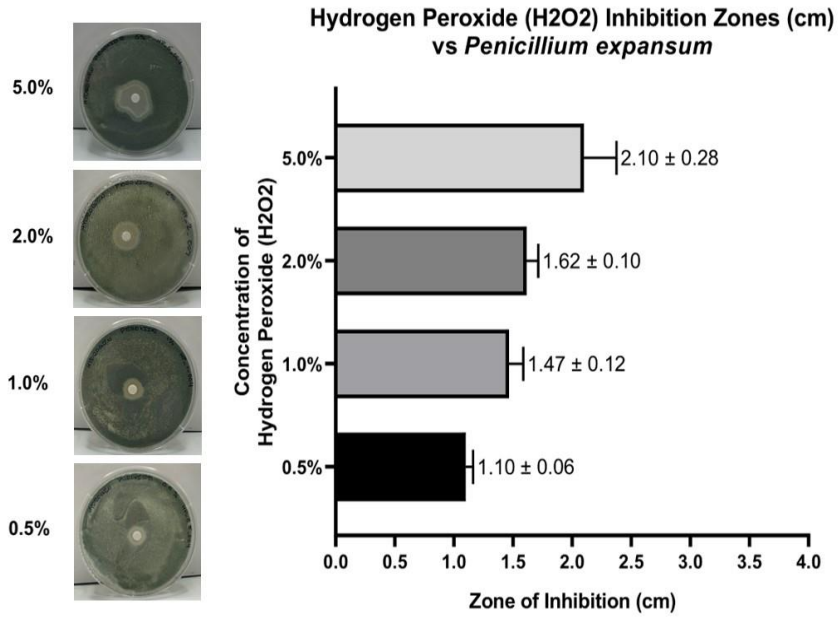

Figure 2. Evaluation of hydrogen peroxide against *Penicillium expansum* using a disc diffusion assay. Zones of inhibition (cm) were measured to quantify antifungal activity. Representative images of plated mold lawns with diffusion discs at varying hydrogen peroxide concentrations are shown on the right. Results are based on twelve independent experiments (n = 12).

Treatment of *P. expansum* with eugenol essential oil resulted in measurable antifungal activity at all tested concentrations, as shown in **Figure 3**. A clear dose-dependent response was observed, with inhibition zone diameters increasing from 0.82 ± 0.42 cm at 1.0% eugenol to 2.37 ± 0.52 cm at 10.0%. At the highest tested concentration (10.0%), eugenol also markedly reduced spore production, as indicated by a visibly less intense greenish mold lawn in representative plated samples (**Figure 3**). These results suggest that eugenol is a promising plant-derived antifungal agent for postharvest applications, particularly for reduced reliance on synthetic chemical sanitizers.

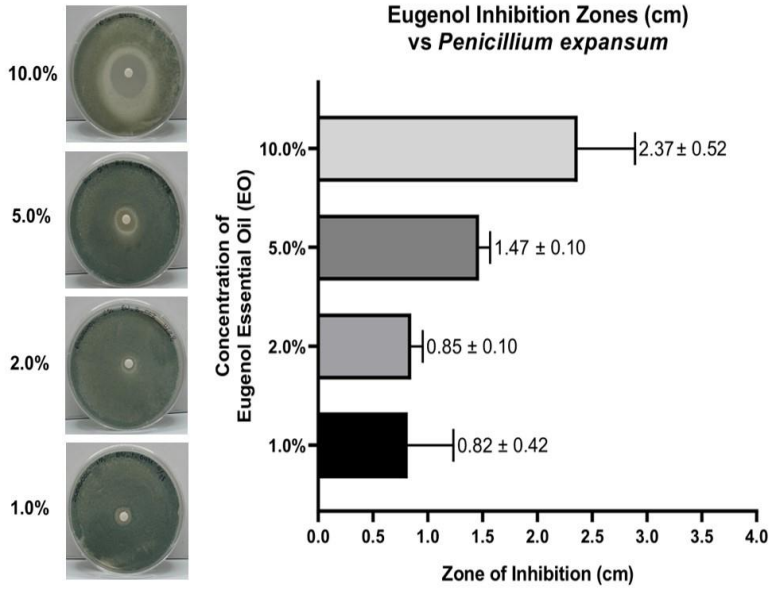

Figure 3. Evaluation of the essential oil eugenol against Penicillium expansum using a disc diffusion assay. Zones of inhibition (cm) were measured to quantify antifungal activity. Representative images of plated mold lawns with diffusion discs at varying eugenol concentrations are shown on the right. Results are based on twelve independent experiments (n = 12).

Table 1 summarizes the comparative antifungal efficacy of hydrogen peroxide, Sporocide®, and eugenol against *P. expansum*. All three agents demonstrated statistically significant inhibitory activity. At the highest tested concentration (5.0%), the two chemical sanitizers exhibited comparable levels of efficacy. However, at reduced concentrations (1.0% and 0.5%), hydrogen peroxide consistently outperformed Sporocide®, indicating greater efficacy under diluted-use conditions. In contrast, eugenol showed significantly lower antifungal activity ($P < 0.05$) than both hydrogen peroxide and Sporocide® at equivalent concentrations of 2.0% and 5.0%. These results highlight hydrogen peroxide as a more robust option for postharvest applications requiring effective mold control at lower sanitizer concentrations, while eugenol oil may be better positioned as a complementary or alternative treatment rather than a direct replacement for conventional chemical sanitizers. Based on these results, all three agents will be advanced to the encapsulation phase to evaluate whether controlled release can enhance antifungal efficacy and extend the duration of antimicrobial activity. This next step will assess the potential of encapsulation to improve performance stability, reduce required application concentrations, and support longer-lasting postharvest mold control.

Table 1. Average inhibition zones (cm) of antimicrobial agents measured by disc diffusion assay with three antimicrobial agents showing most promising antimicrobial effect.

Concentrations	**Control**	Sporocide®	**H_2O_2**	**Eugenol**
0.5%	0 ± 0^{Aa}	0.87 ± 0.15^{Ba}	1.10 ± 0.06^{Ca}	-
1.0%	0 ± 0^{Aa}	1.27 ± 0.08^{Bb}	1.47 ± 0.12^{Cb}	0.82 ± 0.42^{Ba}
2.0%	0 ± 0^{Aa}	1.73 ± 0.31^{Cc}	1.62 ± 0.10^{Cb}	0.85 ± 0.10^{Ba}
5.0%	0 ± 0^{Aa}	2.12 ± 0.13^{Cc}	2.10 ± 0.28^{Cc}	1.47 ± 0.10^{Bb}
10.0%	0 ± 0^{Aa}	-	-	2.37 ± 0.52^{Bc}

[1] Different uppercase letters indicate a significant difference between different types of antimicrobial agents.
[2] Different lowercase letters indicate significant difference between different concentrations of the same antimicrobial agent.

Encapsulation strategies are being actively developed to enhance the delivery, stability, and performance of the selected antifungal agents. In this project, inactivated yeast cells were used as the encapsulation matrix due to their bio-based, edible, and food-compatible properties. Heat treatment was applied to fully inactivate the yeast cells and release intracellular contents, thereby creating internal void spaces within the cell structure that enable antimicrobial loading.

As an initial proof of concept, inactivated yeast cells were soaked in a 10% eugenol solution and subjected to vacuum treatment, resulting in successful encapsulation of eugenols within the yeast cells. Ongoing work will focus on optimizing encapsulation parameters to quantify and improve encapsulation efficiency. The antimicrobial performance of the encapsulated formulations will be evaluated against *P. expansum* and *Listeria innocua*, with *L. innocua* used as a surrogate for *Listeria monocytogenes* to enable rapid screening of encapsulation conditions due to its faster growth rate compared with *P. expansum*.

Proposal Title: Optimizing the lifespan of chlorinated dump tank water via modeling

Report Type: Continuing Project Report

Primary PI: Meijun Zhu
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Cooperators: Multiple apple packing facilities in Wenatchee and Yakima areas

Project Duration: 3 Year

Total Project Request for Year 1 Funding: $114,210

Total Project Request for Year 2 Funding: $96,592

Total Project Request for Year 3 Funding: $99,057

WTFRC Collaborative Costs:

Item	2024-2025	2025-2026	2026-2027
Salaries			
Benefits			
Wages	$3,050.00	$3,141.00	$3,235.00
Benefits	$1,220.00	$1,256.00	$1,294.00
RCA Room Rental	$2,500.00	$2,750.00	$3,000.00
Shipping			
Supplies			
Travel	$350.00	$350.00	$350.00
Plot Fees			
Miscellaneous			
Total	$7,120.00	$7,497.00	$7,879.00

Budget 1

Primary PI: Meijun Zhu
Organization Name: Washington State University
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Item	2024-2025	2025-2026	2026-2027
Salaries	$40,708.00	$42,336.00	$44,030.00
Benefits	$9,382.00	$9,759.00	$10,148.00
Wages			
Benefits			
RCA Room Rental			
Shipping			
Supplies	$29,000.00	$29,000.00	$29,000.00
Travel	$3,000.00	$3,000.00	$3,000.00
Plot Fees			
Miscellaneous	$5,000.00	$5,000.00	$5,000.00
Miscellaneous	$20,000.00		
Total	$107,090.00	$89,095.00	$91,178.00

OBJECTIVES

1. Develop mathematical models to assess the impact of critical processing parameters on chlorine efficacy in controlling *Listeria* during dump tank practices.
2. Validate the developed model with water collected from commercial dump tanks.
3. Monitor the dynamic changes in physicochemical and microbial parameters of processing water in commercial dump tanks.

SIGNIFICANT FINDINGS

1. Free chlorine (FC) positively correlates with *L. innocua* reduction on apples. Higher FC levels also effectively reduce transfer to clean apples and residual levels in water.
2. High chemical oxygen demand (COD) negatively impacts chlorine's ability to reduce bacteria on inoculated apples. Conversely, increased COD levels lead to higher bacterial transfer to clean apples and greater residual contamination in the water.
3. Higher initial contamination levels on apples decrease the overall effectiveness of chlorine.
4. During a 2-minute wash, bacterial transfer to uninoculated apples ranged from 0.83 to 4.00 log CFU/apple, depending on chlorine and COD levels.
5. Response Surface Methodology (RSM) models incorporating FC, inoculation level, and COD as key predictive variables were developed to predict chlorine efficacy in apple dump tank systems.
6. RSM models developed showed high accuracy, with R^2 values of 0.91–0.98, in predicting *Listeria* survival and transfer.
7. The models were further validated using water from real Washington apple packing facilities, confirming their reliability under industrial conditions.

METHODS

Part I. Develop mathematical models for *Listeria* inactivation during dump tank operations

1. Parameter estimation from pre-test survey

A survey on dump tank operation within the Washington apple industry was conducted to gather industry-specific data on critical processing parameters, which guided the selection of operational parameters for our models.

2. Dump tank water simulation

The dump tank water was simulated using decayed apples, fresh squeezed apple juices, and soil from the apple orchards in the central Washington in tap water. The chemical oxygen demand (COD) and various physicochemical parameters, including turbidity, conductivity, total suspension solid (TSS), and pH of the dump tank water, were measured and standardized.

3. Analysis of water physicochemical parameters

The COD of the resulting simulated dump tank water (SDTW) with the Hach method 8000 [1] in a COD reactor (DRB-200, Hach). The pH, oxidation-reduction potential (ORP) and conductivity were measured using Orin Versa Star Pro™ pH/ISE multiparameter meter (Thermo Fisher Scientific). Water turbidity was measured using DR/900 multiparameter handheld colorimeters (Hach Company, Loveland, CO). TSS of water samples was analyzed following Hach Method 8158 [2]. Water conductivity and total dissolved solids (TDS) were determined using an Orion Star A212 conductivity meter (Thermo Scientific, Waltham, MA). Water temperatures were recorded with a Model.9847N digital thermometer (Taylor Water Technologies LLC). The hardness and free chlorine level of water was measured with Lamotte Insta-Test 4 strip.

4. Pilot dump tank

Controlled *Listeria* interventions were carried out in a pilot dump tank (44.5″ L × 20.5″ W × 15.5″ H) equipped with a vacuum pump (~ 1 horsepower) (Fig. 1). At the beginning of each experiment, 102 L of SDTW at room temperature (RT, 22 ± 2 °C) was added to the tank and recirculated at 60 L/min.

Fig. 1. An overview of the pilot dump tank used for testing.

5. Strain selection

A panel of *Listeria innocua* strains, including NRRL B-33197, NRRL B-33314, and NRRL B-33554, was used to prepare a 3-strain cocktail inoculum. These strains were kept in a stock solution of trypticase soy broth supplemented with 0.6% (w/v) yeast extract (TSBYE) and 20% *(v/v)* glycerol at -80°C until use.

6. Apple cultivar selection

Granny Smith apples were used in the pilot intervention studies.

7. Inoculum preparation

Before inoculation, each strain was growth-phase-synchronized twice in TSBYE broth by consecutively culturing at 37°C for 24h, then pelleted by centrifugation and re-suspended to achieve the target population density. To prepare a 3-strain *L. innocua* inoculum cocktail, each respective strain suspension was mixed in equal proportions.

8. Apple inoculation

Apples, free of cuts or bruises and harvested at commercial maturity, were individually inoculated with the 3-strain cocktail of *L. innocua* following our well-established method. The inoculated apples were held at 22 °C (72 °F) for 48h before being introduced to the chlorinated pilot dump tank under various conditions.

9. Pilot dump tank operation

To assess the apple-to-apple cross-contamination scenario, 10 inoculated apples were introduced together with uninoculated apples into chlorinated SDTW for 2 min. For the water-to-apple cross-contamination scenario, 50 uninoculated apples were introduced into chlorinated SDTW spiked with ~ 10^4 - 10^6 CFU/ml of *L. innocua* for 2 min. Spent water samples were collected in triplicates immediately following the 2 min treatments and neutralized with neutralizing buffer. Additionally, a separate set of water samples was collected for turbidity, pH, ORP, conductivity, and FC measurement per our established method.

10. Survival microorganism analysis

Upon completion of each intervention, each apple was individually transferred to a Whirl-Pak bag with 10 mL of neutralizing buffer. Subsequently, apples were hand-rubbed for 80 s to detach microorganisms from apple surfaces. The resulting microbial suspensions were serially diluted, and appropriate dilutions were plated on TSAYE plates per our established method.

Residual populations of *L. innocua* in spent water were determined using both direct plating and membrane filtration methods. The filter membrane was then placed onto CHROMagar™ *Listeria* plate. All plates were incubated at 37 ± 2 °C for 48 h. If survival/count of *Listeria* on apple fruit/water was below the enumerative detection limit, the suspension was tested 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.

11. Chlorine intervention experimental design

Experiments utilized the Box-Behnken design (BBD) [3], with three independent variables: free chlorine (FC) concentrations (ppm, X1), COD (ppm, X2), and the inoculation level of *L. innocua* on apple or in water (log CFU/apple or log CFU/mL, X3), with each variable evaluated at three levels. FC concentrations of 25, 50, and 100 ppm were selected based on commercial practice. Inoculation levels of apples were set at 10^4, 10^5, and 10^6 CFU/apple. COD levels of 250, 1000, and 4000 ppm were chosen to represent a broad range of organic load found in apple process water. FC concentrations, bacterial counts, and COD were transformed into $\log_{10}$ form, and the treatment combinations of BBD design are summarized in Table 1.

Table 1. Treatment layout

Treatment order	X_1	X_2	X_3	FC (ppm)	Inoculation level ($\log_{10}$ CFU/apple)	COD (ppm)
1	-1	-1	0	25	4	1000
2	1	-1	0	100	4	1000
3	-1	1	0	25	6	1000
4	1	1	0	100	6	1000
5	-1	0	-1	25	5	250
6	1	0	-1	25	5	250
7	-1	0	1	25	5	4000
8	1	0	1	100	5	4000
9	0	-1	-1	50	4	250
10	0	1	-1	50	6	250
11	0	-1	1	50	4	4000
12	0	1	1	50	6	4000
13	0	0	0	50	5	1000
14	0	0	0	50	5	1000
15	0	0	0	50	5	1000

12. Mathematical modeling development

Response surface methodology (RSM) models were developed using the *rsm* package (version 2.10.4) in R (version 4.1.0), applying second-order polynomial regression equations. Model adequacy was assessed by calculating the coefficient of determination (R^2). Visualization of response surface were generated using the *rsm* package (version 3.4.2) [4]. Significance of model terms (main effects, interaction terms, and quadratic terms) were evaluated, and regression coefficients were annotated using the following significance codes: ***, $p \leq 0.001$; **, $p \leq 0.01$; *, $p < 0.05$.

13. Statistical analysis

All data were analyzed using one-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test using IBM SPSS version 20.0 (Chicago, IL).

Part II. Validation of developed model using dump tank water from commercial facilities

1. Commercial dump tank water sampling

To evaluate the predictive performance of the developed models, the dump tank water was collected at the end of its operational cycle from four commercial apple packing facilities in Washington State. The collected water was transported to the Food Microbiology lab for *Listeria* intervention testing using the pilot dump tank.

2. *Chlorine efficacy in commercial dump tank water:*

Chlorine interventions were conducted following the same BBD as described in Part I, using the pilot tank and processing water diverted from the commercial tank. Treatments were performed under varying COD levels and FC concentrations. Both apple-to-apple and water-to-apple cross-contamination scenarios were evaluated as described in Part I.

3. *Survival bacterial analysis*

Apple inoculation and the survival of *Listeria* on apples were analyzed as described above.

4. *Validation of the developed model*

Microbial reduction data were used to evaluate the goodness-of-fit of the RSMs developed in Objectives 1. Model predictions were compared with observed microbial counts to assess the accuracy and predictive capability of the models under commercial water conditions.

RESULTS AND DISCUSSION

1. *Commercial apple dump tank operational parameters*

A pre-test survey of commercial apple dump tank practices showed that ~72% of apple packing facilities used municipal water, with water hardness ranged from 0 to 450 ppm, with a mean of 116.6 ppm. The contact time of apples in dump tanks varied from 2 to 7 min (Fig. 2A). The temperature of dump tank water also differed among apple varieties and packing facilities, ranging from 58 °F (14.4 °C) to 100 °F (37.8 °C) (Fig. 2B). Our preliminary data indicated that dump tank water temperatures between 10 and 32 °C (50-90 °F) and contact times between 2 and 7 min had minimal impact on chlorine efficacy. Therefore, the municipal water was used to simulate dump tank water, while temperature and contact time were excluded as dependent variables in the model assessment. A standardized contact time of 2 min and water temperature of 22 °C (72 °F) were used for all model experiments.

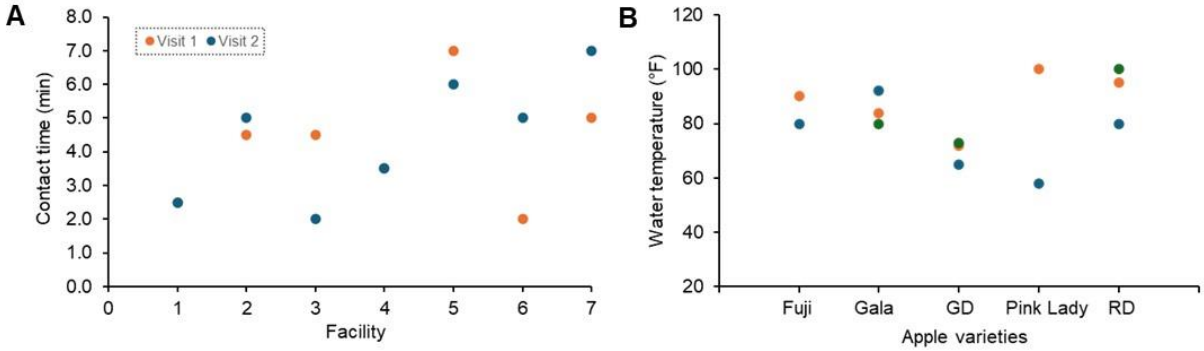

Fig. 2. Operational parameters of apple dump tank based on survey. (A) Contact time (min) across seven facilities during two separate visits. and (B) Water temperature range (°F) recorded for different apple varieties. Colored markers represent temperatures recorded during different observations. GD: Golden Delicious; RD: Red Delicious.

2. *Effectiveness of chlorine in preventing apple-to-apple L. innocua cross-contamination in SDTW*

During the 2-min chlorinated pilot dump tank process, *L. innocua* reduction on inoculated apples ranged from 0.45 log CFU/apple (at 25 ppm FC, 1000 ppm COD, and a 6-log IL) to 1.71 log CFU/apple (at 100 ppm FC, 250 ppm COD, and a 5-log IL). Concurrently, cross-contamination resulted in *L. innocua* transfer ranging from 0.83 log CFU/apple (at 100 ppm FC, 1000 ppm COD, and a 4-log IL) to 4.00 log CFU/apple (at 25 ppm FC, 1000 ppm COD, and a 5-log IL) to uninoculated apples. Residual *L. innocua* populations in the spent SDTW varied from <1 CFU/mL (at 100 ppm FC–1000 ppm COD–4-log IL and 100 ppm FC–250 ppm COD–5-log IL) to 2.78 log CFU/mL (at 25 ppm FC–4000 ppm COD–5-log IL) across all treatments. The regression coefficients for the developed RSM models are presented in Table 2 for the apple-to-apple cross-contamination scenario.

Table 2. Regression coefficients of RSM models predicting *L. innocua* responses in the apple-to-apple cross-contamination scenario.

Coefficients	Apple-to-apple cross-contamination scenario		
	Y_1 (Reduction on IA)	Y_2 (Transfer to UA)	Y_3 (Residual *Li* in water)
Intercept	1.10000**	1.62000**	0.48000
X_1 (FC)	0.27625*	-0.72750**	-0.64500*
X_2 (IL)	-0.18750*	0.24625*	0.23875
X_3 (COD)	-0.21375*	0.41875*	0.51875*
X_1X_2	-0.02750	-0.07750	-0.08000
X_1X_3	0.14500	-0.41250*	-0.67500*
X_2X_3	0.04250	0.21000	0.07250
X_1^2	0.00250	0.10750	0.01625
X_2^2	-0.29500	-0.18500	0.11875
X_3^2	0.18750	0.61000*	0.21875
R^2	0.96	0.98	0.95
$R^2_{adjusted}$	0.84	0.93	0.79
F-value	8.14	19.77	5.96
p-value	0.056	0.016	0.085

IA: inoculated apples; UA: uninoculated apples. *Li: L. innocua*. **, $p < 0.01$; *, $p < 0.05$. R^2: coefficient of determination.

3. Impact of independent variables on responses in the apple-to-apple cross-contamination

The impacts of FC, IL, and COD on the reduction of *L. innocua* on inoculated apples (Y_1) are illustrated in the response surface and contour plots (Fig. 3AB). FC (X_1) showed a positive correlation ($p < 0.05$) with *L. innocua* reduction on inoculated apples, whereas IL (X_2) and COD (X_3) displayed negative correlations with Y_1 ($p < 0.05$) (Fig. 3AB). The regression model for Y_1 performed well, with an R^2 of 0.96 and an adjusted R^2 of 0.84.

FC also exhibited a negative correlation ($p < 0.01$) with both *L. innocua* transfer to uninoculated apples (Y_2) (Fig. 3CD) and residual *L. innocua* levels in spent SDTW (Y_3) (Fig. 3EF). Conversely, IL and COD showed positive correlations with both Y_2 and Y_3, as depicted in the response surface and contour plots (Fig. 3C–F).

4. Impact of independent variables on responses in the water-to-apple cross-contamination

In the water-to-apple scenario, only FC (X_1) was negatively correlated ($p < 0.05$) with *L. innocua* level in spent water (Y_4) and its transfer to uninoculated apple (Y_5) (Fig. 4). Additionally, IL (X_2) exhibited a significant quadratic effect on Y_5, indicating a nonlinear relationship between IL and the extent of microbial transfer to apples (Fig. 4). The regression models developed for Y_4 and Y_5 demonstrated strong predictive performance, with high R^2 values (0.91 - 0.95).

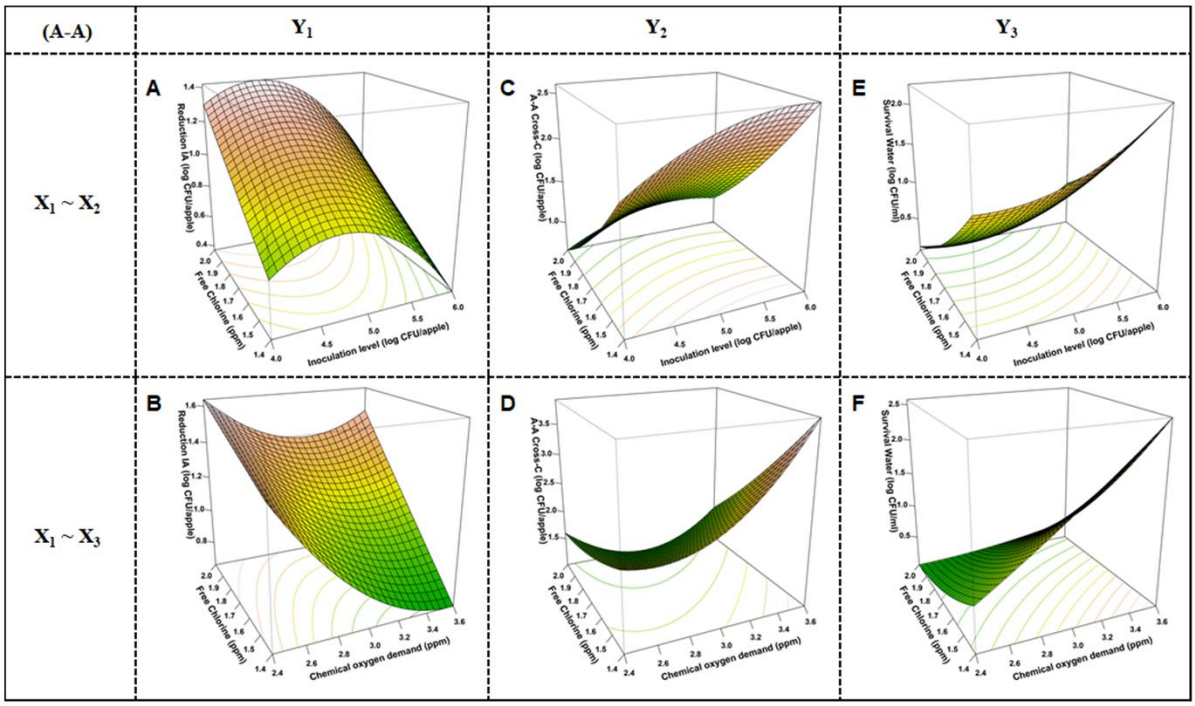

Fig. 3. Response surface and contour plots showing the effect of key variables on *L. innocua* response in the apple-to-apple cross-contamination scenario. (A, C, E) Interaction between FC (X_1) and inoculation level (X_2) at COD (X_3) = 1000 ppm. (B, D, F) Interaction between FC and COD at IL = 5 log CFU/apple. (AB) Y_1, reduction on inoculated apples. (CD) Y_2, transfer to uninoculated apples. (EF) Y_3, levels in spent water. Red and green denote higher and lower response levels, respectively.

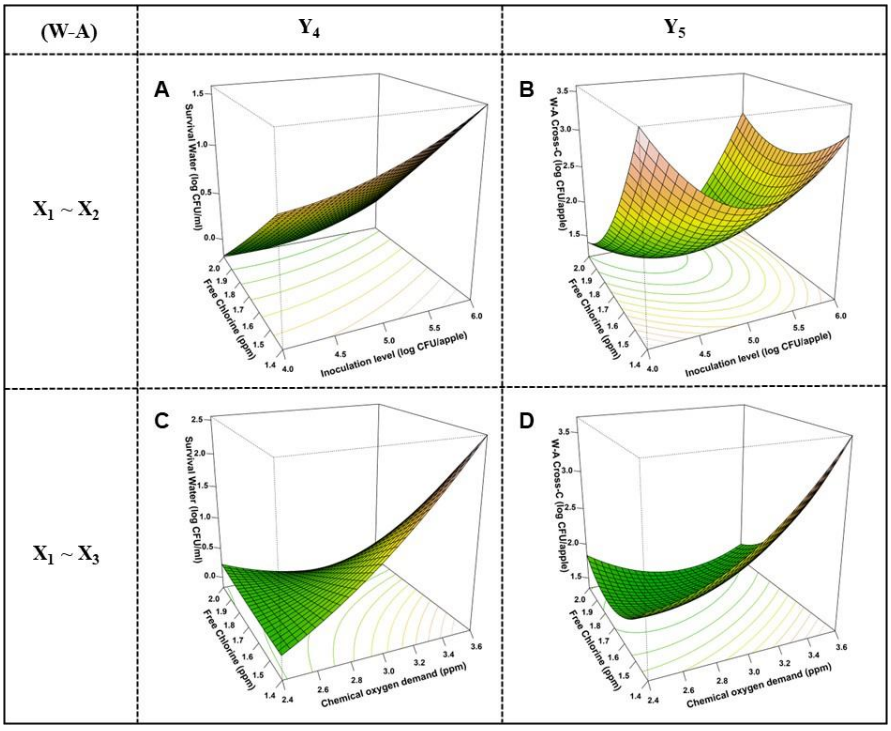

Fig. 4. Response surface and contour plots showing effects of key variables on *L. innocua* responses in the water-to-apple cross-contamination scenario. (A–B) Interaction between FC (X_1, ppm) and inoculation level (IL, X_2, log CFU/apple) at COD (X_3) = 1000 ppm. (C–D) Interaction between FC and COD at IL = 5 log CFU/apple. (A, C) Y_4: survival in spent water; (B, D) Y_5: transfer from water to uninoculated apples. Red and green denote higher and lower response levels, respectively.

5. *Response surface model predictive equations*

The RSM model equations developed to predict *L. innocua* survival outcomes in both apple-to-apple and apple-to-water cross-contamination were summarized in Table 3.

Table 3. RSM equations predicting responses in apple-to-apple and water-to-apple cross-contamination

Responses	Model equation	R^2
Apple-to-apple cross contamination		
Y1	$Y_1 = 1.1 + 0.27625X_1 - 0.1875X_2 - 0.21375X_3 - 0.0275X_1X_2 + 0.145X_1X_3 + 0.0425X_2X_3 + 0.0025X_1^2 - 0.285X_2^2 + 0.1875X_3^2$	0.96
Y2	$Y_2 = 1.62 - 0.7275X_1 + 0.24625X_2 + 0.41875X_3 - 0.0775X_1X_2 - 0.4125X_1X_3 + 0.21X_2X_3 + 0.1075X_1^2 - 0.185X_2^2 + 0.61X_3^2$	0.98
Y3	$Y_3 = 0.48 - 0.645X_1 + 0.23875X_2 + 0.51875X_3 - 0.08X_1X_2 - 0.675X_1X_3 + 0.0725X_2X_3 + 0.01625X_1^2 + 0.11875X_2^2 + 0.21875X_3^2$	0.95
Water-to-apple cross contamination		
Y4	$Y_4 = 1.58 - 0.59875X_1 + 0.31375X_2 + 0.33X_3 + 0.4875X_1X_2 - 0.45X_1X_3 + 0.085X_2X_3 + 0.38875X_1^2 + 0.83375X_2^2 + 0.34625X_3^2$	0.91
Y5	$Y_5 = 0.71 - 0.71875X_1 + 0.3525X_2 + 0.54625X_3 - 0.075X_1X_2 - 0.4025X_1X_3 + 0.19X_2X_3 + 0.13375X_1^2 + 0.25125X_2^2 + 0.05875X_3^2$	0.95

Y_1: reduction on inoculated apples (log CFU/apple). Y_2: transfer to uninoculated apples (log CFU/apple). Y_3: transfer to spent water (log CFU/ml). Y_4: survival in spent water (log CFU/ml). Y_5: transfer to uninoculated apples (log CFU/apple). X_1 = FC. X_2 = IL. X_3 = COD. R^2: model performance indicator.

6. *Validation of developed model using dump tank water from commercial facilities*

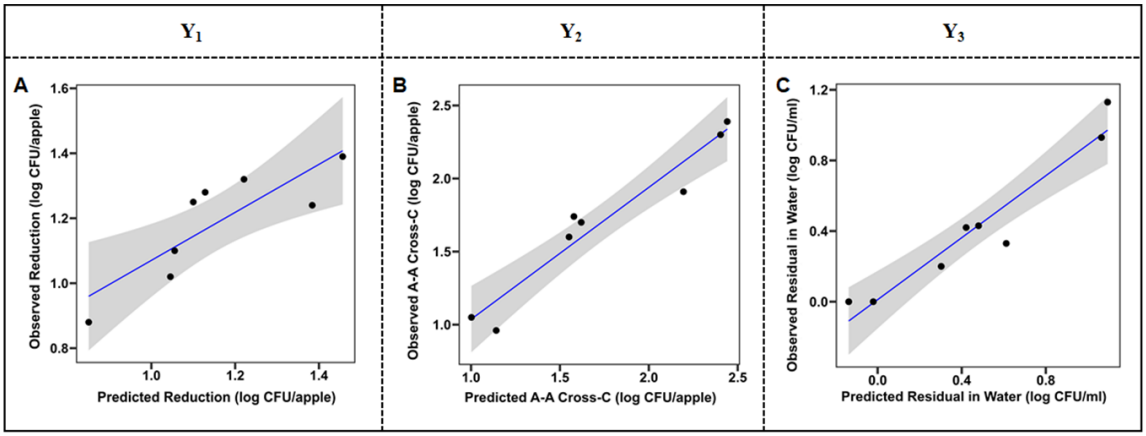

Fig. 5. Predicted versus observed responses in the apple-to-apple cross-contamination scenario using commercial dump tank water. (A) Y_1: reduction on inoculated apples (log CFU/apple); (B) Y_2: transfer to uninoculated apples (log CFU/apple); (C) Y_3: residual in spent water (log CFU/mL).

To evaluate the accuracy and robustness of the RSM models, predictive equations were validated using commercial dump tank water samples with COD levels ranging from 563 to 3287 ppm, collected from four Washington apple packing facilities. Observed responses (Y_1–Y_5) from these validation

experiments showed strong concordance with the corresponding model-predicted values, confirming the models' predictive reliability under commercial dump tank conditions. Predicted-versus-observed response plots for the apple-to-apple and water-to-apple cross-contamination scenarios are shown in Figs. 5 and 6, respectively.

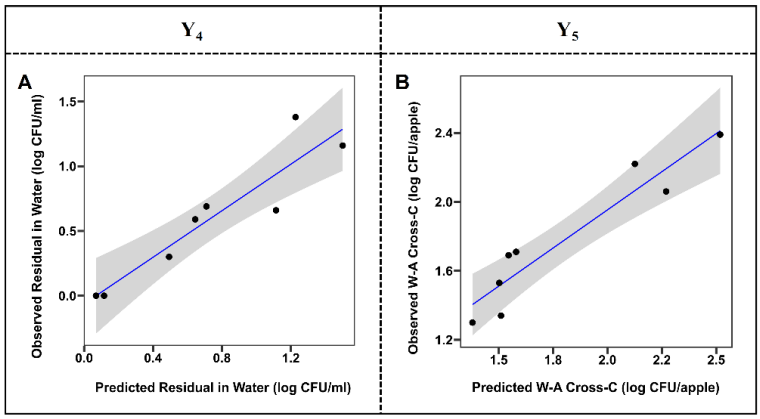

Fig. 6. Predicted versus observed responses in the water-to-apple cross-contamination scenario using commercial dump tank water. (A) Y_4: survival in spent water (log CFU/mL); (B) Y_5: transfer to uninoculated apples (log CFU/apple).

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Project Title: Evaluation of Environmental Monitoring Best Practices for Brush Beds

Report Type: Continuing Project Report – Y1

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Cooperators: Four apple packinghouses in Washington have agreed to cooperate for objective 2. The identities of the operations willing to serve in a cooperator role will be kept confidential.

Project Duration: 2 Year

Total Project Request for Year 1 Funding: $70,836
Total Project Request for Year 2 Funding: $67,604

Other funding sources: None

Primary PI: Claire Murphy
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Supervisor: Naidu Rayapati
Supervisor email address: 509-786-9215

Item	2025	2026
Salaries	$34,694	$36,081
Benefits	$9,696	$11,315
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies	$19,000	$9,500
Travel	$2,500	$1,500
Plot Fees		
Miscellaneous		
Total	**$65,890.0**	**$58,396.0**

Footnotes:
Salaries: requested in years 1 and 2 for an MS student to work on all objectives
Benefits: benefits tied to the MS assistantship
Supplies: $19,000 and $9,500 are requested in years 1 and 2, respectively, to purchase disposal supplies such as pipette tips, swabs, rapid test, microbiological media, Petri dishes, PCR reagents, etc.
Travel: requested for travel related to sample collection and extension activities

Budget 2
CO-PI: Suzette Galinato
Organization: Washington State University
Contract Administrator: Brianna Wagner
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Contract administrator email address: brianna.wagner@wsu.edu
Supervisor: Karen Lewis
Supervisor email address: kmlewis@wsu.edu

Item	2025	2026
Salaries	$3,946	$8,208
Benefits	$1,258	$2,616
Wages		
Benefits		
RCA Room Rental		
Shipping		
Supplies		
Travel	$1,000	$1,000
Plot Fees		
Miscellaneous		
Total	**$6,204.00**	**$11,824.00**

Footnotes:
Salaries: requested in years 1 and 2 for work on objective 3. Wages and benefits are calculated at a yearly increase rate of 4%.
Benefits: Benefits tied to salary
Travel: requested for travel related to sample collection and extension activities

Objectives

1. **Objective 1**: Conduct laboratory experiments on clean and fouled (e.g., wax, background microflora) brush rollers to validate the sensitivity (true positive) and specificity (true negatives) of environmental monitoring swab methods processed using standard FDA-BAM (Food and Drug Administration's Bacteriological Analytical Manual) methods and two rapid tests for detecting different levels of *L. monocytogenes*.
2. **Objective 2**: Implement the methods from Objective 1 in commercial apple packinghouses to evaluate their effectiveness in real-world conditions for *Listeria* spp.
3. **Objective 3:** Perform a cost-benefit analysis for each method, considering equipment, consumables, in-house labor, third-party costs, and testing sensitivity.
4. **Objective 4**: Develop educational materials, including videos and factsheets on environmental monitoring program best practices (e.g., how to collect environmental samples and the pros and cons of incorporating rapid tests).

Significant Findings

Objective 1:

- FDA-BAM, the current standard for *Listeria* spp. detection during environmental monitoring in produce packinghouses, is highly reliable, showing high sensitivity (83–100%) and low false negative rates (0–16.7%) across all brush types and inoculum levels.
- Rapid tests show mixed performance:
    - Visual Rapid Tests perform well at high inoculum but had substantially lower sensitivity at low inoculum, especially on nylon brushes.
    - Antigen-based Rapid Tests consistently had low sensitivity (50%) and are not recommended for routine monitoring due to high false negative risk and limited field usability.
- All methods are highly specific for negative samples (*Bacillus cereus*), with false positive rates of 0% and specificity of 100%, indicating reliable identification of non-*Listeria* bacteria.

Methods

Due to administrative and manufacturing/shipping delays in sourcing brushes, work on Objective 1 is ongoing and is expected to be completed by the end of February 2026. Objective 2 is anticipated to be completed by May 2026, with Objectives 3 and 4 planned for May through December 2026.

Objective 1 (Year 1):

Sample preparation: Nylon (100%) and 50% horsehair/50% polyethylene brushes were purchased new and used clean and artificially fouled (e.g., wax, background microflora).

Pathogen Inoculation: A three-strain cocktail of *Listeria monocytogenes* was inoculated onto brushes at 2 (low) and 4 (high) log CFU/brush to serve as positive controls for evaluating sensitivity and false negative rates. *Bacillus cereus* was used as a negative control because, like *Listeria* spp., it is a Gram-positive, rod-shaped bacterium capable of persisting on surfaces, providing a realistic challenge for detection assays and allowing evaluation of assay specificity. *Bacillus cereus* was also inoculated onto brushes at 2 (low) and 4 (high) log CFU/brush. Additionally, a set of non-inoculated brushes was included as controls.

Swabbing and analysis: Environmental monitoring procedures were used to sample brush surfaces for the presence or absence of *Listeria* spp. on brushes inoculated with *L. monocytogenes*, *Bacillus cereus*, and non-inoculated controls. Three methods were evaluated: the standard FDA BAM method, commonly used by third-party laboratories; a visual rapid test (Hygiena InSite® *Listeria* spp. Rapid

Test); and an antigen-based rapid test (Romer Labs RapidChek *Listeria* spp. Next Day). All swabs were processed according to FDA or manufacturer instructions and confirmed by PCR targeting the *sigB* gene.

Data Analysis: True positive (TP), true negative (TN), false positive (FP), and false negative (FN) results were analyzed across different brush types, surface conditions, bacteria, and inoculum levels to assess the accuracy, reliability, and performance of each test. Sensitivity (ability to correctly identify positives) and specificity (ability to correctly identify negatives), as well as false positive and false negative rates, were calculated to directly compare each rapid test to the standard FDA BAM method.

Objective 2 (Year 2):
Sampling and Processing: Swab samples will be collected from brush rollers (zone 1) as well as from adjacent areas (zones 2–3) in commercial packinghouses using the environmental monitoring methods established in Objective 1. Samples will be transported to the Murphy Lab for analysis to determine *Listeria* spp. prevalence, processed according to FDA or manufacturer instructions, and confirmed by PCR targeting the *sigB* gene.

Evaluation and Analysis: The performance of each method will be assessed based on its ability to detect *Listeria* spp., with particular focus on comparing the sensitivity and specificity of the standard FDA-BAM method versus the rapid tests.

Objective 3 (Year 2):
Economic Feasibility Analysis: We will conduct a cost-benefit analysis to assess the economic feasibility of implementing in-house testing methods into daily operations. This task will leverage the data collected in Objectives 1 and 2 to calculate the net economic benefits of the methods, taking into account any upfront investment and operating costs, and potential benefits, such as cost savings in terms of reduced expenses for sending samples to a testing company and quicker response to initiate a corrective action. The findings will be compared to current procedures (baseline) to determine whether adopting alternative methods is more cost-effective than traditional practices.

Objective 4 (Year 2):
Development and Distribution: Educational materials will be developed based on the findings from previous objectives and existing EMP best practices. This will include instructional videos and concise factsheets covering topics such as swabbing procedures, the application of rapid tests in commercial packinghouses, guides on sample handling, testing methods, and implementation tips. These materials will be distributed through channels such as WSU Extension, the Food Safety Clearinghouse, PD Murphy's email list, industry events (e.g., Food Safety Coffee Group), and partners (e.g., WSTFA).

Results and Discussion:

Previous research on the prevalence of *Listeria* spp. (an indicator organism for *L. monocytogenes*) on food contact surfaces (i.e., zone 1) in apple packinghouses has identified the highest contamination levels on polishing brushes, dryer rollers, and brush rollers under fans *(1)*. Additionally, studies on non-food contact surfaces (i.e., zones 2-3) in apple packinghouses have found *Listeria* spp. and *L. monocytogenes* at elevated prevalences around and under brush beds *(2, 3)*. Brush rollers, in particular, are common harborage sites for *Listeria* spp. due to their complex hygienic design, which retains organic matter, moisture, and nutrients, making them difficult to clean and sanitize. While past and ongoing research has focused on improving cleaning and sanitation practices for brushes *(4, 5)*, there is also a critical need to understand environmental monitoring

program (EMP) options and best practices to ensure these cleaning procedures are truly effective. An EMP is used to detect and manage contamination risks, mainly microbial, in food production environments. The goal of an EMP is to monitor surfaces to identify potential sources of contamination and/or verify the effectiveness of cleaning and sanitization programs. The results of EMP testing help facilities pinpoint high-risk areas, or "hot spots," where contamination is more likely, enabling them to implement corrective actions such as enhanced cleaning, sanitation, or procedural changes. An effective EMP in a packinghouse is essential for mitigating risks and improving the overall safety of fresh produce.

However, EMPs can be costly due to laboratory testing, which is why there is growing interest in rapid, in-house testing methods that can supplement sending samples to external labs. If proven to be both sensitive and specific, these in-house tests can provide quick results, integrate seamlessly into daily operations, and make the process more cost-effective. A recent survey of EMP practices in fresh produce packinghouses, including 27.2% of respondents who packed tree fruit, found that 64.5% of packinghouses had an EMP in place (6). Those without an EMP cited the lack of regulatory or buyer requirements or the costs associated with implementing one, as reasons they did not have (6). These findings suggest that while many packinghouses are adopting EMPs, there is still significant variation in implementation practices across the industry.

Data to date for the current research study found that the FDA-BAM method, the current gold standard for *Listeria* spp. detection for environmental monitoring and the method used by third-party laboratories, consistently demonstrated high reliability across all brush types and inoculum levels in laboratory studies, with sensitivity (percentage of the test to correctly identify positives) ranging from 83.3–100.0% and false negative rates (percentage of positives incorrectly identified as negative) ranging from 0.00 – 16.7% (Table 1). This performance provides a robust benchmark for evaluating alternative environmental monitoring methods. In comparison, the visual rapid test (InSite® *Listeria spp*. rapid test) performed well at high inoculum levels, achieving 100% sensitivity on both brush types, but the test reliability decreased at low inoculum, particularly on nylon brushes, where sensitivity dropped to 16.7%. The antigen-based Rapid Test (RapidChek *Listeria* spp. Nextday) showed limited sensitivity across all conditions (50.0%), indicating a higher risk of false negatives. In packinghouse settings, *Listeria* spp., if present, would usually be present at low levels. Based on these findings, the FDA-BAM is recommended for routine monitoring, while rapid tests may provide preliminary screening in controlled settings; antigen-based rapid tests are not recommended due to low sensitivity and limited field usability.

These results are broadly consistent with findings from a recent study in two ready-to-eat meat facilities in Japan *(7)*. In a recent study that examined alternative methods of environmental monitoring for *Listeria* spp. from 72 samples collected from two ready-to-eat meat production facilities of in Japan, found that 22 (30.6%) and 29 (40.3%) were positive for *L. monocytogenes* and *Listeria* spp., respectively, in the culture-based method (modified FDA-BAM), 16 (22.2%) and 28 (38.9%) with rapid molecular detection system, and 7 (9.7%) and 26 (36.1%) with a visual rapid test (InSite® *L.mono glow*. rapid test – *NOT the same InSite test used in the present study*; *7*). When comparing to the culture based methods, is was found that the molecular detection system showed higher sensitivity (87.5% for *L. monocytogenes*, 85.7% for *Listeria* spp.) and specificity (85.7% and 88.6%, respectively), than the visual rapid test, with a sensitivity for *L. monocytogenes* and *Listeria* spp. of 28.6% and 73.1%, and specificities of 69.2% and 78.3%, respectively *(7)*. While currently data on the utility of *Listeria* spp. rapid test is sparce, both studies demonstrate that culture based (i.e., FDA-BAM) remains the most reliable method for accurate detection of *Listeria* spp., while rapid tests are less sensitive and may miss low-level contamination, limiting their usefulness for routine environmental monitoring.

FDA-BAM, Visual Rapid Tests, and Antigen Rapid Tests all demonstrated excellent specificity for *Bacillus cereus* (used here as a surrogate for non-*Listeria* spp. bacteria because, like *Listeria* spp., it is a Gram-positive, rod-shaped bacterium capable of persisting on surfaces) across all brush types and inoculum levels, with false positive rates of 0.00% and specificity of 100.0% (Table

2). This indicates that all three methods accurately identified negative samples and did not produce false positives under laboratory conditions. While this confirms that the tests are highly specific, it is important to note that antigen-based rapid tests still suffer from low sensitivity for *Listeria* spp. detection and are not practical for in-field use. Similar to the results of this study, Shimojima et al. (7) found that while false-negative samples common, but false-positive samples were rarely found.

Table 1: False negative rate (FNR)[a] and sensitivity[b] of *Listeria monocytogenes* using three environmental monitoring methods for *Listeria spp.* on brushes during laboratory studies

Brush Type	Test Type	FNR	Sensitivity
High inoculation (4 log CFU/brush)			
50% horsehair/50% polyethylene			
	FDA-BAM	0.0%	100.0%
	Visual Rapid Test	0.0%	100.0%
	Antigen Rapid Test	50.0%	50.0%
100% Nylon			
	FDA-BAM	16.7%	83.3%
	Visual Rapid Test	0.0%	100.0%
	Antigen Rapid Test	50.0%	50.0%
Low inoculation (2 log CFU/brush)			
50% horsehair/50% polyethylene			
	FDA-BAM	0.0%	100.0%
	Visual Rapid Test	33.3%	66.7%
	Antigen Rapid Test	50.0%	50.0%
100% Nylon			
	FDA-BAM	0.0%	100.0%
	Visual Rapid Test	83.3%	16.7%
	Antigen Rapid Test	50.0%	50.0%

[a] False negative rate (FNR): proportion of positives incorrectly identified as negative
[b] Sensitivity: Ability of the test to correctly identify positives

Table 2: False positive rate (FPR)[a] and specificity[b] of *Bacillus cereus* using three environmental monitoring methods for *Listeria spp* on brushes during laboratory studies

Brush Type	Test Type	FPR	Specificity
High inoculation (4 log CFU/brush)			
50% horsehair/50% polyethylene			
	FDA-BAM	0.0%	100.0%
	Visual Rapid Test	0.0%	100.0%
	Antigen Rapid Test	0.0%	100.0%
100% Nylon			
	FDA-BAM	0.0%	100.0%
	Visual Rapid Test	0.0%	100.0%
	Antigen Rapid Test	0.0%	100.0%
Low inoculation (2 log CFU/brush)			
50% horsehair/50% polyethylene			
	FDA-BAM	0.0%	100.0%
	Visual Rapid Test	0.0%	100.0%
	Antigen Rapid Test	0.0%	100.0%
100% Nylon			
	FDA-BAM	0.0%	100.0%
	Visual Rapid Test	0.0%	100.0%
	Antigen Rapid Test	0.0%	100.0%

[a] False positive rate (FPR): proportion of negatives incorrectly identified as positive
[b] Specificity: Ability of the test to correctly identify negatives

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