2013 Apple/Apple Crop Protection Research Review

01/29/13

			APPLE COMMITTEE	
Time	**Page**	**PI**	**Project Title**	**Yrs**
8:00		Schmidt	Welcome and introduction	
			Final Reports	
8:15	1	Mattheis	Fruit metabolic responses to controlled atmosphere O_2 and CO_2 stress	3
8:30		Rudell	SCRI update: Biomarkers for post harvest disorders	
8:45	9	Zhu	Genetic controls of apple fruit-specific auxin metabolism: ***Extension***	3
9:00	20	Killinger	Validation of food safety interventions: ***Extension***	3
9:15	31	Ross	Sensory & consumer acceptance of advanced apple breeding selections	3
9:30	43	Peace	Increasing decision confidence in cultivar development	1
9:45	56	Main	Development of an online toolbox for tree fruit breeding	1
10:00	57	Dhingra	Technology platform for fast breeding	1
	58	Dhingra	Breeding in the 21st century: technology platform for fast breeding	
3:30			**Continuing projects 3:30 - 5:30**	
1	69	Evans	Apple scion breeding	3
1	77	Evans	WA2 plant variety rights applications	3
1	79	Auvil	Apple rootstock and scion evaluation	
1	85	Mazzola	Greater system efficiency and fruit quality via soil microbiology: ***Extension***	3
2	92	Rudell	Testing biomarker-based tools for scald risk assessment during storage	3
2	99	Killinger	Enhancing apple packing HACCP programs while ensuring fruit quality	3
2	107	Killinger	Overhead cooling influences on microbial food safety	1
2	114	Bruce	Apple microbial risk factors	3
3	120	Hanrahan	Programs to increase packouts of apples	
3	126	Peck	Implementation/evaluation of apple pollen tube growth models	3
3	134	Salazar	Development of apple bloom phenology and fruit growth models	3
3	142	Castillo	Crop load and canopy management of apple	
4	149	Karkee	3D machine vision for improved apple crop load estimation[1] ***Extension***	2
4	156	Karkee	Design and development of apple harvesting techniques[1]	1

FINAL PROJECT REPORT

Project Title: Fruit metabolic responses to controlled atmosphere O_2 and CO_2 stress

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Michael Young, Glade Brosi, Stemilt Growers

Other funding sources

Agency Name: USDA-NIFA (SCRI)
Amount awarded (Federal + non-Federal): $2.4 million
Notes: D. Rudell is Project Director, J. Mattheis is a Co-PI. The Standard Research and Extension Project, "A diagnostic toolbox for integrated management of postharvest apple necrotic disorders" was submitted (01/12/10) for the current funding cycle. WTFRC and AgroFresh, Inc. are co-sponsors.

Total Project Funding:

Budget History:

Item	2010	2011	2012
Salaries	$43,278	$44,176	$45,093
Benefits	$18,457	$18,933	$19,326
Wages			
Benefits			
Equipment			
Supplies	$2400	$2400	$2400
Travel			
Plot Fees			
Miscellaneous			
Total	$64,225	$65,509	$66,819

Objectives:

1. Identify volatile compounds that accumulate during CA storage.
2. Characterize volatile compound dynamics during storage in atmospheres that induce low O_2 and/or high CO_2 injury.
3. Determine if recognition of changes in volatile compound production during low O_2 or high CO_2 stress has utility for CA system management.
4. Develop sampling protocols to enable detection of biomarkers for scald and other disorders.

The goal of this research is to develop an active fruit monitoring system that alerts storage operators to undesirable CA conditions in real or near-real time. This system would incorporate some if not all existing technologies for storage room monitoring while providing additional measures to identify abnormal fruit metabolism. While the bulk of the proposed research will utilize GC-MS as the analytical system, we anticipate commercialization of this concept could utilize existing instruments and/or expertise already in place at some warehouses, consulting businesses, and ag chemical supply companies. Successful system development and implementation would reduce storage disorder risk that results from CA gas concentrations outside the range tolerable by apple fruit.

Significant Findings

1. CA chamber volatile content differed with cultivar ('Delicious', 'Fuji', 'Granny Smith'), chamber O_2 and CO_2 concentration, and storage duration.
2. Ethanol and ethyl ester accumulation increased with decreased O_2 content ('Delicious') while accumulation of typical 'Delicious' volatiles decreased.
3. 'Fuji' volatile accumulation peaked within 30 days regardless of atmosphere, 'Delicious' within 25 days (CA) or 82 days (RA).
4. Accumulation of ethyl- and methyl esters increased with increased CO_2 content ('Fuji') soon after harvest and prior to an increase in ethanol.
5. CA chamber ethylene concentration decreased with decreased % O_2 or increased % CO_2 concentration; chamber nitric oxide content decreased as storage duration increased.
6. Volatile compounds in commercial CA rooms containing 'Delicious', 'Fuji', and 'Granny Smith' apples were similar to those detected in research CA chambers, and compounds previously identified as possible predictors of 'Granny Smith' superficial scald were detected in both research chambers and commercial CA rooms.

Results and Discussion

'Delicious' low O_2 storage. Fermentation due to insufficient O_2 during CA storage can result in off-flavor development and internal browning. As 'Delicious' is often stored at low O_2 to limit superficial scald development, studies were conducted to characterize volatile compound accumulation during CA storage to assess the potential for non-invasive CA room monitoring for low O_2 stress. Fruit was obtained from the same commercial grower throughout the study. Maturity at harvest was similar in both years except for internal ethylene concentration, higher in 2010 (Table 1).

year	starch	firmness lbs	soluble solids %	titratable acidity %	internal ethylene ppm	weight g
2010	2.3±0.2	15.5±0.4	11.2±0.2	0.250±0.005	10.3±5.57	234±15
2011	2.2±0.2	15.8±0.2	11.9±0.2	0.906±0.330	0.91±0.33	223±7

Table 1. Maturity and quality of 'Delicious' apples at harvest in 2010 and 2011. Fruit obtained from a commercial orchard near Monitor, WA.

The lowest CA O_2 setpoint was determined by monitoring fruit chlorophyll fluorescence using the HarvestWatch system, and the O_2 % where a change in fluorescence was observed was 0.2 and 0.3 in 2010 and 2011, respectively. Fruit were held at these O_2 concentrations to induce fermentation and low O_2 injury as well as at two higher settings and air (2011). Fifty one volatile compounds other than ethylene and nitric oxide were detected and monitored (supplementary tables). Chamber volatile content was impacted by O_2 concentration with compounds associated with low O_2 metabolism, ethanol and ethyl acetate in particular, accumulating as O_2 concentration decreased (Figure 1). Ethanol accumulation occurred sooner after harvest in 2011 compared to 2010, possibly reflecting less capacity for fruit to use ethanol for ester volatile production in 2011 compared to 2010. In both years, CA chamber concentrations for both compounds exceeding 10 nmol L^{-1} were reached for the lowest O_2 setpoints but not in chambers held at higher O_2 settings. Consistent accumulation of ethanol and ethyl acetate above a threshold value when held at an O_2 concentration inducing fermentation would enable a CA room monitoring system to operate with target values for compounds produced during fermentation. Room ethylene and nitric oxide concentrations did not provide an indication of low O_2 stress (Figure 2).

The accumulation pattern for ethanol, ethyl acetate and other volatile compounds varied with harvest year. This difference in relation to typical harvest maturity tests indicates no simple relationship exists between fruit harvested at maturity suitable for long-term CA and response to anaerobic storage conditions. Assuming a consistent headspace threshold value for ethanol and/or ethyl acetate exists that only rooms held under low O_2 stress would exceed, a monitoring system based on the threshold value could alert the storage operator of a problem. In the two years of this study, that alert would occur in less than 3 weeks (2011) compared to 2 months (2010). Responding to the alert (by increasing room O_2) even at 2 months is likely to avoid fruit quality problems as fruit ethanol production would decrease and ethyl volatile production, a source of off-flavors, would also decrease as ethanol is depleted. If a consistent threshold value indicative of low O_2 stress does not exist, a monitoring system based on room to room comparisons could be feasible.

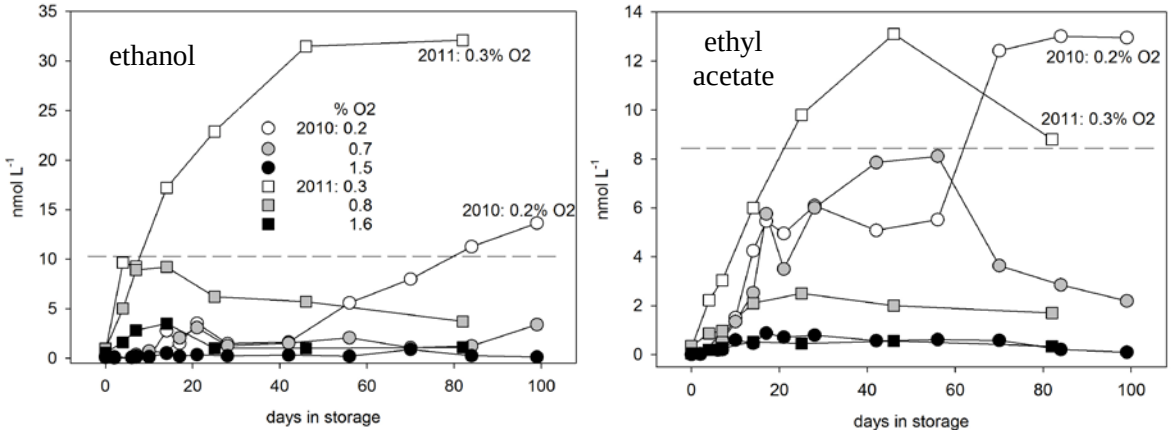


Figure 1. Ethanol and ethyl acetate in CA chambers holding 'Delicious' apples. Fruit harvested in: 2010 stored in 0.2, 0.7, or 1.5% O_2; 2011 stored in 0.3, 0.8, or 1.6% O_2. All fruit held at 33 ^{o}F with 1.5% CO_2. Gas samples for analysis obtained directly from CA chambers.

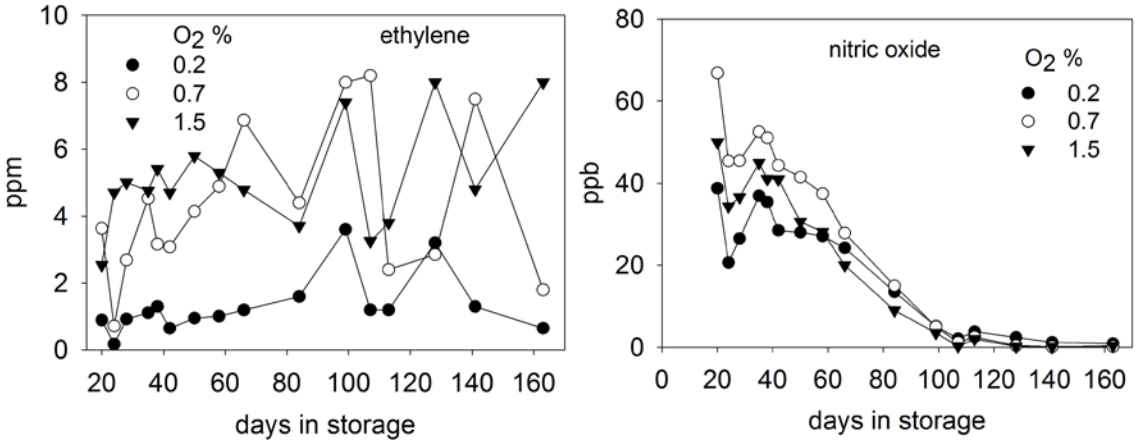


Figure 2. Ethylene and nitric oxide in CA chambers holding 'Delicious' apples. Fruit harvested in 2010 stored in 0.2, 0.7, or 1.5% O_2. All fruit stored at 33 ^oF with 1.5% CO_2. Gas samples for analysis obtained directly from CA chambers.

Apple volatile production proceeds in part based on the amounts of alcohols present in fruit tissue. Fermented fruit accumulate ethanol and therefore produce higher quantities of ethyl-volatiles. These fruit also produce less quantity of volatiles typical of normal ripening as typical alcohols are available in lower amounts, and also due to the lower O_2 setpoint. The combination of more ethyl-volatiles and less typical volatiles leads to off-flavor. This pattern is illustrated by a prominent 'Delicious' aroma volatile, 2-methylbutyl acetate (Figure 3) for which production decreases with decreased O_2 concentration and also with storage duration. The seasonal impact on volatile production is also evident for 2MBA as amounts peaked earlier in storage and to a higher amount in 2010 compared to 2011.

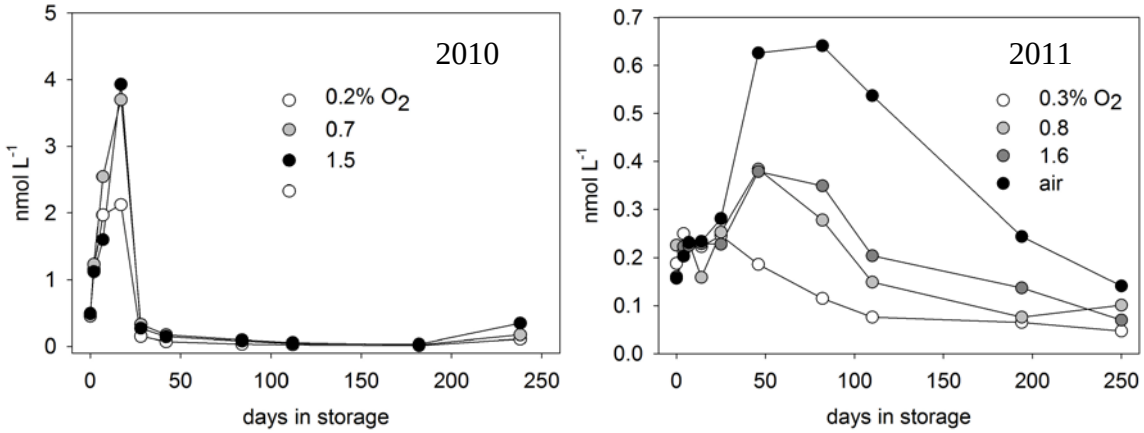


Figure 3. 2-methylbutyl acetate in CA chambers holding 'Delicious' apples. Fruit harvested in 2010 stored in 0.2, 0.7, or 1.5% O_2; 2011 stored in 0.3, 0.8, or 1.6% O_2. All fruit stored at 33 ^oF with 1.5% CO_2. Gas samples for analysis obtained directly from CA chambers.

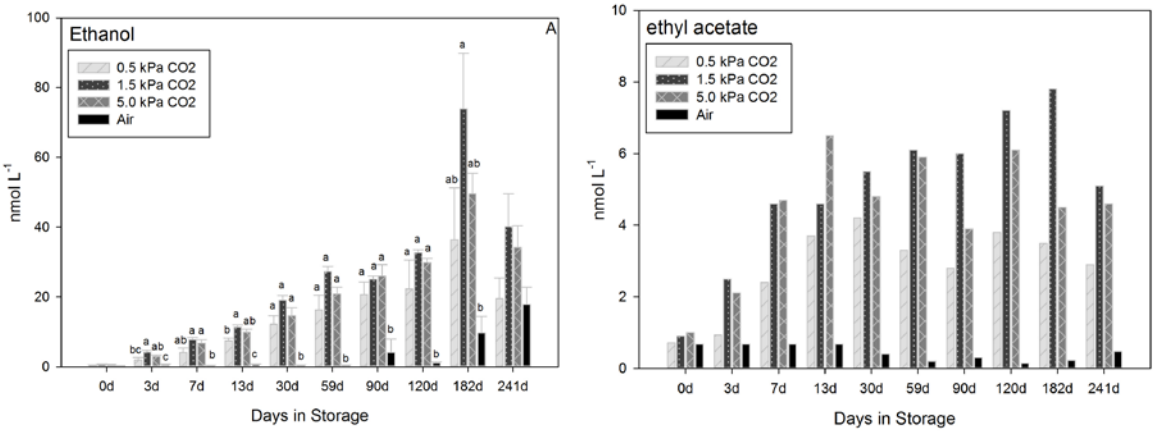
Low O_2 stress conclusions: accumulation of ethanol and ethyl acetate in CA chamber headspace was evident in chambers held at O_2 concentrations that promoted fermentation. A threshold amount for each compound exceeded only in chambers held at 0.2 or 0.3% O_2 was identified based on the 2 study years. This threshold value (10 nmol L^{-1}) could serve as a validation point for further studies to assess the potential for CA room monitoring for the potential for low O_2 injury. Low O_2 setpoints and generation of excess ethanol both limit production of typical 'Delicious' apple ester volatiles.

'Fuji'high CO_2 storage Internal browning due to high CO_2 exposure during CA storage of 'Fuji' occurs via undescribed mechanisms, however, high CO_2 CA storage is known to alter fruit volatile compound production after storage, so studies were conducted to assess volatile accumulation during CA with high CO_2 setpoints. Fruit was obtained from the same commercial grower throughout the study. Fruit at harvest had higher starch, lower firmness, titratable acidity and internal ethylene in 2011 compared with 2010 (Table 2).

year	starch	firmness lbs	soluble solids %	titratable acidity %	internal ethylene ppm	weight g
2010	4.1±0.1	17.2±0.4	16.7±0.1	0.482±0.010	3.01±0.45	279±7
2011	4.9±0.2	15.8±1.9	15.0±0.1	0.405±0.010	1.93±0.57	271±13

Table 2. Maturity and quality of 'Fuji' apples at harvest in 2010 and 2011. Fruit obtained from a commercial orchard near Orondo, WA.

The CA CO_2 settings, 0.5, 1.5, and 5%, were chosen based on previous work where 0.5 and 1.5 did not induce injury whereas injury usually occurs at 5%. All fruit was held at 33 ^{o}F and CA O_2 was 1.5% for all CO_2 treatments. Chlorophyll fluorescence of fruit held in high CO_2 has not proven to provide an indication of stress similar to that occurring as low O_2 conditions are imposed. Internal browning had developed in fruit stored in 5% CO_2 after 1month (2010) and 4 months (2011) but was not observed in other CA treatments. Chambers in which 'Fuji' apples were stored accumulated 51 volatile compounds other than ethylene and nitric oxide. While differences in volatile accumulation were evident due to CA treatment, the magnitude of the differences was not sufficient to clearly indicate the imposed CA conditions were causing stress. Examples include ethanol, ethyl acetate, ethyl butyrate, and methyl butyrate (Figure 4). While ethanol accumulated over time in CA storage, a pattern linking accumulation to stress at 5% CO_2 was not evident. Accumulation of other esters occurred at a faster rate, but no clear patterns associated with 5% CO_2 and injury were observed.



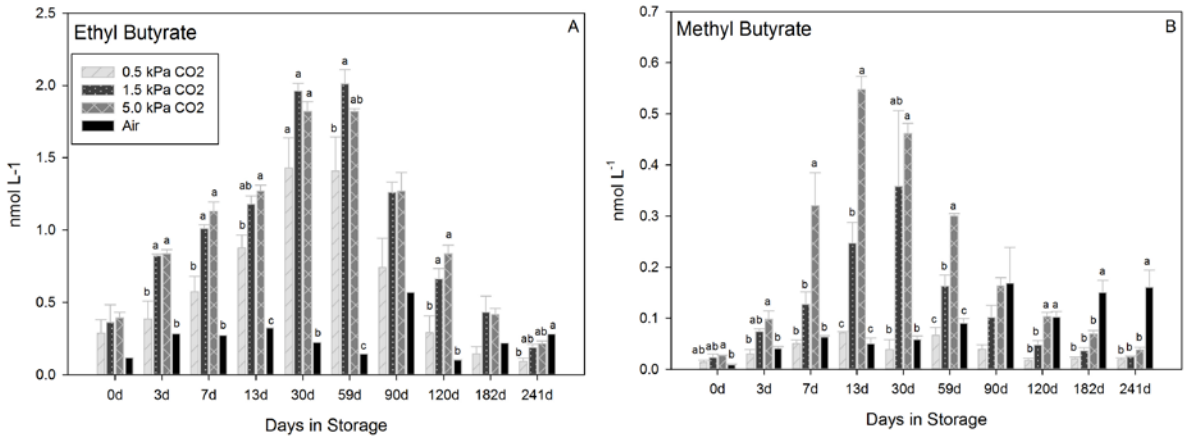


Figure4. Ethanol, ethyl acetate, ethyl butyrate, and methyl butyrate in CA chambers holding 'Fuji' apples. Fruit harvested in 2011 stored in 0.5, 1.5, or 5.0% O_2. All fruit stored at 33 ^oF with 1.5% O_2. Gas samples for analysis obtained directly from CA chambers.

While total accumulation of ethyl and methyl esters was somewhat higher in chambers held at 5% CO_2 compared to lower values, large quantitative differences due to CA treatment were not observed. This finding in both years of the study limits the potential for development of a CA room volatile monitoring system for alerting storage operators to ongoing CO_2-induced fruit stress. The results of the 2 year study are limited in part due to a relatively low incidence of CO_2 injury, even at 5% CO_2. It is unknown what differences if any in volatile accumulation would occur prior to or after CO_2 in situations where a high incidence of disorders occurs.

The results do indicate a pattern of reduced accumulation of some volatiles, illustrated by ethyl- and methyl butyrate, over time in storage. This tendency contributes to the well known diminution of aroma in fruit previously held in long term CA. Should procedures be identified that could enhance the capacity for aroma production during CA, such as manipulation of room O_2 and/or CO_2, monitoring systems similar to that used in this study could provide the basis for enhanced fruit quality management resulting in greater aroma after storage.

'Granny Smith' apple volatile accumulation during air or CA storage. Fruit were stored in air or CA (1% O_2, 1% CO_2) with or without pre-storage DPA (2000 ppm) treatment. Through 8 months storage, volatile compounds typical of 'Granny Smith' were detected with amounts lower in CA chambers. Several volatiles previously related to scald development were detected from fruit stored in air or CA. Accumulation of some esters including butyl butyrate, 2-methylbutyl acetate, and butyl 2-methylbutyrate was greater in chambers held at 1/1 O_2/CO_2 containing DPA-treated fruit. Contrary to what was observed for 'Delicious' and 'Fuji', ester accumulation increased throughout the first 8 weeks CA storage of 'Granny Smith'. Superficial scald symptoms developed on fruit stored in air or CA and volatile accumulation indicative of scald preceded symptom development.

Volatiles accumulating in commercial CA storage. Gas samples from one room each of 'Delicious', 'Fuji', and 'Granny Smith' apples were collected beginning in mid-December from a Stemilt facility in East Wenatchee. All volatiles detected (46) in commercial rooms have been previously identified in research CA chambers containing the same cultivars. Amounts of volatiles detected in all commercial room samples were low compared to samples collected from CA chambers. Of the 3 cultivars, volatile content in the 'Fuji' room was highest followed by 'Delicious' and then 'Granny Smith'. 2-methyl-1-butanol was the most abundant volatile collected in all three rooms, and ester accumulation was low in all rooms. Volatile compounds that have been associated

with development of superficial scald were detected in the commercial ‘Granny Smith’ room. As similar gas volumes were collected from research CA chambers and the commercial rooms, volatile production or accumulation in commercial rooms may be less compared to CA chambers, or headspace volume in rooms versus chambers may be different. Additional sampling from rooms is needed to determine optimum sampling conditions, and additional room sampling of ‘Granny Smith’ rooms at this facility is ongoing in 2012-13.

Executive Summary

A detailed report of the low O_2 and high CO_2 portions of this project is available, send request to james.mattheis@ars.usda.gov.

The low O_2 and high CO_2 studies demonstrate the capacity to identify and quantify apple volatile compounds produced during rather than after CA storage. CA gas composition has a marked impact on volatile accumulation as does storage duration and season of fruit production. For chambers held at very low O_2 concentrations with a risk of fermentation, patterns of volatile accumulation indicative of stress are identifiable. This part of the study demonstrates proof of concept for using storage chamber volatile compound monitoring to identify situations that can result in fruit quality issues leading to postharvest loss. Consistent patterns of ethanol and ethyl acetate accumulation over the initial 2 years of the project support a threshold value for these compounds that could be the subject of validation under scaled up commercial conditions. If validated, a monitoring system could be developed that would alert storage operators when either of these compounds exceeded the threshold value. High CO_2 stress did not result in similar results, possibly due to a low incidence of CO_2 injury even though 'Fuji' apples were held in 5% CO_2.

Both cultivars showed diminution of volatile accumulation over time, a result similar to post storage analyses of fruit volatile production. The capacity to monitor fruit during storage provides a potential means to time alteration of room gas conditions to enhance capacity to produce aroma currently lost as duration in low O_2 increases. Our lab previously demonstrated the utility of this type of dynamic CA where brief periods of increased O_2 during storage resulted in enhanced aroma production after storage. What was lacking in the earlier work was a basis on which to decide when to add oxygen to the room. A decision to add O_2 based on CA room volatile content could potential enhance the effectiveness of this protocol while minimizing loss of other quality components, particularly firmness, in susceptible cultivars. This system may have greater utility in cultivars where firmness management is not the primary objective of the postharvest system. The commercial room sampling to date shows volatile accumulation patterns are similar to those observed in research CA chambers.

The capacity to detect volatiles shown to be likely predictors of superficial scald risk for 'Granny Smith' was also demonstrated in research as well as commercial CA chambers and rooms. Validation of the use of this analysis by Dave Rudell is ongoing. Current work with 2012 crop fruit held initially in 4 now 3 rooms in the Stemilt East Wenatchee facility will provide additional information regarding the dynamics of particular volatiles as a function of storage duration.

FINAL PROJECT REPORT

Project Title: Genetic controls of apple fruit-specific auxin metabolism

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Other funding sources: None
Total Project Funding: $131,000

Budget History:

Item	2010	2011(extended)	Year 3:
Salaries	35,000	36,000	
Benefits	14,000	14,000	
Wages			
Benefits			
Equipment			
Supplies	15,000	15,000	
Travel			
Plot Fees			
Miscellaneous	1,000	1,000	
Total	65,000	66,000	

OBJECTIVES:

1. Elucidate roles for previously identified candidate genes in ethylene, auxin, gibberellin, jasmonate and brassinosteroid metabolism and response during apple fruit ripening.
2. Characterize the relationship between gene expression patterns and specific fruit ripening phenotypes (ripening season, fruit size, fruit texture) in a cross population of 'Honeycrisp' x 'Cripps Pink', as well as other germplasm.
3. Develop a shortlist of candidate genes for hormone metabolism for further validation for use in marker assisted selection.

SIGNIFICANT FINDINGS

1. Most of the selected genes, based on the results from previous microarray or gene chip analysis, showed correlations with apple fruit ripening processes among a wide spectrum of apple germplasm including several commercial cultivars and a segregating population of 'Honeycrisp' x 'Cripps Pink'.

2. The expression patterns of three genes, *MdACS3* (a pre-climacteric ethylene biosynthesis gene), JOM (codes for the enzyme jasmonate O-methyl transferase) and AIP (codes for an auxin induced protein) showed a strong correlation with fruit ripening season.

3. The expression patterns of an AUTR (auxin transporter) encoding gene showed a good correlation with fruit firmness.

4. The expression features of an auxin transporter gene, *MdPIN1-1,* is associated with the timing of climacteric ethylene biosynthesis.

5. Gene activity of *MdGH3.5,* which is related to the availability of active auxin in fruit cortex tissues, showed cultivar-specific regulation patterns during the 10-week on tree maturation and ripening processes.

6. The tissue-specific expression features, i.e. seed-initiated and spreading outward, of the *MdPIN1-1* gene, correspond to starch degradation patterns, and ethylene biosynthesis pathway activation.

7. Overall, variation of auxin metabolism gene activities appear to associate with fruit ripening time or ripening season among apple genotypes studied.

RESULTS AND DISCUSSION

1. Correlations between transcript levels of identified candidate genes and fruit ripening phenotypes within a WSU 'Honeycrisp' x 'Cripps Pink' cross population.

Fruit ripening date, fruit firmness and fruit size were phenotyped based on weekly maturity data for all fruiting trees within 'Honeycrisp' x 'Cripps Pink' cross population. Phenotypic data among individuals were categorized based on the values of ripening date, fruit firmness and fruit size around physiological maturity (average starch pattern index of 3.5, based on 1-6 scale). Fruit from ~12 individual trees (siblings) in each phenotypic group (early or late-ripening; firm or soft fruit; small or large fruit) were selected for the analysis with transcript abundance. Several candidate genes showed correlation with apple fruit ripening processes or quality attributes among individuals in this segregating population. "*" indicated that the transcript abundance of this gene showed statistically significant difference between two groups.

Table 1. Association between gene transcript abundance and sorted phenotypes among individuals in 'Honeycrisp' x 'Cripps Pink' segregating population.

Fruit ripening time

	Early-ripening (Earlier than Sep. 9)	Late-ripening (Later than Oct. 15)	*P* value
*ACS3	20.8 ± 3.5	28.7 ± 3.3	3.20E-05
*JOM	25.6 ± 2.3	31.9 ± 2.3	3.80E-06
*AIP	24.3 ± 1.2	28.3 ± 1.5	1.70E-06
*AUTR	24.5 ± 1.1	22.0 ± 1.2	4.80E-05

Fruit firmness

	Soft fruit (≤ 14.9 Ibs at-harvest)	Firm fruit (≥ 18.2 Ibs at-harvest)	*P* value
JOM	25.6 ± 2.8	28.8 ± 3.5	0.0467
*AUTR	23.0 ± 0.9	24.8 ± 1.2	0.0023
XTH7	24.4 ± 2.4	26.2 ± 2.8	0.1092
BRIP	26.1 ± 3.0	28.4 ± 3.7	0.1495
AQUAPIP	29.3 ± 3.4	29.4 ± 2.1	0.9505

Fruit size

	Small fruit (≤ 2.12 inches of diameter)	Large fruit (≥ 2.82 inches of diameter)	*P* value
*JOM	31.2 ± 2.3	27.9 ± 2.5	0.003
AUTR	23.9 ± 1.1	24.2 ± 1.3	0.4477

Values in the 2nd and 3rd columns are the average and standard deviation of normalized Ct (stand for cycle threshold of quantitative PCR reaction) values for tested candidate genes, which represent the relative abundance of transcripts or the expression level. Each value is the average based on the gene expression data from the fruits of 10-12 individual trees within the same phenotypic group. Fruit cortex tissues of 3 apples were pooled for RNA isolation and qPCR were carried out in triplicate. Anova analysis was performed between the paired groups. $P < 0.01$ would normally be considered significant and $P < 0.001$ highly significant as indicated by "*" in Table 1. ACS3: a pre-climacteric ethylene biosynthesis gene, JOM: jasmonate O-methyl transferase encoding gene, AIP: auxin induced protein gene, AUTR: auxin transporter gene, BRIP: brassinosteroid induced protein gene, XTH7: xyloglucantransferase/hydrolase gene, and AQUAPIP: aquaporin gene.

2. Physiological characterization of four cultivars with distinct ripening phenotypes

To characterize apple genotype-specific maturation and ripening progression, samples harvested weekly and associated fruit maturity data were collected starting in late July for early ripening cultivar 'SweeTango' and a few weeks later for three later ripening cultivars. The four cultivars studied - 'SweeTango' (ST), 'Golden Delicious' (GD), 'Jazz' (JZ) and 'Cripps Pink' (CP) required 122, 148, 150 and 191 days after full bloom (DAFB) to achieve physiological maturity (Table 2). Physiological maturity was considered to be attained when the average starch rating was approximately 3.5 and this harvest date was designated as week 0. Fruit firmness showed a steady decrease in all cultivars as the fruit matured, although the values and rates of change are cultivar-specific. Fruit internal ethylene concentration (IEC) was variable and fluctuated at a low level and characteristic of pre-climacteric fruit.

3. Expression features of a pre-climacteric ethylene biosynthesis gene *MdACS3* as an indicator of ethylene pathway activation.

The expression profiles of *MdACS3* showed steady up-regulation as maturation progressed for all four cultivars (Fig. 1). The detection of measurable *MdACS3* transcript in GD was observed at week -4 and transcript amount increased through week 1. *MdACS3* transcripts in early-season cultivar ST were detectable even earlier than week -4, while consistent detection in CP was delayed till week -3. The peak expression of *MdACS3* was generally observed at week +1, except CP which did not achieve physiological maturity in 2011 before freezing temperatures. The fold change of *MdACS3* transcript abundance varied greatly along the maturation process depending on genotypes.

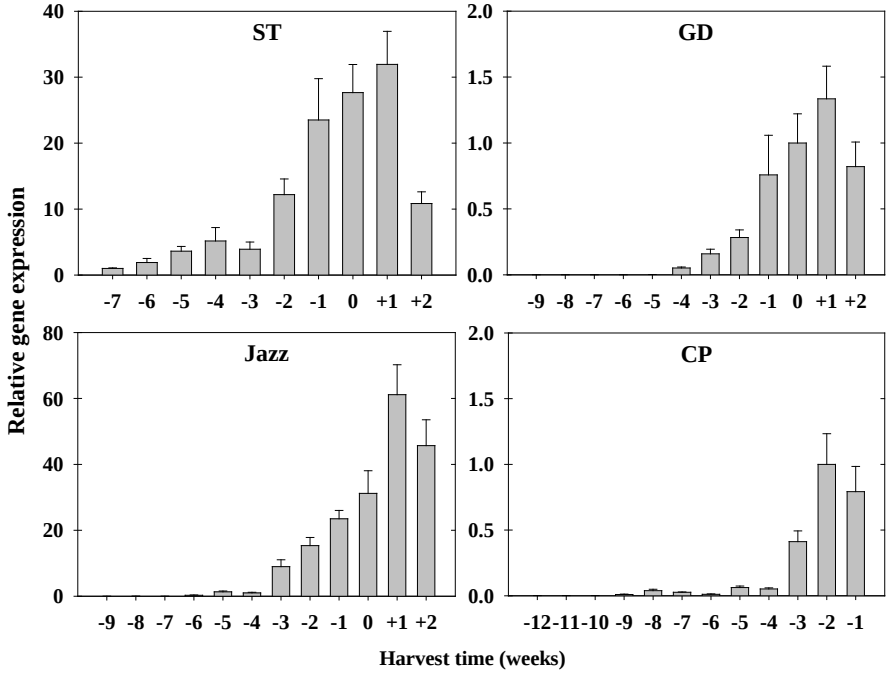

Fig. 1. Expression profile of *MdACS3* during apple fruit maturation and ripening. ST: 'SweeTango', GD: 'Golden Delicious', Jazz: 'Jazz', CP: 'Cripps Pink'. Y axis indicates the fold change in relative gene expression level; X axis indicate week prior to or after physiological maturity (week=0) when fruit were harvested. Each data point represents the means of four replicates with standard error. For better comparison of gene expression levels among cultivars, one comparable Ct value from each cultivar was designated as calibrator for relative gene expression analysis within the sample series for a cultivar. For *MdACS3* gene, week -7 for ST, week 0 for GD, week -4 for Jazz and week -2 for CP were used as calibrator for relative gene expression analysis.

Table 2. Fruit maturity during on-tree development

ST										
Weeks	-7	-6	-5	-4	-3	-2	-1	0	1	2
DAFB	73	80	87	94	101	108	115	122	129	136
IEC (ppm)	0.1±0.05	0	0	0.0±0.03	0.1±0.08	0.1±0.03	0.2±0.04	0.3±0.06	0.7±0.23	0.1±0.02
Firmness (lbs)	17.2±0.4	17.0±0.3	15.9±0.4	15.6±0.4	14.1±0.4	14.0±0.3	13.5±0.3	12.6±0.5	13.2±0.2	13.3±0.4
Starch Index	1.1±0.1	1.4±0.2	1.5±0.1	2.1±0.1	2.1±0.2	2.6±0.2	2.5±0.1	3.4±0.3	4.6±0.1	5.0±0.2
Jazz										
Weeks	-7	-6	-5	-4	-3	-2	-1	0	1	2
DAFB	101	108	115	122	129	136	143	150	157	164
IEC (ppm)	0	0	0.0±0.01	0	0	0.2±0.08	0.1±0.03	1.4±0.74	0.3±0.17	0.1±0.07
Firmness (lbs)	33.1±0.8	30.9±0.8	28.9±0.7	26.7±0.5	25.8±0.6	21.9±0.3	20.6±0.3	19.7±0.4	20.6±0.4	19.7±0.4
Starch Index	1.0±0.0	1.1±0.1	1.0±0.0	1.1±0.1	1.5±0.1	1.9±0.1	2.6±0.2	3.5±0.3	3.7±0.2	5.0±0.1
GD										
Weeks	-7	-6	-5	-4	-3	-2	-1	0	1	2
DAFB	99	106	113	120	127	134	141	148	155	162
IEC (ppm)	0	0.0±0.03	0.1±0.10	0	0.0±0.04	0.0±0.03	0.0±0.01	0.0±0.05	0.1±0.01	0.0±0.01
Firmness (lbs)	17.6±0.4	18.3±0.4	17.7±0.3	17.0±0.4	17.1±0.4	15.9±0.2	16.8±1.0	16.1±0.3	15.3±0.3	15.6±0.4
Starch Index	1.2±0.1	1.1±0.0	1.2±0.0	1.5±0.1	1.7±0.2	2.1±0.1	2.1±0.1	2.3±0.1	2.8±0.2	4.1±0.3
CP										
Weeks	-9	-8	-7	-6	-5	-4	-3	-2	-1	0
DAFB	128	135	142	149	156	163	170	177	184	191
IEC (ppm)	0	0.03±0.04	0	0	0.06±0.06	0.06±0.01	0	0.03±0.02	0.06±0.01	0.16±0.05
Firmness (lbs)	21.5±0.4	20.8±0.5	20.6±0.5	19.8±0.4	19.8±0.3	18.9±0.3	18.9±0.3	18.6±0.3	18.5±0.3	18.0±0.3
Starch Index	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0	1.1±0.0	1.2±0.1	1.4±0.0	2.2±0.1	2.8±0.2

Values of IEC concentration, fruit firmness (Ibs) and starch pattern index (SPI, based on 1-6 scale) of weekly fruit samples represent the average of 15 apples with standard error.

4. Expression profiling of an auxin transporter encoding gene

Due to its unique weak acid character, auxin (indolyl-3-actic acid) requires specific carrier proteins or transporters on the plasma membrane for its distribution and function. In the apple fruit cortex, the transcript of an auxin transporter gene, denoted as *MdPIN1-1*, was readily detected seven to nine weeks before physiological maturity (Fig. 2). The peak expression of *MdPIN1-1* was observed around week -4 in all these cultivars, which corresponded approximately with the timing of *MdACS3* activation in most apple cultivars (see Fig. 1). Some subtle variations of the *MdPIN1-1* expression were identified among early, mid- and late-season cultivars. In early-season cultivar ST a secondary peak of expression was evident at week -1; in both mid-season cultivars JZ and GD a slight down-regulation was observed after week -4. In late-season cultivar CP, the peak transcript abundance was also occurred at week -4 though the overall expression level is low. At any specific time, for example September 21, the expression levels or transcript abundances varied greatly between cultivars, although the values of DAFB were approximately same. The differences at *MdPIN1-1* expression could be one of the important factors that differentiate the timing of ethylene biosynthesis pathway activation, putatively through *MdACS3*, and therefore the rate of maturation and ripening.

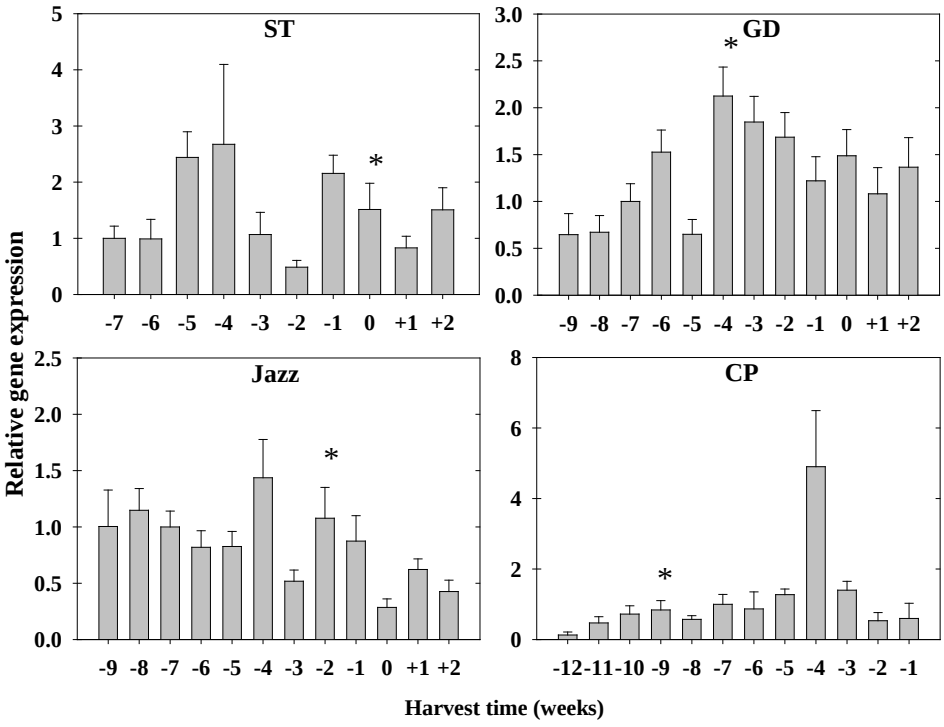

Fig. 2. Expression profile of auxin efflux carrier protein gene, *MdPIN1-1*, during apple fruit maturation and ripening. ST: 'SweeTango', GD: 'Golden Delicious', Jazz: 'Jazz', CP: 'Cripps Pink'. Y axis indicates the fold change of relative gene expression level; X axis indicates week prior to or after physiological maturity (week=0) when fruit were harvested. Each data point represents the means of four replicates with standard error. For better comparison of gene expression levels among cultivars, one comparable Ct value from each cultivar was designated as calibrator for relative gene expression analysis within the sample series for a cultivar. For *MdPIN1-1* gene, week -7 samples for all four cultivars were used as calibrator for relative gene expression analysis.

5. Auxin homeostasis gene

GH3 proteins in plant tissues control free or biologically active IAA levels by deactivating IAA. A previously identified apple GH3.5 gene had down-regulated expression patterns as fruit maturity progressed in all four cultivars studied (Fig. 3). In early-season cultivar ST, stronger reductions in transcript levels were observed from week -6 to week -5, and week -3 to week -2; similar patterns were also observed in GD. Overall, considerably higher transcript levels were detected in early stages in all cultivars studied, but more dynamic changes in GH3.5 transcript abundance were found in both JZ and CP as fruit maturity increased.

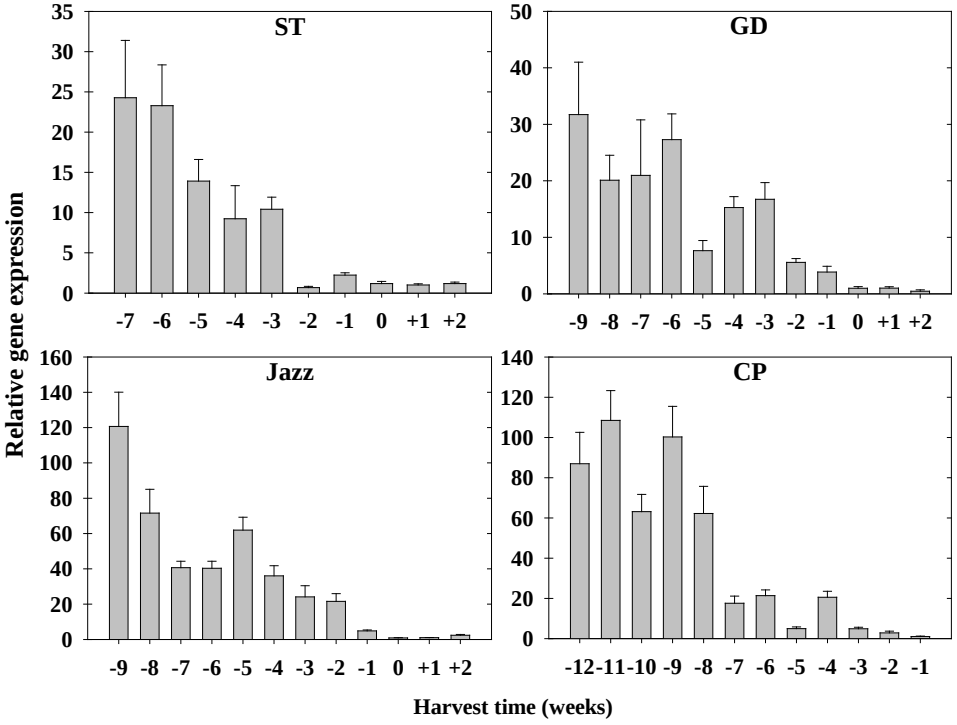

Fig. 3. Expression profiles of an *MdGH3.5* genes during apple fruit maturation and ripening. ST: 'SweeTango', GD: 'Golden Delicious', Jazz: 'Jazz', CP: 'Cripps Pink'. Number on Y axis indicate the fold change of relative gene expression level; number on X axis indicate the weeks when samples were harvest and "-" means week before physiological maturity and "+" means week after physiological maturity. Each data point represents the means of four replicates with standard error.
For better comparison of gene expression levels among cultivars, one comparable Ct value from each cultivar was designated as calibrator for relative gene expression analysis within the sample series for a cultivar. For *MdGH3.5* gene, week +1 for ST, GD and Jazz, and week -1 for CP were used as calibrator for relative gene expression analysis.

6. Tissue-specific expression of auxin transporter genes and ethylene biosynthesis genes

The temporal and spatial expression features, i.e. in different tissues of seed, core, cortex and peel and along the maturation processes, of auxin transporter genes *MdPIN1-1* and a pre-climacteric ethylene biosynthesis gene *MdACS3* were examined using mid-season cultivar 'Jazz' (Fig. 4). For *MdPIN1-1*, a stronger expression was observed in seed and core tissues and a relatively weak expression was found in the cortex and peel. For *MdACS3*, a strong expression was observed in core and cortex but

no expression was detected in seed tissues. This expression pattern is correlated to the starch degradation process observed in maturing fruit. This data supports a scenario of auxin-ethylene interaction that the developing seed is a primary source of auxin which is transported to core and cortex; auxin triggers the expression of *MdACS3* initially in the core, then in cortex and finally in peel as auxin is transported to these tissues. As the result of *MdACS3* expression and internal ethylene accumulation, *MdACS1* expression begins and sustained ethylene production promoting fruit ripening occurs. If confirmed, genetic differences in the capacity and rate of auxin metabolism (biosynthesis, transport and homeostasis) could determine the timing of ethylene biosynthesis pathway activation as illustrated in Figure 5 of proposed model on the roles of auxin and its interaction with ethylene biosynthesis pathway in apple fruit.

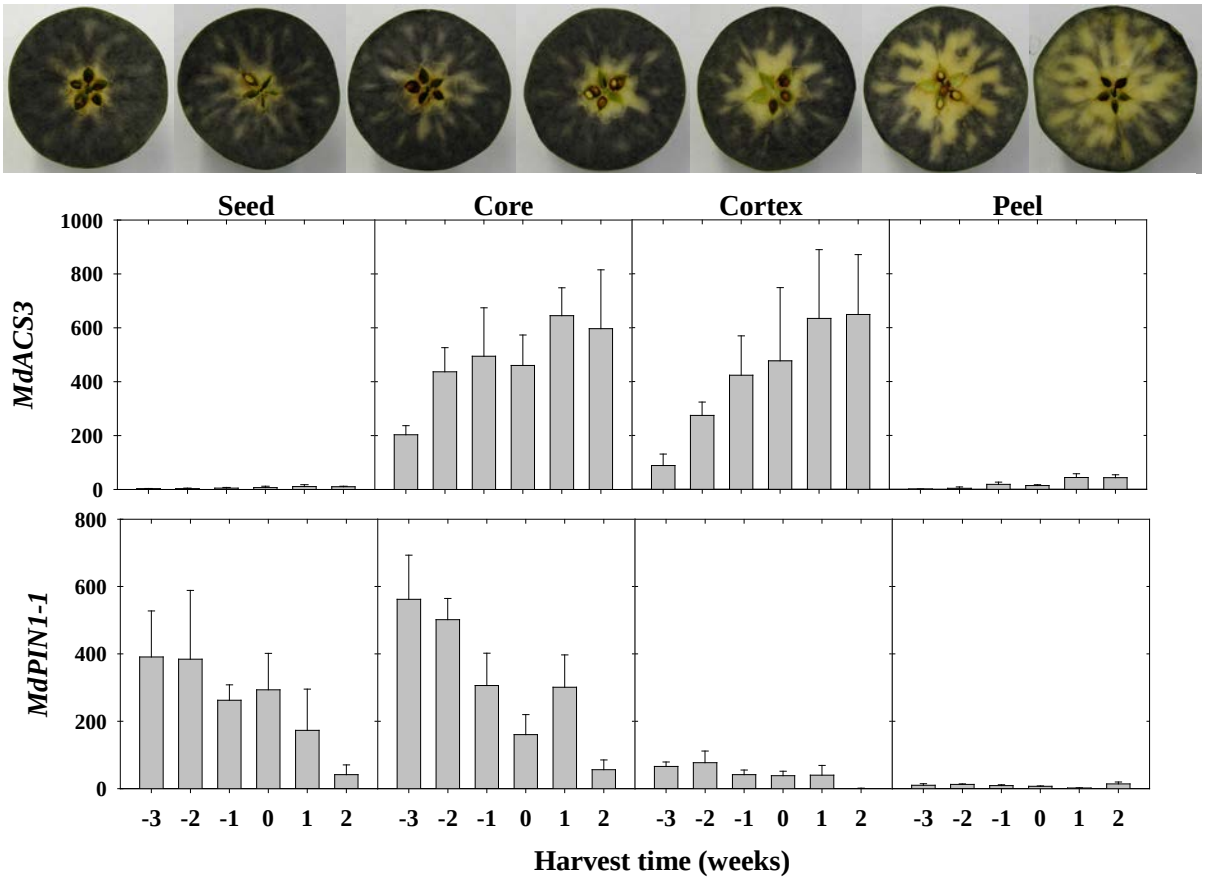

Fig. 4. Center-initiated ripening patterns as suggested by the starch clearing is correlated with "from center to out-layer" gene activities for both auxin transporting function *MdPIN1-1*and ethylene biosynthesis gene *MdACS3* in four different 'Jazz' apple tissues during apple maturation and ripening. The number indicated the weekly samples with week 0 as physiological maturity; "-" means before and "+" means after week 0.

7. A simplified model to illustrate the interaction between ethylene and auxin

While apple fruit ripening is likely not solely regulated by ethylene, little is known regarding other molecular mechanisms regulating fruit ripening and quality attributes, especially those with plant hormone interactions. For example, all apple genotypes produce ethylene during ripening but production duration and strength vary with cultivar. The very basic question is what is behind the

timely activation and the strength of ethylene production? The results from this study indicate auxin may function to activate ethylene biosynthesis pathway, and therefore regulate onset of apple fruit ripening time or the ripening season.

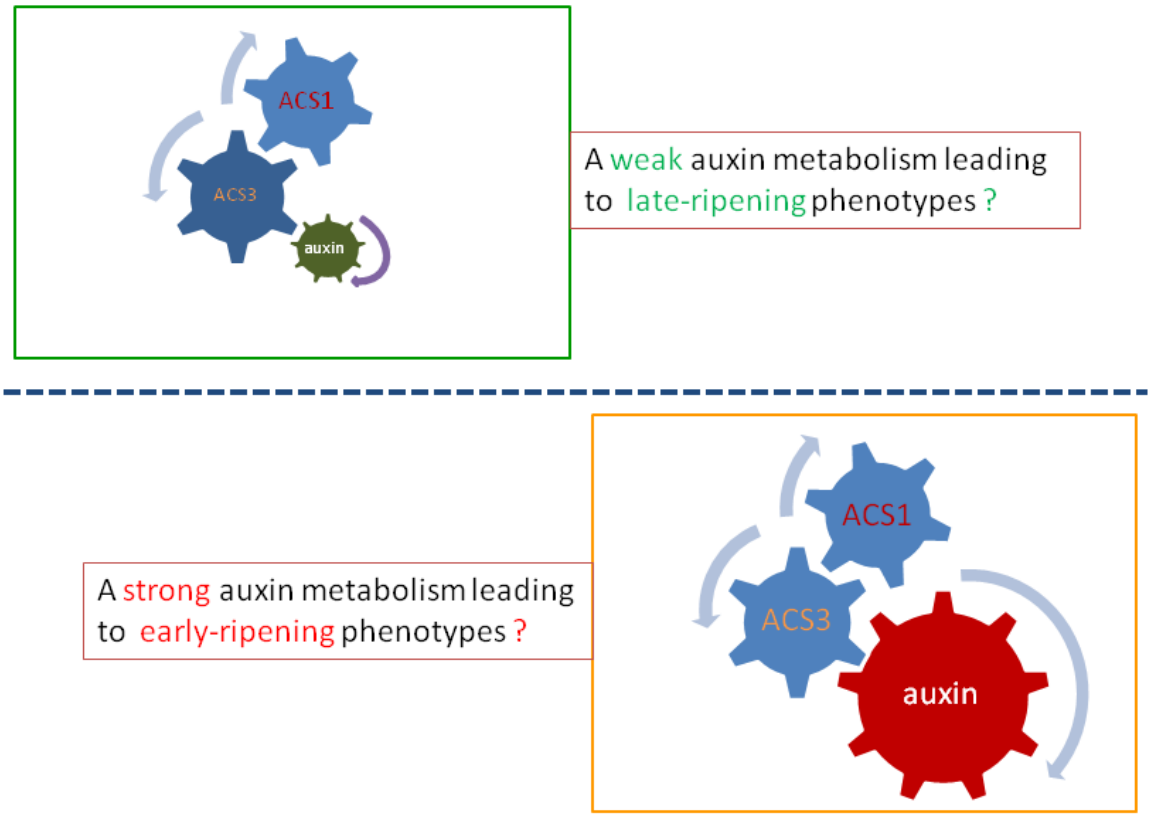

Fig. 5. A simple model describing the relationship between auxin metabolism and ethylene biosynthesis pathway in maturing apple fruit

8. What this study means to the tree fruit industry?

From current analysis, several candidate genes, especially those genes regulating auxin metabolism, showed strong association with the apple fruit ripening season. Understanding the molecular regulation of auxin in apple fruit could potentially generate innovative technology for managing pre-harvest maturation process and postharvest fruit quality. Elucidating the specific mechanism of plant hormone interactions and their roles on cultivar-specific fruit quality and maturation/ripening patterns is an essential step in developing useful molecular breeding tools, such as gene-specific “functional molecular markers”, for improved precision and efficiency in the apple breeding processes.

EXECUTIVE SUMMARY

Apple cultivars exhibit variation in maturation and ripening physiology and fruit quality traits. The ripening season of apple vary up to 3 months even within elite cultivars. In a breeding segregation population, depending on the parent combination, up to 8-10% of the individuals are those which are unable to fully ripen in North Central Washington State. The phenotype of the apple ripening date/season is a simple but economically important horticultural trait, which can also substantially influence at-harvest quality and postharvest storability. Understanding the molecular regulation for this trait is critical for breeding locally-adapted new apple cultivars. Ethylene has been well-established for its regulating roles in apple fruit ripening; but ethylene may not account for every aspect of fruit ripening and quality. This study was built on two previous experiments in our lab: 1. The transcriptomics study of fruit ripening using a gene chip (microarray) containing 24,000 apple genes: among genes identified, those functioning in metabolism and response of auxin were a primary group of differentially regulated genes. Therefore, it was proposed that auxin metabolism plays a key role in the timely activation of the ethylene production and ripening season for an apple cultivar; 2. An earlier study on a pre-climacteric ethylene biosynthesis gene *MdACS3*: the activation of *MdACS3* was observed around 4 weeks prior to onset of climacteric ethylene production regardless of the actual ripening season. The primary goal of the current study was to get insight into the relationships between auxin metabolism and the timing of ethylene biosynthesis initiation in maturing apple fruit.

Among siblings of 'Honeycrisp' x 'Cripps Pink' progeny, several candidate genes showed strong correlation between apple fruit ripening and quality phenotypes (fruit ripening time, firmness and size) and transcript abundance around physiological maturity. The genes functioning in auxin metabolism, specifically, auxin transport and auxin homeostasis regulation, were investigated for their roles in the activation of ethylene biosynthesis among four cultivars with distinct ripening seasons, 'SweeTango', 'Golden Delicious' 'Jazz' and 'Cripps Pink'. The results suggest that availability of biologically active auxin is a primary contributor to activate the ethylene pathway, and subsequently initiate the apple ripening process. The majority of candidate genes tested showed expression patterns during the fruit maturation and ripening consistent with this hypothesis. The tissue-specific expression patterns of the auxin transporter gene in seed, core, cortex and peel correspond with the "center originated" ethylene biosynthesis gene expression and starch degradation patterns in ripening fruit, adding evidence that auxin availability is essential for timely activation of the ethylene biosynthesis pathway. These results provided data for understanding the molecular aspects of apple ripening regulation in addition to ethylene. The results have been presented at several scientific symposiums, including Sixth Rosaceous Genomics Conference (Trento, Italy 2012); American Society of Horticulral Sciences (Miami, FL 2012) and Washington State Annual Horticultural meeting (Yakima, WA 2012). The data has been submitted for peer-reviewed publication.

Supplementary Table 1. Volatiles monitored in storage chambers containing 'Delicious' apples. Compounds listed in groups as clustered by response patterns during storage.

ethyl propanoate	butyric acid	2-methylbutyl propanoate	methyl hexanoate
ethyl-2-methylbutyrate	pentanal	2-methylbutyl butyrate	1-pentanol
ethyl hexanoate	heptanal	propyl 2-methylbutyrate	1-butanol
ethyl pentanoate	hexanal	propyl propanoate	acetone
ethyl butyrate	propyl hexanoate	propyl acetate	1-hexanol
1-propanol	hexyl propanoate	butyl butyrate	butyl 2-methylbutyrate
methyl butyrate	butyl hexanoate	butyl propanoate	decanal
methyl acetate	hexyl butyrate	pentyl acetate	6-methyl-5-hepten-2-one
methyl-2-methylbutyrate	hexyl 2-methylbutyrate	propyl butyrate	6-methyl-5-hepten-2-ol
ethyl acetate	ethyl octanoate	2-methylpropyl acetate	2-methyl-1-propanol
ethanol	estragole	butyl acetate	2-methyl-1-butanol
acetaldehyde	octanal	2-methylbutyl acetate	
	2-ethyl-1-hexanol	hexyl acetate	
	nonanal		
	benzaldehyde		

Supplementary Table 2. Volatiles monitored in storage chambers containing 'Fuji' apples. Compounds listed in groups as clustered by response patterns during storage.

2-methylbutyl butyrate	ethyl butyrate	1-pentanol
butyl butyrate	ethyl propanoate	1-butanol
2-methylbutyl propanoate	ethyl pentanoate	2-methyl 1-butanol
hexyl acetate	ethyl hexanoate	1-hexanol
pentyl acetate	ethyl 2-methyl butyrate	1-propanol
2-methylbutyl acetate	ethyl acetate	2-methyl 1-propanol
butyl acetate	methyl butyrate	acetone
butyl propanoate	methyl hexanoate	butyl 2-methylbutyrate
2-methylpropyl acetate	methyl 2-methylbutyrate	6-methyl-5-hepten-2-ol
propyl acetate	pentanal	benzaldehyde
propyl propanoate	heptanal	acetaldehyde
propyl butyrate	hexanal	ethanol
propyl 2-methyl butyrate	butyric acid	methyl acetate
6-methyl-5-hepten-2-one	2-ethyl-1-hexanol	decanal
hexyl propanoate	nonanal	
propyl hexanoate	octanal	
butyl hexanoate		
hexyl butyrate		
hexyl 2-methylbutyrate		
ethyl octanoate		
estragole		

FINAL PROJECT REPORT

Project Title: Validation of fresh apple packing food safety interventions

PI:	Karen Killinger, Ph.D.	**Co-PI (2):**	Richard Dougherty, Ph.D.
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Drs. Dong Hyun Kang and Gene Kupferman also served as co-investigators in previous years of this project.

Cooperators:

Deborah Carter, Technical Issues Manager of the Northwest Horticultural Council. Contact information: (509) 453-3193, carter@nwhort.org

Several chemical suppliers have donated supplies, other resources and time to discuss aspects of this project and ensure relevancy to the apple packing industry.

An equipment donation from Aquapulse systems was made to WSU with estimated value of $10,000 arrived in 2011 for chlorine and chlorine dioxide experiments.

Several apple packing facilities have participated in packing plant studies or with insight on packing conditions to ensure that laboratory results relate to large scale commercial treatments.

Total Project Request: Year 1: $50,990 (awarded) **Year 2:** $53,030 (awarded) **Year 3:** $55,152 (awarded).

Other funding sources

Agency Name: Washington State USDA Specialty Crop Block Grant Program
Amt. awarded: $55,868

Notes: This project supported a literature review, initial laboratory experiments to select methodology for apple inoculation and an educational meeting with the tree fruit industry. The literature review provided information on the state of knowledge regarding antimicrobial interventions for whole, fresh apples, which was found to be relatively limited. Additionally, the review of literature indicated that methodology used in evaluating antimicrobial interventions for apples varied significantly between studies. Therefore, microbial studies were conducted to assist in selection of methods for apple inoculation, such as preparation of apples prior to inoculation, inoculation methods and media and drying time. These experiments developed a foundation for methodology that was utilized in Years 1 and 2 and the proposed work of Year 3 in order to provide the industry with scientific information using standardized methods that will allow for comparison of results. An educational food safety meeting, "Safety of Northwest Produce" was conducted with 100 participants, primarily from the Washington tree fruit industry, and provided important opportunities to discuss research needs with industry representatives.

Budget 1
Organization Name: WSU **Contract Administrator: Carrie Johnston**
Telephone: (509) 335-7667 **Email address: mdesros@wsu.edu**

Item	2009	2010	2011
Salaries	22,014	22,895	23,811
Benefits	1,560	1,622	1,688
Wages	5,100	5,304	5,516
Benefits	816	849	883
Equipment	0	0	0
Supplies	16,500	17,160	17,846
Travel	5,000	5,200	5,408
Plot Fees	0	0	0
Miscellaneous	0	0	0
Total	50,990	53,030	55,152

Footnotes:
[1] Additional funds are not requested

Objectives:

1) Perform laboratory validation studies to examine foodborne pathogen and indicator organism reduction by antimicrobial treatments currently used in the apple packing industry
2) Validate antimicrobial interventions under industrial packing line conditions using indicator organisms
3) Conduct appropriate food safety extension outreach with the apple packing industry

Significant Findings:

- Our study represents the most robust examination of the effectiveness of peroxyacetic acid (PAA), chlorine and chlorine dioxide on whole, fresh apples using industry-relevant concentrations and application durations as indicated by currently available scientific literature. Many food safety experts utilize a 2-3 log reduction as a benchmark when evaluating the effectiveness of a single antimicrobial intervention in a given process.

- Laboratory experiments on PAA involved treatments using water and 40, 60 and 80ppm PAA with application times typical for current industry practices (5 seconds of direct application followed by exposure times of 10, 25, 40 and 60 seconds) to represent spray bar application times and time on a conveyance system after the spray bar. Three replications for generic *E.coli* and pathogenic *E.coli* O157:H7 were completed.

    - Treatments at or near 80ppm PAA were significantly different than water and all but one treatment at 60ppm PAA were significantly different than water. PAA treatments produced a 90% microbial reduction (0.7 - 1.4 $\log_{10}$) for generic *E.coli* and pathogenic *E.coli*.

- Commercial experiments for PAA examined a single spray bar at 40, 60 or 80ppm PAA (2 seconds average direct, application time) and double spray bar at 80ppm PAA (9 seconds average direct application time). Four replications were conducted and results from two replications using generic *E. coli* are presented.

    - A single spray bar at 40, 60 or 80ppm PAA produced less than a 90% reduction in generic *E. coli* (0.3 - 0.5 $\log_{10}$ reduction), which was not different than the generic *E. coli* levels on the inoculated controls.
    - A double spray bar using 80ppm PAA (9 seconds average direct application time) produced a 0.8 $\log_{10}$ reduction in generic *E. coli* which was significantly different than the inoculated control, but not significantly different than water.

- Laboratory results using increased direct PAA application times (30, 60 or 120 seconds) demonstrated an increased, 90 - 99% (1.5 - 2 $\log_{10}$) reduction of generic *E. coli* and pathogenic *E. coli* O157:H7. These laboratory results indicate greater reductions using PAA in commercial settings are possible.

- Laboratory experiments examined chlorine dioxide, using oxidation-reduction potential (ORP) in millivolts (mV) for measurement of chlorine dioxide activity. Treatment combinations included water and concentrations of chlorine dioxide that yielded three ORP levels (665, 750, 850mV) were applied at 3 application times (2, 3.5, and 5 minutes) representing application time in dump tanks.

    - Four replications for non-pathogenic (generic) *E.coli* and pathogenic *E.coli* O157:H7 were completed. Chlorine dioxide (665, 750, and 850mV) treatments at all application times responded similarly, producing less than a 90% reduction (0.5 to 0.7 $\log_{10}$ cfu/mL) of generic *E.coli* and *E.coli* O157:H7.

- Laboratory experiments examined chlorine, using ORP in mV for measurement of chlorine activity. Treatment combinations included a water treatment, and concentrations of chlorine that yielded three ORP levels (665, 750 and 850 mV ORP), and three application times (2, 3.5 and 5 minutes) to represent time of application in dump tanks.

    - Based on industry input of common practices, an additional treatment of phosphoric acid buffer (approximate pH 3.5) was included to represent treatment of apple varieties when chlorine is not used in the dump tank.
    - Five replications for generic *E. coli* and pathogenic *E. coli* O157:H7, respectively, were completed. Chlorine (665, 750 and 850mV ORP) and phosphoric acid treatments responded similarly producing less than a 90% reduction (0.5 to 0.8 $\log_{10}$) reduction of generic *E.coli* and *E.coli* O157:H7.

- Bacterial adherence to apples was not consistent over the four year study. However, there was no difference between the response of pathogenic *E. coli* O157:H7 or generic *E. coli* to chlorine, phosphoric acid or chlorine dioxide.

    - In two years of the study, experiments indicated pathogenic *E. coli* O157:H7 had greater adherence to apples than generic *E. coli*; whereas in two years of the study, pathogenic *E. coli* O157:H7 and generic *E. coli* adhered at similar levels to apples.
    - In one experiment using only generic *E. coli*, reduced levels of adherence on apples were observed.
    - Seasonal variation and apple physiology may influence bacterial adherence to apples.

Results and Discussion:

Objective 1.

<u>Laboratory Examination of Peroxyacetic Acid (PAA) at Spray Bar Application Time and Exposure Durations.</u>

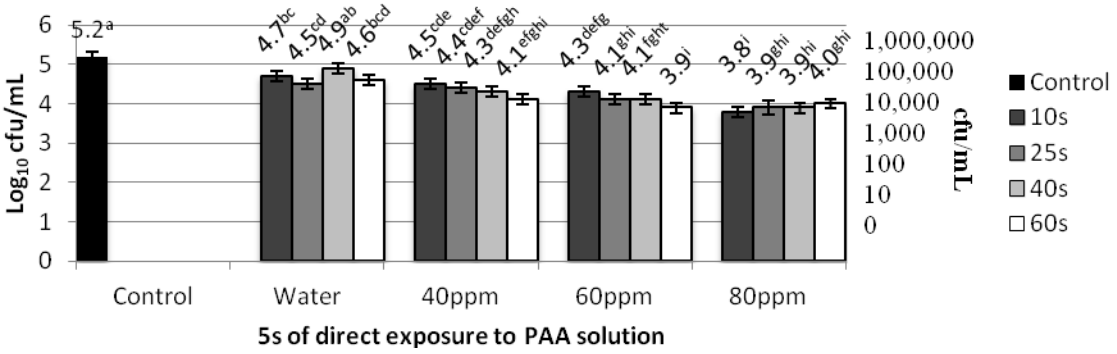
Three replications examining generic *E. coli* and pathogenic *E. coli* O157:H7 on washed, Gala apples were performed. The compounds and concentrations tested included: water and peroxyacetic acid at 3 concentrations (40, 60 and 80ppm). Apples were placed directly in water or PAA treatments for 5 seconds of application time followed by removal, and 4 exposure durations were examined (10, 25, 40 or 60 seconds) to mimic time on a conveyance system, based on industry

input and plant visits. For the experiments, 5 apples were examined in each of the 16 treatment combinations. Uninoculated and inoculated control treatments were also examined.

For inoculated control treatments, the levels of generic *E. coli* and pathogenic *E. coli* O157:H7 on the apple surface after inoculation were similar. Therefore, in this experiment, bacterial adherence was similar between generic and pathogenic *E. coli* O157:H7. There was no difference in response to PAA treatments between generic and pathogenic *E. coli*. At any concentration of PAA (40, 60, or 80ppm) exposure duration did not significantly increase bacterial reduction.

At concentrations and exposure durations typically used by the industry, the laboratory study indicated PAA treatments reduced bacterial levels about 90% (0.7 - 1.4 $\log_{10}$ reduction) (Figure 1). Treatments at or near 80ppm PAA were significantly different than water and all but one treatment at 60ppm PAA were significantly different than water.

Figure 1. Average bacterial levels (generic *E. coli* and *E. coli* O157:H7) on apple surfaces after inoculation and direct application of peroxyacetic acid (40ppm, 60ppm, 80ppm) for 5s followed by air-drying exposure times (10s, 25s, 40s, 60s) in a laboratory study. Values reported in $\log_{10}$ colony forming units (cfu)/ml scale (5=100,000 cfu/ml, 3=1000cfu/ml).

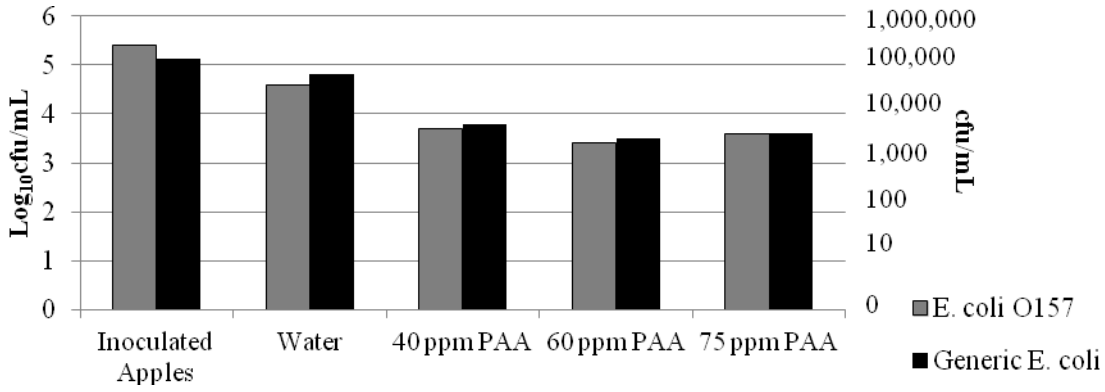


a-i For treatments not sharing a common superscript differ($p<0.05$).

Laboratory Examination of Peroxyacetic Acid (PAA) at Application Times of 30 – 120 seconds.

Three replications were performed examining generic *E. coli* and pathogenic *E. coli* O157:H7 on washed, Gala apples. The compounds and concentrations tested included: water and peroxyacetic acid at 3 concentrations (40, 60 and 75ppm). Apples were placed in water or PAA treatments for longer, direct application times (30, 60 and 120 seconds). Overall in this experiment, *E. coli* O157:H7 and generic *E. coli* appeared to have similar responses to treatments in this experiment. For some treatments, duration of application (30, 60 or 120 seconds) did not appear to affect microbial reduction. For generic *E. coli* and pathogenic *E. coli* O157:H7, when longer, direct application times (30-120 seconds) of peroxyacetic acid were used, a 90-99% reduction (1.5 – 2 $\log_{10}$ reduction) was observed (Figure 2).

Figure 2. Average *E. coli* O157:H7 and generic *E. coli* levels (log_{10} colony forming units/ml, cfu/ml) on apples after microbial inoculation and treatment with 40, 60 and 75 ppm peroxyacetic acid (PAA). Values are averaged over application times of 30, 60 and 120 seconds. Values are reported in log_{10} scale (5=100,000 cfu/ml, 3=1000cfu/ml).



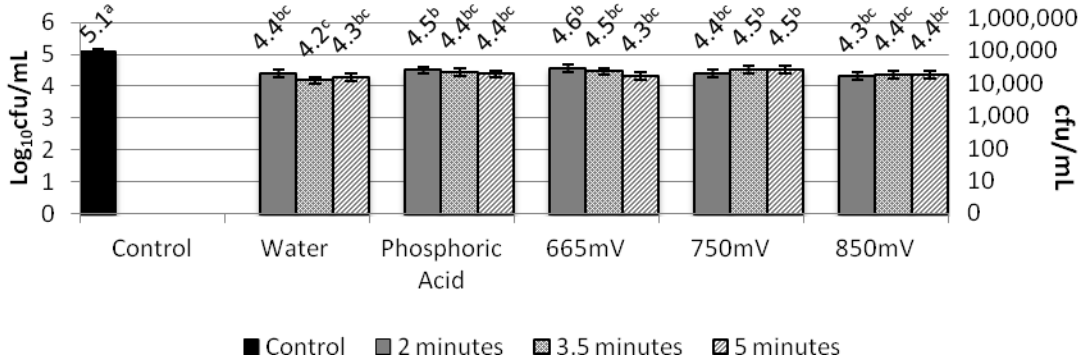
Laboratory Examination of Chlorine and Phosphoric Acid Treatments at Dump Tank Application Times.

Five replications examining generic *E. coli* and pathogenic *E. coli* O157:H7 on washed, Gala apples were performed. Industry input indicated that a treatment of phosphoric acid alone should be included to mimic dump tank treatment of apple varieties that do not involve the use of chlorine. The compounds and concentrations tested included: water, phosphoric acid (pH 3.5) and three concentrations of chlorine as measured by oxidation-reduction potential (ORP) (665, 750 and 850 mV). To mimic dump tank application, three application times were examined (2, 3.5 and 5 minutes).

For inoculated control treatments, levels of pathogenic *E. coli* on apples were higher than levels of generic *E. coli*. Therefore, in this experiment, adherence of pathogenic *E. coli* O157:H7 was higher after initial inoculation compared to non-pathogenic, generic *E. coli*. However, there was no difference in the response of pathogenic *E. coli* O157:H7 and generic *E. coli* to the chlorine treatments. Also, application time (2, 3.5 and 5 minutes) did not appear to significantly affect bacterial reduction.

For generic *E. coli* and pathogenic *E.coli* O157:H7, all treatments including water had significantly lower bacterial levels than the inoculated controls (Figure 3). All chlorine treatments (665, 750 and 850mV ORP) produced bacterial reductions similar to water, less than a 90% reduction (0.5 - 0.9 log_{10} reduction). Similarly, phosphoric acid produced results that were similar to water for generic *E. coli* and *E. coli* O157:H7, less than a 90% reduction (0.6 - 0.9 log_{10} reduction). While the laboratory data indicated that microbial levels were not reduced on apple surfaces when compared to water, it is likely that chlorine controls cross-contamination risk by controlling microbial levels in the treatment solution.

Figure 3. Average bacterial levels (generic *E. coli* and *E. coli* O157:H7) on apple surfaces after inoculation and direct application of chlorine (target ORP levels 665mV, 750mV, 850mV) and phosphoric acid (pH 3.5) for 2, 3.5 and 5 minutes in a laboratory study. Values reported in log_{10} colony forming units (cfu)/ml (5=100,000 cfu/ml, 3=1000cfu/ml).



a-c treatments that do not share a common superscript differ ($p<0.05$).

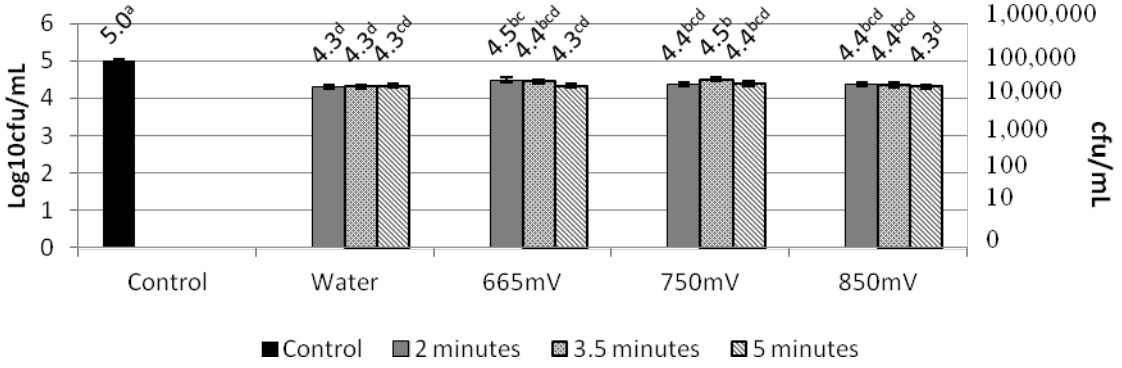
Laboratory Examination of Chlorine Dioxide Treatments at Dump Tank Application Times.

Four replications examining generic *E. coli* and pathogenic *E. coli* O157:H7 on washed, Gala apples were performed. The compounds and concentrations tested included: water and three concentrations of chlorine dioxide measured by ORP (665, 750 and 850mV). To mimic dump tank application times, three application times were examined (2, 3.5 and 5 minutes).

For inoculated control treatments, levels of pathogenic *E. coli* on apples were higher than generic *E. coli*. Therefore after initial inoculation in this experiment, adherence of pathogenic *E. coli* O157:H7 was higher compared to non-pathogenic, generic *E. coli*. However, there was no difference in the response of pathogenic *E. coli* O157:H7 and generic *E. coli* to the chlorine dioxide treatments. Application time (2, 3.5 and 5 minutes) did not appear to significantly affect bacterial reduction.

For generic *E. coli* and pathogenic *E.coli* O157:H7, all treatments including water had significantly lower bacterial levels than the inoculated controls (Figure 4). All chlorine dioxide treatments (665, 750 and 850mV ORP) for generic *E. coli* and pathogenic *E.coli* O157:H7 were similar to water treatments and produced less than a 90% reduction (0.5 to 0.7 log_{10} reduction). While the laboratory data indicated that microbial levels were not reduced on apple surfaces when compared to water, it is likely that chlorine dioxide controls cross-contamination risk by controlling microbial levels in the treatment solution.

Figure 4. Average bacteria levels (generic *E. coli* and *E. coli* O157:H7) on apple surfaces after inoculation and direct application of chlorine dioxide (target ORP levels 665mV, 750mV, 850mV) for 2, 3.5 and 5 minutes in a laboratory study. Values reported in log_{10} colony forming units (cfu)/ml (5=100,000 cfu/ml, 3=1000cfu/ml).



a-d treatments that do not share a common superscript differ ($p<0.05$).

Objective 2.

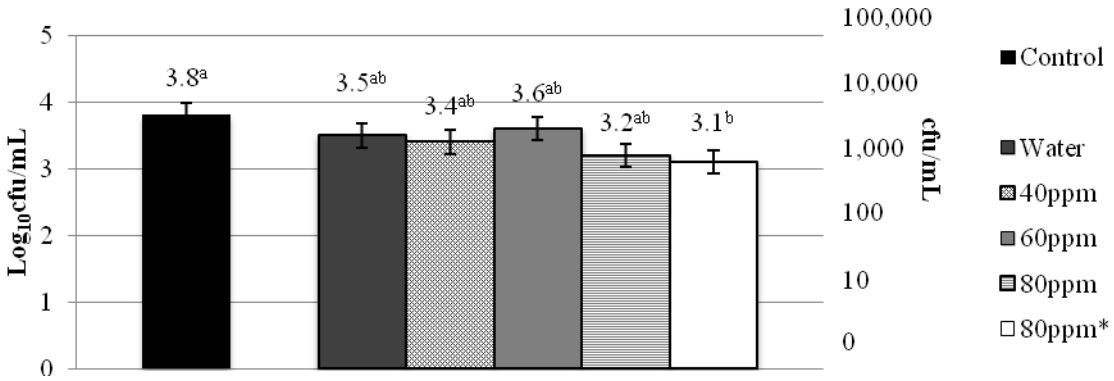
Commercial Examination of Peroxyacetic Acid Spray Bar Applications.

Four replications at a commercial packing facility were performed to assess PAA spray bar applications. Data from the first two replications indicated that generic *E. coli* adherence was reduced in 2011 compared to 2009 and 2010 using the same methods; this observation coincided with an observation from an industry partner that fungicide residue adherence on apples was also reduced in the same year. Therefore, for replications 3 and 4, the concentration of the inoculum was doubled.

The use of different nozzles was identified as a potential influencing factor. Experiments focused on examining 40, 60 and 80ppm PAA using high pressure nozzles (the more common industry practice) in all four replications. A low pressure nozzle treatment at 40ppm PAA was included in the first two replications. Data suggested that the high and low pressure nozzles did not affect reduction of generic *E. coli* levels, so this treatment was removed in order to examine increased application time using a double spray bar at 80ppm PAA application in two replications.

Data examining inoculated apples prior to and after the spray bar will be discussed from the last two replications as this data was the most consistent due to fluctuations in microbial levels as noted above (Figure 5). All treatments achieved less than a 90% reduction (<0.1 log cfu/ml). Only the double spray bar treatment was significantly different than the inoculated control but was not significantly different than the water treatment. The water treatment produced a 0.3 log reduction and treatment with 40ppm PAA and 60 ppm PAA produced a 0.4 and 0.2 log reductions, respectively. However, 80ppm PAA achieved a 0.6 log reduction. Average direct application time under the single spray bar treatment was 2 seconds (range 1-4 seconds). The double spray bar treatment with 80ppm PAA achieved a 0.7 log reduction with an average direct application time under the double spray bar of 9 seconds (range 6-13 seconds). Equivalent treatments examined in the laboratory studies achieved greater bacterial reductions (between 90-99%); therefore, greater reductions using PAA in commercial settings are possible.

Figure 5. Average generic *E. coli* levels on apple surfaces after inoculation and direct application of peroxyacetic acid (40ppm, 60ppm, 80ppm, 80ppm* double spray bar) for in a commercial study. Values reported in log_{10} colony forming units (cfu)/mL scale (5=100,000 cfu/ml, 3=1000cfu/ml).



a-c For treatments not sharing a common superscript differ($p<0.05$).

***Double spray bar application**

Objectives 1 and 2.

Levels of bacterial indicator organisms.

All of the experiments above involved measuring background bacterial levels. Almost all experiments involved Gala apples to reflect average wax levels among typical varieties grown in the Pacific Northwest; when Galas were not available Braeburn apples were used. Information on apple handling varied. In all experiments, unwaxed apples were used. In a few experiments, organic apples were used. In most experiments, pre-sized apples were used; these apples received a chlorine antimicrobial treatment.

In total, 200 apples were examined for total coliforms and generic *E. coli*, and 170 apples were examined for aerobic plate counts. Aerobic bacterial counts could not be recovered from organic apples due to mold growth on the tryptic soy agar. It is acknowledged that these results represent a relatively small sample size.

Indicators of fecal contamination (total coliforms and generic *E. coli*) were not detected on 86.5 – 98.5% of the apples, respectively. Higher levels (100-1000 cfu/ml) of total coliforms were detected on 4% of the apples and higher levels (100-1000 cfu/ml) of generic *E. coli* were detected on 0.5% of the apples. These levels are similar to other published literature. For overall bacterial levels (aerobic plate counts), 71.7% averaged 100 or fewer cfu/ml, 20% averaged 1,000 cfu/ml and 8.2% averaged 10,000-100,000 cfu/ml. These values align with or are lower than other published literature. The data indicate that fecal contamination levels and overall bacterial levels on apples are not consistent, and the risk of pathogen contamination exists.

Table 1. Average values for background microflora and percent of total apples examined for aerobic bacteria, total coliforms and generic *E. coli* on the surface of whole fresh apples. Values reported in log_{10} colony forming units (cfu)/mL and equivalent value of colony forming units provided.

Microbial level	Total Coliform (n=200)	Generic *E.coli* *(n=200)*	Aerobic Bacteria (n=170)
Not Detected	173 (86.5%)	197 (98.5%)	0 (0.0%)
<1 Log *<10 colonies*	8 (4.0%)	2 (1.0%)	15 (8.8%)
1 Log *10 colonies*	10 (5.0%)	0 (0.0%)	58 (34.1%)
2 Log *100 colonies*	7 (3.5%)	1 (0.5%)	49 (28.8%)
3 Log *1,000 colonies*	2 (1.0%)	0 (0.0%)	34 (20.0%)
4 Log *10,000 colonies*	0 (0.0%)	0 (0.0%)	8 (4.7%)
5 Log *100,000 colonies*	0 (0.0%)	0 (0.0%)	6 (3.5%)
6 Log *1,000,000 colonies*	0 (0.0%)	0 (0.0%)	0 (0.0%)

Objective 3.

Food Safety Outreach and Education. Two workshops were conducted and attended by several representatives from the tree fruit industry. The workshop "Farm Production Practices for Food Safety" featured 4 national speakers, 3 regional speakers and 4 WSU faculty. Over 70 participants attended the workshop. Presentations were also delivered at the Washington State Horticultural Association meetings in 2010, 2011 and 2012.

The workshop "Safety of Northwest Produce" featured 1 national speaker, 3 regional speakers and 2 WSU faculty. Over 100 participants attended, including growers and packers, processors, suppliers, sales and marketing representatives, laboratory technical staff, trade association representatives, consultants and extension personnel. Participants reported increased knowledge in 7 food safety topics. Half to two-thirds of participants agreed that the information gained at the workshop would be useful in communicating to others in their operation about food safety, working with other groups on food safety and conducting food safety training in their operations. Some participants indicated that as a result of the workshop they would do the following: refine or implement food safety practices, share food safety information with employees, communicate the importance of food safety and share food safety information with management.

Executive Summary:

The objectives of the study were to conduct laboratory and commercial experiments to examine the ability of commonly used antimicrobial compounds in the apple packing industry to reduce foodborne pathogen contamination risk and conduct appropriate extension outreach. Selected antimicrobials were: peroxyacetic acid (PAA), chlorine, phosphoric acid and chlorine dioxide at concentrations and application times relevant to the industry. Laboratory studies examined the response of pathogenic *E. coli* O157:H7 and compared the response of generic *E. coli*. Only generic *E. coli* (non-pathogenic) were examined in commercial studies. Therefore, the laboratory comparison was important to understand differences between pathogenic and generic *E. coli* so that accurate estimation of pathogen reduction with commercial scale interventions could be performed.

Laboratory studies examining peroxyacetic acid (PAA) simulating typical industry practices, concentrations of 40, 60 and 80 ppm and 5 seconds of direct application resulted in up to a 90% (0.7 - 1.4 $\log_{10}$) reduction of pathogenic *E. coli* O157:H7 and generic *E. coli*. A commercial study examining typical industry practices with PAA, a single spray bar of 40, 60 or 80ppm PAA and 2 seconds average direct, application time produced less than a 90% reduction in generic *E. coli* (0.3 - 0.5 $\log_{10}$ reduction). A double spray bar at 80ppm PAA (9 seconds average direct, application time) produced a significant, but less than 90% reduction (0.8 $\log_{10}$ reduction) in generic *E. coli*. Laboratory studies using longer, direct application times (30, 60 and 120 seconds) of PAA at 40, 60 and 80ppm PAA produced a greater reduction, up to 99% (1.5-2 $\log_{10}$) for pathogenic *E. coli* O157:H7 and generic *E. coli*. Therefore, enhanced reductions using PAA in commercial settings are possible. A 99% (2 log) bacterial reduction for a single intervention would likely be viewed more favorably as an antimicrobial intervention by regulatory officials.

Laboratory studies examined chlorine and chlorine dioxide using typical industry practices, concentrations yielding 665, 750 and 850mV oxidation reduction potential (ORP) and direct application times of 2, 3.5 and 5 minutes as well phosphoric acid (pH 3.5) at direct application times of 2, 3.5 and 5 minutes. All treatments resulted in a less than 90% reduction (0.5 – 0.8 $\log_{10}$). While the laboratory data indicated that microbial levels were not reduced on apple surfaces when compared to water, it is likely that chlorine and chlorine dioxide control cross-contamination risk by controlling microbial levels in the treatment solution.

An important finding of the study was that the adherence of pathogenic *E. coli* O157:H7 and generic *E. coli* are not always similar, with pathogenic *E. coli* O157:H7 having greater adherence to apples in some cases. An increased understanding of the response of pathogenic *E. coli* on apples and the potential influence of apple physiology on bacterial interactions would provide the industry with advanced knowledge to enhance food safety efforts.

A limited examination of initial microbial levels on apples was performed. Information on treatment of the apples prior to receiving was not always available; many of the apples had been pre-sized and treated with a chlorine antimicrobial. Indicators of fecal contamination (total coliforms and generic *E. coli*) were not detected on 86.5 – 98.5% of the apples, respectively. Higher levels (100-1000 cfu/ml) of total coliforms were detected on 4% of the apples and higher levels (100 cfu/ml) of generic *E. coli* were detected on 0.5% of the apples.

Although the presence of fecal contamination was not often detected, there is a need to ensure that when higher levels of fecal contamination are encountered, the contamination risk is controlled by preventing cross-contamination and reduced through the use of effective antimicrobial interventions. Based on the data in this study, current apple packing interventions likely reduce pathogen risk less than 90%; therefore, there is a need to enhance current antimicrobial interventions to more effectively reduce pathogen risk.

FINAL PROJECT REPORT

Project Title: Sensory and consumer acceptance of advanced apple breeding selections

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Total Project Funding: $87,155

Budget History:

Item	Year 1: 2010	Year 2: 2011	Year 3: 2012
Salaries[1]	22,014	22,895	24,542
Benefits[2]	1,881	1,956	1,897
Equipment			
Supplies[3]	4,000	4,000	4,000
Travel			
Plot Fees			
Miscellaneous			
Total	$27,865	$28,851	$30,439

Footnotes:
1 Salaries: One MS graduate student was supported by this research (9 month salary)
2 Benefits: includes health insurance and medical aid
3 Supplies: includes chemical reagents, sensory panels supplies (consumables consisting of paper plates, towels, cuspidors, forks, plastic wrap, tape, saltines, photo copies, participation incentives).

ORIGINAL OBJECTIVES:
The overall objective of this study was to study newly developed Washington State apple selections and characterize the sensory properties of these new selections. The sensory properties of these apple selections were then related to consumer acceptance. Specific objectives were to:
1) Profile new apple selections using trained panelists. Working with Dr. Kate Evans, we identified promising apple selections ready to move to the next phase of development. Panelists were trained to describe important sensory properties of apples. The trained panel then developed sensory profiles for each of the new apple selections.
2) Consumer panel evaluation of new selections of Washington State apples. Consumers evaluated each apple selection for their liking of various sensory attributes.

SIGNIFICANT FINDINGS:
In **2010**, a series of commercial apple varieties were profiled in order to establish baseline profiles for these apples.

- Fuji – high perceived sweetness, apple flavor, firmness
- Gala – moderate sweetness, high firmness
- Honeycrisp – high crispness, moderate sweetness, high apple flavor
- Pink Lady – moderate sweetness, sourness, apple flavor
- Granny Smith – high sourness, astringency and firmness; low sweetness
- The analytical measures of crispness, firmness and mealiness (using the DigiTest), sweetness (percent soluble solids), sourness (titratable acidity) supported the results found by the trained panel.

In **2011**, the apple selections that were evaluated varied in their sensory properties and consumer acceptance based on apple selection, pick date and growing site. Specifically:

- WA5 (Brewster Pick 2), WA5 (Quincy Pick1) and Honeycrisp were rated highly by the trained panel for texture attributes and were highly acceptable by a consumer panel.
- For WA5 (Brewster), harvest time had a significant impact on texture attributes, with Pick2 being significantly higher in texture intensity. Consumers found significant differences in texture attributes and overall acceptance (Pick 2 higher).
- Growing location also influenced consumer acceptance with WA5 (Pick1); apples grown in Quincy had higher acceptance for crispness and firmness compared to those grown in Brewster.
- Honeycrisp (Pick2) was highly rated for sensory attributes as evaluated by the trained panel, but had low mealiness and astringency.
- WSU7 (Quincy, Pick1 and 2) were high in juiciness and firmness, moderately high in sweetness, high in sourness and high in apple flavor intensity as evaluated by the trained panel.
- For WSU7 (Quincy), harvest time had a significant impact only on juiciness acceptance, with Pick2 having a higher juiciness acceptance compared to Pick1.

In **2012**, apple selections were profiled by trained panelists and consumers.

- Spring 2012: WA2 and WA38 were compared in a pairwise comparison to Gala in a consumer test in Spokane. Consumers (n=120) at River Park Square (shopping center in Spokane WA) were asked which of the two apples they preferred for the attributes of appearance, taste/flavor and texture and to indicate the sample they preferred overall. Consumers significantly preferred WA2 over Gala for all attributes. WA38 was also compared to Gala in the consumer testing in Spokane WA. Results showed that consumers significantly preferred WA38 to Gala based on appearance and texture. Consumers (n=100) in Pullman WA identified WA2 fruit as having greater overall acceptance compared to Gala ,

with acceptance of firmness, crispness and juiciness being statistically significant. Consumers in Pullman WA also found WA38 to be more acceptable based on texture attributes and overall acceptance.

- Spring 2012: Trained panelists evaluated 8 apple selections (Gala as the control), with several of the apple selections being stored under different storage conditions: Jazz (MCP +/-), WA38 (MCP +/-), WA2 (RA/CA), WSU19 (MCP) and Ambrosia (MCP). Some differences were observed due to the application of MCP during storage. Gala rated lower in many sensory attributes compared to the other apples. Pullman consumer panel (n=100) evaluation of these apples showed that WA2 (RA, CA), Jazz+MCP and WSU19 (MCP), WA38 (MCP) were rated highly for overall acceptance. Ambrosia, Gala and Jazz (-MCP) were liked less by the consumers.

- Fall 2012: Consumers (n=80) and trained panelists evaluated 8 Phase 2 apple selections (Gala as the control). On Day 1, significant differences in overall acceptance of the apple selections were found, with WSU 52 being liked significantly more overall than WSU 61 and the Gala control. A similar pattern was found for apple flavor intensity, with WSU 52 being liked significantly more than WSU 50, WSU 61 and the Gala control. The acceptance of the taste attributes of sweetness and sourness also differed among apples. The sweetness and sourness of WSU 52 was liked more than these same attributes in Gala. For the texture attributes, WSU 64 and WSU 50 were liked the most for firmness, crispness and juiciness, while WSU 61 and Gala were liked the least for these texture attributes. On Day 2, WSU 81, WSU 92, WSU 82, WSU 65 and Gala (control) were evaluated. Fewer differences were noted. Apple flavor intensity differed among apples, with the Gala apple being the lowest in liking (as observed in Day 1). Gala was also lower in acceptance based on the attributes of juiciness and sweetness.

- Fall 2012: Trained panelists evaluated the following selections: WSU 50, WSU 52, WSU 61, WSU 64, WSU 65, WSU 81, WSU 82, WSU 92, and Gala. WSU 82 was the sweetest selection and only significantly differed from WSU 52 and WSU 50, which were the two least sweet. Unlike with sweetness, there was a high variation between apples in terms of sourness. WSU 92 was the most sour variety and significantly differed from the four least sour varieties, WSU 61, WSU 82, Gala, and WSU 81. WSU 64 was the highest in apple flavor and only significantly differed from Gala. WSU 50 had the highest crispness intensity and Gala had the lowest. Gala was also significantly different from WSU 50, WSU 65, WSU 92, and WSU 64 for crispness. WSU 64, WSU 50, WSU 92, and WSU 65 were the most firm varieties. All varieties had at least medium levels of juiciness, with WSU 50 being the highest, and WSU 81 and Gala being the lowest.

RESULTS AND DISCUSSION:

Spring 2012

Methods:

Apples: The selections included in this study were selected in collaboration with the WTFRC: Jazz (MCP +/-), WA38 (MCP +/-), WA2 (RA/CA), WSU19 (MCP), Ambrosia (MCP) and Gala (CA+MCP). In order to minimize the effects of environment, all the fruit was sourced from the breeding program Phase 3 orchards in Quincy and nearby commercial orchards by the WTFRC staff. The samples were delivered to Pullman one week prior to the consumer and trained panel assessment and stored in a cold room at 37F until needed. A chain of custody document was completed for each sample. Fruit from each sample were also tested by the WABP lab in Wenatchee prior to assessment.

Trained Panel Evaluations: The trained panel was composed of 10 individuals. The panelists were trained over 15 hours using techniques described by Meilgaard et al. (1999). The apple attributes were selected using reported literature and previous studies performed in our lab. Panelists were trained to recognize apple flavor (sweetness, sourness, apple flavor intensity and astringency) and texture (firmness, crispness, juiciness and mealiness). Evaluations took place in individual sensory booths equipped with laptop computers for recording data. Following training sessions, apple selections were presented to each panelist for evaluation in replicate. Panelists were presented with 1/8 of the apple under study. The apple selections were randomly presented to the panelists at room temperature and under white lighting conditions. Panelists were asked to indicate the intensity of the apple attributes described above. Results were collected using Compusense 5.0 software (Guelph, ON) and analyzed using ANOVA and Tukey's HSD.

Consumer Panel Evaluation: Consumer evaluations were conducted in two sites: Pullman WA (February 21 and 23 2012) and Spokane WA (March 3 2012) in River Park Square, a popular shopping center located downtown Spokane. For all consumer panels, consumers were recruited using advertising, posters and e-mail. In Pullman, evaluations took place in individual sensory booths equipped with laptop computers. Consumers (n=100) were presented with 1/8 apple of the apple selections. Honeycrisp and Gala were presented as controls. Consumers indicated their overall acceptance and the acceptability of flavor (sweetness, sourness, and apple flavor intensity) and texture (firmness, crispness, and juiciness) attributes for each apple selection. All attributes were evaluated by the panel using a 7-point scale (1 = dislike very much, 7 = like very much). Results were collected and analyzed as described above. Due to the number of samples, two days of consumer evaluations were conducted. On Day 1, consumers evaluated WSU19(-MCP), Ambrosia(+MCP), WA2 (CA), Jazz(+MCP) and Gala (+MCP control). On Day 2, consumers evaluated WA38(+/-MCP), WA2(RA), Jazz(-MCP) and Gala (+MCP control).

In Spokane, consumers (n=120) were presented with two pairs of apple samples: WA2 (CA/-MCP) and Gala(+MCP control)and WA38 (CA/-MCP) and Gala(+MCP control). For each pair of samples, consumers were asked which of the two apples they preferred for the attributes of appearance, taste/flavor and texture and to indicate the sample they preferred overall. They were presented with a slice of apple for tasting but were also shown a whole apple in order to judge appearance. In both Pullman and Spokane, consumers were from diverse ethnic backgrounds and ranged from 18 to 70 with approximately 38% of subjects under the age of 35. Approximately 60% of subjects were female and the majority of consumers ate apples at least once to several times a week and grew up in the Northwest U.S.A.

Results

Consumer Panel Evaluations: In the Spokane consumer testing, consumers preferred WA2 over Gala for all attributes (Table 1). When comparing WA38 to Gala, consumers significantly preferred WA38 based on appearance and texture ($p<0.05$).

Table 1. Consumer scores indicating the preferred sample in a pair comparing WA 2 (CA/-MCP) to Gala (CA/+MCP). Data represent 120 consumer responses with * $p<0.05$.

	Number of consumers preferring each sample		
	WA 2	Gala	Total
Overall	88*	32	120
Appearance	85*	34	119
Taste/Flavor	82*	38	120
Texture	88*	32	120

In the Pullman WA consumer testing, 100 consumers were asked to score different attributes on a 7-point hedonic scale on a number of different apples including the same samples of WA 2 (CA/-MCP) and Gala (CA/+MCP). Once again, WA2 fruit had a greater overall acceptance compared to Gala and was preferred for all attributes, with firmness, crispness and juiciness being statistically significant (Table 2).

All of the apple selections were evaluated by consumer panels in Pullman WA. Due to the number of samples, two consumer panels on two separate days were conducted.

Day One The separation of the different apple selections based on specific attributes is shown in Table 2. Based on overall acceptance, the Gala control was rated the lowest compared to the other apple selections. Based on texture, WA2CA and Jazz+MCP were more accepted by consumers for firmness, with Gala (CA/+MCP) being the least accepted. A similar trend was observed for crispness while for juiciness, the WSU19-MCP and the Gala (CA/+MCP) apples were rated the lowest in acceptance. Based on sourness, results showed that the apple selections of WSU19-MCP, WA2CA and Jazz+ MCP were the most accepted with similar results found for sweetness acceptance.

Day Two The separation of the different apple selections evaluated by the consumers on Day 2 is shown in Table 3. Based on overall acceptance, the WA2 (RA) and WA38 (+MCP) apples were the most accepted by the consumers. A similar pattern was observed for acceptance of apple flavor intensity. For texture attributes, Gala (CA/+MCP) was the least accepted based on firmness, as observed with the Day 1 apples. Gala (CA/+MCP) was also least accepted for crispness and juiciness, again results that were observed when comparing Gala (CA/+MCP) to the Day 1 apple selections. Regarding acceptance of sweetness, the most accepted apples were WA38(-MCP) and WA2(RA).

Table 2. Mean separation (Tukey's HSD) for consumer (n=100) acceptance of sensory attributes of five apple selections on Day 1 using a 7-pt hedonic scale (with 1= dislike very much and 7=like very much). Within each attribute, different letters indicate a significant differences ($p<0.05$).

Apple attributes	**WSU 19 -MCP**	**Ambrosia +MCP**	**WA 2CA**	**Gala** (CA/+MCP)	**Jazz+MCP**
Overall acceptance	5.6a	5.3ab	5.6a	5.1 b	5.3ab
Apple flavor intensity	5.3a	4.6 b	5.2ab	4.7 b	5.0ab
Firmness	5.4 c	5.6bc	6.1a	4.9 d	5.9ab
Crispiness	5.7a	6.0ab	6.2a	5.1 c	6.0ab
Juiciness	5.2 c	5.7ab	6.1a	5.3 bc	6.0a
Sweetness	5.7a	5.2 b	5.4ab	5.1 b	5.3ab
Sourness	5.2a	4.4 c	5.1ab	4.6 bc	4.8abc

Table 3. Mean separation (Tukey's HSD) for consumer (n=100) acceptance of sensory attributes of five apple selections on Day 2 using a 7-pt hedonic scale (with 1= dislike very much and 7=like very much). Within each attribute, different letters indicate a significant differences ($p<0.05$).

Apple attributes	**WA38+MCP**	**WA38-MCP**	**WA2RA**	**Gala**(CA/+MCP)	**Jazz-MCP**
Overall acceptance	5.1 c	5.7ab	6.0a	5.0 c	5.2 bc
Apple flavor intensity	4.8 b	5.5a	5.7a	4.5b	4.7 b
Firmness	5.9a	6.0a	6.1a	4.8 b	5.8a
Crispiness	6.1ab	6.2ab	6.3a	5.0 c	6.0 b
Juiciness	5.9ab	6.1a	6.1a	5.1 c	5.6 b
Sweetness	4.9 b	5.5a	5.8a	4.9 b	5.0 b
Sourness	4.4 c	5.1ab	5.3a	4.6a	4.6 c

Trained Panel Evaluations: The intensities of several of the sensory attributes evaluated by the trained panelists are shown in the following figures. For sourness, Ambrosia was found to be the least intense. For the texture attributes of crispness, firmness and juiciness, Gala was the least intense.

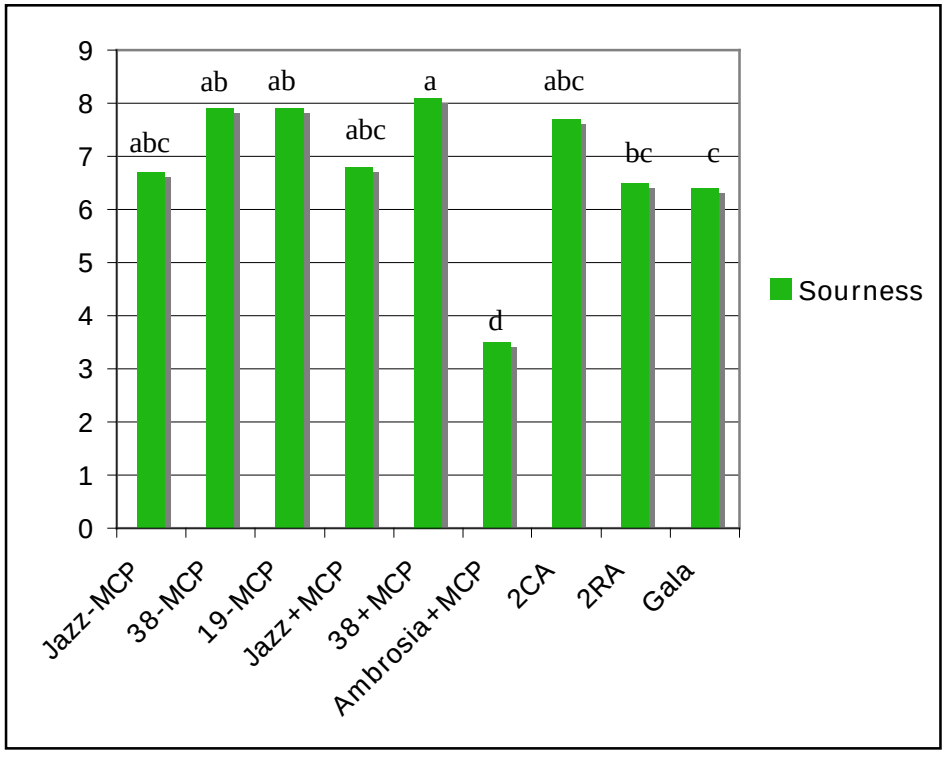

Figure 1. Separation of the 9 apples as evaluated by the trained panelists for sourness intensity (n=13).

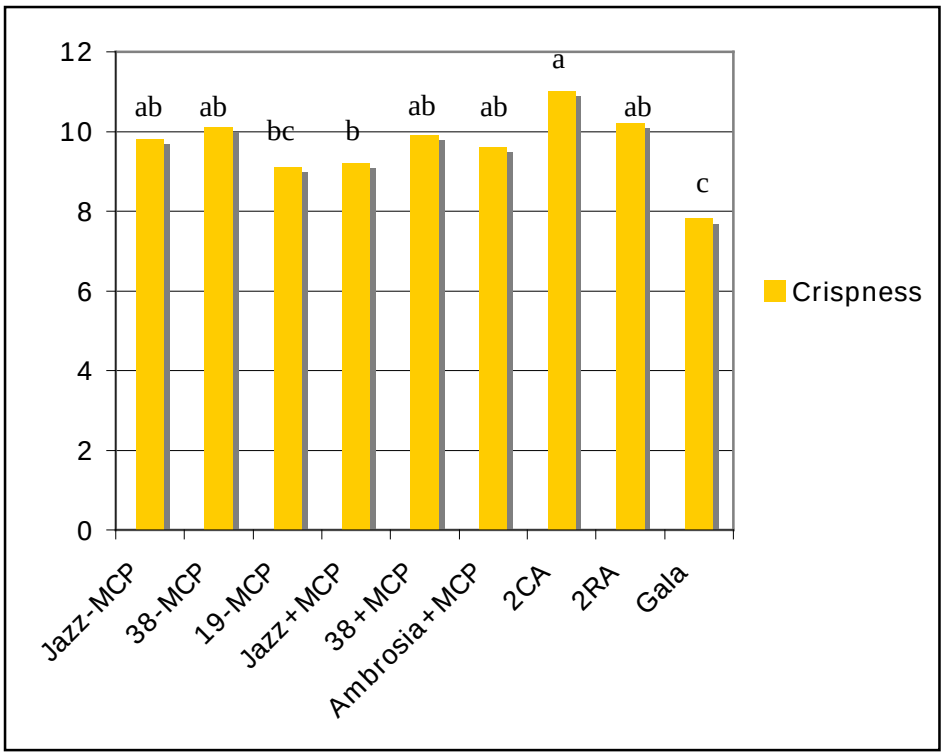

Figure 2. Separation of the 9 apples as evaluated by the trained panelists for crispness intensity (n=13).

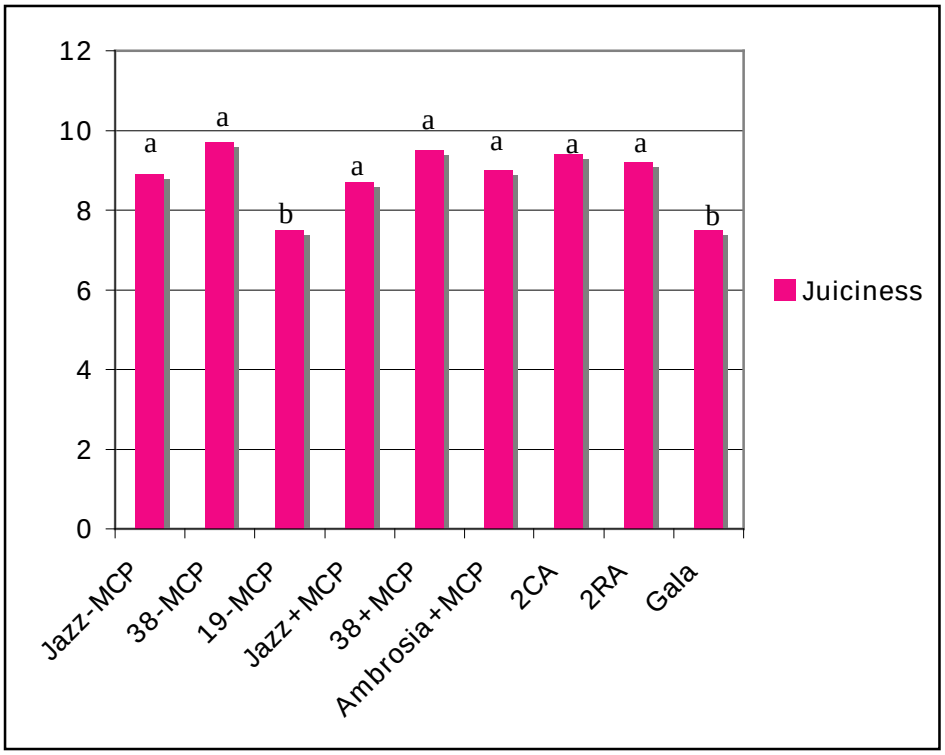

Figure 3. Separation of the 9 apples as evaluated by the trained panelists for juiciness intensity (n=13).

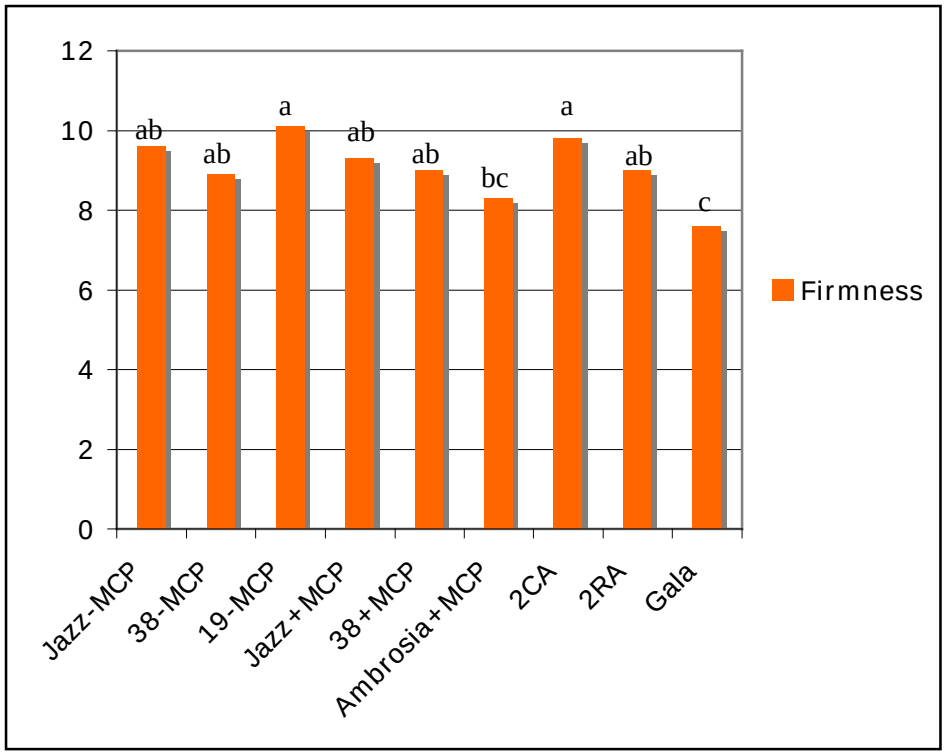

Figure 4. Separation of the 9 apples as evaluated by the trained panelists for firmness intensity (n=13).

Fall 2012

The methods used in Fall 2012 were similar to those in Spring 2012. The Phase 2 apple selections evaluated were: WSU 50, WSU 52, WSU 61, WSU 64, WSU 65, WSU 81, WSU 82, WSU 92, and Gala. The consumer panels differed in that that they were only conducted in Pullman. For the consumer panels, on Day 1, 80 consumers were presented with WSU 52, WSU 64, WSU 50, WSU 61 and Gala (control). On Day 2, 80 consumers were presented with WSU 81, WSU 92, WSU 82, WSU 65 and Gala (control). The Gala control apples were supplied by the WTFRC.

Consumer Panel Evaluations: On Day 1, significant differences in overall acceptance of the apple selections was noted (Table 4). WSU52 was liked significantly more overall than WSU 61 and the Gala control but with similar overall liking as WSU64 and WSU50. A similar pattern was found for apple flavor intensity, with WSU52 being liked significantly more than WSU50, WSU61 and the Gala control. The acceptance of the taste attributes of sweetness and sourness also differed among apples. The sweetness and sourness of WSU52 was liked more than these same attributes in Gala. For the texture attributes, WSU64 and WSU50 were liked the most for firmness, crispness and juiciness, while WSU61 and Gala were liked the least for these texture attributes. Compared to Day 1, the apples evaluated on Day 2 did not show as many differences (Table 5). No significant differences were noted among apples for overall acceptance, firmness, crispness, or sourness. Apple flavor intensity did differ among apples, with the Gala apple being the lowest in liking (as observed in Day 1). As also seen in Day 1, Gala was lower in acceptance based on the attributes of juiciness and sweetness.

Table 4. Mean separation (Tukey's HSD) for consumer (n=80) acceptance of sensory attributes of five apple selections on Day 1 using a 7-pt hedonic scale (with 1= dislike very much and 7=like very much). Within each attribute, different letters indicate a significant differences ($p<0.05$).

Apple attributes	**WSU 52**	**WSU 64**	**WSU 50**	**WSU 61**	**Gala (control)**
Overall acceptance	5.7a	5.5ab	5.5ab	5.0b	5.0b
Apple flavor intensity	5.8a	5.3ab	5.2b	5.1b	4.5c
Firmness	5.6ab	5.8a	5.9a	4.7c	5.2b
Crispiness	5.9ab	6.1a	6.1a	4.9c	5.5b
Juiciness	6.1a	5.8ab	5.7ab	5.5bc	5.2c
Sweetness	5.6a	5.4a	5.4a	5.3ab	4.9b
Sourness	5.3a	4.8ab	5.2a	4.9a	4.3b

Table 5. Mean separation (Tukey's HSD) for consumer (n=80) acceptance of sensory attributes of five apple selections on Day 2 using a 7-pt hedonic scale (with 1= dislike very much and 7=like very much). Within each attribute, different letters indicate a significant differences ($p<0.05$).

Apple attributes	**WSU 65**	**WSU 92**	**WSU 81**	**WSU 82**	**Gala (control)**
Overall acceptance	5.4	5.5	5.6	5.5	5.2
Apple flavor intensity	5.4a	5.3ab	5.4a	5.0ab	4.8b
Firmness	5.7	5.7	5.4	5.4	5.6
Crispiness	6.1	5.9	5.7	5.7	5.9
Juiciness	6.0a	5.8ab	5.9ab	6.0a	5.5b
Sweetness	5.3ab	5.2ab	5.5a	5.4ab	5.0b
Sourness	5.0	4.8	5.0	4.6	4.8

Trained Panel Evaluations: WSU82 was the sweetest selection and only significantly differed from WSU52 and WSU50, which were the two least sweet selections. Unlike with sweetness, there was a high variation among apple selections in terms of sourness (Figure 5). WSU92 was the most sour and significantly differed from the four least sour selections, WSU61, WSU82, Gala, and WSU81. All the apple selections had low intensities and little variation for astringency. WSU52 was the most astringent, with a mean intensity of 5.6, and significantly differed from the three least astringent selections, Gala, WSU65, and WSU81. There was also little variation between apples in terms of apple flavor intensity. WSU64 the highest in apple flavor and only significantly differed from Gala. All the texture attributes were found to have medium to medium-high intensities except for mealiness, which exhibited low intensity in all apple selections. WSU50 had the highest crispness intensity and Gala had the lowest crispness intensity (Figure 6). Gala was also significantly different from WSU50, WSU65, WSU92, and WSU64 for crispness. WSU64, WSU50, WSU92, and WSU65 were the most firm selections and did not significantly differ from each other (Figure 7); however, they were significantly different from the rest of the selections. All selections had at least medium levels of juiciness (Figure 8), with WSU50 being the highest, with a mean of 10.6 intensity, which significantly differed from WSU81 and Gala, the two least juicy selections. The mealiest selection was WSU81 and the least mealy selection was WSU50.

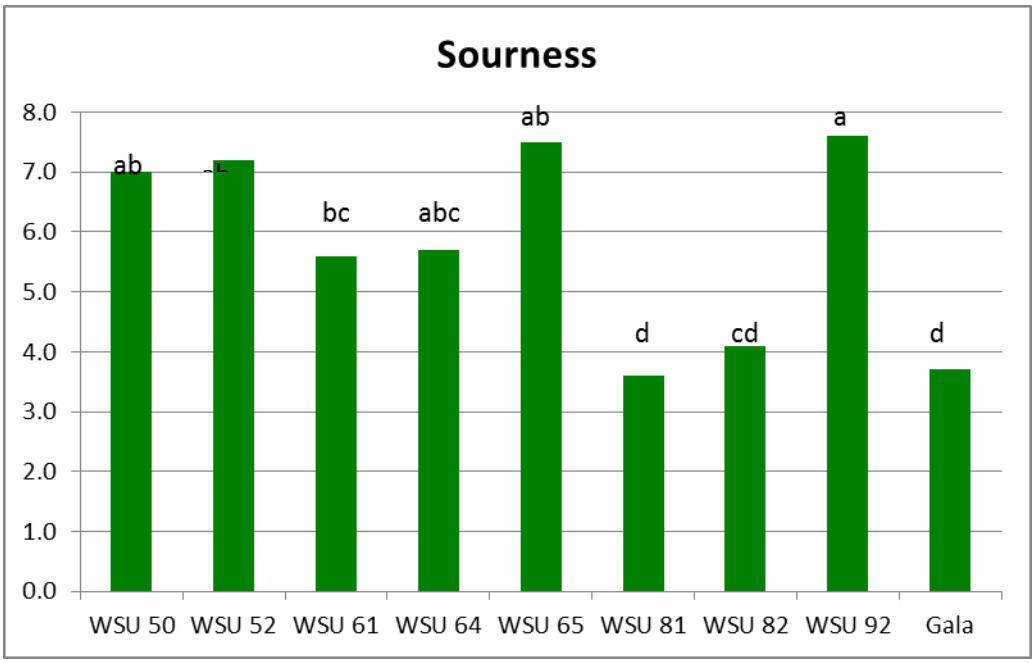

Figure 5. Separation of the 9 apples as evaluated by the trained panelists for sourness intensity (n=10).

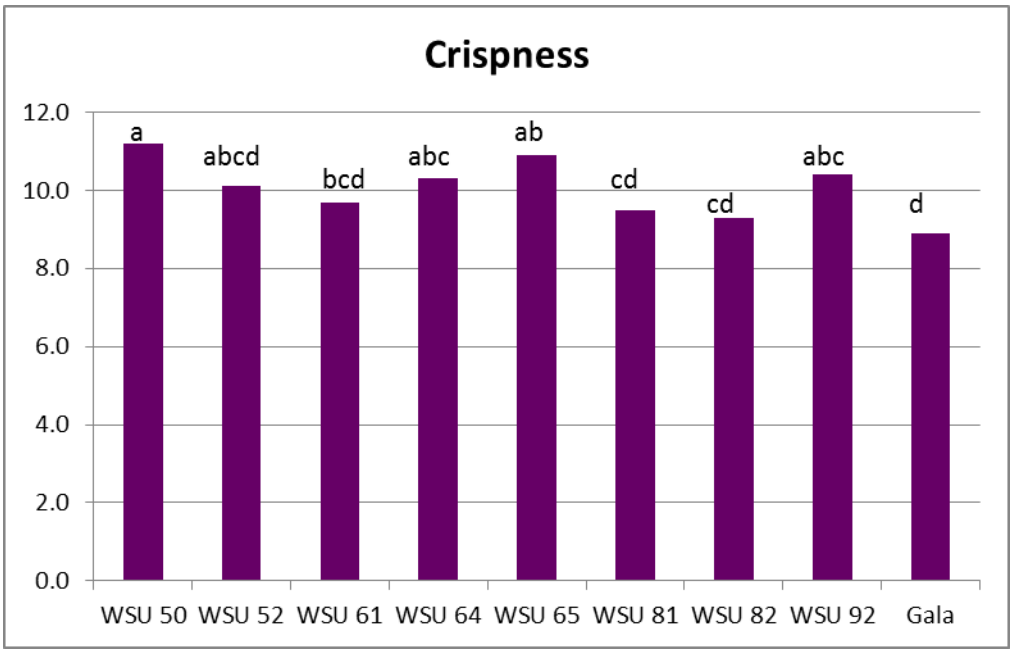

Figure 6. Separation of the 9 apples as evaluated by the trained panelists for crispness intensity (n=10).

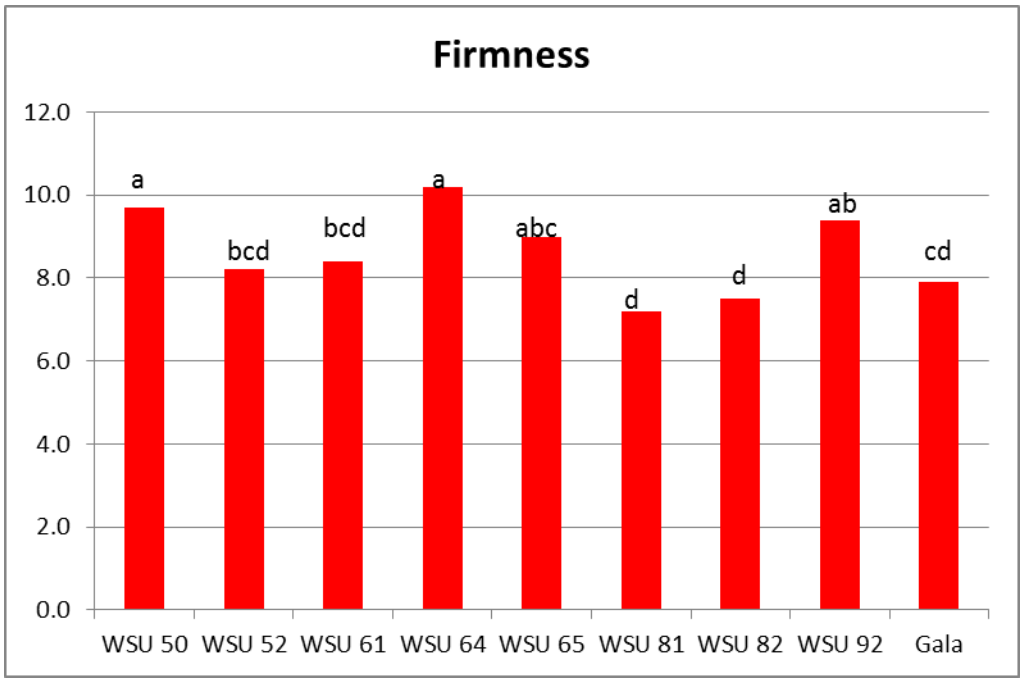

Figure 7. Separation of the 9 apples as evaluated by the trained panelists for firmness intensity (n=10).

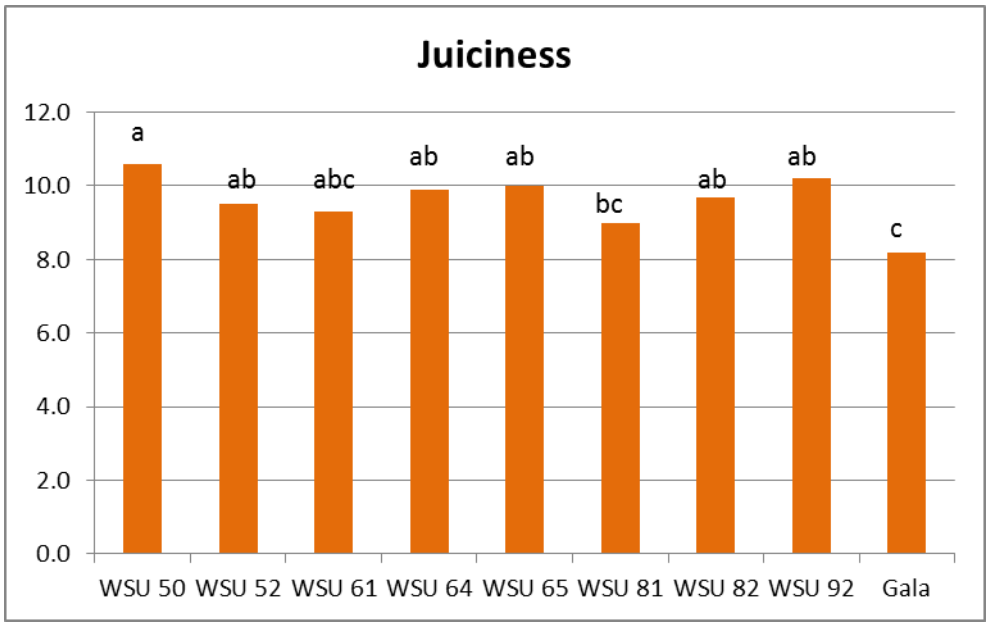

Figure 8. Separation of the 9 apples as evaluated by the trained panelists for juiciness (n=10).

EXECUTIVE SUMMARY

Significant Progress and Future Directions

The fruit industry is going through a transformation in which the consumer is given more consideration than in the past. Consequently, greater pressure has been placed on breeders and growers to develop new apple cultivars that meet the needs of the consumers. To gather consumer acceptability information, as well as detailed characterizations of the apples, sensory evaluation of new apple selections is required.

This project characterized new apple selections developed by the Washington State University Apple Breeding Program (WABP). These selections showed promise for commercialization based on the preliminary evaluation by the breeding team. However, in order to aid decisions regarding the commercialization of an advanced selection from the breeding program, further formal sensory evaluation studies were conducted on the fruit. The first step was to characterize the sensory properties of the apples using a trained sensory panel. To determine consumer acceptability, thus helping with future commercial success, the apple selections were also evaluated by consumer panels to determine the acceptance of various sensory attributes of the apples including texture, flavor, taste and appearance. These results have assisted the WABP and the WTFRC in identifying which apple selection should be recommended for commercialization and provided some information regarding the optimum storage regime required.

This project built upon several years of previous collaborative research in which researchers at the WSU sensory evaluation facility (Pullman) worked with apple breeders at the WSU Tree Fruit Research & Extension Center (Wenatchee) to perform sensory evaluation of promising apple selections. The sensory and consumer data on the advanced and elite selections from the WABP provided useful feedback to the breeding team, confirming decisions about which selection or selections to take forward for release and commercialization.

Summary of Findings:

- Established a baseline profile of five commercially available apple selections.
- Described specific sensory differences in apple selections due to pick date and growing site. For some apples, an earlier pick date maintained the sensory quality of the apples, which corresponded to a high consumer acceptance. However, for other apples such as WSU7 (Quincy), harvest time had a significant impact only on juiciness acceptance, with Pick2 having a higher juiciness acceptance compared to Pick1.
- Growing location influenced sensory properties. For example, WA5 grown in Quincy had higher consumer acceptance for crispness and firmness compared to those grown in Brewster.
- WA2 and WA38 were compared to Gala in consumer tests in Spokane and Pullman WA. Results showed that WA2 fruit was significantly preferred over Gala for appearance, taste/flavor and texture by consumers at River Park Square (shopping center in Spokane WA). WA38 was also compared to Gala in consumer testing in Spokane WA. Results showed that consumers significantly preferred WA38 to Gala based on appearance and texture. Similar results were found in Pullman WA consumer tests which found WA2 and WA38 to be more acceptable than Gala based on texture attributes and overall acceptance.
- Trained panelists evaluated 8 apple selections (Gala as the control), with several of the apple selections being stored under different storage conditions. Results showed the effect of the presence of 1-MCP on the sensory properties of some selections and varieties.
- Early testing shows that WSU64, WSU50 (high crispness and juiciness) and WSU81 are promising new selections based on their acceptance of sensory properties.
- Three years of consumer and trained panel sensory evaluation studies confirm that overall acceptance of the apple is related primarily to its texture attributes, followed by flavor.

FINAL PROJECT REPORT

Project Title: Increasing decision confidence in cultivar development and adoption

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Cooperators: Tom Auvil and Jim McFerson (WTFRC); Fred Bliss (Davis, California); Eric van de Weg (Plant Research International, Netherlands); Dorrie Main & Sook Jung (WSU, Pullman); Sujeet Verma and Yingzhu Guan (WSU PhD students)

Other funding sources

Agency Name: WTFRC Apple Review
Amount awarded: $642,160 (2012-2014)
Notes: "Apple Scion Breeding Program" PI: Kate Evans. Co-PI: Peace. The foundational program on which this "Decision Confidence" project was built. Develops new cultivars and engages interested industry members.

Agency Name: WTFRC Apple Review
Amount awarded: $59,740 (2012)
Notes: "Further development of an online toolbox for tree fruit breeding" PI: Dorrie Main. Co-PIs include Evans and Peace. Expansion of functionalities for the WABP's database. Decision Confidence project used these data and identified and proposed useful new functionalities.

Agency Name: USDA-CSREES, Specialty Crop Research Initiative
Amount awarded: $7,200,000 + same matched by universities and industry (9/09 – 8/13)
Notes: "RosBREED: Enabling marker-assisted breeding in Rosaceae". PI: Amy Iezzoni. Co-PIs include Peace, Evans, Main, Bink, and van de Weg. Relevant outputs from RosBREED project for include new genetic tests and dense genome-wide marker data mined for "genomic selection" opportunities.

Agency Name: USDA-CSREES, Specialty Crop Research Initiative
Amount awarded: $2,000,000 + same matched by universities and industry (9/09-8/13)
Notes: "Tree Fruit GDR: Translating Genomics into Advances in Horticulture". PI: Dorrie Main. Co-PIs include Evans and Peace. "Breeder's Gateway" and "Grower's Gateway" efforts of that project benefited from specific examples of follow-through such as in this Decision Confidence project, allowing programming resources in the GDR project to be leveraged here.

Total Project Funding: $89,963

Budget History:
Budget 1: Washington State University

Item	2012
Salaries[1]	8,007
Benefits[1]	4,019
Wages[2]	13,000
Benefits[2]	1,937
Equipment	0
Supplies – molecular[3]	12,000
Travel – in-state	2,000
Travel – international[4]	3,000
Plot Fees	
Miscellaneous[5]	0
Total	43,963

[1] Salary for 3 months for genetic screening technician Terrence Rowland, 0.25 FTE, 50.2% benefits.
[2] Timeslip employment for PhD students Sujeet Verma and Yingzhu Guan and field and fruit lab technicians in Wenatchee, with 14.9% benefits for enhanced phenotyping efforts.
[3] $7000 for genome scans (96 WABP individuals to enhance that available from RosBREED data) and $5000 for fill-in data and new markers for specific trait locus genetic tests on WABP individuals in Peace lab, Pullman.
[4] Trip for PI Dr. Peace to Australia for late project discussions with co-PI Dr. Hardner and Australian apple industry reps.
[5] No cost to current project: funds remaining from 2011 project will be used for consultancy fees of co-PI Dr. Bink as in 2011 proposal; funds remaining from 2011 workshop (which did not involve Drs Bink, van de Weg, or Bliss in person due to unavailability) will be used to hold a workshop in summer 2012 (Activity 4).

Budget 2: Australia Crop Genetic Services Pty Ltd

Item	2012
Salaries[1]	40,000
Benefits	
Wages	
Benefits	
Equipment	
Supplies	
Travel[2]	6,000
Plot Fees	
Miscellaneous	
Total	46,000

[1] Consultancy fees for activity 1 and 2 analyses by co-PI Dr. Hardner, 40% time of one year
[2] $6000 for travel Australia to Washington for one week

RECAP ORIGINAL OBJECTIVES

Overall goal: Improve release and adoption decisions about the WABP's new scion cultivars by revealing and communicating genetic potential for commercial performance.

Specific objectives:

1. Improve efficiency and predictability of advanced/elite selection trials by optimizing field experimental design.
2. Put rapidly accumulating DNA information to immediate use by describing genetic potential of new cultivars in the context of commercial production.
3. Demonstrate to the WA apple industry the efficacy and opportunities of a combined field-design and DNA-based approach to decision-making.

SIGNIFICANT FINDINGS

Opportunities for increasing decision confidence in new cultivar development in the WABP and new cultivar adoption by the Washington apple industry were refined and enacted or explored, tested, and adapted to WSU's program for new cultivar development, release, and management.

Trial information: Evaluation and delivery of opportunities for improving efficiency of WABP field trials to accurately predict genetic potential (Objective 1)

- Sensory trait evaluation methods used in WABP P2 trials are robust; selection for any one trait is not likely to lead to detrimental consequences for other traits; and sensory evaluations may be relied on completely and instrumental measures removed
- Eating quality contributed more to overall fruit quality than did appearance quality; the former was highly influenced by crispness and juiciness and not firmness or acidity, while the latter was moderately influenced by russet amount and ground color but not size (P2)
- Doubling the number of individuals assessed in P2 would increase the probability of detecting elite selections; to achieve this for the same resource cost, incomplete designs could be used so that not all entries are assessed at each location
- Fruit only need to be evaluated following storage (or only at harvest) to adequately predict genetic potential; fruit evaluation at any age is suitable for predicting relative genetic potential of selections for most traits providing the average (main) effect of age is taken into account in the analysis; there is little value in increasing number of years of assessment to more than 2 years assuming use of 3 locations and 3 harvests per year for each entry; and there is little value in doubling the number of fruit assessed for each sample from 5 to 10
- Identifying storage disorder fatal flaws prior to P3 would provide a large efficiency gain
- Objective predictions of genetic potential for fruit quality traits based on all available information is recognised as the best approach in WABP trials, but the technology for streamlined delivery of this information each year requires further software development

DNA information: Evaluation and delivery of opportunities for use of DNA information to improve confidence in genetic potential prediction (Objective 2)

- Use of available DNA tests continued for crossing and seedling selection, providing large estimated resource savings.
- Our DNA test for acidity, crispness, and juiciness was used extensively in 2012 for seedling selection and also for crossing decisions.
- Our DNA tests for storability were used primarily in crossing decisions, helping to enrich new generations with superior alleles, which is the most efficient use of a trait-predictive DNA test. In addition to reducing the need for seedling DNA testing for the ethylene genes, generating a higher proportion of seedlings that are genetically superior for storability provides more opportunity to select for superiority in other traits.

- >4000 seedlings predicted to be inferior for the above traits were eliminated. Their early culling provides an estimated future resource savings of $60K. Such resource savings can be redirected to focus on those plants with the best predicted chance of becoming new cultivars.
- Cultivars and selections have been placed in genetic categories describing predicted performance for bitter pit susceptibility, sunburn susceptibility, skin color, sweetness, and acidity. These advances in DNA information were made possible by the RosBREED project. New DNA tests for these traits are therefore now available for WABP use in 2013 onwards.
- An informal international collaboration was initiated to explore the promising approach of Genomic Selection for the WABP using RosBREED data.

Information to/from industry: Refinement of a WABP–industry information delivery and feedback system for increasing potential for commercial success of new WABP cultivars (Objective 3)

- The useful Trait target checklist approach will be advanced in late January
- The effectiveness of the many avenues used by the WABP for delivering information on genetic potential of new cultivars will be investigated in a dedicated socio-economics study in the planned RosBREED 2 project.

RESULTS & DISCUSSION

1. ***Improve efficiency and predictability of advanced/elite selection trials by optimizing field experimental design***
= Evaluation and delivery of opportunities for improving efficiency of WABP field trials to accurately predict genetic potential

1a. Adjust for main effects
Adjustment for main effects of year of assessment, age, and location were routinely included in quantitative analyses of P2 trials in 2012, as recommended from 2011 findings.

1b. Increase replication
Following indications from initial results suggesting that increased replication of fruit number would increase accuracy of genetic potential of traits with low heritability such as instrumental sweetness, apples were juiced singly and soluble solids were recorded rather than the WABP typical practice of pooling juice from each sample of five apples. However, further analysis of this trait revealed that it was unlikely that this level of increased replication would have the desired effect so sampling reverted to the typical less time-consuming practice after the first few weeks of the 2012 season.

1c. Enhanced fruit quality phenotyping
Juice samples from some WABP P2 selections and all the RosBREED germplasm from WSU, University of Minnesota, and Cornell University were obtained and will be run through a Gas Chromatograph before the end of this project to obtain profiles for individual sugars and acids by PhD student Yingzhu Guan, breeding trainee in the RosBREED project. Some evaluation of further germplasm of the Apple Germplasm Library at the Sunrise orchard was completed, concentrating on presence/absence of sunburn.

1d. Genetic correlations among traits
Genetic correlation among most traits is low, which suggests that selection for one trait will not lead to detrimental consequences for other traits. In addition, these results suggest that HARDNESS and CRISPNESS are not being confounded by sensory assessors – they are appropriately being assessed as separate traits. Following are various findings for specific traits (in capitals, as they are labeled in WABP fruit evaluations).

Genetic potential of entries (P2 selections and standard varieties in the trials) for the composite fruit quality rating of OVERALL fruit quality rating was more highly correlated with EATING QUALITY rating than APPEARANCE QUALITY rating. Therefore, eating quality

contributed more to assessors perception of overall fruit quality than did appearance quality. Genetic potential for the composite fruit quality rating of overall EATING QUALITY was moderately correlated with CRISPNESS and JUICINESS, weakly correlated with SWEETNESS and AROMATIC TASTE, and poorly with HARDNESS and TARTNESS. Therefore, assessor perceptions of eating quality were most influenced by CRISPNESS and JUICINESS, and very little influenced by HARDNESS or TARTNESS. Genetic potential for the composite fruit quality rating of overall APPEARANCE was moderately correlated with RUSSET and GROUNDCOLOR, indicating that both traits contribute equally to the WABP's consideration of overall appearance, but SIZE was not important.

Genetic potentials were similar for SWEETNESS and AROMATIC TASTE, and were also similar for CRISPNESS and JUICINESS; therefore, each trait within the pair could be controlled by similar genes and selection for one positively influences the other. A moderate negative relationship observed between HARDNESS and SIZE means that selections with smaller fruit tended to have firmer fruit or perhaps are more difficult to rate for HARDNESS due to limited flesh amount. Correlation between Digi-Test CN and CRISPNESS may be improved if variability in size is taken into account.

As expected there was a high genetic correlation ($r > 0.8$) between TITRATABLE ACID and TARTNESS, and SUGAR ACID RATIO and TARTNESS; however, the genetic correlation between Digi-Test M1 and HARDNESS, Digi-Test M2 and HARDNESS, Digi-Test CN and CRISPNESS, and BRIX and SWEETNESS was only moderate ($r = 0.5$-0.7). Correlation between Digi-Test CN and CRISP may be improved if variability in size taken into account.

1e. Connectedness among P2 trials

Models of alternative design for P2 trials indicated that doubling the number of individuals assessed in P2 increased the probability of detecting elite selections. Genetic analysis of P2 data indicated that the interaction between genetic potential and location was low. Therefore, for the same resources, incomplete designs could be used so that not all entries are assessed at each location, meaning more entries could be tested for the same resources. However, the risk of losing information from the loss of a location was felt to be too great to implement this design.

1f. Tree age effects

Age did not affect the overall mean of a trait, except Digi-Test FRUITWEIGHT. There is little evidence from the analysis that indicates the *relative* genetic potential of an entry to other entries differs among ages. There were few traits where the genetic potential was significantly affected by tree age (RUSSET and APPEARANCE SUMMARY) but the only a few entries changed rank and the influence was not large and there was no discernible pattern in the results.

1g. P3 sources of variation

A datafile of all variables assessed in P3 has been prepared for future analysis; however, the design of P3 trials is not as robust for statistical analyses compared to P2 trials. The November 2012 workshop concluded that P3 trials provide only informal estimates of mean performance and P3 is best focused on (i) fatal flaw detection, and (ii) providing familiarity with selection to breeder and potential adopters, particularly for traits with large environmental (and perhaps externally manageable) influences, although detection of the genetic potential for these types of traits are difficult without robust designs. However, many selections in P3 have been rejected based on a fatal flaw; therefore, it would be more efficient to accurately identify these fatal flaws earlier in the selection process. Options for improving methods to identify storage disorder fatal flaws prior to P3 were discussed during the November 2012 workshop; however, some participants suggested further research is required to develop strong recommendations for the adoption of these new methods.

1h. Streamlined delivery of quantitative genetic analyses

The mean genetic potential (for a trait) of a selection that is predicted based on all available information is widely accepted as the most objective and best estimate of the genetic potential of an individual. In the November workshop, the value to WABP of objective predictions of the

genetic potential for fruit quality traits was recognised. The accuracy of genetic potential predictions will increase as more data is available from ongoing annual assessments.

This project (and the previous 2011 decision confidence project) has developed the technology required for predicting genetic potential. However, to support adoption of this technology by WABP further work is need to extend software that has been developed for research purposes to operation software that can be routinely implemented by WABP staff and deliver user-friendly informative output.

1i. Sampling assessment

The two major sources of variation for all traits in WABP P2 trials was determined to be (1) genetic variation, i.e., differences among selections due to their differing genetic makeup, and (2) variation among samples after all other effects had been removed. The former indicates that the trials contain material with differences in genetic potential from which superior selections can be selected. The latter indicates that increasing replication will improve accuracy of predicting genetic potential, especially for certain traits like SWEETNESS and AROMA. Sensory trait evaluation methods used in WABP P2 trials are robust and any bias in individual evaluations are removed by taking the entry mean across multiple harvests, years, and locations, as indicated by the strong average effect of an entry across location, ages, years of assessment, and harvests, and the lack of large higher order interactions between entry and any of these factors.

The lack of significance and interaction for any fruit quality trait between entry and *storage condition* (i.e., fresh or two months storage) for most entries suggests that fruit only need to be evaluated following storage (or only at harvest) to adequately predict genetic potential. This conclusion was made because there was no significant difference between the ranking of entries for each trait assessed on fresh fruit or on fruit after two months storage except for HARDNESS and CRISPNESS. Golden Delicious was more susceptible than other cultivars evaluated to loss of CRISPNESS and HARDNESS after two months storage.

The lack of evidence of any significant and meaningful interaction between entry and *age* for any fruit quality trait suggests that evaluation at any age is suitable for predicting genetic potential, providing the average (main) effect of age is taken into account in the analysis.

A model of the accuracy of predicted genetic potential was developed to evaluate the impact of alternative sampling designs for P2 trials. There was little increase in accuracy of predicting genetic potential by increasing number of years of assessment to more than 2 years, assuming use of 3 locations and 3 harvests per year for each entry. Assuming each observation is a sample of 5 fruit, the accuracy of predicting genetic potential was not greatly improved by doubling the number of fruit assessed for each sample.

Use of instrumental measures for indirect prediction of genetic potential for sensory fruit quality traits was less accurate than direct selection on the sensory traits themselves, assuming the sensory trait evaluated is equivalent or closer to the sensory trait used by consumers to determine appeal. As instrumental methods are not available for all sensory traits (hence some sensory evaluation is required) and there is no large cost saving in not assessing some sensory traits if some sensory evaluation is required, there seems no strong logic to replace sensory with instrumental evaluation. This may be revised if the correlation between Digi-Test CN and CRISPNESS can be improved, and an instrumental means of assessing SWEETNESS, GROUNDCOLOR, and RUSSET can be found that has a high genetic correlation with these traits that are the main determinants of OVERALL fruit quality rating.

2. ***Put rapidly accumulating DNA information to immediate use by describing genetic potential of new cultivars in the context of commercial production***

= Evaluation and delivery of opportunities for use of DNA information to improve confidence in genetic potential prediction

2a. *Ma* locus information – refinement of use in breeding

The WABP's use of the *Ma* locus DNA test that partially predicts seedling performance for the valuable traits of **crispness**, **juiciness**, and **acidity** (and perhaps also firmness and several other traits) was increased in 2012 over previous years. Large resource savings were calculated to have been made by incorporating this DNA information into parent and seedling selection decisions, assuming that the selections made according to this test will result in superior material in the breeding orchard. Descriptions below exemplify what can be achieved with any DNA test.

In *parent selection*, the DNA test was used in 2012 to enrich for alleles that tend to confer in superior levels in these traits, especially from Honeycrisp, and to avoid the accumulation of inferior alleles in P1 breeding populations. This use of the *Ma* locus DNA test in supporting crossing decisions helped refine the creation of P1 populations for which a higher proportion of seedlings are expected to perform at and above target levels for these traits. In a few years when the P1 seedlings produce fruit in the breeding orchard and a higher proportion of seedlings achieve selection thresholds used in previous years, the choice can be made to (1) concordantly increase the number of "keepers" entering P2 or (2) to raise the bar for one or more traits thereby maintaining a similar number of "keepers" that have even better genetic potential for the DNA-tested traits than has been possible with conventional parent selection approaches. Note that such DNA-informed parent selection has been performed since ~2006 using the DNA tests of ethylene genes for fruit storability (2b. below), and so the resulting seedling populations that are now beginning to fruit should, on average, have improved genetic potential for storability than in previous years. We have not yet begun after-the-fact formal evaluation of how well DNA-screened seedling populations are performing compared to efforts before this "revolution". Another advantage of DNA testing in parent selection is the reduced need for DNA testing on seedlings for those particular tests used to guide crossing.

In *seedling selection*, more than 7000 seedlings were DNA-tested in 2012 (mostly for the *Ma* locus and a little for one of the ethylene genes) – approximately double the number screened in 2011for the *Ma* locus. Almost 60% of seedlings were judged by the DNA tests to be genetically inferior and were culled. This number of strategically screened seedlings represents more than half the young seedlings raised in 2012 and an estimated net resource savings in the next few years of $60,000 by avoiding the costs of raising those suspected inferior seedlings and instead concentrating efforts on those plants with the best predicted chance of becoming new cultivars. Progress in late 2011 and early 2012 in understanding the relative effects of the multitude of *Ma* locus genotypes present in breeding germplasm increased the number of target desirable and undesirable marker genotypes.

The treasure trove of data from the RosBREED project (obtained from high-resolution genome scanning and multi-year standardized phenotyping at massive, unprecedented scales) is being mined for value to the WABP. For example, predictions for acidity levels according to genotypes at the *Ma* locus on chromosome 16 have now been refined by consideration of a second major locus (the "*Acidity*" or "*A*" locus) on chromosome 8. When effects on acidity are analyzed separately, both loci are observed to have a low, a medium, and a high acidity genotype (Figure 1), making each DNA test a useful semi-predictor of acidity levels among individuals.

A more informative and detailed picture is achieved when the two loci are examined jointly (Figure 2). While in general the two loci combine additively, the interesting exceptions are the high-low (*Ma-A*) and low-high combinations pertaining to genetic categories 7 and 3, respectively. Genetic category 7 is associated with low acidity (bland-tasting fruit) rather than the expected medium to high acidity considering both loci. Or if only the *Ma* locus was considered, high acidity would be expected. Similarly, predictions according to the "*A*" locus are highly dependent on the configuration of the *Ma* locus. Therefore, when breeding for medium- to high-acid new cultivars, either locus alone is not a suitable predictor; a combined test based on both the *Ma* and "*A*" loci would be preferred. Such a combined test is now available for WABP parents and elite selections.

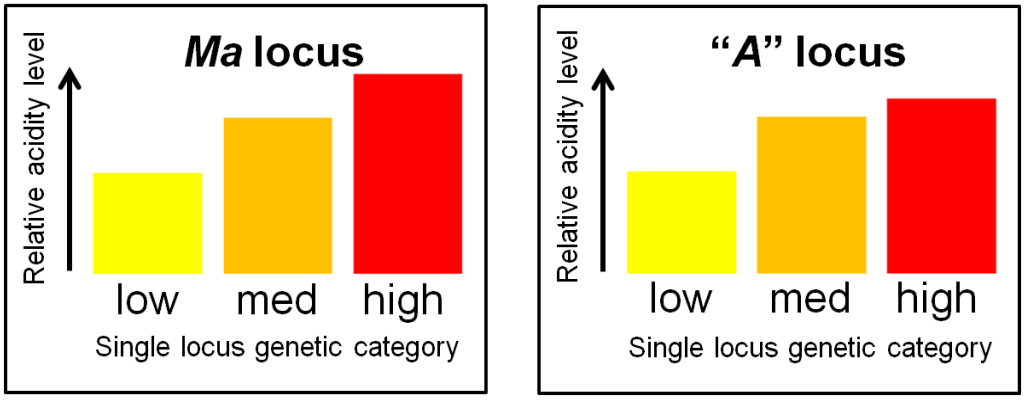

Figure 1: Relative acidity levels according to two regions in the apple genome independently influencing fruit acidity, the Ma *locus and the "*A*" locus. Effects were determined from a dataset generated in the RosBREED project based on observations on more than 600 individuals (pedigree-linked cultivars, selections, seedlings, and ancestors) representing U.S.-wide apple germplasm, Washington certainly included, that have been (1) evaluated for fruit quality for two years at harvest and after 10 weeks and 20 weeks of cold storage and (2) genome scanned with the high-resolution 8K International RosBREED Consortium Apple SNP array.*

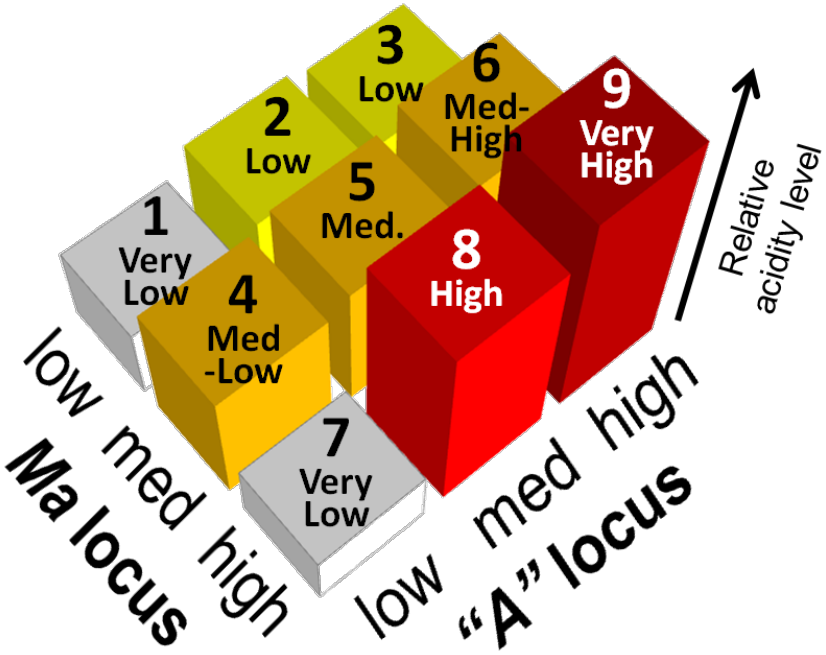

Figure 2: Relative acidity levels of nine genetic categories defined by DNA markers at two regions in the apple genome jointly influencing fruit acidity, the Ma *locus and the "*A*" locus. Descriptive labels from "Very Low" to "Very High" were given based on quantitative differences that were consistently observed within each year-and-storage-regime dataset and averaged across the entire dataset. The entire dataset was that described for Figure 1. Calculations were made with the simple case of just two contrasting alleles (high acid, low acid) for each locus. In the future, further distinctions could be made within these alleles for sub-types with intermediate and/or more extreme effects, but for now these two-allele models are suitable as they already explain a large degree of genetic variation for acidity in breeding germplasm.*

The nine acidity genetic categories were consistent over storage and over two years, as described below. Specific predictions for titratable acidity (TA) across three storage regimes were calculated based on the available two seasons of data (2010 and 2011) from the RosBREED project. Such predictions can be expressed as the probability that an individual belonging to one of the nine acidity genetic categories is above or below a defined TA threshold (Figure 3). These results are on a finer scale to those of Figure 2: while the latter considered both years and all storage regimes at once, the predictions in Figure 3 separate each year-storage dataset but are still grouped according to the *Ma-A* genetic categories. The Very Low categories (7 and 1) often have probabilities of greater than 50% that fruit will be at least 0.1 units below the average TA observed for a given year and storage regime, and often have probabilities of less than 10% that fruit will be at least 0.1 units above the average (Figure 3) – in other words, they tend to have bland fruit. In contrast, in the Very High category (9), those probabilities are reversed – tart fruit.

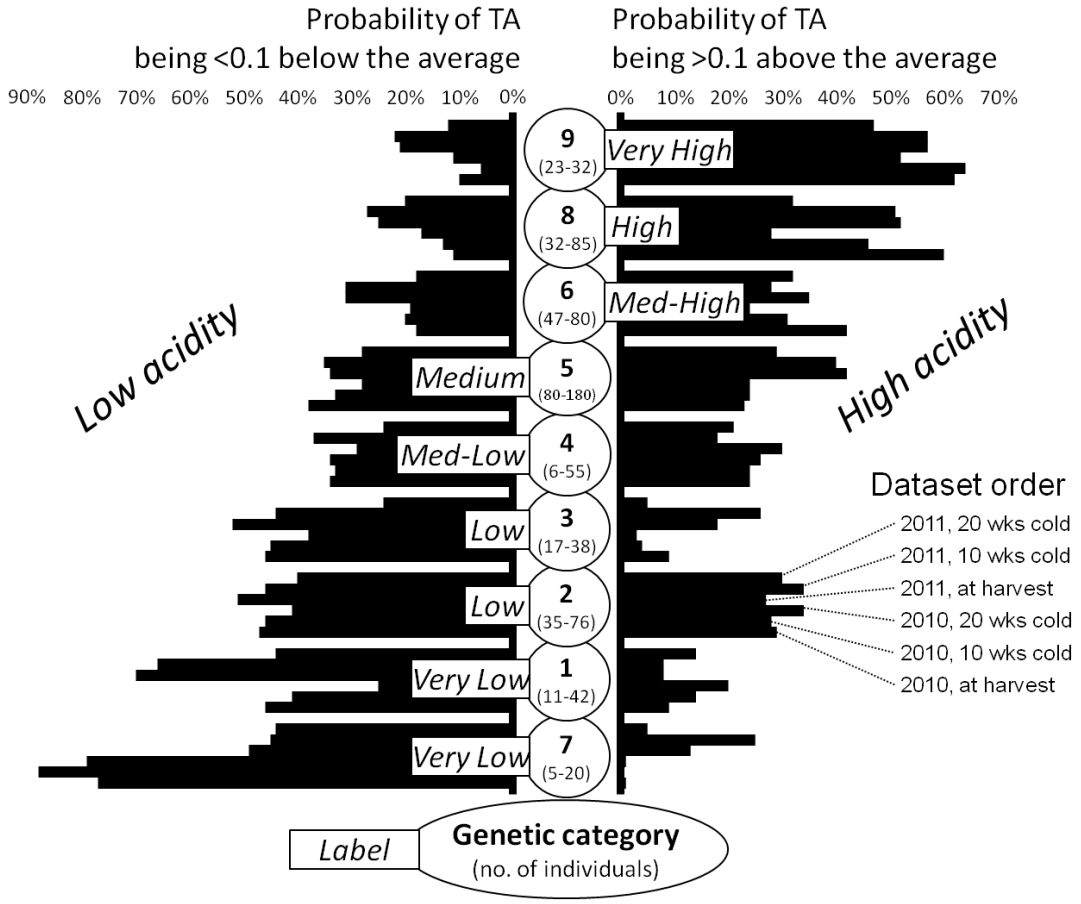

Figure 3: Estimated probability of an apple cultivar (or selection or seedling) achieving thresholds for titratable acidity (TA) before and after cold storage, according to genetic categories defined by two acidity loci. The dataset was that described for Figure 1. The trait acidity loci, Ma *and "*A*", and their nine genetic categories are those described in Figure 2. Probabilities were calculated by converting all observations in each category into Normal distributions and using the NORMDIST function in Excel with thresholds of 0.1 units (%) of TA above and below the average TA of all individuals in a given year and storage regime. Results for sensory acidity ("tartness", scored on a 1-5 scale) were very similar to TA, as expected because these two measures of acidity were highly positively correlated.*

Interestingly, no commercial cultivars were observed to belong to either acidity extreme (Very Low or Very High, categories 7, 1, or 9), and few belonged to the Low or High categories (2, 3, and 8). The bulk of commercial cultivars were in the Medium acidity genetic categories (4, 5, and 6). However, half the seedlings arising from any random cross will be in the non-Medium categories and therefore could be avoided (or higher acidity purposely targeted) by using the DNA information described here in crossing decisions and/or in greenhouse seedling screening. This DNA information on the *Ma* and "*A*" loci regarding acidity will be incorporated in 2013 breeding decisions.

2b. *ACS* and *ACO* gene information – refinement of use in breeding

As in previous years, the two DNA tests for **fruit storability** based on the ethylene genes, *ACS* and *ACO*, were used in *parent selection* to avoid creating populations with a high degree of seedlings containing alleles for high ethylene levels that tends to be associated with poorer storage potential. Numerous elite WABP selections, genotyped for these genes, are now available that serve as efficient parents. This creation of seedling populations enriched for low ethylene alleles helping to confer extended storability has meant that the use of these DNA tests in *seedling selection* is much reduced from previous years. This outcome highlights the positive connection between these two levels of DNA testing. Another way to look at it is that most DNA tests should have a limited shelf life for seedling selection as long as the DNA information is being even more efficiently used for parent selection. All else being equal, it takes much less effort to make a cross resulting in no inferior seedlings at a particular region in the genome than it does to make a different cross that requires screening and culling of the inferior seedlings. In addition, the efficient crosses result in more seedlings superior at the targeted genomic region, providing greater opportunity to select for superiority in other traits and genomic regions.

2c. Use of further DNA information in the WABP

Further DNA information has been gained from the RosBREED dataset for various fruit quality traits (2d. below). WABP parents and most P2 and P3 selections have been placed into genetic categories determined by genomic regions influencing traits such as susceptibility to bitter pit, susceptibility to sunburn, and amount of blush/stripe cover. In addition to use in *parent selection* beginning in the 2013 crossing season, this DNA information will now advise breeding decisions in *elite selection advancement*.

2d. Predictability of further genetic tests

Important external appearance traits of **susceptibility to bitter pit**, **susceptibility to sunburn**, and **skin color attributes** (amount of blush/stripes, relative degree of blushes vs. stripes, and cover hue) were chosen for in-depth genetic analysis using the RosBREED dataset. The WABP component of the RosBREED dataset was specifically investigated for bitter pit and sunburn susceptibilities to find those genomic regions influencing these traits as they occur in this region. Genomic regions were indeed found, and genetic categories developed for them. For example, two bitter pit loci were identified and combined to produce four informative genetic categories (Figure 4).

For **sweetness**, PhD student Yingzhu Guan found some genomic regions influencing the various measured aspects of sweetness (SSC, sensory 1-5 scores, and amounts of individual sugars) within the RosBREED project, but these regions have yet to be converted into predictive DNA tests. Similarly, PhD student Sujeet Verma found several genomic regions influencing crispness, juiciness, and/or acidity (in addition to the *Ma* locus and "*A*" locus described in 2a.) but DNA tests targeting them have not yet been developed. Many **other fruit quality traits** measured in the RosBREED project on breeding germplasm representative of the WABP, including **susceptibility to various other disorders**, await analytical attention.

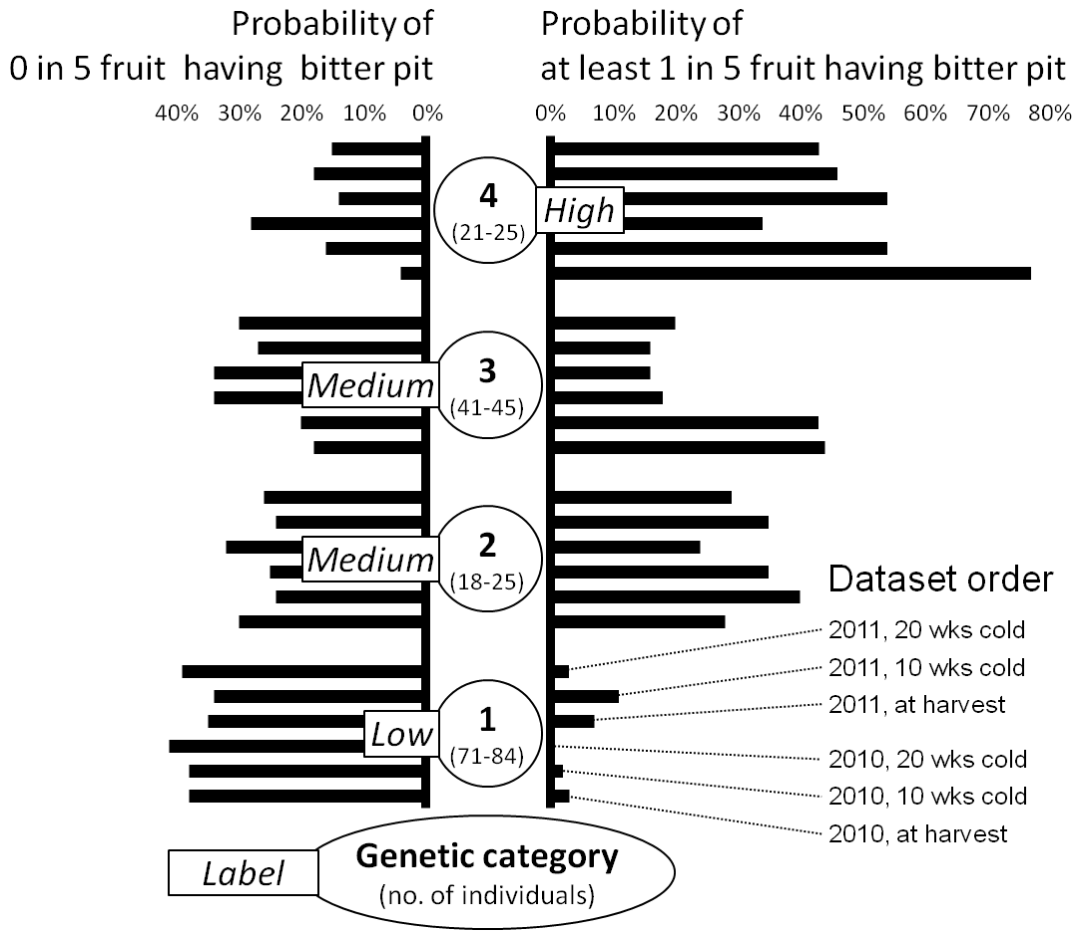

Figure 4: Estimated probability of an apple cultivar (or selection or seedling) achieving thresholds for bitter pit incidence before and after cold storage, according to genetic categories defined by two bitter pit loci, Bp-1 *and* Bp-2. *Four genetic categories were developed jointly for the two bitter pit loci. The dataset was that described for Figure 1; probabilities were calculated as described for Figure 3.*

2e. Genomic Selection

The 2012 November workshop extensively discussed the use of thousands of DNA markers across the whole genome for constructing Realized Relationship (RR) coefficients and Genomic Selection methods for improving the accuracy of predicting genetic potential of entries. Preliminary work on the application of RR approaches to RosBREED data was undertaken but hampered by incomplete datasets and inconsistencies in results between pedigree and RR methods. An informal collaboration between RosBREED, Plant & Food Research (New Zealand), and University of Queensland (Australia) was initiated to move this research forward to provide a stronger foundation for the implementation in operational breeding. Genomic selection is being applied operationally in New Zealand apple breeding to greatly reduce the number of entries entering advanced testing field trials. Conditions required for implementation of RR or GS are: technical expertise, dense marker array (further research required on how dense), large training populations with accurate phenotyping, and recalibration of training population after several generations of breeding. Results from the RosBREED analysis will be used to further evaluate the opportunities for WABP.

3. ***Demonstrate to the WA apple industry the efficacy and opportunities of a combined field-design and DNA-based approach to decision-making***
= Refinement of a WABP–industry information delivery and feedback system for evaluating and increasing potential for commercial success of new WABP cultivars

3a. Target trait checklist
The checklist, with its list of traits, target levels (thresholds), and standard cultivar anchors to transparently align breeding targets, selection thresholds, and new DNA test development, has two main useful features over previously used approaches to prioritizing among traits (1) it considers trait levels rather than traits themselves, and (2) it splits trait levels into two categories: Essential (or Needs) and Bells & Whistles (or Opportunities). Discussions in the November workshop focused on what the numbers really mean, when to best use it, and how it aligns with socio-economics results coming from the RosBREED project. This topic will be discussed further with the Breeding Program Advisory Committee (BPAC) and WTFRC & industry representatives in the Genomics, Genetics, and Breeding meeting in late Jan 2013 immediately prior to the WTFRC Apple Research Review.

3b. Information delivery and feedback
Information about new WABP cultivars was delivered through Good Fruit Grower articles (Hanrahan et al., issue 63[11]; Evans and Barritt, issue 63[14]), storage season presentations in Yakima and Wenatchee which included fruit tasting, field days (with fruit), fact sheets, posters, fruit and presentations at grower meetings, and the delivery of gift boxes to numerous industry contacts throughout the year. The effectiveness of each avenue will be investigated in the planned RosBREED 2 project in a dedicated socio-economics study, with involvement by Dr. Desmond Layne. The industry advice channels for the WABP were restructured during 2012 with the formation of the BPAC and a Cultivar Licensing Committee (CLC) who would take over the different roles of the IAC. BPAC members had their first opportunity to taste some Phase 2 selections at the November meeting. Initial discussions outlining technology transfer options for the WABP have already taken place with Dr. Layne with a view to greatly increasing the web presence of the WABP once Dr. Layne takes up his Extension Leader appointment to WSU in February.

EXECUTIVE SUMMARY

Choice of scion-rootstock combinations is the single most profit-influencing decision that a grower can make. While planting standard scion cultivars is an option to mitigate risk, new cultivars from breeding efforts provide sustained genetic solutions to flaws in standard cultivars or to address market opportunities.

The Washington Apple Breeding Program (WABP) creates new genetic combinations, selects among them to find winners, and then releases superior new cultivars to the Washington apple industry. This program is resource-hungry in running costs, land, expertise, and time, and success is not assured. But the potential payoffs for the Washington apple industry are enormous and justify the huge investment. We sought to increase decision confidence in new cultivar development and adoption by ensuring the breeding program is aiming for the right targets, is accurate and efficient in its execution, and is effective in conveying successful outcomes to industry clients.

Detailed investigation of selection trial design, especially of Phase 2, identified numerous strengths that underscore progress to date in developing superior new cultivars. Some opportunities were identified to consider for further streamlining routine operations. Such opportunities include:

- reducing redundancies on sensory and instrumental measures of the same essential trait
- focusing more on those traits contributing to the decision to move selections forward
- evaluating fruit only after storage
- reducing the number of years of fruit evaluation
- doubling the number of selections in Phase 2 trials, which could be achieved with the same resources by use of incomplete trial designs
- identifying storage disorder fatal flaws prior to Phase 3
- software development to provide breeder access to routine complex analyses of all breeding data so that genetic potential predictions are objectively based on all available information

DNA information was used to improve confidence in genetic potential prediction. The RosBREED project channeled further DNA information into the WABP. Successes include:

- use of increasingly refined DNA tests for storability, crispness, juiciness, and acidity in crossing and seedling selection to provide large estimated resource savings ($60K from marker-assisted seedling selection, probably even more for marker-assisted parent selection)
- genetic categorization of cultivars and selections to describe predicted performance for bitter pit susceptibility, sunburn susceptibility, skin color, sweetness, and acidity
- availability of new DNA tests from 2013 onwards for the above-mentioned traits and others

The WABP–industry information delivery and feedback system for increasing potential for commercial success of new WABP cultivars was addressed in 2012. Advances include:

- the WABP's use of many avenues for delivering information on genetic potential of new cultivars, the effectiveness of which is planned for investigation in a dedicated socio-economics study in RosBREED 2
- Dr. Des Layne being hired into the role of tree fruit extension team leader at WSU

More detailed reports on project components, including a summary of the November workshop, are available to apple industry members on request.

NEW PROJECT PROPOSAL **PROPOSED DURATION**: 1 year

Project Title: Further development of an online toolbox for tree fruit breeding

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Cooperators: Amy Iezzoni (MSU), Gennaro Fazio (USDA-ARS), Fred Bliss (UC Davis)

Total Project Request: **Year 1:** $59,740

No report submitted

FINAL PROJECT REPORT

Project Title: Breeding in the 21st century: technology platform for fast breeding

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City/State/Zip:	Pullman/WA/99164		

Cooperators: Ralph Scorza and ARS team at the Ag Station in Virginia, Nnadozie Oraguzie, Cameron Peace, Fred Bliss and Jim McFerson

Other funding sources

Agency Name: National Science Foundation
Funding amount: $8000 for undergraduate student support

Total Project Funding: $24,673

Budget History:

Item	**2012 –** Funding request for 10 months
Salaries	
Benefits	
Wages[a]	10,500
Benefits	4,473
Equipment	
Shipping	
Supplies[b]	3,000
Travel	1,000
Plot Fees[c]	700
Miscellaneous[d]	5,000
Total	**$ 24,673**

Footnotes:

a. Technical help – Time slip employee for plant handling, transgenic experiments, micropropagation and assisting in crosses, data collection etc.
b. Tissue culture supplies and reagents
c. Greenhouse/tissue culture facility use
d. Greenhouse materials/plant screening/transgenics and breeding/grafting program costs

OBJECTIVES

Note: This final report is for the project funded in May 2012 for 10 months which builds upon ongoing work in our programs since 2011 with support from the WTFRC.

The WSU Apple Breeding Program (WABP) has identified reduction of generation cycle as a priority in order to rapidly combine desirable traits in new varieties.

The primary objective of this project is to *reduce the generation cycle* in breeding so that time and resources can be saved in combining multiple traits faster to produce the varieties desired by the farmers and the consumers. The techniques being developed in this project are already being implemented in apple variety development worldwide; failure to invest in this technology will result in the loss of a competitive advantage within the WABP. Tree fruit breeding programs individually use a physiological, genetic or transgenic approach to reduce generation cycle.

The three approaches of reducing generation cycle that are being tested in our programs are:

1. Environmental and horticultural approach

For this approach, seedlings are grown under modified environmental conditions to accelerate growth and development with the goal of breaking juvenility in a shorter time. Seedlings for this aspect were selected from crosses that had already been made for the WABP with a reasonable level of diversity including desirable fruit quality traits and seasonality [e.g. Akane (early variety) and Fuji (late variety)] whilst not using Cripps Pink. These are baseline comparison seedlings which are not expected to be precocious.

2. Genetic approach

For this approach, varieties known to induce early flowering in their offspring (precocity) are used as parents. Parental overlap was intentionally maintained with individuals used in objective 1 for a direct comparison. Cripps Pink (Elite cultivar) and *M. zumi* (Wild donor) were used as the precocious parent.

We have made significant progress in using modified environmental conditions to accelerate growth of own-rooted WABP seedlings. Physiological manipulation of seedling growth and development in the greenhouse is primarily being carried out at WSU Pullman greenhouse. The horticultural practice of grafting seedlings onto precocious rootstocks is routinely used within the WABP.

3. Transgenic intermediate approach

This approach uses a quick flowering transgenic plant as an intermediate so that seedlings from any cross can flower within a year reducing the generation cycle to one year or less. This technique is particularly useful in enabling the breeder to incorporate diverse germplasm into the breeding program and rapidly go through several generations of selection (to remove the poor characteristics that frequently accompany novel traits from wild germplasm). The transgene can then be selected out of the final generation cross resulting in a non-transgenic selection with no regulatory issues attached.

Specifically, the objectives for the 10-month project were:

1. Maintain WABP seedlings (non-precocious and precocious) in the greenhouse and, monitor and regulate growth and development to record flowering timelines
2. Micropropagate and multiply WA 38 and WSU 48 for transformation experiments
3. Establish *M. zumi* in tissue culture for micropropagation
4. Perform transformation experiments with Royal Gala, WA38 and WSU 48

SIGNIFICANT FINDINGS

Significant findings for objective 1

- Apple seedlings follow a unique growth pattern under our modified environmental conditions different from what has been reported for growth in the orchard.
- Careful observations were made over the last year and a preliminary phenotype-guided growth model has been established that guides decisions for fertilization, pruning and administration of cold treatment to meet chilling requirement. This model is incomplete as most of the seedlings are currently in cold treatment or just emerging from that.
- A clear impact of genetic background under modified environmental conditions on the growth rate is visible based on number of nodes generated per month.

Significant findings for objective 2 and 3

- Modified sanitizing and operating protocols were developed to counter the large pathogen load infesting the elite WSU selections and *M. zumi* accessions.

- Tissue culture protocols for maintaining WA 38, WSU 48 and *M. zumi* have been established. Each individual requires a unique combination of phytohormones and salts in tissue culture for robust growth and development.

Significant findings for objective 4

- Transformation protocols for Royal Gala as a standard for transgenic experiments have been established and several putative transgenic Royal Gala plants are ready to be tested for transgenic status.
- Micropropagation of WA 38 is underway for transformation experiments.

RESULTS & DISCUSSION

Detailed results and discussion follow this table that summarizes the progress and milestones achieved as an indicator of success in the funded project.

Time Frame	Objectives	Progress	Milestones
May 2012 – March 2013	1. Maintain WABP seedlings (non-precocious and precocious) in the greenhouse and, monitor and regulate growth and development to record flowering timelines	Careful phenotyping of seedling growth and development has provided decision making guidance for fertilization, irrigation, pruning and chilling treatment	A preliminary greenhouse growth model established
	2. Micropropagate and multiply WA 38 and WSU 48 for transformation experiments	Tissue culture protocols refined and established. Plant sanitization protocols established. More plant material will be established in tissue culture from fresh material in the Spring	Micropropagation procedures established.
	3. Establish *M. zumi* in tissue culture for micropropagation	Individuals from *M. zumi* have been established in suitable custom tissue culture media	Plantlets established in tissue culture.
	4. Perform transformation experiments with Royal Gala, WA 38 and WSU 48	Several transformation experiments performed with Royal Gala. WA 38 and WSU 48 micropropagation is ongoing for subsequent transformation experiments	Methods to generate transgenic Royal Gala plants established.

Objective 1: Maintain WABP seedlings (non-precocious and precocious) in the greenhouse and, monitor and regulate growth and development to record flowering timelines.

Status: A total of 510 seedlings; 85 WABP seedlings from crosses made in 2010 derived from non-precocious parents and 427 WABP seedlings derived from crosses made in 2011 derived from precocious parents are currently in the WSU Pullman facilities. The 2010 seedlings provided us a test set of how to grow and maintain apple seedlings in the greenhouse. We utilized the observations in successfully growing 427 seedlings representing 8 parental combinations with desirable traits (Figure 1A and B – please see at the end of the document).

Observations: Post-germination in March 2011, majority of the seedlings grew rapidly to a size of 18 to 24 inches within 8 weeks and by November 2011 many seedlings touched the greenhouse roof

indicating a height of 6 to 8 feet on average. It was interesting to note that some of the seedlings segregated for compact size. Some were super dwarfs (Fig 1C) while a few individuals were dwarf (Fig 1D). It is important to note that such seedlings would not have survived in a nursery operation but have been carefully maintained in the greenhouse and may possess unique fruit traits. While this is still speculation, the hallmark of any breeding exercise is the available genetic diversity and survival of maximum number of diverse seedlings bodes well for generating individuals with a unique combination of traits.

Growth and Development: Seedlings derived from each cross exhibit variable phenotypes in shoot growth with some seedlings being vigorous. This could be genetic or the impact of environmental conditions. Seedlings from each cross were randomly separated into Group A and Group B. In November 2012, Group A seedlings were placed in the cold to mimic natural dormancy conditions while Group B seedlings remained in the greenhouse. At the time of this report, Group A seedlings have completed dormancy and are now emerging from it (Fig 2A) and Group B seedlings were defoliated in early December for initiating dormancy. This process resulted in the production of unusual bud "clusters" on one tree (Fig 2B). It is not clear what these are but they appear abnormal and are similar to flower bud clusters in cherry. In summary, growth and development under modified environmental conditions are allowing survival of unusual seedlings with diverse growth and development behaviors.

Model of Plant Development: Careful observations of the ~500 seedlings over the last year have resulted in the establishment of a preliminary model that can be used to identify phenotypes and plantlets cycled through dormancy conditions to reduce generation times. The model identifies three developmental stages which are easily recognizable. First stage represents vigorous growth characterized by actively growing shoot tips. In the second stage, the shoot tips become less aggressive and start producing bract-like structures. Finally, when the plant is ready for dormancy the shoot tip appears like a rosette. In the final stages the leaves come off easily from the stem. At this point, the tree should be moved into the cold with slow reduction of temperature and light duration. Figure 3A represents this concept graphically; it shows one typical growth cycle. A plant goes through this cycle once when grown outside in an orchard. One could accommodate 2 or 3 such cycles in greenhouse/cold room conditions (Fig 3B). We intend to utilize this model to move seedlings through growth cycles and reduce generation time. It is critical to note that each individual seedling will behave differently requiring detailed and everyday attention in such experiments. While labor-intensive, it is a worthy approach as it can save several years in variety development and could be particularly useful to fully exploit seedlings coming through DNA-assisted selection. Once the investment has been made in the seedling using the portfolio of key markers we expect to have in the near future, we would predict the number of successful (un-culled) seedlings will be reasonably few; at this point, seedling death through transplanting to the nursery would be a greater loss so it is likely the WABP will need to use greenhouse facilities to rear these valuable seedlings. Being able to speed up this growth and maturation process using the model developed will increase the efficiency of the application of DNA-assisted selection and should ultimately reduce costs.

2. Micropropagate and multiply WA 38 and WSU48 for transformation experiments
In spring 2011 there was limited amount of plant material available. Despite that, several explants were established in tissue culture. Fungal and bacterial infection decimated the collection with a few surviving samples. This required the establishment of rigorous sanitization protocols. In addition to the use of bleach we used a sonication device to ensure that air bubbles created due to surface irregularities are dislodged. This resulted in substantial reduction of post-sanitization infection.

For micropropagation of WA 38 and WSU 48, five different apple micropropagation media for Royal Gala and Geneva rootstocks available in the lab were tested. While the experiment requires more

time, observations made thus far have resulted in the identification of suitable media formulation for WA 38 and WSU 48 growth and micropropagation.

Fresh plant material will be used in spring 2013 to initiate more new cultures.

3. Establish *M. zumi* in tissue culture for micropropagation
As was the case with WA 38 and WSU 48, a limited amount of plant material was available for *M. zumi*. However a few individuals have now been established in tissue culture; the repeated sanitization due to the high bacterial and fungal load on the initial plant material has resulted in reduced growth and vigor. Fresh plant material in spring 2013 will be used to establish larger numbers of plantlets in tissue culture.

4. Perform transformation experiments with Royal Gala, WA 38 and WSU 48

There are several groups that have successfully reported on genetic transformation of apples however techniques used are extremely variety/genotype-specific and none of the transformation protocols reported are for individuals that are relevant to the WABP. Royal Gala has been a workhorse for our transformation work. Our first goal was to test our methods and replicate what others have accomplished.

So far, three genetic transformation experiments in Royal Gala have been performed with considerable success. Transformation was performed using protocols provided by Jay Norelli at the USDA-ARS station in West Virginia. At present there are over 60 preliminary transgenic plants (Figure 4) that are undergoing rooting in antibiotic selection medium. In the next few weeks, their transgene integration status will be confirmed via molecular analysis.

While it is encouraging to see the level of success with Royal Gala, transformation of WA 38 and WSU 48 may require protocol modifications given the fact that different composition of growth media is required for the WSU genotypes for growth and micropropagation compared to Royal Gala.

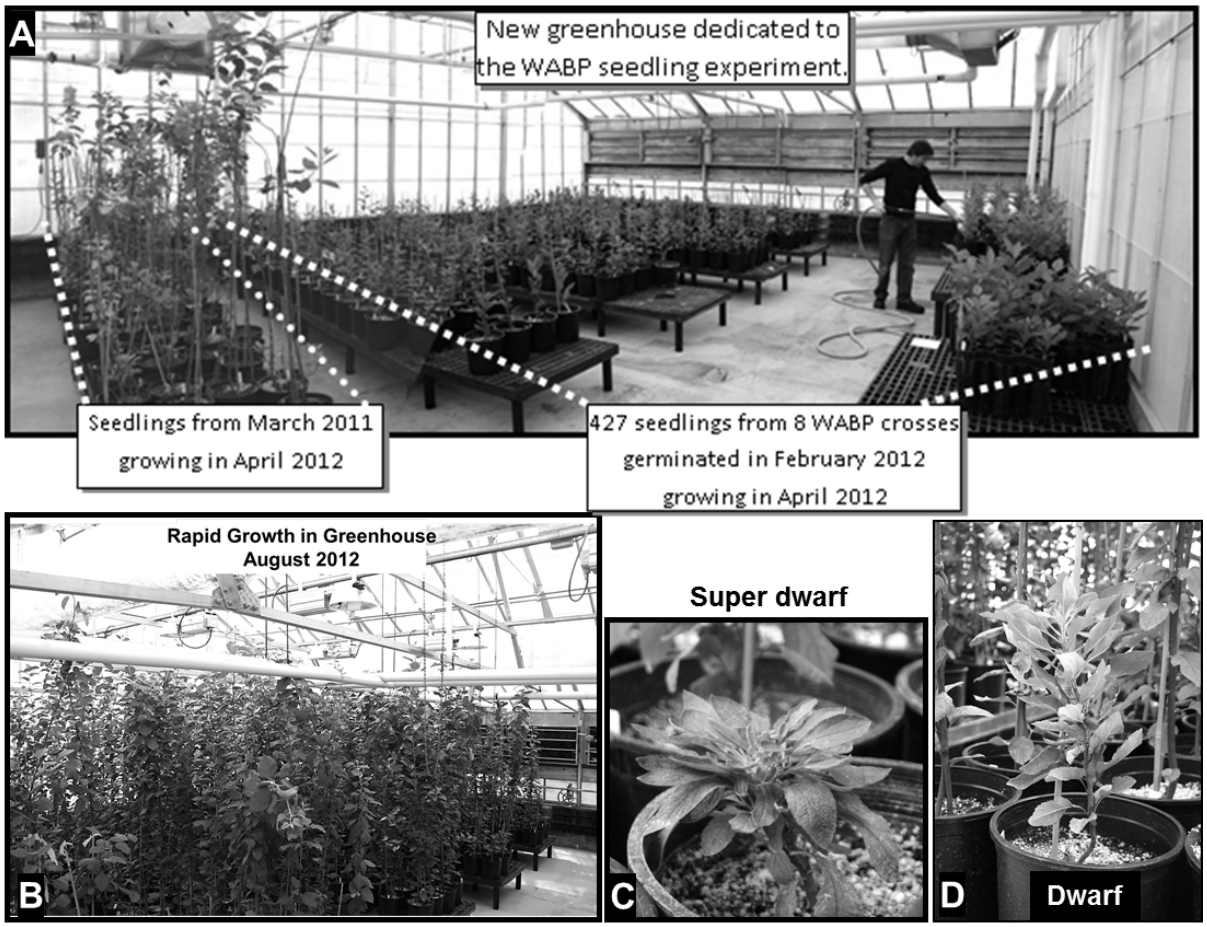

Figure 1: WABP seedling growth in WSU Greenhouse. A. 510 seedlings in the greenhouse. Notice the robust seedling growth as recorded in April 2012. B. The seedling stand is 6-8 ft tall in August 2012. C. A super dwarf seedling in the same population in September 2012. D. A dwarf seedling in the same population in September 2012.

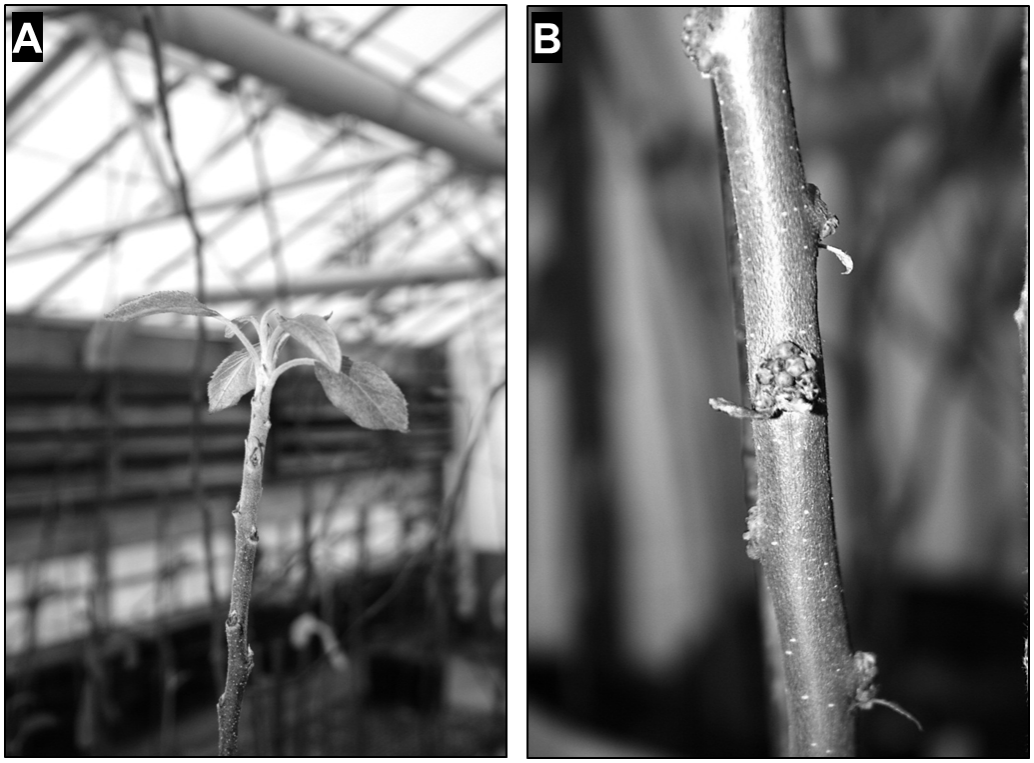

Figure 2: A. Resumption of growth in Group A seedlings after being moved out of dormancy. B. Appearance of unique cluster buds in one of the individuals.

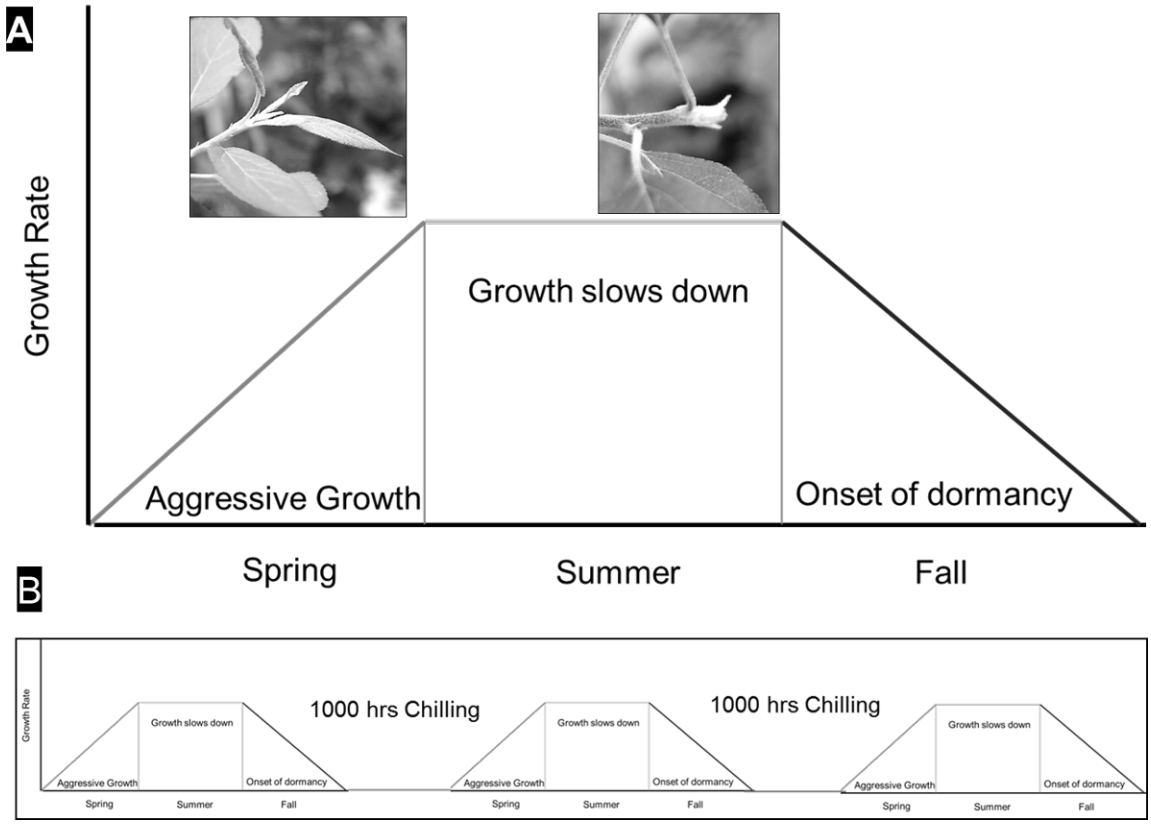

Figure 3: Model of plant growth. A. Representative graph of growth rates in the spring, summer and fall. Images of shoot tips taken at the time of aggressive growth and slowing down of growth are shown. This growth cycle is completed in one year in the orchard B. It is proposed that three such growth cycles could be completed in one year in the greenhouse.

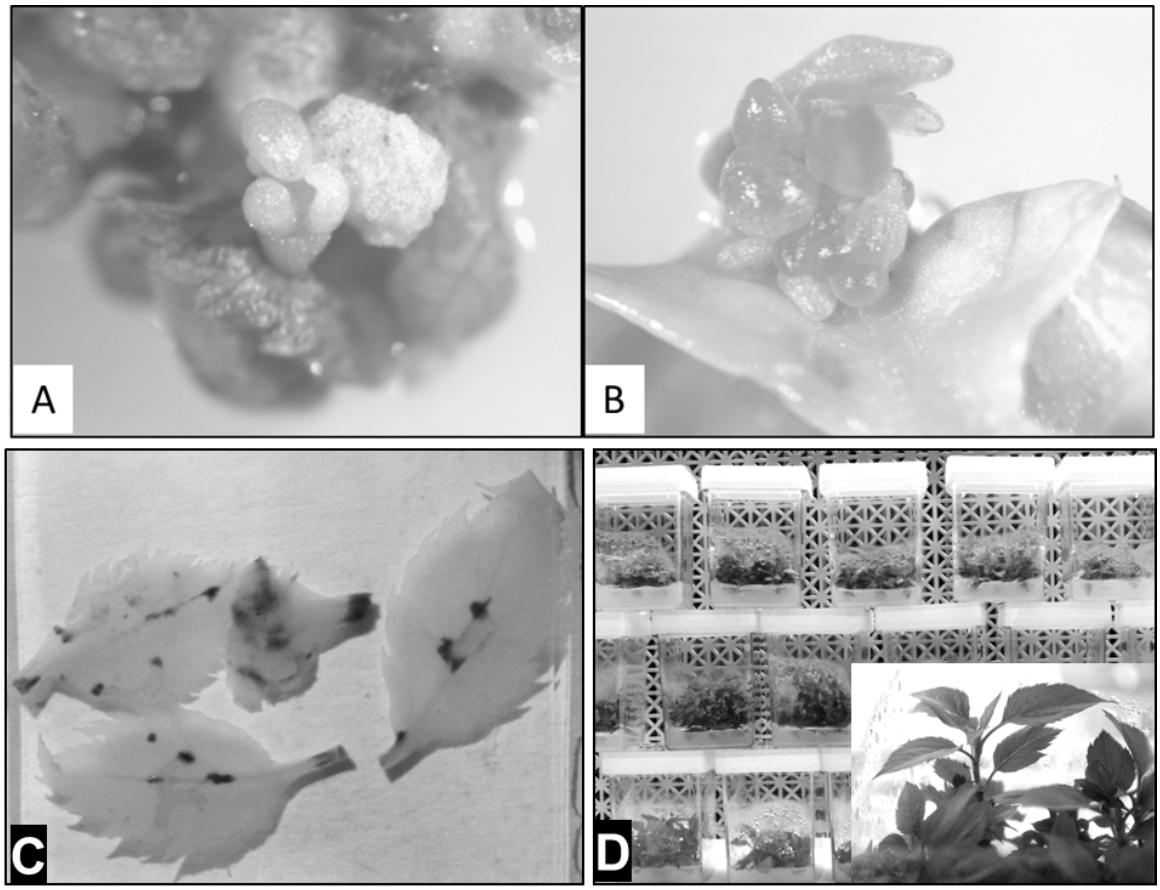

Figure 4: Transgenic experiments in Royal Gala. A: A putative transgenic shoot emerging from apple leaf tissue on antibiotic selection. B: Another transgenic event on another leaf explant. C. Dark spots indicate expression of transgenic protein in parts of the plant where the cells express the transgene. D. Representative transgenic plantlets thriving on very high concentration of antibiotic that allows only those plants to survive that harbor the transgene. Close up of one such plant is shown in the inset.

EXECUTIVE SUMMARY

The aim of this project was to progress earlier work initiated to *reduce the generation cycle* in breeding so that time and resources can be saved in combining multiple traits faster to produce the varieties desired by the growers and the consumers. This reduction in generation time has been identified as a priority in the WSU apple breeding program (WABP) in order to allow the rapid combination of desirable traits in new varieties.

Significant progress has been made in our ability to establish the required plant material in tissue culture (a key first step for the transformation process required for the fast generation cycling). Transformation experiments with our model variety Royal Gala also appear to be successful. Progress in understanding how to manipulate apple seedlings to rapidly go through juvenility in the greenhouse is underway; this model is however incomplete as most of the seedlings are currently in cold treatment or just emerging.

Summary of findings

Apple seedlings follow a unique growth pattern under our modified environmental conditions different from what has been reported for growth in the orchard. Careful observations were made over the last year and a preliminary phenotype-guided growth model has been established that guides decisions for fertilization, pruning and administration of cold treatment to meet chilling requirement. A clear impact of genetic background under modified environmental conditions on the growth rate is visible based on number of nodes generated per month.

Modified sanitizing and operating protocols were developed to counter the large pathogen load infesting the plant material required for initiating tissue culture. Tissue culture protocols for maintaining our target accessions have been established. Each individual requires a unique combination of phytohormones and salts in tissue culture for robust growth and development. Transformation protocols for Royal Gala as a standard for transgenic experiments have been established and several putative transgenic Royal Gala plants are ready to be tested for transgenic status. Micropropagation of WA 38 is underway for transformation experiments.

Future directions

We propose to continue this work focusing on the physiological manipulation of seedling growth and development as this will be a key tool in moving forward elite seedlings following the extensive DNA-assisted selection that we envisage will become a routine part of the WABP as more markers become available. A regeneration protocol is the next step required in establishing WA 38 as a quick-flowering transgenic that will serve as the intermediate parent in future rapid generation cycling crossing within the WABP.

CONTINUING PROJECT REPORT **YEAR**: 1 of 3
WTFRC Project Number: AP-12-103A

Project Title: Apple scion breeding program

PI:	Kate Evans	**Co-PI (2):**	Cameron Peace
Organization:	WSU TFREC	**Organization**:	WSU-Horticulture
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Address:	1100 N. Western Ave	**Address**:	PO Box 616414
City/State/Zip:	Wenatchee/WA/98801	**City/State/Zip**:	Pullman/WA/99164

Cooperators: Bruce Barritt, Professor Emeritus, WSU; Yanmin Zhu, USDA-ARS Wenatchee; Amit Dhingra, Dorrie Main, Carolyn Ross, WSU Pullman; Tom Auvil, Ines Hanrahan, WTFRC; Roger Adams, Willow Drive Nursery, Ephrata; Craig Hardner, Australian Crop Genetic Services, Brisbane, Australia; Fred Bliss, Davis, California.

Total Project Request: Year 1: 213,580 **Year 2: $206,640** **Year 3:** $211,812

Other funding sources

Agency Name: WTFRC Apple Review
Amount awarded: $59,740 (2012-2013)
Notes: "Further Development of an online toolbox for tree fruit breeding" PI: Main. Co-PIs: Evans, Jung, Peace, Oraguzie. Establishment of bioinformatics and databasing support to facilitate the translation of genomics information into application in WSU tree fruit breeding programs. Synergistic project for application of bioinformatics to tree fruit crops.

Agency Name: WTFRC Apple Review
Amount awarded: $89,000 (2012-2013)
Notes: "Increasing decision confidence in cultivar development and adoption" PI: Peace. Co-PIs: Hardner, Evans, Bink. Synergistic project to improve release and adoption decisions about the WABP's new cultivars.

Agency Name: WTFRC Technology Review
Amount awarded: $23,777 (2012-2013)
Notes: "Breeding in the 21st century: Technology platform for fast breeding" PI: Dhingra. Co-PI: Evans. Synergistic project to implement fast-track breeding to elite selections from the WABP.

Agency Name: USDA-CSREES Specialty Crops Research Initiative
Amount awarded: $2,000,000 + equal matching from universities, industry (Sep 2009 – Aug 2013)
Notes: "Tree Fruit GDR: Translating genomics into advances in horticulture". PI: Main. Co-PIs include Evans and Peace. Synergistic project for application of bioinformatics to tree fruit crops.

Agency Name: USDA-CSREES, Specialty Crops Research Initiative
Amount awarded: $7,200,000 + equal matching from universities, industry (Sep 2009 – Aug 2013)
Notes: "RosBREED: Enabling marker-assisted breeding in Rosaceae". PI: Iezzoni. Co-PIs include Peace, Main, and Evans. A synergistic project to establish sustainable marker-assisted breeding infrastructure for U.S. Rosaceae crops, using the Marker-Assisted Breeding Pipeline concept that involves Pedigree-Based Analysis.

WTFRC Collaborative expenses:

Item	2012	2013	2014
Salaries	18,830	**18,830**	19,395
Benefits	6,626	**7,232**	7,940
Wages[1,2]	52,500	**47,250**	48,668
Benefits[1,2]	7,650	**6,885**	7,573
RCA Room Rental	8,400	**8,400**	8,400
Shipping	0	**0**	0
Supplies	3,000	**3,000**	3,000
Travel[3]	6,875	**7,000**	7,500
Plot Fees	0	**0**	0
Total	103,881	**98,597**	102,476

Footnotes:
[1]Lab volume decreased by 50%, Field by 30%.
[2]Lab and Field volume decreased by 10%.
[3] In-state travel to research plots which are spread out across the state.

Budget 1
Organization: WSU-TFREC **Contract Administrator:** Carrie Johnston & Kevin Larson
Telephone: 509.335.7667, 509.663.8181 **Email address:** carriej@wsu.edu;kevin_larson@wsu.edu

Item	2012	2013	2014
Salaries[1]	54,642	**56,828**	59,101
Benefits	21,694	**22,562**	23,464
Wages[2]	20,800	**21,632**	22,497
Benefits	3,099	**3,223**	3,352
Orchard establishment supplies	19,000	**20,000**	19,000
Genotyping supplies	11,000	**13,000**	15,000
Travel[3]	15,000	**15,000**	15,600
Plot fees	8,400	**8,800**	8,800
Total	153,635	**161,045**	166,814

Footnotes:
[1] Salaries for Agricultural Research Technologist (Bonnie Konishi@ 1.0FTE) and salary for 3 months for genetic screening technician (Terence Rowland @ 0.25FTE)
[2] Wages for time-slip labor for orchard establishment and trait phenotyping.
[3] In-state travel to research plots which are spread out across the state.

Budget 2
Organization Name: Willow Drive **Contract Administrator:** Roger Adams
Telephone: 509 787 1555 **Email address:** roger@willowdrive.com

Item	2012	2013	2014
Seedling propagation	34,720	**42,095**	38,420
Phase 2 trees	4,425	**3,500**	3,500
Phase 3 trees	20,800	**0**	3,078
Plot Fees	0	**0**	0
Total	59,945	**45,595**	44,998

Objectives:

1. Produce, through conventional and DNA-informed breeding methods, promising selections and subsequently elite selections with outstanding eating quality and commercial potential.
2. Using both objective (instrumental) and subjective (sensory) evaluation techniques to develop selections with outstanding eating quality.
3. Use extensive performance evaluation in combination with genetic markers to validate and implement new DNA tests for key fruit and tree traits.

Significant Findings:

1. Eleven new crosses were made in 2012 with approximately 45,000 seeds produced in the WSU Apple Breeding Program (WABP). Some of these seeds will be held in reserve for future years.
2. Seedlings from approximately 14,000 seeds from 2011 crosses were grown in the greenhouse, 2,000 of which were screened for fire blight resistance.
3. A new seedling planting system was successfully used for seedlings tested with DNA markers. Approximately 7,300 seedlings were screened with DNA markers for fruit quality; just over 4,200 were culled leaving the remaining 3,100 to be transplanted to Willow Drive nursery along with the other 2,600 seedlings that were not screened.
4. Seedlings at Willow Drive were propagated on M.9 rootstocks for future orchard evaluation. More than 4,300 seedling/M.9 trees were produced in the nursery for planting in Phase 1 seedling orchards in 2013.
5. The final count of new Phase 1 trees planted in 2012 was approximately 3,800.
6. Promising selections already in Phase 2 trials (planted in 2005, 2006, 2007, 2008, 2009, 2010 and 2011) at three evaluation sites in Central Washington were evaluated for productivity and fruit quality.
7. Two new promising selections were planted at three evaluation sites in Phase 2 trials in 2012.
8. Seventeen promising selections made in 2011 were propagated in 2012 for planting in 2014 Phase 2 trials at three diverse sites in Central Washington.
9. Samples of fruit from eight Phase 2 selections were profiled by the sensory panel at WSU-Food Science and Human Nutrition under the supervision of Dr. Ross in December 2012.
10. The Cultivar Licensing Committee met in 2012 to develop a release strategy for WA 38 and future releases from the WABP.
11. More than 280 reference cultivars and individuals from breeding progenies based at the Sunrise orchard have been phenotyped in detail and extensively genotyped as part of the U.S. apple reference germplasm set for the SCRI RosBREED project, to be combined with similar data from other U.S. breeding programs for powerful analyses. Individual sugar and acid profile data have also been collected on all the RosBREED apple samples by Dr. Evans's graduate student Yingzhu Guan in collaboration with Dr. Rudell (USDA-ARS). Co-PI Peace co-authored a publication on the genome-wide apple SNP genome scanning capability developed as a part of RosBREED's core genomics resource development. The standardized phenotyping protocol for apple fruit quality was published by Dr. Evans with Dr. Peace and other RosBREED collaborators.

Methods:

<u>Objective 1 - Breeding:</u>

a. Marker-assisted parent selection will be used to determine the most suitable combinations of parents for crossing to achieve our aim of a portfolio of new improved apple varieties. Using data from the SCRI-funded RosBREED project and the Peace lab, facilitated by the new Breeders Toolbox for breeding database interfacing developed by the Main lab, we will choose the optimum cross combinations from among available germplasm.

b. Crosses will be made each spring, most likely aiming at annual production of around 20,000 seeds. Following vernalization, seedlings will be germinated and grown in the greenhouse at the TFREC. To optimize efficiency and accuracy of sample collection, leaf samples will be collected in the greenhouse from some of these seedling progenies and sent to the Peace lab for DNA testing. Genetic tests used will depend on the particular cross combination. Some progenies will be inoculated with fire blight to enable phenotypic selection for resistance. Only the un-culled seedlings will be planted in the nursery and then budded onto M.9 rootstock for further evaluation.

c. Budded trees will be planted at the TFREC Columbia View orchard for Phase 1 trials (Figure 1) where their resulting fruit will be evaluated. Selection in the orchard will be initially based on fruit appearance (primarily color, uniformity, freedom from defects) followed by eating quality (primarily firmness, crispness, sugar/acid balance).

d. Promising selections will be propagated onto either M.9 or G.41 rootstock and placed in replicated Phase 2 trials (five trees/selection) at three diverse sites in central Washington. Data will be collected on fruit quality, productivity and tree health. DNA samples will be collected from all Phase 2 selections for screening with predictive markers to provide DNA-based information on genetic potential to enhance subsequent selection decisions.

e. Outstanding selections will be propagated as "elites" for Phase 3 trialing with an aim of approximately 75 trees in each of four diverse grower sites in central Washington. Phase 3 is conducted in cooperation with the WTFRC, managed by Tom Auvil. Harvested fruit will be subjected to a range of storage treatments managed by Ines Hanrahan. Certified, virus tested material will be produced for Phase 3 elite selections and distributed to nurseries.

f. Outstanding selections will be proposed for commercialization, patent data will be collected and submitted and the nursery mother trees will be confirmed as true-to-type by genetic fingerprinting.

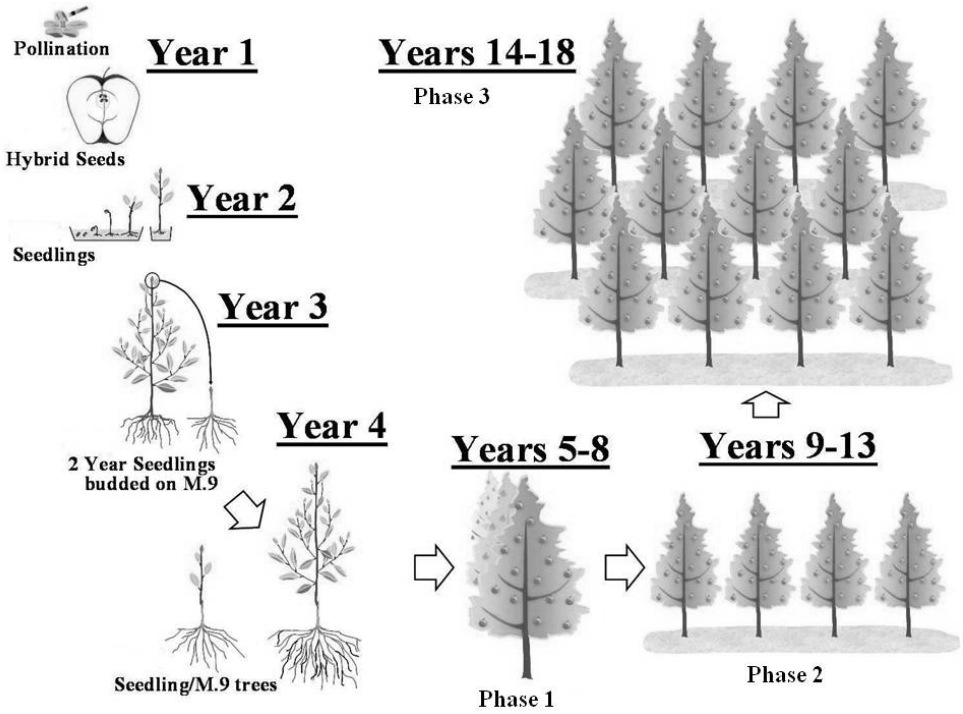

Figure 1: Breeding program schema showing the progression of selections through the program.

Objective 2 - Sensory/instrumental evaluation:

a. Using the labeling system initiated in the Phase 1 orchard, fruit samples collected will be bar-code labeled to correspond to the source tree, the pick number and the harvest date. These labels will then remain with the fruit as it is evaluated in the fruit laboratory thus minimizing mixing of samples and data-entry errors.
b. Ten fruit samples will be divided into five fruit for instrumental evaluation and five for sensory evaluation. The fruit for instrumental evaluation will be tested for maturity using the Cornell starch chart. Texture, size and weight will be recorded with the Mohr® DigiTest and the remaining fruit will be juiced for soluble solids concentration and titratable acidity measurements.
c. Sensory analysis will usually be performed by a team of four, producing a detailed breakdown of appearance and eating quality attributes. The breeding team was trained in sensory profiling by the Ross lab in Pullman in 2010.
d. First-season seedling fruit will be stored in regular atmosphere storage at the TFREC at 34^{o}F for two months prior to evaluation. If a sample achieves the appropriate overall rating, the same seedling tree will be harvested at more than one pick date the following year (subject to fruit availability). Second- and third- season samples will be evaluated at harvest as well as after two months storage. If sufficient fruit is available, a four month stored sample will also be evaluated.
e. Fruit evaluation will continue as selections move forward through Phases 2 and 3, with samples taken at up to four pick dates and evaluated at harvest and, after two and four months of regular storage. Larger volumes of fruit from Phase 3 will be drenched with a post-harvest fungicide, tested with and without a 1-MCP treatment and stored in regular and controlled-atmosphere storage using the Stemilt facility. Fruit out of storage will be tested in the WTFRC lab as well as the TFREC lab.
f. Fruit from promising selections from Phases 2 and 3 will be sent to the Ross lab in Pullman for trained sensory panel evaluation and consumer panel evaluation.

Objective 3 - DNA-informed selection development:

a. Marker-locus-trait associations will be developed and adapted to the WABP for important fruit quality traits including firmness, crispness, juiciness, acidity, soluble solid concentrations and fruit color principally from data collected as part of the SCRI-funded RosBREED project. Genetic tests will be further validated in the WABP using the multi-year data collected to date on advanced and elite selections. Tree traits such as precocity, vigor, disease and insect resistance and cross compatibility will also be targeted.
b. Full marker characterization will be performed on all elite selections to increase decision confidence in advancing selections to commercial release.

Results & Discussion:

Objective 1 - Breeding:

2012 was an excellent year for crossing with approximately 45,000 seeds being produced in 11 different cross combinations. Some of these seeds will be held in reserve for future years. Seedlings from approximately 14,000 seeds from 2011 crosses were grown in the greenhouse. Due to the pending retirement of Dr. Larry Pusey from the USDA-ARS facility in Wenatchee, the breeding team learned how to prepare fire blight inoculum required to

screen seedlings for resistance. Two thousand seedlings were screened for resistance in the greenhouse.

The new seedling planting system was successfully used for seedlings tested with DNA markers. Seedlings were planted in a "96-plant" format to mirror the DNA lab's "96-sample" (12x8) plate system, thus greatly reducing the possibility of data transfer error and reducing the number of labels required. Approximately 7,300 seedlings were screened with DNA markers for fruit quality; just over 4,200 were culled leaving the remaining 3,100 to be transplanted to Willow Drive nursery along with the other 2,600 seedlings that were not screened.

Approximately 3,800 new Phase 1 trees were planted at the TFREC Columbia View orchard for evaluation. The total number of seedlings in this Phase is now just over 24,000, reduced from the previous high level of 35,000, as other blocks of Phase 1 seedlings were also removed having come to the end of their evaluation period. Selection in the orchard was particularly difficult this year with the high level of smoke in the orchards and had to be based primarily on fruit appearance with eating quality tested back in the lab. Higher than normal levels of ethylene in the orchards also affected fruit ripening.

Two hundred and forty trees of WSU 46 were shared between three Phase 3 sites in spring 2012. Storage trials continued on fruit from six other elite selections harvested in 2011(conducted by I. Hanrahan). Selections WA5 and WSU 19 were discontinued due to internal breakdown issues in storage. Longer term storage of WA 2 and WA 38 continued to show promise with fruit receiving positive feedback when tasted at a field day in Wapato and the Sunrise field day (both in August 2012).

One hundred and seventy new trees of WA 38 were top-worked onto rootstocks in the Quincy and Prosser Phase 3 sites. The trees are growing well (particularly in Quincy where they are already starting to fill the trellis).

Five hundred and thirteen boxes of Phase 3 fruit were harvested in 2012 (conducted by T. Auvil) including a greater volume of WSU 17 and 36. These two selections will be tested through to six months in controlled atmosphere this season with and without 1-MCP treatment.

Following its release by WSU's Cultivar Release Committee, the patent application was submitted for WA 38 and more certified virus tested material was distributed to Washington nurseries. The first CVT mother trees were planted in 2012.

Samples of leaf tissue were collected from CVT mother trees of all WSU selections/releases currently held by the nurseries for trueness-to-type testing. Unfortunately due to a freezer problem, samples were lost and had to be re-collected late in the season. Early fingerprint data identified one or two possible problem trees; samples will be re-tested in 2013.

Objective 2 - Sensory/instrumental evaluation:

Bar-coded fruit samples continued to be of benefit in reducing sample mixing and data-entry errors. The fruit testing protocol was changed slightly in 2012. Data analysis from the supporting WTFRC-funded project "Increasing decision confidence in new cultivar development and adoption" showed that Phase 2 selections ranked in the same order of acceptability at harvest as after two months storage. Consequently, to reduce tasting fatigue, only instrumental tests were performed on these samples at harvest, saving considerable time and effort during the busiest season of the program. Also after the typical two months of air storage, fruit samples were stored for a further week at room temperature before sensory and

instrumental evaluation was performed; a more severe screening than previous years but one more reflective of the final consumer experience.

Fruit from eight Phase 2 selections were sent to the Ross lab, Pullman for trained sensory panel evaluation and consumer panel evaluation as part of the WTFRC-funded project "Sensory and consumer acceptance of advanced apple breeding selections".

Objective 3 - DNA-informed selection development:

Many genomics regions for valuable traits have been identified for WABP germplasm and will soon be used in breeding decisions for greater efficiency, accuracy, and creativity in developing new cultivars for Washington. These regions were discovered, or confirmed for those previously reported in other germplasm, using RosBREED data and analyses collected and conducted for the WABP in large part by our RosBREED-funded graduate students. The students have focused on sweetness (Yingzhu Guan) and acidity, crispness, and juiciness (Sujeet Verma). One region, the *Ma* locus, associated with all three latter traits, has been used to drive forward improvements in Pedigree-Based Analysis software, through close collaboration between Verma and our long-standing colleagues at Plant Research International, Netherlands, Eric van de Weg and FlexQTL™ software developer Marco Bink. Finally, co-PI Peace has used RosBREED data for the *Rf* locus, largely responsible for differences in skin color, to advance concepts and methods for translating genomic regions associated with important traits into predictive tools (Figure 2). Next to go through this pipeline of discovery, validation, and translation to practical application will be the critical Washington production-limiting traits of sunburn and various storage disorders.

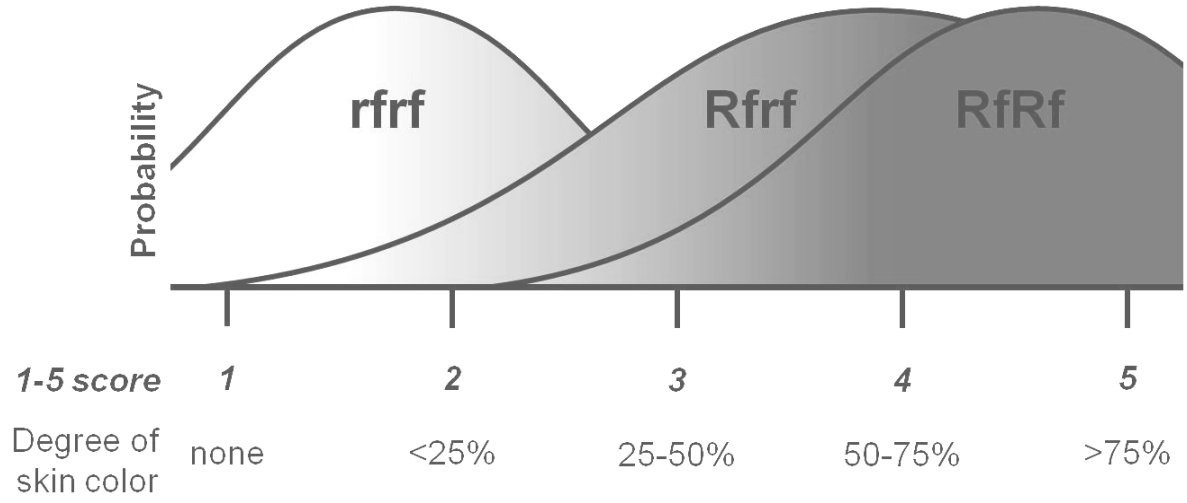

Figure 2: A predictive DNA test for fruit skin color – an example of how DNA information can be used in breeding. *DNA markers at the* Rf *locus can distinguish WABP breeding parents and selections as being one of three genotypes, each with a predicted degree of skin color. Using these distributions, the outcome of any potential cross can be predicted as the proportion of seedlings expected to exhibit a target degree of skin coloration. For example, for Gala × Honeycrisp, 44 seedlings per 100 created are expected to bear fruit with blush or stripes covering at least half the fruit surface, while for Enterprise × Honeycrisp it would be 65/100. So the first cross is only two-thirds as efficient as the second for the target outcome of genetic predisposition to higher-blushed fruit. (Note that if the first family was actually created, the one-quarter of all seedlings expected to be rfrf could be detected and culled by running the DNA test across all seedlings.)* ***We are developing such predictive models for all DNA tests available for important WABP traits.***

WABP Publicity – posters, presentations and fruit samples

Gift boxes of WA 2 and WA 38 were delivered to numerous industry contacts throughout the year with follow-up calls to collect feedback. Storage data and fruit of WA 2 and WA 38 were presented on August 22nd in Yakima and August 23rd in Wenatchee. Fruit was also available for tasting at the rootstock field day (Wapato, 8.22.12) and the Sunrise field day (8.23.12). A field day at the Quincy Phase 3 site was open to the public on September 25th.

Talks and posters:

- June 2012 – Dr. Evans presented "The potential impacts of genetics, genomics and breeding on organic fruit production" at the ISHS Organics meeting, Leavenworth, WA.
- July 2012 – Dr. Evans presented two talks during an invited visit to Western Australia, "WSU apple breeding program" at the Department of Agriculture and Food, Manjimup, and "Apple breeding in the Pacific Northwest" at the University of Western Australia, Perth.
- Examples from the WABP of marker-assisted breeding development and application were used in talks during 2012 at: Wiley Research Expo, Pullman, WA (Jan), FruitBreedomics 1st annual meeting, Prague, Czech Republic (Feb), RosBREED 3rd annual meeting, East Lansing, MI (Mar), 2nd International Symposium on Biotechnology of Fruit Species, Napier, New Zealand (Mar), American Society for Horticultural Science annual conference, Miami, FL (Aug), 6th Rosaceous Genomics Conference, San Michele, Italy (Oct), Yakima Pomology Club meeting, Pullman, WA (Sep), and the University-Industry Consortium, San Diego, CA (Oct).
- Two posters describing work associated with the WABP were presented at WSU's Academic Showcase, Pullman; fruit were also available for tasting (March).
- Posters on marker-assisted breeding in the WABP were presented at: the American Society for Horticultural Science annual conference, Miami, FL (Aug, 2 posters), and the 6th Rosaceous Genomics Conference, San Michele, Italy (Oct, 2 posters).

CONTINUING PROJECT REPORT **YEAR**: 2 of 3
WTFRC Project Number: AP-11-104

Project Title: 'WA 2' plant variety rights applications

PI:	Kate Evans	**Co-PI:**	Tom Kelly
Organization:	WSU-TFREC	**Organization**:	WSURF
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Address:	1100 N. Western Ave	**Address**:	1610 NE Eastgate Boulevard, Ste 650
City/State/Zip:	Wenatchee WA 98801	**City/State/Zip**:	Pullman WA 99163

Cooperators: Clean Plant Network, Prosser; Tom Auvil, WTFRC

Total Project Request: **Year 1:** 9,150 **Year 2:** 10,650 **Year 3: 3,440**

Other funding sources: None

WTFRC Collaborative Expenses: None

Budget 1
Organization Name: WSU; WSURF **Contract Administrator:** Kevin Larson; Tom Kelly
Telephone: 509 663 8181; 509 335 1210 **Email address:** kevin_larson@wsu.edu; kellytj@wsu.edu

Item	2011	2012	2013
Supplies	250	250	250
Travel	0	0	0
Miscellaneous	0	0	0
- **Quarantine costs**	8,000	0	0
- **PVR application fees**	900	10,400	3,190
Plot Fees	0	0	**0**
Total	9,150	10,650	**3,440**

Footnotes:
Supplies will cover collection and postal charges for distribution of propagating wood plus the issue of phytosanitary certificates.

OBJECTIVES

1. To establish certified virus tested (CVT) material of 'WA 2' in the Plant Variety Rights (PVR) process in selected territories (see "Methods" section below) as a prelude to applying for PVR in those territories, which is the only way to protect 'WA 2' outside the USA and control its possible release.
2. To apply for PVR for 'WA 2' in the EU and in the countries mentioned below, as is feasible, by project's end.

SIGNIFICANT FINDINGS

- Propagating wood was sent again to the EU from the University's Sunrise orchard; propagation was successful and trees are growing in quarantine.
- CVT propagating wood was sent to Chile, South Africa, Australia and New Zealand in winter 2011 from the Fruit Tree Clean Plant Network in Prosser for establishing trees in quarantine. Grafts in Australia failed; more wood will be sent as soon as possible.
- The first stage of the application for PVR in the EU is being prepared ready to submit in spring 2013.

METHODS

CVT propagating wood of 'WA 2' was sent from the Fruit Tree Clean Plant Network in Prosser (and WSU's Sunrise orchard if acceptable) to selected quarantine establishments in the EU, Chile, New Zealand, Australia, and South Africa. Quarantine is expected to take up to two years depending on the territory. Trees will be propagated for PVR testing and the initial application submitted in a timely manner as appropriate.

Agreements are in place and discussions have been initiated with in-country agents for each of these territories (a requirement for PVR applications). A strategy for testing 'WA 2' and controlling possible release will also be discussed with a view to having some of the PVR costs covered in this proposal reimbursed or at least some of the future annual PVR payments taken over by licensees. Beyond that, any unreimbursed funds spent under this proposal for obtaining and maintaining PVR protection would be recoverable from future royalties of that cultivar coming in to WSURF.

RESULTS & DISCUSSION

Under the agreements, agents acting on behalf of WSURF within each of the territories noted above will oversee the quarantine process and PVR application for 'WA 2'.

Propagating wood was sent to the EU quarantine establishment in the Netherlands from Sunrise orchard in April 2011; the EU, unlike other areas, will accept wood from the University orchard rather than having to go through the Clean Plant Network. Unfortunately these grafts failed, possibly as the grafting was late in the season, so wood was sent again early in 2012. This time, the grafts were successful and trees are growing well in quarantine.

Propagating wood from the Clean Plant Network in Prosser distributed to the other territories in winter 2011 as requested. Grafts failed in Australia so further propagating wood is being sent winter 2012/13.

The application for PVR in the EU is being prepared ready for submission in spring 2013. PVR applications for Chile, South Africa, Australia and New Zealand are expected to also be submitted in a timely manner.

CONTINUING PROJECT REPORT **YEAR**: 2013

Project Title: Apple rootstock and scion evaluation

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WTFRC Staff cooperators: Ines Hanrahan, Felipe Castillo, Tory Schmidt, Jim McFerson

Collaborators: Dr. Kate Evans, WSU-TFREC, Wenatchee,
Dr. Gennaro Fazio, USDA-ARS, Geneva, New York

Cooperators: Dave Allan, Rachel Crane, Del Feigal, Ron Wilcox, Dale Goldy, Tim Welsh, Jose Ramirez, Hans Groenke

Total Project Request: **Year 1:** 129,855 **Year 2:** 120,100 **Year 3:** 110,700

WTFRC Collaborative expenses:

Item	(2012)	(2013)	(2014)
Salaries[2,3]	30,500	31,500	32,500
Benefits[2,3]	10,360	10,700	11,500
Crew Wages[3]	59,750	50,000	40,000
Crew Benefits[3]	9,970	8,500	6,800
Stemilt RCA room	8,400	8,400	8,400
Shipping	0		
Supplies[4]	3,000	3,000	3,000
Travel[1]	7,875	8,000	8,500
Miscellaneous	0		
Total	129,855	**120,100**	110,700

Footnotes:
[1]Fuel and maintenance
[2]Salaries and benefits for Auvil, Hanrahan, Schmidt, and Castillo apportioned to this project.
[3]Harvest, storage and fruit quality lab labor for Phase 3 apple scion
[4]Trees for Phase 3 apple scion plots
Note: budget is for informational purposes only.

OBJECTIVES:

1. Evaluate apple rootstocks, particularly disease resistant rootstocks, in commercial settings in Washington State.
2. Integrate the processes of evaluation and industry implementation of new rootstocks and scions.
3. Refine protocols and improve efficiency for P3 scion evaluation program.

Scion evaluation accomplishments

- 177 trees at Quincy and 169 trees at Prosser were grafted to WSU 38 from discontinued Phase 3 (P3) genotypes. The Quincy trees are trained to a single leader and Prosser are two leader or twin stem trees. Most of the Quincy grafts grew 8 feet vertically and branched well. The Prosser site generally grew both leaders 3 feet with side branching.
- Fruit was harvested for industry samples. Storage samples for laboratory examination were reduced to late season CA for WA 2 and WSU 38.
- Pollination / fruit set experiments were set up and conducted by WABP on WA 2 and WSU 38.
- Gala was harvested and stored for WSU sensory program
- WSU 19 was discontinued due to internal browning.
- WSU 17 has issues with splitting in the stem bowel prior to harvest. The fruit is large, and varies in color considerably from site to site. It can have a vivid pink color. It is a low acid genotype. Breeding Program Advisory Committee (BPAC) may consider discontinuing WSU 17.
- Planted WSU 46 at three sites with about 80 trees each. Trees were small whips and may take another season to get canopy volume to crop.
- WSU 36 in Prosser and Mattawa had large size fruit on second leaf trees. It will require another season or two to assess its potential.
- Held a field day first week of October in Quincy and two post harvest report meetings in August.

Rootstock findings and activities

- G.41, G.890, G.935, G.30 G.214, G.935 are all replant disorder tolerant and fire blight resistant.
    - G.30 is difficult to propagate and has limited availability.
    - G.935 and G.30 are not woolly aphid resistant.
    - G.41 suffered from broken unions on budded trees in the nursery during 2012.
        - As with many new product introductions, occasional production problems may arise that may preclude full delivery of orders. These production problems are similar to the problems encountered when M.9 rootstock was first grown and budded in large quantities in the early 1990's.
- Yields of the Geneva replant tolerant genotypes continue to increase over control /commercial standards in replant sites.
- Precocity of the more vigorous Geneva genotypes (G.30 and G.890) remain high even as the production canopy matures. The crop density of these genotypes are significantly better than the Malling stocks they replace (M.26, M.7 and M.106)
- Production of Geneva rootstocks will increase significantly in each of the next few seasons, with increased volume of finished tree delivery 2016-17-18.
- G.890 and G.214 were grown in volume via tissue culture for spring 2013 and 2014 planting in stool beds.
- Fumigation in small plots or rocky soils has become difficult. Replant tolerant, especially the more vigorous and precocious rootstocks may provide a solution to replant sites. Also, vigorous

scions such as WSU 38 may also assist mitigating replant disorders' negative effect on canopy development.
- Two field days were presented in Wapato.

- In Washington State, G.210 is planted in only two Gala trials in Wapato. The replant tolerance indicated is impressive. t is not as large as G.30 and grows similar volumes of fruit as G.30. G.210 is commercially released and is worthy of more grower evaluation.
- G.222 is a good M.9 type rootstock with fire blight and woolly aphid resistance. It is not as replant tolerant in the limited number of trials in Washington State.

Table 1: Summary of 10 years of rootstock trials by WTFRC

	04 Gala Wapato	**04 Gala Chelan**	**'04 HC Naches**	**03 HC Frenchmn**	**03 HC Chelan**	**06 Fuji Vantage**	**06 Gala Wapato**	**'06 Fuji Brewster**	**08 HC Royal**
Bud 9	Replant	Replant	Replant	Root	Root		Replant		rootstock
3041 = **G.41**	Replant	Replant		Root	Root	PnP	Replant		rootstock
4214 = **G.214**	Replant	Replant	Replant	Root	Root	PnP	Replant	Replant	rootstock
5935 =**G.935**	Replant	Replant	Replant	Root	Root	PnP	Replant	Replant	rootstock
G.11	Replant	Replant		Root	Root	PnP	Root	Replant	rootstock
G.16	Replant	Replant	Replant	Root	Root	PnP	Replant		rootstock
M.26	Replant	Replant	Replant	Root	Root				
Nic.29	Replant	Replant		Root	Root				rootstock
Pajam 2	Replant	Replant		Root	Root				
M.9	Replant	Replant	Replant	Root	Root				
G.65									rootstock
M.27									rootstock
4202 = **G.202**			Replant			PnP		Replant	
G.30			Replant	Root	Root	PnP	Replant		
4011						PnP		Replant	
4019						PnP			
4172						PnP			
4288						PnP			
5046				Root	Root	PnP	Replant		
5202 = **G.222**						PnP	Replant	Replant	
5257						PnP	Root	Replant	
5463						PnP	Root	Replant	
Mark						PnP			
Sup 4						PnP			
M 9 Emla				Root	Root		Replant	Replant	
Pajam 1				Root	Root				
5757							Root		
5890 = **G.890**							Root	Replant	
5012							Replant		
6210 = **G.210**							Replant		
M.7								Replant	rootstock

Replant = Trial has seperate Fumigated and NON Fumigated units
Rootstock = Only Fumigated plots
PnP = Plant in Place benchgrafts in a well

prepared and fumigated site.

Rootstock hypersensitive to virus, not recommended
Geneva stocks in **Bold** font are commercially propagated
Shaded are replant tolerant.
Semi-Dwarf selections – many discontinued genotypes are not shown

Figure 1: 2006 Wapato Gala accumulated yield in bins per acre 2007-2012

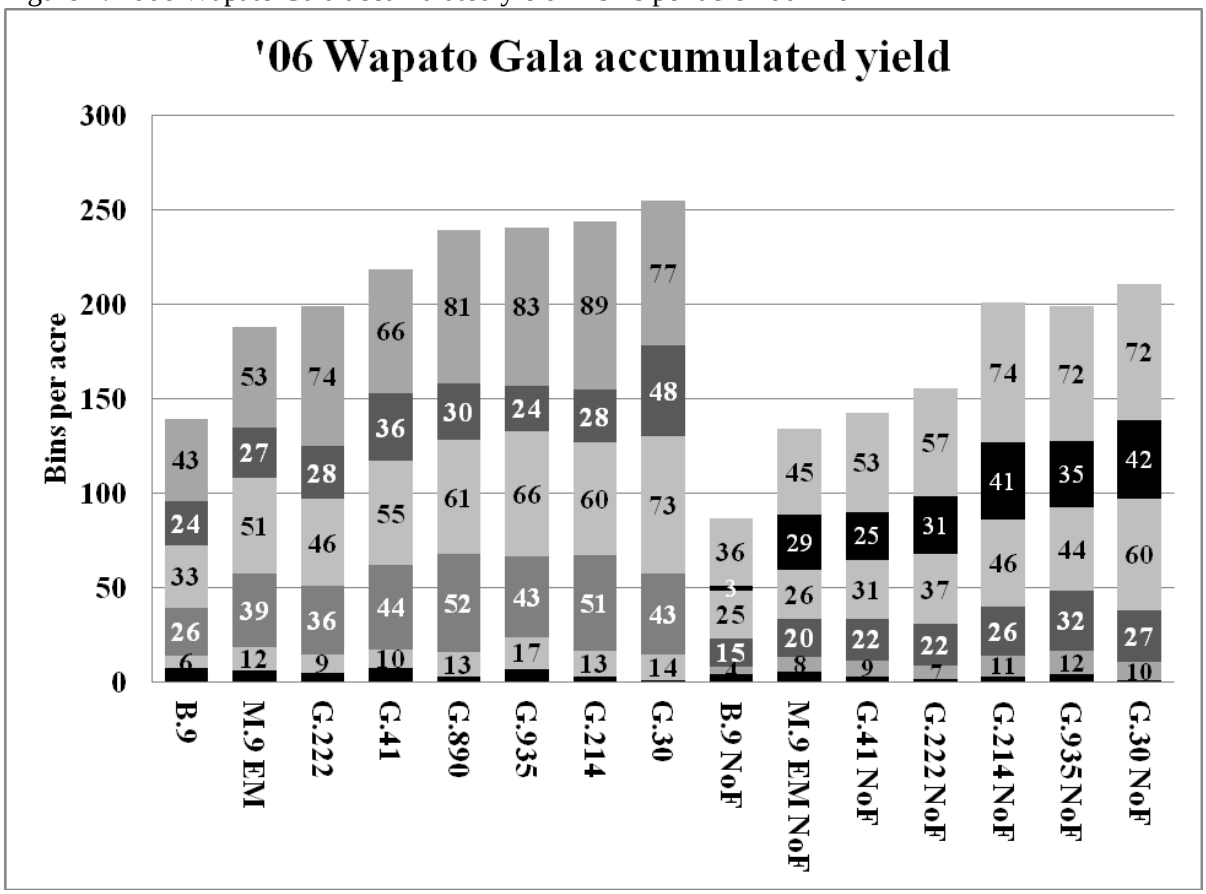


Figure 2: 2006 Wapato Gala Trunk Cross Sectional Area (cm^2) in 2012

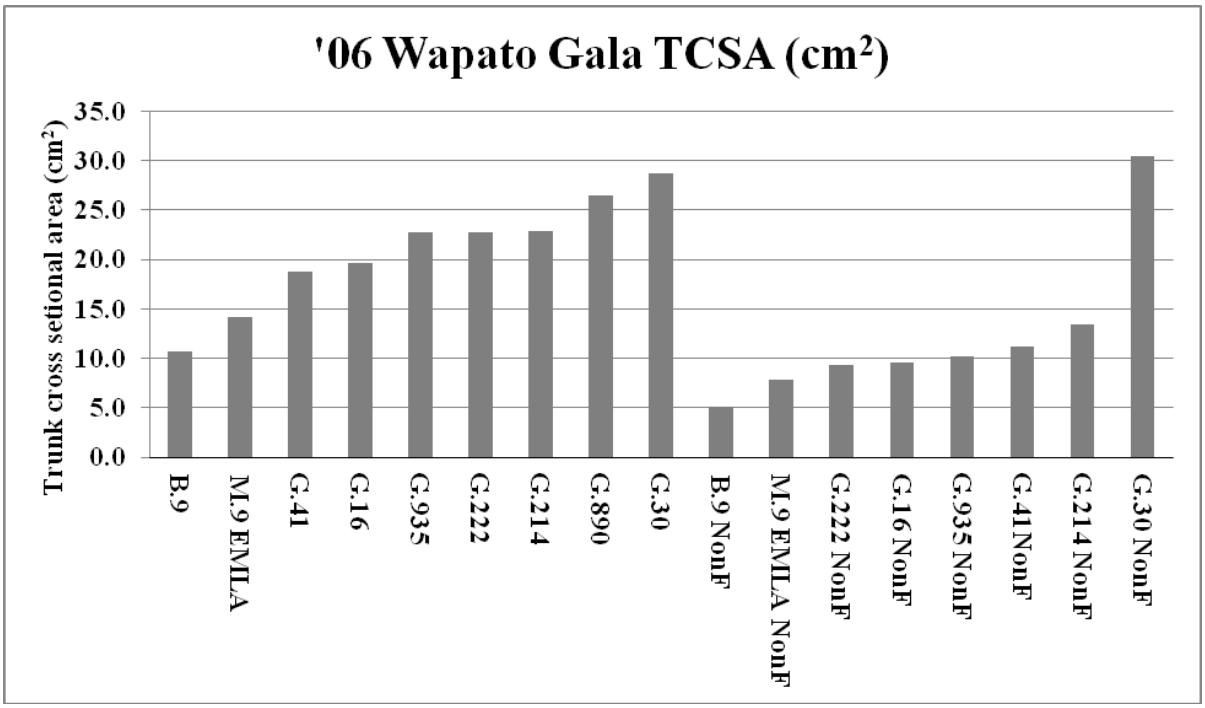


Figure 3: 2006 Vantage Fuji accumulated yield in bins per acre 2008-2012

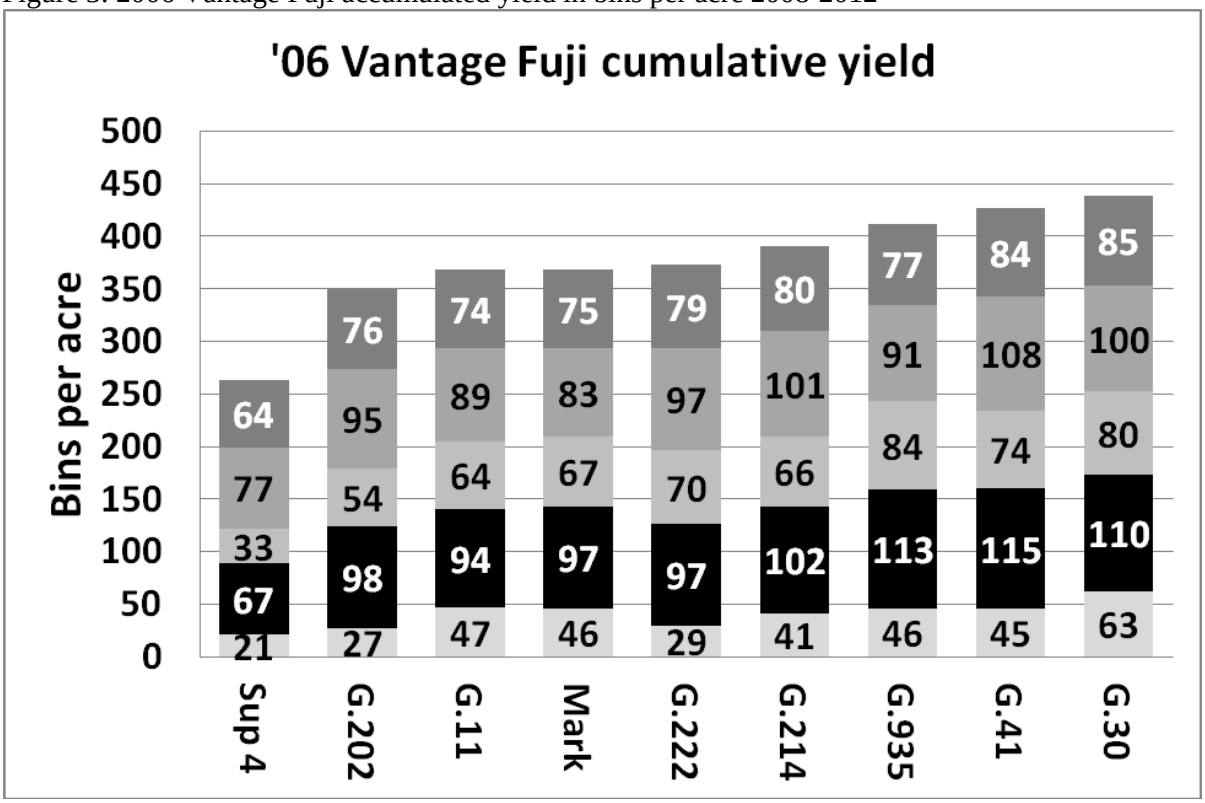
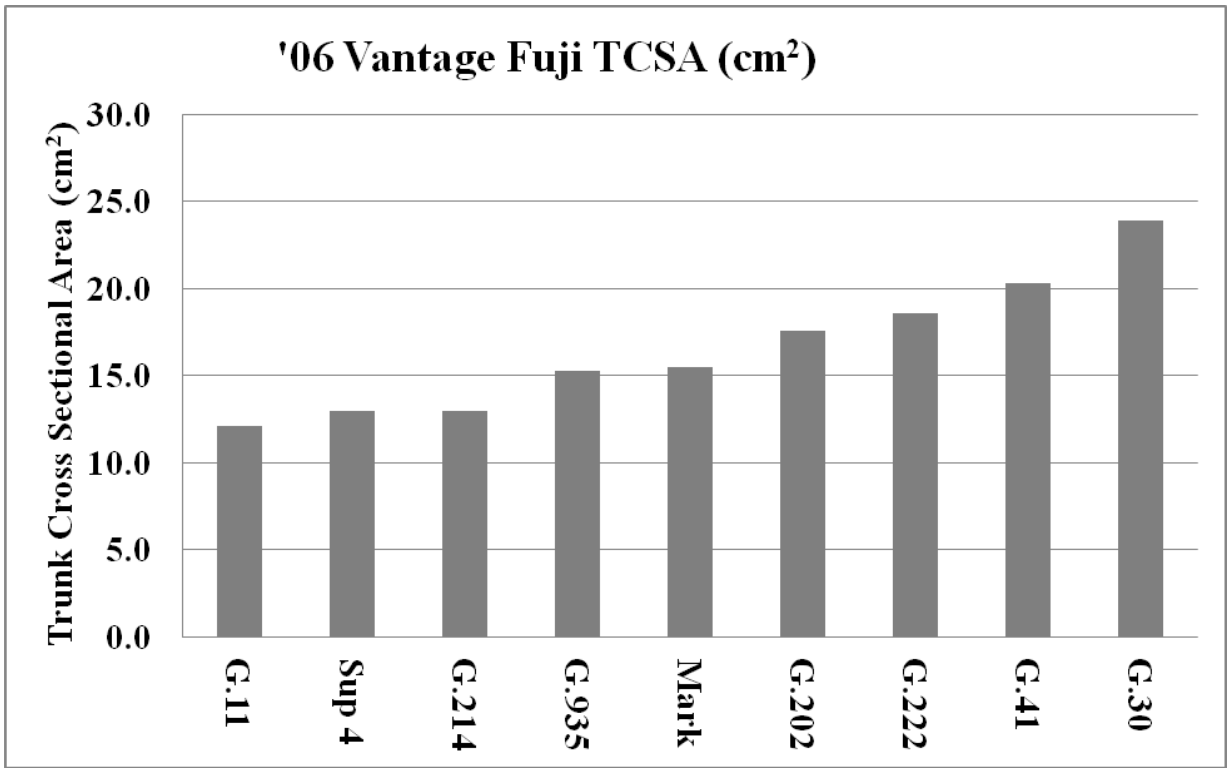


Figure 4: 2006 Vantage Fuji Trunk Cross Sectional Area (cm^2) in 2012



The rootstock trials were placed in various production and management systems. The performance of the replant tolerant Geneva rootstocks has been very similar: G41, G.214, G.935, G.30 out yield the commercial standards of M.9 EMLA and Mark. The unfumigated plots of the replant tolerant rootstocks have cumulative yield equal to or greater than the fumigated standard of M.9 emla. The exception has been G.41 in the Wapato Gala trial due to an irrigation problem in the first two seasons of the trial.

Cornell University has a data summary for commercially released genotypes at the following website: http://www.cctec.cornell.edu/plants/GENEVA-Apple-Rootstocks-Comparison-Chart-120911.pdf

There are significant discrepancies between New York data and Washington State data.

- The estimate of canopy volume is much larger in New York than in Washington State. G.890 and G.30 are obviously larger than M.9 in Washington State, but they are not M.7 or M.106 class. There is a significant increase in vigor in the M.9 clones from M.9 Fl56 to M.9 Nic29. G.890 and G.30 are one vigor class step up from vigorous M.9, and are more productive than the vigorous M.9 clones.
- Cornell rates G.202 as replant tolerant. Washington data is at best inconclusive.
- Cornell rates G.222 as NOT replant tolerant. Washington data indicates that G.222 is not susceptible to replant disorder. G.222 has not been adequately evaluated for replant tolerance in Washington State. It does propagate well and Cornell rates as very fire blight resistant and woolly apple aphid resistant.
- G.969 has been released however there is not any data in Washington State and is characterized as a vigor class larger than G.30 with replant and fire blight resistance.

CONTINUING PROJECT REPORT **YEAR**: Extension

Project Title: Greater system efficiency and fruit quality via soil microbiology

PI:	Mark Mazzola	**Cooperator:**	James Mattheis
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City:	Wenatchee	**City:**	Wenatchee
State/Zip	WA 98801	**State/Zip:**	WA 98801

Total Project Request: $208,547 **Year 1:** 66,927 **Year 2:** 69,830 **Year 3:** 71,790

Budget 1

Organization Name: USDA-ARS **Contract Administrator:** Chuck Meyer
Telephone: 510-559-5769 **Email address**: chuck.myers@ars.usda.gov

Item	Year 1	Year 2	Year 3	Year 4
Salaries[a]	47,690	49,120	50,594	
Benefits	15,737	16,210	16,696	
Wages				
Benefits				
Equipment				
Supplies	3,500	4,500	4,000	
Travel			500	
Miscellaneous				
Total	66,927	69,830	71,790	0

Footnotes: Project has been approved for a no-cost extension

OBJECTIVES:

The current research program directly addresses the 2012 apple research priority concerning soil microbiology and its linkage to health and productivity. Specifically, elements of this program seek to identify relationships between soil indices, such as mineral nutrition and plant response, and to better understand interactions between the rhizosphere microbiome (all elements of microbiology) and growth and yield, and relationship to rootstock performance/development.

SIGNIFICANT FINDINGS:

- Pyrosequencing analysis can provide new insight into the structure and function of orchard soil microbial communities and serves as a powerful means for the identification of microbial species or groups that will affect soil health and orchard productivity.

- Diversity and abundance of mycorrhizal fungi detected in roots was significantly greater for trees grown in Brassicaceae seed meal amended or fumigated soil than when grown in non-treated replant soil. The most prominent mycorrhizal groups associated with healthy trees were of unknown identity.

- Fungi of the genus *Oidiodendron* were only detected in the rhizosphere of trees cultivated in Brassicaceae seed meal amended soils; this is significant in terms of sustained soil health as these fungi have been reported to provide biological control of *Phytophthora* root and crown rot of apple.

- Yield performance of Gala/M9 and Gala/G11 was identical and rhizosphere microbial communities were similar for both rootstocks in fumigated replant orchard soil.

- In contrast, yield performance and rhizosphere microbial community composition exhibited a rootstock-dependent response in Brassicaceae seed meal amended soil; Gala/G11 outperformed Gala/M9; however, yields for trees on both rootstocks were superior to that in fumigated soils.

- Application of Brassicaceae seed meal soil amendment did not alter fruit quality as assessed by firmness, relative to control or fumigation treatments after 4 months cold storage in air.

- The process of ammonia oxidation, the rate limiting state in the nitrification process, was dominated by the activity of ammonia oxidizing archaea, rather than bacteria, in three orchard soils of central Washington State.

GOALS & ACTIVITIES

During the final year of this project a primary objective will be to conduct further bioinformatic analysis of the data set generated through pyrosequencing of rhizosphere bacterial/archaeal/fungal communities as a means to characterize the functional biology responsible for enhanced soil health and root development at the WSU-Sunrise orchard. Studies will continue to examine the effect of fertility management options on retention or loss of nitrogen from orchard soils with the goal of reducing microbial driven nitrogen loss through input management. Specifically the goal will be to link gene activity to resulting N form and concentrations in different orchard soils as a means of characterizing the relative dominance of edaphic or genetic factors in determining N availability. Thirdly, the effect of fertility management options on the dynamics of nematode communities, which are important factors in N cycling and consistently have been correlated with “soil health” and biological resilience of soil systems, will be characterized through application of DNA-based studies

including T-RFLP, real-time quantitative PCR and sequencing of clone libraries. It is anticipated that these findings will be used to identify appropriate fertility treatments to be applied at the Sunrise orchard in fall of 2013 to address objective 4 of this project plan.

METHODS:

Samples were collected from the rhizosphere of trees grown in fumigated and control soil, and a root-stimulating (brassicaceae seed meal) soil amendment at the WSU-Sunrise orchard (sandy texture; 1.2% organic matter [OM]). Soil was collected from the rhizosphere of two trees in each 10 tree block with five replicate blocks per soil treatment and two rootstocks; M9 and G11. DNA was isolated and purified to standardized concentration and amplified using primers specific from bacteria/archaea (926F-1392R) and fungi (ITS1F-ITS4R) in pyrosequencing reactions. Reactions resulted in the generation of 5000 to 10000 sequences/microbial group/sample (72 samples). Community profiles among treatments will be compared and contrasted, and analyzed in the context of relative root development, disease suppression, growth and yield of apple.

Soil fauna have demonstrable effects on plant root development. While numerous studies have examined the effect of fertilizers on bacterial community size and function (Hallin et al., 2009), few have assessed these same effects on the soil fauna. Synthetic fertilizers commonly depress soil microbial diversity and activity. Development of treatment strategies to augment rather than suppress the soil fauna populations will contribute positively to tree productivity both directly through biotic mechanisms that lead to a more resilient ecosystem and indirectly via mineralized nitrogen production. The effect of different N inputs on orchard soil nematode communities will be examined. Orchard soils will be treated with mineral ($CaNO_3$ or urea) or organic (chicken manure, canola meal or mustard meal) nitrogen inputs at the rate of 70 lbs per acre. Soils will be planted to apple and sampled every three weeks for a period of 18 weeks with three replicates per soil treatment. DNA will be isolated from each soil sample and amplified using the nematode specific primer pair. Nematode communities will be analyzed by T-RFLP analysis to determine whether significant qualitative changes result from these treatments. If significant differences are observed the specific nature of these changes will be determined by sequencing of a clone library generated using the amplified nematode DNA from each soil.

The link between DNA contents and activity in the field is not always conclusive and therefore, increased gene copies do not always mean that activity will be increased. Studies will be conducted to assess the relative dominance of edaphic factors and genetic factors in the efficient retention of N in soil systems. To date, we have completed analysis of the abundance and activity of genetic factors involved in N cycling for two orchard soils as influenced by nitrogen amendments (WSU Sunrise, sandy texture; 1.2% OM; RF, sandy loam, 4.2% OM). Analysis of the third orchard soil (GC, gravelly sandy loam, 3.3% OM is currently in progress. In addition, rhizosphere soils from these treatments have been collected and determination of ammonium (NH^{+4}) and nitrate (NO^{-3}) concentrations is currently in progress. To assess the relative correspondence between gene abundance and function in these soils, potential ammonia oxidation, an estimation of the production of nitrite in soil, will be determined. These data will provide guidance on the relative activity of the bacterial and archaeal ammonia oxidation genes (*amoB* and *amoA*), which have been quantified in these soils.

RESULTS & DISCUSSION:

Pyrosequencing analysis of rhizosphere fungal community

The effect of soil treatment and rootstock on composition of the rhizosphere fungal community was assessed through pyrosequencing analysis. The trees were Gala grafted to G11 or M9 rootstock and

were planted in May 2010 into replant soil at the WSU-Sunrise orchard which was not treated (control), fumigated (Telone-C17) or amended with *Brassicaceae* mustard seed meal (*Brassica juncea/Sinapis alba*). Roots and associated soil were collected in October, 2011 and DNA isolation and pyrosequencing was conducted through 2012. The analysis yielded approximately 1 million total sequences representing 568 different fungal species. The abundance and diversity of arbuscular mycorrhizal fungi (Glomeromycota) detected in the apple rhizosphere was greater when trees were grown in fumigated or seed meal amended soils, than when grown in non-treated replant soil at the WSU-Sunrise orchard (**Fig. 1**). The findings also demonstrate that a greater diversity of fungi have potential to form mycorrhizal associations with apple than is commonly reported. Although the Glomerales (primary genus *Glomus*) dominated the mycorrhizal population detected in the apple rhizosphere when grown in non-treated replant soil, multiple orders of mycorrhizal fungi were detected when trees were grown in fumigated soil or seed meal amended soils. The order Diversisporales, which includes important mycorrhizal fungi within the genera *Acaulospora*, *Gigaspora* and *Scutellospora*, were prominent in the rhizosphere of M9 when cultivated in fumigated or seed meal amended soils. Of significance is the observation that the dominant group of mycorrhizal fungi detected in the rhizosphere of apple when cultivated in seed meal amended or fumigated soils represented a group of unknown identity.

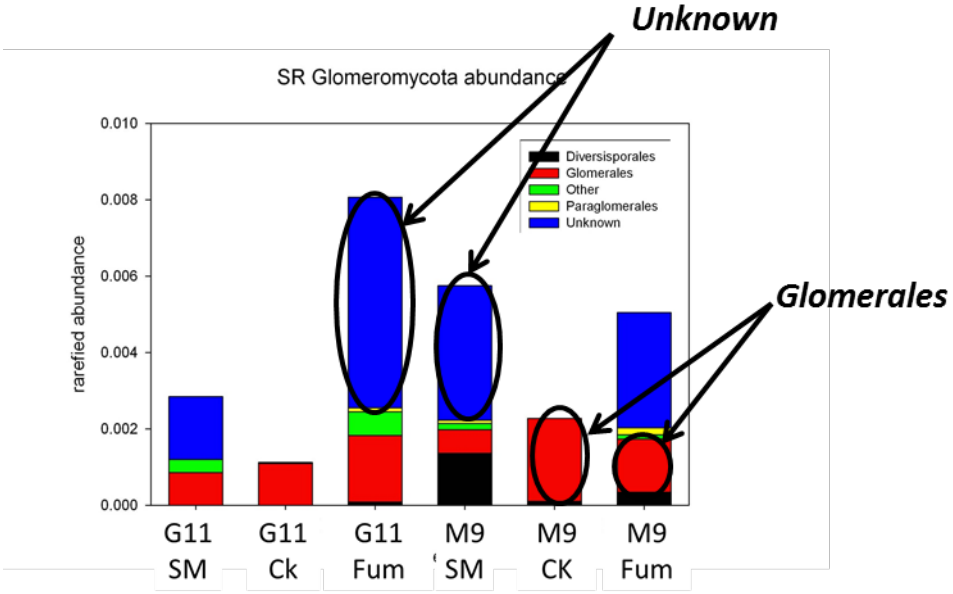


Fig. 1. Abundance and diversity of mycorrhizal fungi as detected through pyrosequence analysis of DNA isolated from the rhizosphere of apple at the WSU-Sunrise orchard. SM=Brassicaceae seed meal; Ck=no treatment control; Fum=Telone-C17 fumigation.

Soil treatments had significant effect on composition of the ascomycete fungal community detected in the apple rhizosphere. Interestingly, many genera of ascomycetes possessing members that cause various foliar and shoot diseases of apple (*Dothideomycetes* and *Septogloeum*) were present at greater abundance in the rhizosphere of trees cultivated in the control and fumigated orchard soils. This may suggest that the seed meal treatment induces the development of a more competitive soil microbial environment that limits re-colonization by fungi that are introduced on leaf litter/tree debris.
A significant, and potentially important finding relative to long-term orchard resilience to disease development, was the observation that members of the genus *Oidiodendron* sp. were only detected in the rhizosphere of apple cultivated in seed meal amended soil (**Fig. 2**). The importance of these

fungi resides in the fact that they have been reported to provide biological control of *Phytophthora cactorum*, causal agent of Phytophthora crown and root rot in apple.

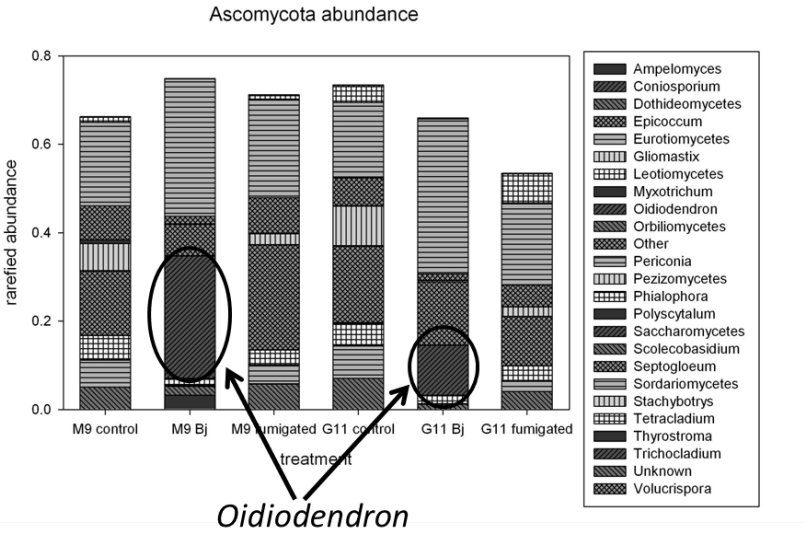


Fig. 2. Composition of the ascomycete fungal community in the rhizosphere of apple as influenced by rootstock and soil treatment determined by pyrosequencing analysis.

Pyrosequencing analysis of rhizosphere bacterial community

Pyrosequencing analysis yielded over 1 million bacterial sequences which represented over 1219 different bacterial species. There were clear associations between bacterial community composition and relative tree growth and yield performance as influenced by both soil treatment and rootstock. When cultivated in fumigated soil, yields from Gala/M9 and Gala/G11 were virtually identical, and were significantly greater than that attained in non-treated replant soil (**Fig. 3**). The bacterial communities detected in the rhizosphere also were highly similar when these two rootstocks when cultivated in fumigated soil (**Fig.** 4). In contrast, when cultivated in seed meal amended soil the rhizosphere bacterial community composition of these two rootstocks were dissimilar and Gala yields were significantly greater when grafted on G11 than on M9 rootstock. For both rootstocks, yields attained in seed meal amended soils were greater than that attained in fumigated soils, and bacterial communities detected in the rhizosphere of trees cultivated in fumigated and seed meal treated soils were highly dissimilar.

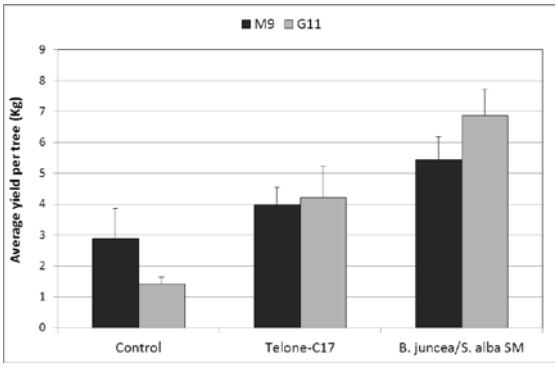


Fig. 3. 2012 Yields at WSU-Sunrise orchard from a Gala block planted in May 2010. Bars represent one standard deviation of the mean.

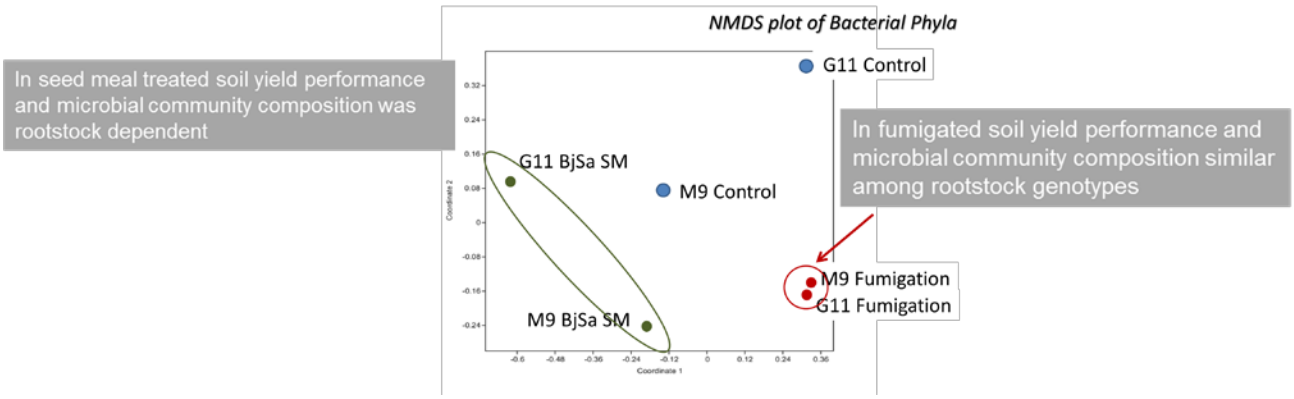


Fig. 4. Pyrosequencing analysis of rhizosphere microbial communities indicated that rootstock-dependent yield performance in seed meal amended soils is linked with differences in bacterial community composition. No differences in yield performance or microbial community composition were observed among rootstocks in fumigated soils. This finding suggests that long-term pathogen suppression and enhanced tree performance in SM treated soils are biologically determined.

The effect of soil treatments on fruit quality was assessed. Seed meal amendment at planting did not alter fruit quality in terms of firmness. After 4 months storage under normal atmosphere at 40 F, no significant differences in Gala fruit firmness were observed among soil treatment or rootstock (**Table 1**). Differences in fruit color were observed. Gala fruit from trees grown in the non-treated control replant soil exhibited enhanced color development relative to fruit from either the fumigated or seed meal amended soil. This finding indicated that the difference in fruit color may have resulted from a stress response rather than differences in fertility management as fumigated and non-treated plots received the same fertility input.

Table 1. Effect of soil treatment on Gala fruit quality parameters

Soil treatment/rootstock	Pressure (lbs.)	hue	chroma
Control M9	16.33±1.57	68.5±15.1a	33.9±2.6a
Seed meal M9	16.20±2.08	71.3±23.3ab	35.5±3.9b
Fumigation M9	16.39±2.04	76.2±17.6b	35.5±3.3b
Control G11	16.71±1.97	62.0±26.4a	36.1±4.0a
Seed meal G11	16.21±1.82	72.6±20.2b	35.1±3.4a
Fumigation G11	16.78±1.49	80.3±18.8c	34.8±3.5a

Values are means ± one standard deviation; within a rootstock, means followed by a different letter are significantly different.

Significance: Preliminary analysis of pyrosequencing data from apple rhizosphere microbial communities has revealed several previously uncertain or unknown attributes of orchard soil biology and the effects of management on potential for long-term soil health. The mycorrhizal community detected in the apple rhizosphere possessed far greater diversity than that commonly inferred, and is dominated by unknown or uncharacterized species. Given that most commercial mycorrhizal inoculants are dominated by *Glomus* spp., and the vast majority of native isolates detected in apple roots in this study were not, the utility, appropriateness and/or efficiency of such products must be questioned. The seed meal amendment system used in this study selected for multiple components of the fungal and bacterial community that may provide long-term disease suppression. Our data from the WSU-Sunrise and other field trials support this inference. Rootstock genotype will also influence

rhizosphere microbial community composition; thus rootstock genotype must be considered when attempting to manage long-term orchard health.

Studies continued in multiple orchard soils to monitor the presence of specific microbial genes that function in the cycling of nitrogen. The goals are to gain an understanding of the active microbial populations responsible for nitrogen cycling in orchard soils and the effect of different fertility inputs on the abundance and diversity of these microbial groups, to relate the comparative abundance of these populations to potential loss of nitrogen from orchard soils, and to identify inputs or methods that could minimize such loss. Interestingly, ammonia oxidizing microbial community was dominated by archaea, rather than bacteria in all three orchard soils examined (**Fig. 5**).

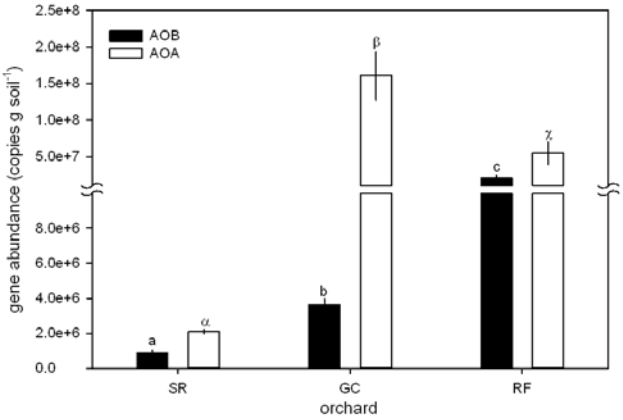
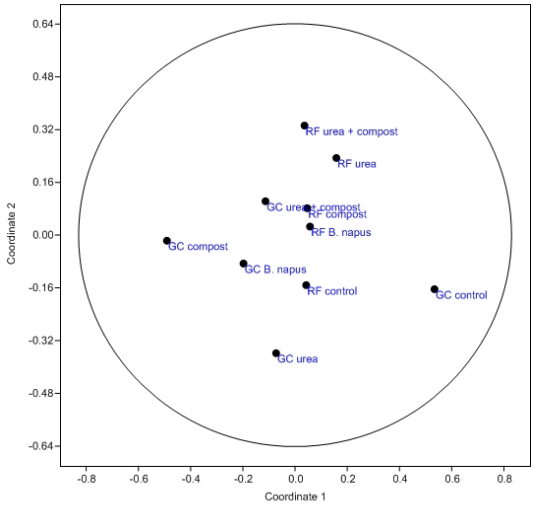


Figure 5. Bacterial (AOB) and archaeal (AOA) ammonia-oxidizing *amoA* gene abundance in non-amended SR, GC and RF orchard soils. Values are mean ± SE (n = 7). Means with different letters are significantly different at $P < 0.05$ based upon Tukey's honest significance test for comparisons between soils.

The genetic composition of the AOB (Fig. 6) and AOA (data not shown) communities did not differ significantly among orchard soils or in response to fertility inputs.

Figure 5. Effect of fertilizers on ammonia-oxidizing bacterial community composition in GC and RF orchard soils as assessed by non-metric multi-dimensional scaling (NMDS) analysis of T-RFLP data. Treatments include non-amended control, *Brassica napus* seed meal (B. napus), plant-based compost (compost), urea, and urea with compost (urea + compost). All samples fell within the 95% confidence interval ellipses and therefore are not significantly different.



Significance: Within a confined climatic zone (NC Washington) the genetic composition of the ammonia-oxidizing community did not differ among orchard soils of varying fertility levels and texture suggesting that practices developed for management of this community should have application across diverse soil systems. Archaea rather than bacteria dominate the ammonia-oxidizing microbial community and must be considered when developing efficient fertility management programs.

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

Project Title: Testing biomarker-based tools for scald risk assessment during storage

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Cooperators: Dr. Bruce Whitaker

Total Project Request: **Year 1:** $24,750 **Year 2:** $24,750 **Year 3: $24,350**

Other funding sources

Agency Name: NIFA, USDA (Grant no. 2010-51181-21446**)**
Amt. awarded: $1,483,438 (federal total over 4 years)
Notes: Specialty Crops Research Initiative grant to develop biomarker based storage management tools for multiple apple postharvest physiological disorders was awarded during the last cycle. David Rudell (Project Director) will manage and participate in the project and James Mattheis (Co-PI) and Yanmin Zhu (Co-PI) will also participate. This is a multi-state, multi-national project. The proposed project extends and compliments the activities of this SCRI project.

Agency Name: AgroFresh, Inc.
Amt. awarded: $232,253 (for Rudell and Mattheis role in SCRI project over 4 years)
Notes: Cash donation to support activities and objectives outlined in the Specialty Crops Research Initiative grant to develop biomarker based storage management tools for multiple apple postharvest physiological disorders was awarded during the last cycle (see above).

Budget

Organization Name:USDA-ARS **Contract Administrator:** Chuck Myers

Telephone: (510)559-5769 **Email address:** Chuck.Myers@ars.usda.gov

Item	2011	2012	2013
Salaries			
Benefits			
Wages	$15,038	$15,038	$5,263
Benefits	$4,962	$4,962	$1,737
Equipment			
Supplies[1]	$1,000	$1,000	$1,000
Travel			
Miscellaneous[2]	$3,750	$3,750	$3,750
Miscellaneous[3]			$12,600
Total	$24,750	$24,750	**$24,350**

Footnotes:

[1]Liquid nitrogen for sample processing and instruments; sample vials.

[2]25% service and maintenance for laboratory instruments used in project.

[3]Storage rental fee (2 rooms at $6,300 each per season)

Objectives:

1. Determine if scald risk assessment tools indicate when delayed CA imposition leads to high scald risk and high scald incidence.
2. Indicate if and when scald risk is high during CA storage based on risk assessment tools and determine if storage conditions can be changed to alter biomarker levels and scald incidence.
3. Assess effectiveness of scald-risk assessment tools in pilot scale and commercial CA storages.

Goals and activities for the next year:
Validation of real-time scald risk monitoring of multiple lots of Granny Smith in pilot scale CA rooms to test when and if scald risks are high. A preliminary test is also monitoring scald risk of the same lots in a commercial CA room. We are also testing user-friendly techniques for monitoring biomarkers.

SIGNIFICANT FINDINGS:

1. Validated 21 of 25 scald risk assessment biomarkers (SRABs) as predictors of scald risk.
2. Levels of 13 SRABs most effectively reflected scald risk altered by delaying CA imposition.
3. SRAB levels correlated with scald risk as early as 3 months after CA storage imposition. scald was first detected at 9 months.
4. Changes in SRAB levels can detect scald risk early in fruit stored under suboptimal atmospheres and storage outcome improved (reduced scald) by subsequently adjusting CA conditions.
5. Changes in SRAB levels directly reflect how the fruit is reacting to the CA and crop protectant conditions imposed.

METHODS:

Equipment and Cooperative Summary: Tissue sampling, processing and analysis of SRABs using analytical instrumentation (gas and liquid chromatography-mass spectrometry) will be performed at ARS-TFRL, Wenatchee. Storage experiments will be performed both at ARS-Wenatchee and in commercial storages. Additional chemical identification and molecular characterization will be performed in cooperation with Dr. Bruce Whitaker (BARC, USDA-ARS, Beltsville, MD). New information will be disseminated through published articles in peer reviewed journals as well as poster and oral presentations at industry meetings and professional conferences.

Experimental Plan
Procedures:
Year 1
Relationships between delayed CA and levels of candidate scald biomarkers
'Granny Smith' apples were harvested 1 month prior to commercial harvest to improve the likelihood of maximum scald susceptibility. Firmness, internal ethylene concentration, soluble solids, and titratable acidity were evaluated at harvest. Apples were stored at 33 ^{o}F in air for 0-1 month upon which subsets will be transferred to CA (1% O_2: 1% CO_2). Scald risk assessment biomarker (SRAB) levels were evaluated monthly from 0-6 months. Scald incidence and severity were evaluated immediately after removal from storage (from 2 to 10 months) and following 7 days at 68 ^{o}F.

Year 2

Test whether biomarker levels and scald incidence can be altered by altering CA conditions mid-storage.

'Granny Smith' apples were harvested 1 month prior to commercial harvest. Fruit maturity and quality were evaluated at harvest. Apples were stored at 33 ^{o}F in CA at 1% or 5% O_2 (all 1% CO_2). Scald risk assessment biomarker levels were monitored monthly. When biomarker levels indicate scald risk is increasing, a portion of the fruit stored at 5% O_2 was pulled down to 1% O_2. Biomarker evaluation will continue to indicate if levels return to "normal" levels. Scald was evaluated in all treatments at 0 and 7 days (at 68 ^{o}F) after 3, 6, and 9 months storage.

Year 3

Biomarker-based scald risk-assessment under scaled-up test conditions

'Granny Smith' apples were taken from 4 commercial lots with varying scald-susceptibility histories. Multiple bins of fruit from each lot are being stored in commercial pilot scale rooms (40 bin) or larger rooms at 33 ^{o}F in CA at 0.5% or 2% O_2. Scald risk assessment biomarkers will measured monthly from 1-9 months and one of the 2% O_2 rooms adjusted down to 0.5% when and if scald biomarkers indicate. Scald will be evaluated in all treatments at 0 and 7 days (at 68 ^{o}F) after monthly.

Biomarker-based scald risk-assessment under commercial conditions

'Granny Smith' apples were sampled from 4 lots (the same lots as above) from an organic commercial long-term CA room. Scald risk assessment biomarkers will be measured monthly from 1-9 months (or until the room is emptied). Scald will be evaluated in all treatments at 0 and 7 days (at 68 ^{o}F) monthly.

RESULTS & DISCUSSION

Scald risk assessment following delayed CA storage and DPA treatment

'Granny Smith' scald was reduced or eliminated by DPA drenches. Scald symptoms developed on air-stored fruit at 6 months and on CA stored fruit at 9 months. Delayed (up to 1 month) CA had little relationship with final scald incidence and severity after 10 months in 1% O_2 plus 7 days at 68 °F. Levels of 22 out of 25 scald risk assessment biomarkers (SRABs) increased at least 3 months prior to scald symptom development at 9 months in CA storage. Levels of 13 SRABs were highly reflective of scald final incidence and severity; very little increase in fruit that received treatments that didn't develop scald, and increasing levels as final scald levels increased. For example, elevated scald risk was actually detectable by measuring SRAB N24 at 2 months in air, air+DPA, and CA fruit not treated with DPA, all treatments which developed scald by 10 months storage.

Rather than relying on changing levels of just 1 or 2 SRABs, we envision monitoring multiple SRABs to provide a more complete risk assessment. Using multiple SRABs may be a useful way to monitor many different indicators of the causes and effects of scald, painting a more complete picture of the condition of the peel during storage. For instance, many of the SRABs that closely reflect final scald levels may reflect oxidative stress that ostensibly leads to scald development. By monitoring these SRABs, we are actually providing a direct assessment of damage caused by adverse storage conditions that lead to scald.

It remains to be seen whether differences in SRABs occur among susceptible and non-susceptible lots or under commercial CA conditions. Preliminary evidence indicates gene expression SRABs exists, this work is a result of the associated SCRI Postharvest Toolbox project. Some of these gene expression SRABs may provide earlier risk assessments than existing metabolic SRABs.

We also will employ easy to use analytical platforms for monitoring SRABs in the final year of this project.

Real-time scald assessment (experimental chambers)

Experiments were conducted to determine whether detection of high scald risk can be used to indicate when storage environment should be changed and whether these changes actually extend the symptom free life of the product. Experimental CA chambers containing Granny Smith apples were held at 0.5 or 5% O_2, fruit was also held in air. SRABs were monitored monthly. Changes in levels of 3 SRABs indicated scald risk in fruit held at 5% O_2 at 2 months and in 6 SRABs after 3 months (Table 1). One of the 5% O_2 chambers was adjusted to 0.5% O_2 at 3 months + 2 weeks. Scald was present on air stored fruit starting at 3 months and on 5% O_2 stored fruit starting at 6 months + 7 days at 68 °F. Scald incidence and severity was substantially reduced at 6 months + 7 days and less so at 9 months + 7 days by altering the storage conditions when risk was detected (Fig. 1). Results indicate that scald risk monitoring reflects fruits' response to the storage environment. Also, scald outcome can be improved, once risk is accurately assessed, by optimizing conditions farther into CA storage than previously considered.

Scald Risk Report		
SRAB	**Change (2M)**	**Change(3M)**
N24	**46**	**936**
N30	--	--
N34	--	**169**
N110	--	--
N122	--	--
N124	--	--
N157	--	**5**
N172	--	**29**
N210	--	--
N211	--	--
N220	--	--
N222	--	--
NC49	--	--
MHOH	--	**702**
MHO	--	**13**
N187	--	--
NHB31	--	--
N248	--	--
NHA34	--	--
Sitogl182	--	--
Camgl182	--	--
Sitogl183	--	--
Sitogl180	--	--
N192	--	--
Sitogl160	--	--

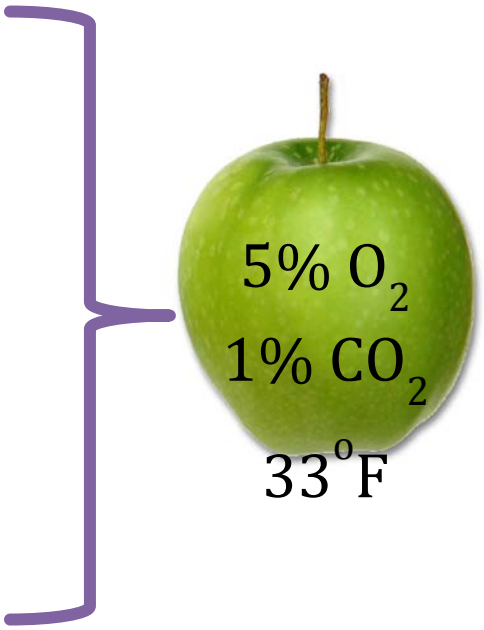

Table 1. Fold changes in levels of SRABs in Granny Smith peel during CA storage in 5% O_2, 1% CO_2. Increased SRAB levels indicate adjusting the atmosphere to 0.5% O_2:1% CO_2 at 3 months was necessary. This improved the storage outcome after 6 months.

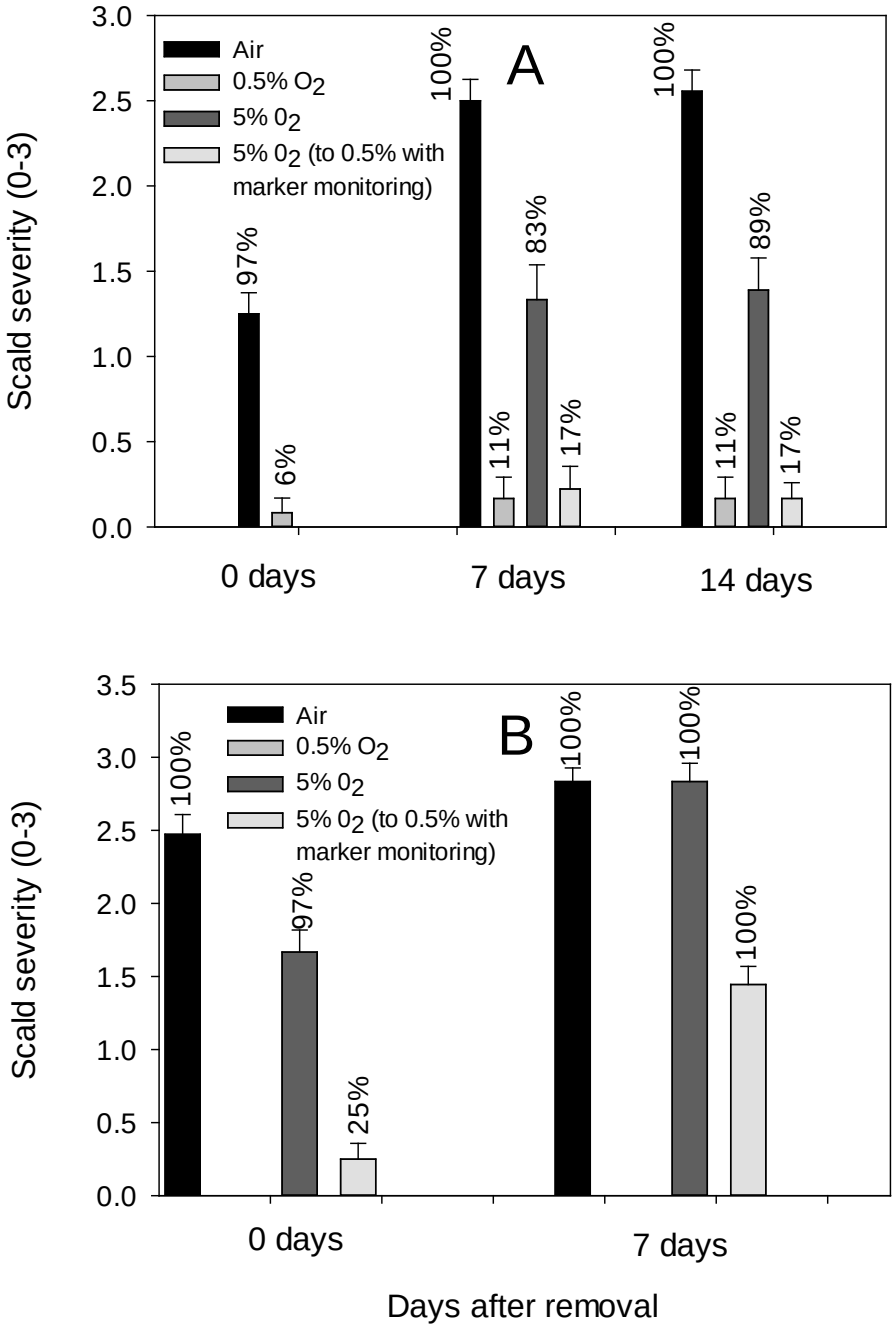

Fig 1. Scald incidence (% scalded fruit above bars) and severity (% peel with scald 0 = none; 1 = 0-25%; 2 = 25-50%; 3 = greater than 50%) during the a shelf-life period at 68 °F after 6 (A) and 9 (B) months storage at 33 °F in: air, 0.5% O_2:1% CO_2, 5% O_2:1% CO_2, 5% O_2:1% CO_2 (adjusted to 0.5% O_2:1% CO_2 at 3 months). Adjusting the atmosphere at 3 months reduced scald incidence and severity after 6 months storage + 7 and 14 days shelf-life but only severity after 9 months.

Real-time scald assessment (pilot and commercial rooms)---in progress
SRAB monitoring of multiple lots of fruit under pilot (research rooms) and commercial conditions is currently underway to indicate whether risk assessment tools can rank risk among different lots of fruit and whether it works in larger rooms. Last season's results are being reproduced using starting by monitoring research rooms set at 2% O_2 (rather than 5) and reducing O_2 to 0.5% when and if SRAB levels suggest there is a risk. More affordable and accessible means of measuring SRABs are also being evaluated.

Other progress
In collaboration with Dr. Bruce Whitaker, a group of unknown candidate SRABs and other apple peel chemicals in our data base have been identified as *p*-coumaryl acyl esters. These apple peel chemicals are part of the waxy surface layer and are likely part of the wax structure. A few of these compounds have potential as SRABs.

CONTINUING PROJECT REPORT **YEAR**: 1 of 3
WTFRC Project Number: AP-12-102

Project Title: Enhancing apple packing HACCP programs while ensuring fruit quality

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Cooperators:

Richard Kim, Ph.D., Pace International. Dr. Kim designed and conducted experiments related to fruit quality during a commercial trial. He also reported results related to those experiments.

Several chemical suppliers have donated supplies, other resources and time to discuss aspects of this project and ensure relevancy to the apple packing industry. Several apple packing facilities have agreed to or expressed interest for participating in packing plant studies to validate interventions to ensure that laboratory results relate to large scale production treatments.

Total Project Request: Year 1: 72,313 **Year 2: 72,947** **Year 3:** 77,161

Other funding sources

Agency Name: Center for Produce Food Safety
Amt. requested/awarded: Not yet determined

WTFRC Collaborative expenses:

Item	2012	2013	2014
Wages[1]	6,221	6,470	6,728
Benefits[1]	1,555	1,617	1,682
Miscellaneous			
Total	7,776	8,087	8,410

Footnotes:
[1] Wages and benefits for assistance from WTFRC crew.

Budget 1
Organization Name: WSU **Contract Administrator: Carrie Johnston**
Telephone: (509) 335-4564 **Email address: carriej@wsu.edu**

Item	2012	2013	2014
Salaries[1]	44,856	**38,051**	41,668
Benefits	6,457	**7,966**	9,530
Wages		**4,080**	4,243
Benefits		**82**	85
Equipment[2]		**3,000**	0
Supplies[3]	19,000	**17,500**	19,555
Travel[4]	2,000	**2,268**	2,080
Plot Fees			
Miscellaneous			
Total	72,313	**72,947**	77,161

Footnotes:
[1] Graduate student, technical support and undergraduate students in Pullman.
[2] Incubator, freezer or other laboratory equipment.
[3] Fruit, chemicals, measurement devices, microbial supplies, freezer, and analysis fees.
[4] Travel to Wenatchee and Yakima for commercial studies and fruit collection.

Objectives:

1) Evaluate the most effective microbial controls for HACCP systems individually and in combination using indicator organisms under commercial conditions
2) Identify and evaluate potential alternative approaches or novel compounds to enhance microbial control
3) Perform laboratory studies to examine pathogen and fungicide adherence to different apple varieties and examine the effects of storage over several harvest seasons
4) Conduct appropriate food safety extension and outreach with the apple packing industry

Significant Findings:

- Commercial scale PAA spray bar applications resulted in a >90% reduction of generic *E. coli* (1.4 to 1.5 $\log_{10}$ reduction) when 60-80ppm was directly applied to apples for at least 30 seconds with or without soap application.

- Three replications at a commercial facility examined dump tank applications of water, phosphoric acid, chlorine, chlorine dioxide and PAA were performed.
    - All treatments produced significant reductions (near or greater than 90%; 0.9-1.7 log reductions) in generic *E. coli* compared to the inoculated control. Phosphoric acid (pH 3.1-3.4) and high and low ORP chlorine treatments were statistically similar to the water treatment. High and low ORP chlorine dioxide treatments were slightly, but statistically different from the water treatment. PAA at 80ppm produced 90% bacterial reduction (1.1 $\log_{10}$) compared to the water control

- Laboratory experiments (3 replications) focused on PAA for dump tank application and lactic acid for spray bar application. PAA treatments included water and 2 concentrations of PAA (60 and 80ppm) applied at 3 application times (2, 3.5, and 5 minutes) representing exposure time in dump tanks. Lactic acid treatments included water and lactic acid (1 and 2%) applied at 3 application times (5, 15, and 30 seconds) followed by a 10 second exposure time.
    - PAA (60ppm and 80ppm) treatments responded similarly producing 1.2 to 1.6 $\log_{10}$ cfu/mL (90%) reduction of generic *E. coli* and *E. coli* O157:H7.
    - Pathogenic *E. coli* O157:H7 and generic *E. coli* responded similarly to both 1 and 2% lactic acid with a 90% reduction (0.8 - 1.0 $\log_{10}$); however this response was the same as water.

Methods:

Year 1, Objective 1:

Commercial PAA Spray Bar Studies. Three replications of commercial study was performed investigating the effectiveness of PAA in spray bar applications. The treatments were: water, neutral soap and water, 60 and 80ppm PAA, as well as 60 and 80ppm PAA with neutral soap and water. Apples were placed on the brush bed at the sorting table; the order of spray bars were 1) soap 2) PAA (average 21 seconds of direct application time) followed by conveyance (average time 50 seconds) 3) water 4) PAA (average 7 seconds of application time). PAA concentration (ppm) was monitored using a test kit. Temperature and pH were also measured for all treatments.

Apples were inoculated with non-pathogenic (generic) *E. coli* at WSU and transported in coolers containing ice to the packing facilities. Inoculated controls were measured immediately upon arrival at the packing facility and after the completion of each day. Forty inoculated apples were placed on the brush bed and samples were collected after points 2-4 listed above. For the water

treatment and soap and water treatments, twenty apples for each treatment were collected after point 2 above. Apples were sampled immediately after collection, and the diluent was chilled for transport to WSU. Samples were diluted appropriately and plated on VRBA for enumeration of generic *E. coli*.

Commercial Scale Dump Tank Studies.

Examination of chlorine, chlorine dioxide and peroxyacetic acid in low organic load dump tanks.
In Year 1, three replications were conducted at a commercial packing facility to assess chlorine, chlorine dioxide and PAA. Project planning and performance involved industry representatives, chemical suppliers and WSU personnel. Concentration of chlorine and chlorine dioxide solutions were monitored by oxidation-reduction potential using both a stationary and portable meter; pH of all treatments were also monitored. The concentration of PAA was measured using a PAA ppm probe (Prominent); the target concentration of PAA was 80ppm and ranged from 80 to 91ppm . The target pH for the phosphoric acid pH was 3.5 (range 3.1 to 3.4). The target ORP levels for chlorine and chlorine dioxide were 750 and 850mV. The portable pH/ORP meter readings were not consistent with the stationary pH/ORP meter readings. It was difficult to accurately establish ORP levels of the chlorine and chlorine dioxide treatments; therefore, the target 750mV treatment was labeled the low ORP treatment and the target 850mV treatment was labeled the high ORP. Apples were inoculated at WSU with generic (non-pathogenic) *E. coli* and transported on ice in coolers to the packing facility. For each treatment, 35 inoculated apples were placed into the dump tank and were collected prior to the sorting table. Apples were sampled immediately after collection, and the diluent was chilled for transport to WSU. Samples were diluted appropriately and plated on VRBA for enumeration of generic *E. coli*.

Pace International performed experiments on apples inoculated with fruit pathogens for two of the replications. Inoculums of single isolates of *Penicillium expansum* (CLX3354) and *Mucor piriformis* (A259) were prepared and final concentrations were adjusted to 2×10^6 (6 log) conidia/ml with a hemacytometer. The conidial suspensions were kept in an ice chest until used. For decay evaluation, organic 'Red Delicious' apples purchased from a commercial packinghouse were surface-disinfested with 70% ethanol and air-dried. Fruit were wounded in the middle of the equator with a sterile finish-nail head (4 mm in diameter and 3 mm in depth). Dump tank treatments (10 L) was collected in a 20-L plastic bucket. A 10 ml spore suspension was added into the bucket and agitated with a stainless steel whisk for 5 min. Apples were co-inoculated with *P. expansum* and *M. piriformis* in the first trial, and a separate inoculation was made in the second trial. For each treatment, sets of twenty apples were treated with three sets per treatment; a set of twenty apples was placed in a plastic mesh bag for exposure to the treatment-inoculum solution. Apples in the mesh bag were immersed in the solution for 30 seconds. Fruit were air-dried for 15 min after dipping and then placed on sterilized apple trays. Three trays for each treatment were placed in a cardboard apple box and stored at room temperature. After 2 weeks, apples were evaluated for the development of blue mold and Mucor rot. Percent incidences of decays were calculated and data were transformed prior to being examined by analysis of variance (ANOVA) using SAS (Version 9.2, SAS Institute, Cary, NC).

Examination of chlorine in high and low organic load dump tanks. In Year 1, one replication was conducted at a commercial packing facility to assess chlorine in low and high organic load dump tanks; additional replications will be performed in Year 2. Project planning and performance involved industry representatives and WSU personnel. The following points in the system were examined for antimicrobial reductions: 1) dump tank treated with chlorine 2) hyper-wash treated with chlorine dioxide 3) flume system treated with high and low ORP chlorine. Seventy-five inoculated apples were placed into the dump tank and were collected prior to the hyper-wash cabinet (20 apples), after the hyper-wash (20 apples), and after the flume system (35 apples). The levels of chlorine and chlorine dioxide were measured using stationary and portable meters for pH and ORP (mV). Methods for preparation and sampling of apples are described above.

Year 1 Objective 2:

PAA and Lactic Acid Laboratory Studies. In Year 1, laboratory work was designed to examine PAA at application times similar to dump tank applications and lactic acid at application times similar to spray bar treatments. Additionally, chemical supplier input indicated that two PAA products (12 and 15% PAA) are commonly used in the industry. Compounds and concentrations tested at exposure times of 2, 3.5 and 5 minutes included: water, 60ppm and 80ppm of 12% PAA. An additional treatment at 80ppm using the 15% PAA was examined, however, the only application time was 5 minutes. For lactic acid experiments, apples were placed directly in lactic acid (1% or 2%) or water for 5, 15, or 30 seconds followed by removal and an exposure time of 10 seconds to mimic time on a conveyance system. For each treatment combination, 5 apples were examined. Both pathogenic *E. coli* O157:H7 and non-pathogenic (generic) *E. coli* were examined. Appropriate dilutions were plated on sorbitol MacConkey (SMAC) agar for enumeration of *E. coli* O157:H7 and violet red bile agar (VRBA) for enumeration of generic *E. coli*. Plates were incubated at 95˚F and enumerated manually.

Years 2 and 3, Objective. 1. In Year 2, further replications will be performed as described above to complete the examination of chlorine in high and low organic load dump tanks. In Year 2, for peroxyacetic acid spray bar applications, additional commercial partners will be identified to confirm results observed in Year 1 as described above. It would also be useful to expand the study to include treatments examining the effects of acidic and basic soaps in comparison to neutral soaps. The direction of Year 3 experiments will depend on the results of Year 2.

Years 2 and 3, Objective 2. In Year 2, the project team will coordinate with potential project partners to examine alternative antimicrobial treatments, such as ozone, acidified water, chlorine stabilizers or organic acids in laboratory and commercial experiments. Methods will be similar as described above.

Years 2 and 3, Objective 3. A set of experiments will be performed to examine adherence of relevant bacteria and fungicides to different apple varieties stored for different periods in controlled atmosphere. Factors that will be examined include: season/year (3 years), apple variety (up to 6 varieties), fungicide type (3 fungicides), and length of controlled atmosphere storage (3 storage periods). Apples from orchards where pre-harvest fungicides were not applied will be utilized. The following varieties will be considered for examination and selected with input from the industry: Golden Delicious, Granny Smith, Gala, Fuji, Honeycrisp and Red Delicious. Factors to consider in variety selection include: wax levels, acidity, economic value to the industry and typical antimicrobial treatment applications, among others. It is anticipated that at least 3 different sources of apples for each variety will be examined (3 replications). Determination of disorders will be conducted at harvest; only fruit free of disorders will be utilized in the experiments described above. It is likely that fruit will be collected at identified orchards and drenched in a laboratory setting using appropriate methods (Errampalli et al., 2005; Xiao et al., 2011).

Untreated apples will be collected for examination of initial bacterial and mold levels and for use as a control group (untreated). To examine fungicide adherence, at least one of the following fungicides will be examined in Year 2 with expansion to other fungicides in Year 3: fludioxonil (Scholar), pyrimethanil (Penbotec) and thiabendazole (Mertect). Recommended concentrations by manufacturer labels will be used. After fungicide application, apples will be examined prior to storage as well as after five and eight months of storage in controlled atmosphere. Fungicide residue measurement will be contracted to a qualified laboratory for analysis with a gas chromatograph. To examine bacterial adherence, three strain cocktails of each of the following bacteria will be examined separately: generic *E. coli*, *E. coli* O157:H7, and *Salmonella*. Apples for bacterial attachment studies will be selected and equivalent to apples from the untreated treatment in the fungicide adherence studies to maintain consistency for examination of quality and safety aspects. Each variety will be examined at the time of harvest prior to storage, as well as after five months and eight months of

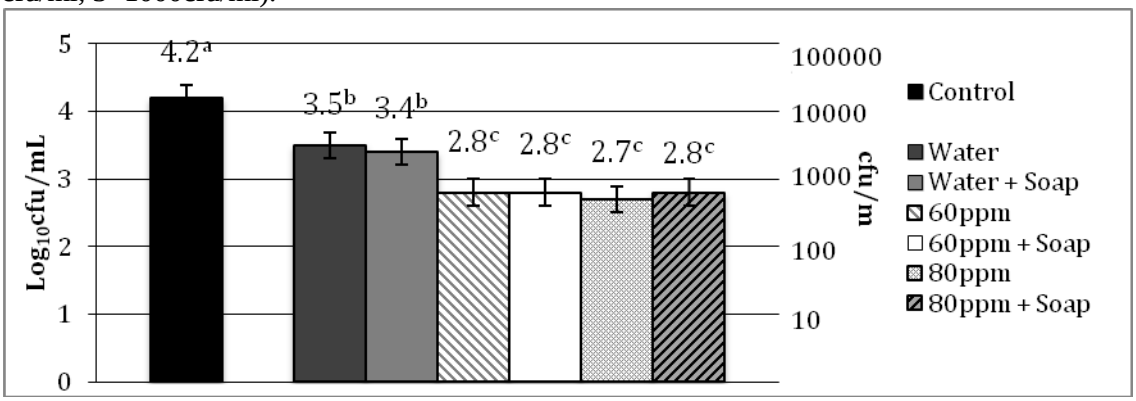
controlled atmosphere storage. To examine the potential influence of factors associated with fruit physiology, characteristics of the apples will also be examined. Quality measurements will include common maturity testing at harvest and after storage. Measurements of natural wax levels (Belding et al., 1998; Schreiber and Riederer, 1996) and lenticel size and density will be performed (Turketti et al., 2011). The need for examination of lenticel breakdown will also be considered (Turketti et al., 2011).

Results and Discussion:

Objective 1.

Commercial PAA Spray Bar Studies. Three replications were performed (Figure 1). PAA (60ppm and 80ppm) and PAA (60ppm and 80ppm) with soap were similar to each other, >90% reduction (1.4 to 1.5 $\log_{10}$ reduction) and were significantly greater than water alone as well as the soap with water treatment. PAA at 60 to 80ppm with or without soap and an average direct contact of 30 seconds can achieve greater than a 90% reduction.

Figure 1. Average Generic *E. coli* levels on apple surfaces after inoculation and direct application of water, water and soap, peroxyacetic acid (60ppm, 80ppm) and peroxyacetic acid with soap (60ppm, 80ppm). Values reported in $\log_{10}$ colony forming units (cfu)/ml scale (5=100,000 cfu/ml, 3=1000cfu/ml).



a-e Treatments not sharing a common superscript differ ($p<0.05$).

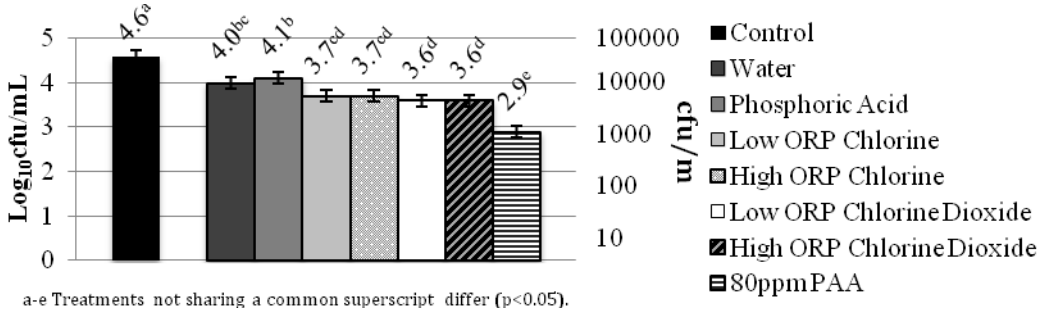
Commercial Scale Dump Tank Studies.

Examination of chlorine, chlorine dioxide and peroxyacetic acid in low organic load dump tanks. A commercial study was performed to examine the effect of chlorine, chlorine dioxide, phosphoric acid, and peroxyacetic acid in a dump tank. Three replications examining generic *E.coli* inoculated apples were performed (Figure 2). All treatments significantly reduced generic *E. coli* levels compared to the inoculated controls. However, phosphoric acid and chlorine (low and high ORP) were similar to water for reduction of generic *E. coli*, less than a 90% reduction (0.5 to 0.9 $\log_{10}$ reduction). Chlorine dioxide (low and high ORP) produced a slight, but statistically significant reduction of generic *E. coli* compared to water; however, this reduction was similar to that observed for chlorine. During this study, the gas vapors produced by the chlorine dioxide treatments created an uncomfortable work environment, therefore, this concern would have to be addressed for use in a commercial facility. The 80ppm PAA treatment achieved a significant bacterial reduction, greater than 90% (1.7 $\log_{10}$ cfu/ml reduction). Based on the results from this study, chlorine (low and high

ORP) and phosphoric acid achieved less than a 90% reduction, chlorine dioxide (low and high ORP) achieved a 90% reduction and 80ppm PAA achieved almost a 99% reduction in generic *E.coli.*

Results on fruit quality were not consistent between replications. In the first replication, the high and low ORP chlorine treatments as well as the high ORP chlorine dioxide treatment had significantly less decay from *Penicillium expansum* when compared to the water control treatment; however, in the second replication, only the high chlorine dioxide treatment had significantly less *Penicillium expansum* decay. In the first replication, the high and low ORP chlorine and chlorine dioxide treatments had significantly less *Mucor piriformis* decay compared to the water control treatment; however, in the second replication, peroxyacetic acid and the high ORP chlorine dioxide treatment had significantly less *Mucor piriformis* decay than the water control.

Figure 2. Average generic *E. coli* levels on apples after inoculation and direct application of water, phosphoric acid (pH 3.5), chlorine (low and high ORP) and chlorine dioxide (low and high ORP) and peroxyacetic acid (80ppm) in a commercial study. Values reported in $\log_{10}$ colony forming units (cfu)/ml (5=100,000 cfu/ml, 3=1000cfu/ml).



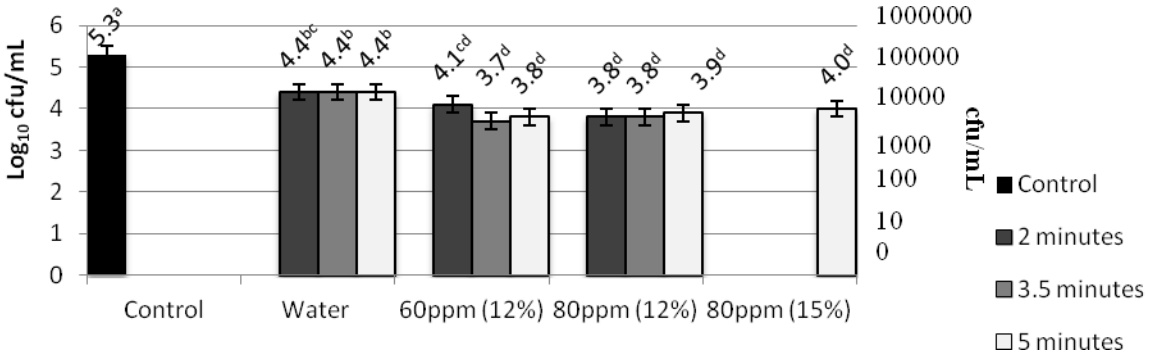
Examination of chlorine in high and low organic load dump tanks.

A commercial study was performed to examine the effect of chlorine in a high or low organic load dump tank. One replication examining generic *E. coli* inoculated apples has been completed. Initial results indicated that the reductions achieved by chlorine in this system may be greater than the antimicrobial effects of the replicated trial described above. Therefore, completing sufficient replications of this experiment in Year 2 are a high priority.

Objective 2.

PAA Laboratory Study. PAA (60 and 80ppm) was examined in a laboratory setting using application times that are relevant to commercial systems. Three replications examining generic *E.coli* and pathogenic *E.coli* O157:H7 were performed, respectively (Figure 3). Exposure time (2, 3.5, and 5 minutes) did not significantly affect bacterial reduction. Generic *E. coli* adhered to the apples to a greater extent than pathogenic *E. coli* O157:H7. There was no difference in the response of pathogenic *E. coli* O157:H7 and generic *E. coli* to the antimicrobial treatments. All PAA treatments (60ppm and 80ppm PAA-12% and 80ppm PAA-15%) were similar to each other for reduction of generic *E. coli* and pathogenic *E. coli* O157:H7. All PAA treatments significantly reduced generic and pathogenic *E. coli* compared to water, with the exception 60ppm PAA with a contact time of 2 minutes. All PAA treatments produced over a 90% reduction in generic *E. coli* and pathogenic *E. coli* O157:H7 (1.2 to 1.6 $\log_{10}$ cfu/ml). Previous research in our laboratory indicated that 30 seconds of direct exposure time achieved a 90% bacterial reduction (greater than 1 log for pathogenic *E. coli* O157:H7 and 1.5 log for generic *E. coli).* Therefore, application of PAA beyond 2 minutes may not enhance bacterial reductions.

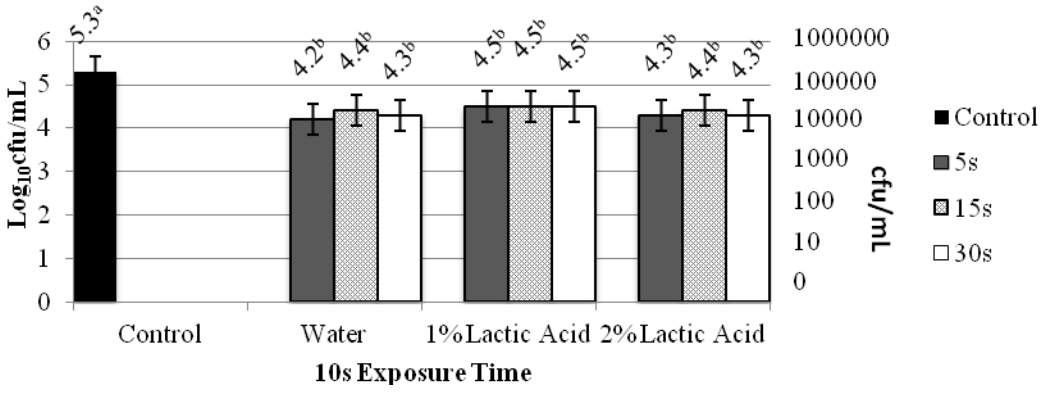
Figure 3. Average bacterial levels (Generic *E. coli* and *E. coli* O157:H7) on apple surfaces after inoculation and direct application of peroxyacetic acid (60ppm, 80ppm) for 2, 3.5 and 5 minutes in a laboratory study. Values reported in $\log_{10}$ colony forming units (cfu)/ml scale (5=100,000 cfu/ml, 3=1000cfu/ml).



a-d Treatments not sharing a common superscript differ ($p<0.05$).

Lactic Acid Laboratory Study. Three replications examining generic *E. coli* and pathogenic *E. coli* O157:H7 were performed (Figure 4). Exposure time (5, 15 or 30 seconds of direct contact followed by 10 seconds of exposure time) did not significantly affect bacterial reduction. There was no difference in the response to antimicrobial treatment of pathogenic *E. coli* O157:H7 or generic *E. coli*. All lactic acid treatments (1% and 2%) for generic *E. coli* and pathogenic *E. coli* O157:H7 produced reductions that were similar to reductions achieved with water, about a 90% reduction (0.8 - 1.0 $\log_{10}$ reduction). These results indicated that 30 seconds of exposure time was insufficient for lactic acid to achieve bacterial reductions on apples.

Figure 4. Average bacterial levels (Generic *E. coli* and *E. coli* O157:H7) on apple surfaces after inoculation and direct application of lactic acid (1%, 2%) for 5, 15 and 30seconds followed by 10s exposure time in a laboratory study. Values reported in $\log_{10}$ colony forming units (cfu)/ml scale (5=100,000 cfu/ml, 3=1000cfu/ml).



a-b Treatments not sharing a common superscript differ ($p<0.05$).

CONTINUING PROJECT REPORT **YEAR**: 1 of 2
WTFRC Project Number: AP-12-108

Project Title: Overhead cooling influences on microbial food safety

PI: Karen Killinger, Ph.D.
Organization: WSU/School of Food Science
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Co-PI (2): John Scott Meschke, Ph.D.
Organization: University of Washington
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City/State/Zip: Seattle, WA 98105-6099

Cooperators: Discussions with industry partners are at several stages, including active participation, discussion of potential sites and sources of apples, and working with industry organizations to identify interested cooperators.

Total Project Request: **Year 1:** $120,000 **Year 2:** None requested

Other funding sources

Agency Name: Center for Produce Food Safety and/or USDA Initiative
Amt. requested/awarded: Not yet determined
Notes:

WTFRC Collaborative expenses:

Item	2012	2013	
Wages	6,400	6,658	
Benefits	1,600	1,664	
Total	8,000	**8,322**	

Footnotes:

Budget 1
Organization Name: WSU **Contract Administrator: Carrie Johnston**
Telephone: (509) 335-4564 **Email address: carriej@wsu.edu**

Item	2012	2013	
Salaries[1]	24,905		
Benefits	7,671		
Equipment[2]	10,000		
Supplies[3]	74,907		
Travel[4]	2,517		
Plot Fees			
Miscellaneous			
Total	120,000	0	

Footnotes:
[1] Graduate student, technical support and undergraduate students in Pullman.
[2] Freezer and laboratory equipment for inoculation methods.
[3] Fruit, chemicals, measurement devices, microbial supplies and analysis fees.
[4] Travel to central Washington for inoculation studies and fruit collection.

OBJECTIVES

1) Select and validate appropriate, non-pathogenic surrogate organisms as well as inoculation methods in the laboratory to evaluate food safety risk associated with overhead, evaporative cooling water application.

2) Investigate foodborne pathogen and surrogate survival in laboratory studies and non-pathogenic surrogate survival in field studies to examine risks associated with standard overhead cooling water application practices.

SIGNIFICANT FINDINGS

- Candidate strains were identified through a literature review. Strains isolated from Washington irrigations water sources will also be considered. Candidate strains will be further narrowed in order to select strains for validation of growth characteristics.
    - At least 54 strains of pathogenic *E. coli* O157:H7 strains
    - At least 101 *Salmonella spp.* strains
    - At least 22 non-pathogenic surrogates
- Three replicated growth curves of non-stressed (healthy) cells were performed for two candidate strains of pathogenic *E. coli* O157:H7 and generic *E. coli*.
    - Strains were similar in growth overall, but the use of 12-18 hour cultures may be optimal for use in inoculation studies.
- Three sampling locations were selected for preliminary evaluation of irrigation water quality. All sampling sites were irrigation ponds. Further confirmation of these findings is needed in Year 2.
    - Fecal coliform and generic *E. coli* levels at some sampling locations appeared to be influenced by sampling date.
    - Overall, sampling site within a sampling location did not appear to influence levels of fecal coliform and generic *E. coli*. Collecting composite samples from multiple locations at an irrigation pond may accurately reflect water quality of the pond.

METHODS

Year 1. Objective 1. Strain Selection. Strain selection for use in laboratory and field experiments will involve several phases: 1) strain selection and acquisition 2) growth curves performed with non-stressed (healthy) cells 3) growth curves performed under several stress conditions. An extensive literature review was performed. Three replicated growth curves were performed with two strains of pathogenic E. coli O157:H7 (ATCC 43890, SEA 13B88) and two non-pathogenic (generic) E. coli strains (ATCC 11775 and ATCC 25922). For the growth curves, samples were collected at the following time points: 0, 2, 4, 6, 8, 10, 12, 15, 18, 21 and 24 hours. Samples were measured for

absorbance at 600nm in a spectrophotometer and the sample was serially diluted and spread plated on tryptic soy agar plates.

Year 1. Objective 2. Evaluation of potential field sites through examination of fecal coliform and generic *E. coli* levels in open surface waters used for overhead cooling and on apple surfaces.
A limited number of sampling sites were identified for preliminary water testing. Three sampling locations were monitored for three sampling dates in October and November 2012. All sampling locations were irrigation ponds. At each sampling location, at least three sampling sites were monitored. Water samples were tested for total coliforms, fecal coliforms and *E. coli* using the IDEXX Colilert®-18, Quanti-Tray®/2000 system. Two 100ml duplicates were taken from each water sample, and each was mixed with a packet of Colilert®-18. The duplicates were then poured into a Quanti-Tray®/2000 and sealed using the IDEXX Quanti-Tray® sealer. For each water sample, one Quanti-Tray® was incubated at 35°C for 18-22 hours to test for total coliforms and *E. coli*, while the second Quanti-Tray® was incubated at 44.5°C for 18-22 hours to test for fecal coliforms. After 18-22 hours, the Quanti-Trays® were removed from incubation. Using the IDEXX Quanti-Tray®/2000 MPN table, the most probable number (MPN) of total coliforms, fecal coliforms and generic *E. coli* was generated for each duplicate.

Year 2. Based on the acquisition of additional funding, the scope of the proposed work may need to be narrowed. As the project proceeds, input from the industry will be important to determine appropriate directions.

Objective 1. Strain selection. To identify the most appropriate strains for laboratory and field studies, additional candidate pathogen isolates (*E. coli* O157:H7 and *Salmonella*) and non-pathogenic surrogates will be evaluated for growth under standard laboratory conditions (non-stressed conditions). Additionally, E. *coli* O157:H7 and *Salmonella* isolates from Washington open surface waters will be considered for selection. Strains exhibiting similar growth characteristics will then be evaluated under selected stress conditions (acid stress, starvation, use of open surface irrigation water medium). At least three isolates of pathogenic *E. coli* O157:H7, three *Salmonella* isolates and three surrogates will be selected for inoculation studies.
Evaluation of inoculation methods. Methods for pre-harvest apple inoculation will be optimized. It is anticipated that methods of spot and spray inoculation will be compared to dip inoculation with apples. Factors during apple inoculation that need to be examined include the effect of droplet size for spray inoculation, the amount of inoculum, and the effect of drying after inoculation. The methods of inoculation will be examined for the ability to deliver consistent levels of inoculum to apple surfaces. Ideally, a single method will be identified that can deliver both low and high levels of inoculum to apple surfaces. Microbial recovery can also be examined. The methods examined will depend on the selected surrogates. The extent and breadth of initial method verification will be guided by results from initial experiments. Initially inoculation methods will be examined in a controlled, laboratory environment; based on laboratory results, the most promising inoculation methods will be examined under field conditions.

Objective 2. Survival of Pathogenic and Surrogate Strains in Laboratory Studies. Selected, validated pathogen and surrogate survival on apples will be examined under conditions mimicking overhead cooling water application on healthy and injured fruit in the laboratory. Two potential routes of contamination exist. Pathogens may be present on the apple prior to overhead cooling or pathogens may be present in the overhead cooling water and transferred to the apples during application. In the laboratory, foodborne pathogen and surrogate survival will be examined incorporating both potential contamination routes and the influence of overhead cooling water application. First, to examine pathogens that may be present at the time of overhead cooling water application, pathogens and surrogates will be inoculated at selected time periods prior to application of water to mimic overhead cooling. Survival will be monitored for designated periods. In the second experiment, pathogens and surrogates will be mixed into overhead cooling water and applied to apples. Survival will be monitored for designated periods to reflect common overhead cooling application times prior to harvest. The laboratory studies will target which key factors may be most influential for microbial reduction under field conditions. The influence of apple variety, UV and shading are factors that may be useful to examine in controlled laboratory conditions prior to development of field studies.

Objective 2. Evaluation of potential field sites through examination of fecal coliform and generic *E. coli* levels in open surface waters used for overhead cooling and on apple surfaces. Input from industry representatives will assist in identification of potential field sites. Additional sites are needed to represent the breadth of the growing region for apples in Washington. Preliminary sampling of water used for overhead cooling will be continued in March or April of 2013. The design of this portion of the study will depend on the number of potential sites identified and the willingness of growers to participate in the study. If growers are willing to share or collect monitoring data of surface waters utilized for overhead cooling, the need for sampling and cost of sampling may be reduced.

Objective 2. Survival of Surrogate Strains in Field Studies. Field studies will examine validated, non-pathogenic surrogate survival under conditions relevant to application of overhead, cooling water. Laboratory results will help focus study design; however, large sample sizes will be needed to achieve statistical validity for field studies. Factors that will be considered in the study design will include: field location, apple variety, shade influences, microbial presence prior to overhead cooling water application and length of time between overhead cooling application and harvest. Appropriate environmental factors (temperature, humidity, UV exposure, rainfall) will be monitored. In the field, to examine pre-existing contamination, selected surrogates will be inoculated onto fresh apples at selected time periods prior to application of overhead cooling water. Overhead cooling water will be applied and apples will be examined for surrogate survival at designated time points prior to harvest (ex. 0, 1, 3, 7 and 14 days after overhead cooling water application). To examine contamination introduced through overhead cooling water application, surrogate organisms will be mixed with overhead cooling water and applied at different time periods prior to harvest (ex. 0, 1, 3, 7 and 14 days). Apples will be examined for surrogate survival at designated time points prior to harvest.

RESULTS AND DISCUSSION

Objective 1. Validation of surrogate strains. The literature review identified at least 54 strains of pathogenic *E. coli* O157:H7 strains, 101 *Salmonella spp.* strains, 18 non-pathogenic surrogates for *E. coli* O157:H7 and 4 *Salmonella spp.* surrogates. Further literature review was performed to assist in narrowing the list of potential strains for further analysis. Currently, at least 14 strains of pathogenic *E. coli* O157:H7 strains, 2 *Salmonella spp.* strains, 8 non-pathogenic surrogates for *E. coli* O157:H7 and 4 *Salmonella spp.* surrogates are under consideration.

Replicated growth curves (Figures 1-4.) using healthy cells have been completed for pathogenic *E. coli* O157:H7 (ATCC 43890, SEA 13B88) and two non-pathogenic (generic) *E. coli* strains (ATCC 11775 and ATCC 25922). The data indicated that the pathogenic *E. coli* O157:H7 (ATCC 43890, SEA 13B88) and two non-pathogenic (generic) *E. coli* had similar growth curves when under optimal conditions for growth. However, three of the strains had variability observed at the 24 hr reading, indicating that when preparing inoculations for future studies; harvesting cells for use in a bacterial inoculum may be more consistent when cells are incubated for 12-18 hours prior to inoculum preparation.

Figure 1. Levels of pathogenic *E. coli* O157:H7 ATCC 43890 incubated in buffered peptone water at 98.6ºF and measured at times 0, 2, 4, 6, 8, 10, 12, 15, 18 and 24 hrs. The y-axis is provided in log colony forming units (cfu) per milliliter (ml) and numerical value (log 6 = 1,000,000 cfu/ml ; log 8=100,000,000 cfu/ml).

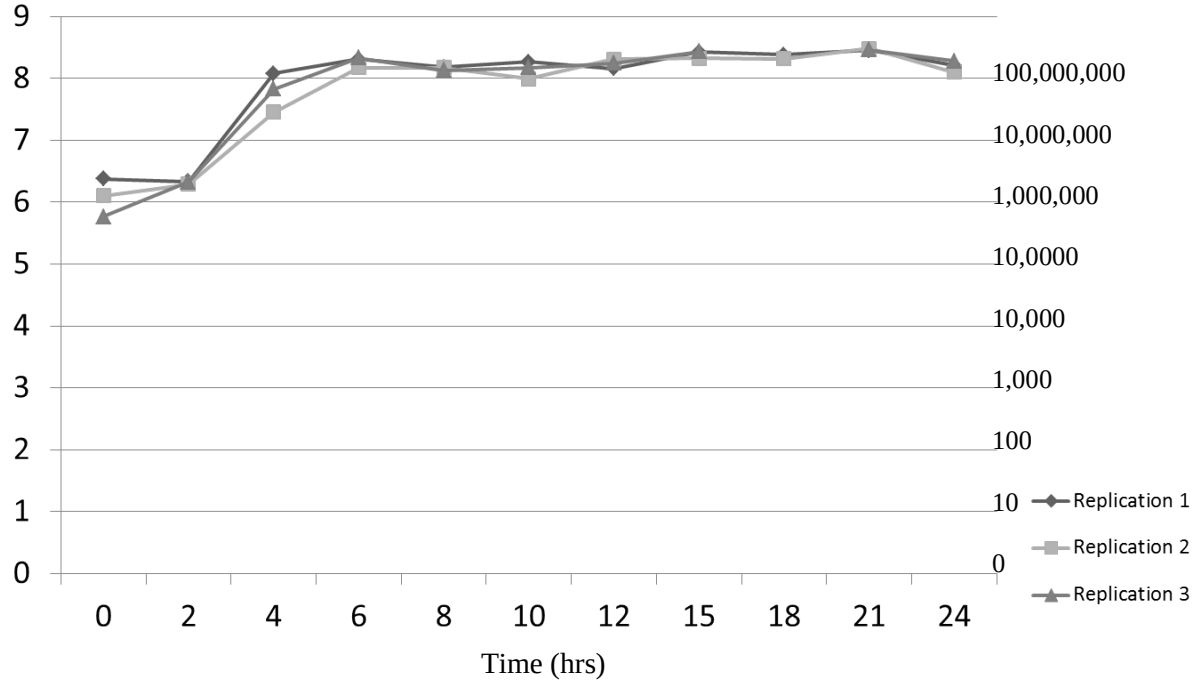


Figure 2. Levels of pathogenic *E. coli* O157:H7 SEA 13B88 incubated in buffered peptone water at 98.6ºF and measured at times 0, 2, 4, 6, 8, 10, 12, 15, 18 and 24 hrs. The y-axis is provided in log cfu/ml and numerical value (log 6 = 1,000,000 cfu/ml ; log 8=100,000,000 cfu/ml).

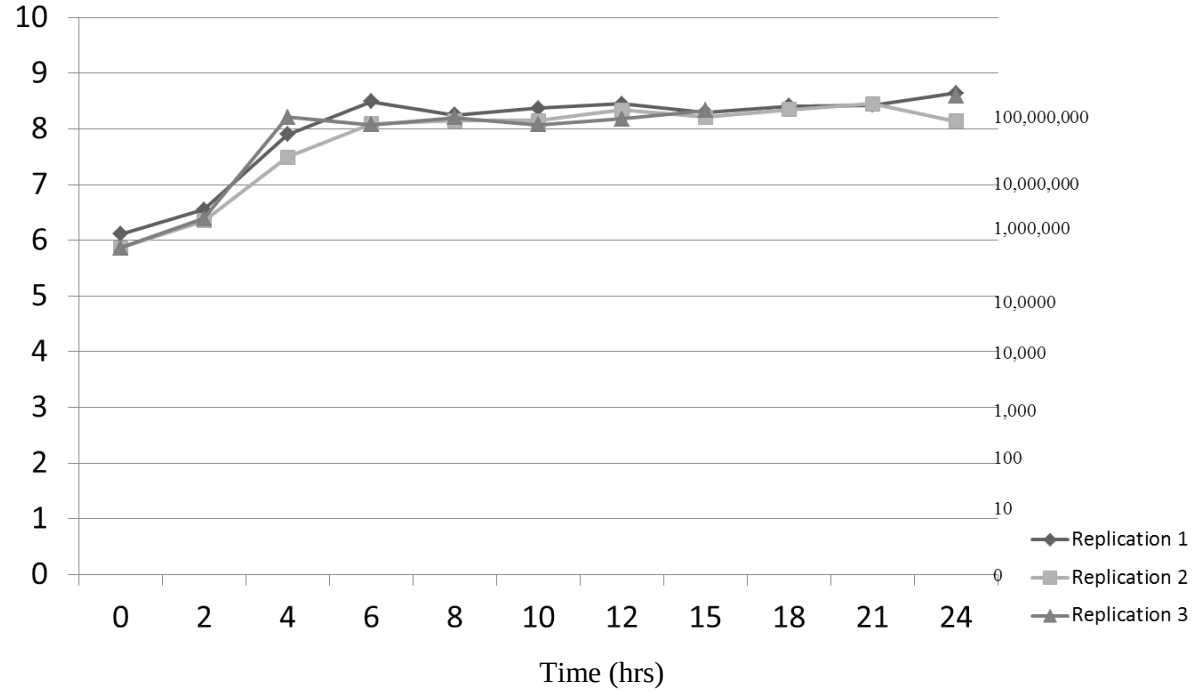


Figure 3. Levels of generic *E. coli* ATCC 11775 incubated in buffered peptone water at 98.6ºF and measured at times 0, 2, 4, 6, 8, 10, 12, 15, 18 and 24 hrs. The y-axis is provided in log cfu/ml and numerical value (log 6 = 1,000,000 cfu/ml ; log 8=100,000,000 cfu/ml).

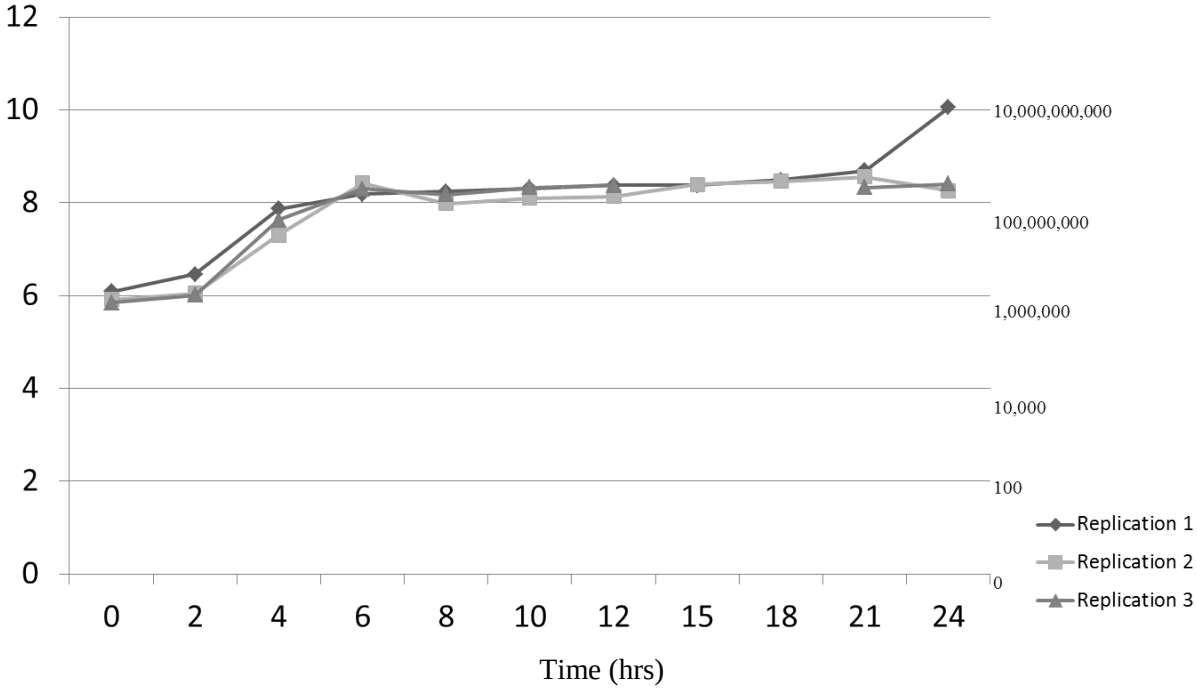
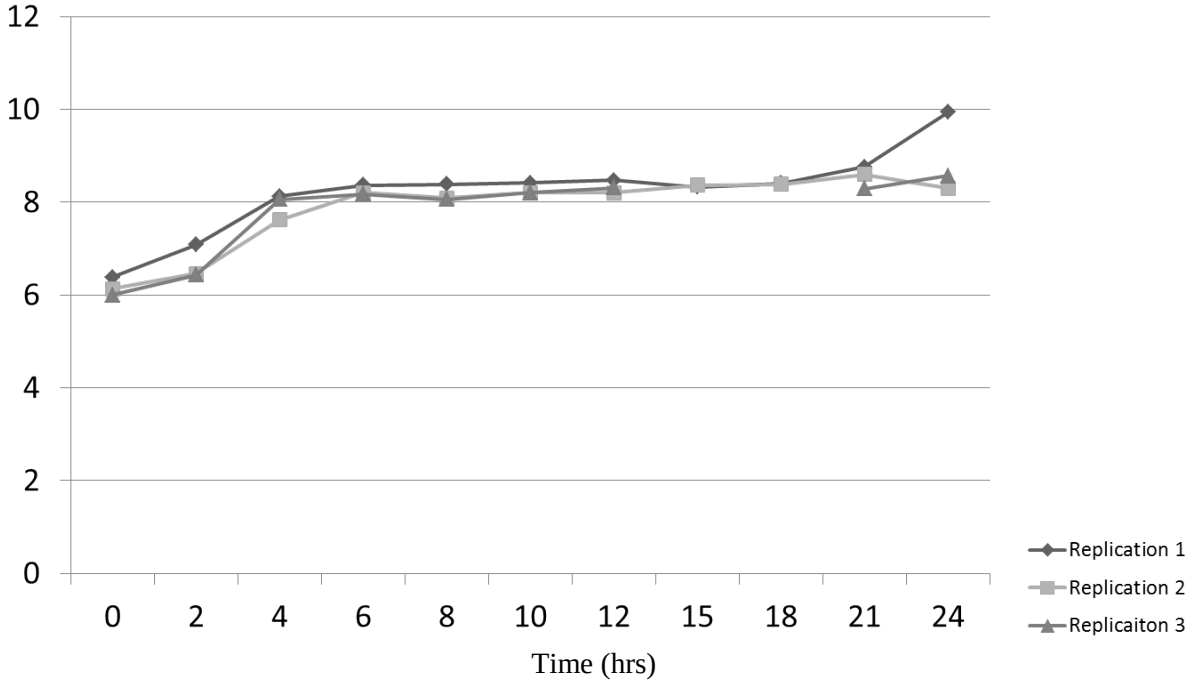


Figure 4. Levels of generic *E. coli* ATCC 25922 incubated in buffered peptone water at 98.6ºF and measured at times 0, 2, 4, 6, 8, 10, 12, 15, 18 and 24 hrs. The y-axis is provided in log cfu/ml and numerical value (log 6 = 1,000,000 cfu/ml ; log 8=100,000,000 cfu/ml).



Objective 2. Evaluation of potential field sites through examination of fecal coliform and generic *E. coli* levels in open surface waters used for overhead cooling and on apple surfaces.

Fecal coliform level and generic *E. coli* levels at all sampling sites were relatively low (< 100 MPN/100ml fecal coliforms or generic *E. coli*). For one sampling site, variability in fecal coliform levels and generic *E. coli* levels was observed between sampling dates, whereas the other two sites were fairly consistent in indicator organism levels between sampling dates. Overall, levels of indicator organisms did not vary widely between sampling sites on a specific sampling date. Therefore, collection of a larger composite sample from multiple sites within an irrigation pond may be an accurate assessment of overall water quality from the irrigation pond.

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

Project Title: Apple microbial risk factors

PI: Richard Pleus
Organization: Intertox, Inc.
Telephone: (206) 443-2115
Email: rcpleus@intertox.com
Address: 600 Stewart St., Ste. 1101
City/State/Zip: Seattle, WA 98101

Cooperators: NONE

Total Project Request: **Year 1:** $26,993.96 **Year 2: $39,388.04** **Year 3:** $425.00

Other funding sources

Agency Name: The Center for Produce Safety
Amt. awarded: $66,807

WTFRC Collaborative expenses: None

Budget 1
Organization Name: Intertox Inc **Contract Administrator: Roxann Thomas**
Telephone: 206-443-2115 **Email address: rthomas@intertox.com**

Item	2012	2013	2014
Salaries	17,698.62	21,016.40	
Benefits	8,141.38	9,667.44	
Wages			
Benefits			
Subcontract		938.00	
Supplies	254.45	(44.45)	
Travel	899.51	310.65	425.00
Expert Panel		7,500.00	
Miscellaneous			
Total	$26,993.96	$39,388.04*	$425.00

Footnotes: Of the 2012 funded amount $36,123 only $26,993.96 was expensed, leaving $9,129 for 2012. We would like to request adding the remaining dollars on the 2012 budget to the 2013 budget. The money not spent in 2013 consists of $7,500 for the expert panel review of the risk assessment model and $938 for IDS work with Cascade Analytical to obtain 2012 packinghouse data. The expert panel review work will occur mid-year 2013 after additional data from individual packinghouses and other researchers is incorporated into the risk assessment model. With regard to the IDS contractual piece, the decision was made to wait until early 2013 to acquire data from Cascade Analytical in order to obtain a complete year of 2012 testing data as opposed to a partial year of data. Also left is a small balance in wages and benefits.

OBJECTIVES

1. **Gather pathogen testing data and information about mitigation measures from apple growers.**

   According to the USDA's National Agricultural Statistical Services, Washington State has more than 3,000 apple growers with an estimated 167,489 acres of apples. The original schedule for Objective 1 was to collect apple grower data on pathogen testing and mitigation measures by April 2012. In early 2012, it was determined that because of the large number of growers, contacting all of them would be extremely difficult given the project timeline and resources. As of April 2012, Intertox had collected testing data from apple growers representing an estimated 18% of Washington state acreage. To increase access to grower data, in January 2013, Intertox will use a survey instrument at a Washington grower shipper meeting to interview as many growers as possible in order to obtain information that is representative of the entire Washington state grower population. Included in the survey will be a request for growers to provide access to their testing data. Intertox Decision Sciences (IDS) will then follow-up and collect data throughout 2013, until the completion of the project, to allow for most robust dataset possible.

2. **Correlate pathogen levels in water used in fresh market apple production and packing operations at different points in the system to levels measured on apples before they leave the packinghouse.**

   We began our correlation analysis using data indicating pathogen presence/levels at various points along the packing line. Many companies, however, only began regular testing in the past two to three years, meaning there are a limited number of test results per company. To increase the number of results per company, 2012 data will be obtained and added to the database in the first quarter of 2013. IDS is also working to increase the number of Washington apple growing and packing companies participating in the project. In 2012 IDS contacted and obtained agreement from several additional packing house/ harvest companies to participate in the project and provide data. Data will be collected throughout 2013 until the completion of the project to allow for the most robust dataset possible.

   Along with packing house test results, other 3rd party research data will be used for the correlation analysis. We are working with the Washington Tree Fruit Research Commission (WTFRC) and the Northwest Horticultural Council (NHC) to obtain access to other individual packing facility research, currently underway, that is correlating contamination at various points along the packing line with microbial levels on finished product. If sufficient data from both test results and 3rd party research is still not available, Intertox will model the potential for apples to become contaminated if contamination is introduced or present in the packing line. We expect to complete and document the correlation analysis in the first quarter of 2013 and then to continue to refine it throughout 2013 as more data become available.

3. **Characterize potential exposure to pathogens from consumption of fresh market apples and describe potential human health effects associated with these exposure levels; combine the results to estimate the risk of becoming ill from eating contaminated apples.**

   As of December 31st, 2012, a draft exposure assessment that includes a description of the human health effects from foodborne illness caused by *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* — the three pathogens of concern addressed in this study — has been completed and is being reviewed by Intertox scientists. By January 31, 2013 the exposure assessment will be available for review by select industry representatives.

4. Prepare a written risk assessment report about the findings of Objectives 1-3.

Using microbial testing data and information from Washington companies and research studies, we have constructed a quantitative microbial risk assessment (QMRA) model using Microsoft Excel and Palisade @Risk software to estimate potential exposure levels to these pathogens and the risk of becoming ill from eating contaminated fresh market apples. The draft model is based on potential contamination and growth/reduction of pathogens occurring at various times in the field-to-fork continuum: in the field, during transport, during packing (e.g., washing/ treatment/ cross-contamination), and during retail and home storage. The model estimates exposures for several population groups, including adults and children, using national fresh apple consumption rates, and incorporates probabilistic methods to characterize the uncertainty and variability in model inputs as well as outputs (e.g., where possible, input parameters such as temperature, time, etc., are included). For each pathogen, risks are characterized as the probability and severity of illness along with the total estimated number of cases. Currently, we are developing model distributions and testing calculations. Model parameters are estimated based on published studies and observed orchard/ packing activities. In 2013 we will continue our development of the exposure assessment and complete development and testing of the QMRA. In the next reporting period, we will test a range of inputs and evaluate the veracity of model outputs. We will also conduct quality control (QC) of all inputs and outputs. Finally, we will generate a draft risk assessment report, using available data, which presents the methods, inputs, and assumptions used in the model, and tabulates model output for each scenario and population. The risk assessment report including the findings from Objectives 1, 2 and 3 is scheduled for completion April 30, 2013.

5. Submit the risk assessment model and report for review to an advisory panel consisting of the WTFRC, the Northwest Horticultural Council and other experts in the field of quantitative microbiological risk assessment.

Objective 5 is scheduled for completion August 31, 2013.

SIGNIFICANT FINDINGS

There is no change to the original project schedule relating to findings from Objectives 1, 2 and 3. As per the original schedule, the findings will be included in the risk assessment report scheduled for completion April 30, 2013.

METHODS

Gather pathogen testing data and information about mitigation measures from apple growers.

Hazard Identification is the first step of the risk assessment process and includes gathering information about the pathogens of potential concern, their presence on apples, and the nature of adverse outcome (infection or illness) associated with consumption of contaminated apples. Microbial hazards will be identified from contaminated apple products, research studies on apples, and if available, pathogen testing data from growers and packers. As part of a 2012 Washington State and CPS-funded project, we worked with Washington apple packing companies to gather microbial testing data and information about their food safety programs and mitigation practices. In partnership with the WTFRC and NHC, we developed and distributed a questionnaire to packing facilities asking for information about their food safety programs and their willingness to anonymously share data gathered as part of their programs. Intertox is using similar methods for this project to survey and gather data from apple growers.

Correlate pathogen levels in water used in fresh market apple production and packing operations at different points in the system to levels measured on apples before they leave the packinghouse.

Because water is used extensively in apple production and packing operations, water quality is a particularly important risk factor to consider when examining the risk of contamination in fresh market apples. Apples come in contact with water applied at various points from the orchard to the packing house including during irrigation and application of pesticides, in drench tanks and flumes, and from spray bars. We will use regression analysis to test for correlations between pathogen levels measured in water applied during production and packing operations and levels measured on apples ready for shipping. The analysis outcome will provide information about how water applied throughout the system may contribute to levels on the end product. In the absence of sufficient correlative data to estimate end product contamination, predictive modeling may also be used to estimate end product contamination based on the estimated presence of contaminant sources along the packing line (e.g., similar to the method used by Duffy and Schnaffer, 2002, to predict levels in tree-picked apples and dropped apples from estimated levels of infected animal droppings or contaminated manure).

Characterize potential exposure to pathogens from consumption of fresh market apples and describe potential human health effects associated with these exposure levels; combine the results to estimate the risk of becoming ill from eating contaminated apples.

Characterizing exposure (exposure assessment) provides an estimate of the levels of pathogen that could be consumed on fresh market apples, i.e., the probability of contamination and the potential dose of the contaminant. The risk assessment estimates the pathogen dose based on several sources of information:

- Data on the frequency of detection and levels of pathogens measured on apples ready to ship (end product testing), and, where such data are lacking, estimates of potential pathogen levels based on levels measured in water, if available data are sufficient to establish these correlations (see Objective 2, above).
- Information on the impact of food handling, processing, and storage conditions on the overall potential exposure.
- Information on the rate of consumption of apples averaged over time, e.g., from the FDA Total Diet Study or the USDA Continuing Survey of Food Intake by Individuals (CSFII), which report food consumption from a representative sample of the U.S. population. In addition, information on patterns of consumption related to socio-economic and cultural background, ethnicity, seasonality, age differences (population demographics), regional differences, and consumer preferences and behavior will be considered.
- Foodborne illness outbreak data from CDC's OutbreakNet – Foodborne Outbreak Online Database.

The dose-response assessment describes the likelihood that an individual will become ill from eating an apple contaminated by a pathogen of concern. A dose-response assessment estimates the relationship between the exposure level (dose) and the frequency and severity of illness or other adverse effect (response). In this step of the risk assessment, we will identify and establish dose-response curves that describe the relationship between exposure to a given pathogen and infection or illness, and describe the characteristics of the host, pathogen, and food matrix that contribute to an individual's response to a pathogen. Where possible, we will characterize the uncertainty and variability in the dose-response estimates, and present these parameters as probability density functions (PDFs) for incorporation in the probabilistic risk assessment model. The results of the exposure assessment and dose-response assessment will be combined to yield a risk estimate (risk characterization) that describes the nature and magnitude of risks associated with the pathogen(s) of concern, including the attendant uncertainties. Estimates of risk will be

illustrated graphically as probabilistic distributions that characterize the probability of effects on public health, incorporating information on the variation inherent in the exposure and hazard estimates, as well as the uncertainty associated with these estimates.

As part of the probabilistic assessment, sensitivity analysis will be conducted to show which variable(s) in the risk assessment equations contribute most to the overall uncertainty/ variability in the output. This will allow future resources to be focused on investigation of those variables that contribute most to uncertainty in the output.

Prepare a written risk assessment report about the findings of Objectives 1-3.

The final deliverable for the project will be a comprehensive written risk assessment for the production and packing of fresh market apples. The report will be based on the four components of a risk assessment (Hazard Identification, Exposure Assessment, Hazard Characterization/Dose-Response Assessment, and Risk Characterization) as described in previous sections. A description of the methods, the results of the risk assessment, and the study limitations will be thoroughly discussed.

Submit the risk assessment model and report for review to an advisory panel consisting of the WTFRC, the Northwest Horticultural Council and other experts in the field of quantitative microbiological risk assessment.

In consultation with WTFRC and NHC, we will ask at least one expert in food safety microbiology to join the advisory panel to review and provide feedback on the risk assessment model. The expert panel member(s) will be chosen based on their experience in the field of quantitative microbiological risk assessment models developed for fresh produce. Panel member(s) will be asked to provide guidance and input on the model itself as well as feedback on the final written report.

RESULTS & DISCUSSION

Objective 1: Intertox worked with the NHC and the WTFRC to size the grower population in preparation for survey distribution. Since not all growers are members of the NHC, Intertox is also working with other Washington organizations and plans to distribute the survey(s) during one association's annual meeting in January 2013. Prior to fielding the grower survey, Intertox visited several orchards to better understand their microbial testing programs and operational concerns. IDS continues to solicit growers and packing houses that did not contribute data for the first project to provide their testing data for this project.

Objective 2: We continue to search for and gather data indicating pathogen presence/levels at various points along the packing line. We are also analyzing individual company validation study data for applicability. In addition, we are using existing research study data to build a model depicting the potential for contamination along the packing line to result in contaminated finished product.

Objective 3: In September, Intertox scientists visited several Washington apple orchards and processing facilities, and observed orchard, storage, and packing conditions that could influence the potential for contamination and pathogen growth/reduction. This information is being used to develop potential consumer exposure scenarios. Information and data on the U.S. consumption of fresh market apples was collected, analyzed, and summarized. Factors that contribute to the contamination risk (hazards) during production, harvest and packing are identified and discussed. The exposure assessment includes a description of the human health effects from foodborne

illness caused by *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* – the three pathogens of concern addressed in this study.

Using the microbial testing data and information from Washington companies and research studies, we constructed a draft QMRA model using Microsoft Excel and Palisade @Risk software to estimate potential exposure levels to these pathogens and the risk of becoming ill from eating contaminated fresh market apples. The model considers potential contamination and growth/reduction of pathogens occurring at various times in the field-to-fork continuum: in the field, during transport, during packing (e.g., washing/ treatment/ cross-contamination), and during retail and home storage. Exposures are being estimated for several population groups, including adults and children, using national fresh apple consumption rates. The model incorporates probabilistic methods to characterize the uncertainty and variability in model inputs as well as outputs (e.g., where possible, input parameters such as temperature, time, etc., are included). Model parameters are being estimated based on published studies and observed orchard/ packing activities. For each pathogen, risks will be characterized as the probability and severity of illness along with the total estimated number of cases. Currently, we are completing development of model distributions and testing calculations.

CONTINUING PROJECT REPORT **YEAR:** 2012
WTFRC Project Number: N/A

Project Title: Programs to increase packouts of apples

PI: Ines Hanrahan
Organization: Washington Tree Fruit Research Commission
Telephone: 509-669-0267
Email: hanrahan@treefruitresearch.com
Address: 104 N 1st St., Suite 204
City/State/Zip: Yakima, WA, 98901

Cooperators:
WTFRC internal program: Manoella Mendoza, Tory Schmidt, Udel Mendoza, Felipe Castillo
Other scientists: James Mattheis, Janie Countryman, David Rudell
Product suppliers: Pace International, Fine Americas Inc.
Grower collaborators: AFC Vantage, Columbia Reach, Allan Bros., Weippert ranches

Other funding sources
All supplies and chemicals were donated by industry suppliers.

Budget 1
Organization Name: WTFRC **Contract Administrator: Kathy Schmidt**
Telephone: 509 665 8271 **Email address: Kathy@treefruitresearch.com**

Item	2011	2012	2013
Salaries	7,867	7,867	7,867
Benefits	2,439	2,439	2,439
Wages	7,585	13,382	4,640
Benefits	3,778	9,691	3,360
Equipment + supplies	100		
RCA rental	12,600	2,100	
USDA rental	0		
Travel	2,013		
Total gross costs	36,382	35,839	18,306
Reimbursements	(7,000)	(7,000)	
Total net costs	**29,382**	**28,839**	**18,306**

Footnotes:
Entire budget is based on fiscal year August 1st, 2011– July 31st, 2012.
Salaries: include proportional time spent on outlined projects for Hanrahan, Castillo, Schmidt
Wages: covers time slip expenses, benefit rate at 42%
Supplies: fruit donated, storage boxes and trays donated
RCA rental: numbers based on fiscal year (@ approx. $6,300/room/year)
Reimbursements: monetary contributions by chemical suppliers

NOTE: Budget for informational purposes only. Research is funded through WTFRC internal program

OBJECTIVES

1. Determine the time course of delayed sunburn development in storage.
2. Investigate horticultural practices to mitigate delayed sunburn.
3. Determine time course of Honeycrisp soft scald development.
4. Develop a Honeycrisp starch scale for Washington State.
5. Determine the effectiveness of a farnesene analog to enhance red color in apples.
6. Expand collaborative efforts with other research programs working on fruit quality management.

SIGNIFICANT FINDINGS

1. Delayed sunburn appears between 2-3 months of storage, even on clean fruit.

2. Delayed sunburn development is influenced by fruit position in canopy and shade netting will decrease delayed sunburn.

3. Soft scald develops after 2 weeks in storage, with maximum symptom expression at 2- 3 months of storage. Early symptoms resemble bruising.

4. A starch scale is available under: www.treefruitresearch.com.

5. Red color was not improved in Honeycrisp and Fuji apples in 2012.

6. Ongoing collaborative efforts across disciplines, institutions, and regions leverage funding and increase relevance and impact of research.

METHODS

Delayed sunburn: Trials were set up to test the onset of delayed sunburn in storage as influenced by in season sunburn protectant application (Raynox, Eclipse) or shade net use in three Granny Smith orchards. Only clean fruit from the middle of the canopy was placed in cold rooms with RA and CA conditions and was evaluated periodically for up to 9 months. Some fruit was treated with 1-MCP. In addition, in one site clean fruit from one location was harvested from four different positions in the tree (upper, lower, east, west) and subjected to the same storage conditions. Individual fruit were graded for delayed sunburn according to the Schrader/McFerson system (0 = clean, 6 = necrosis). Postharvest evaluations included common fruit maturity parameters, background color grade, sunburn, and russet incidence.

Honeycrisp physiological disorders: In 2011, we harvested fruit sequentially (3 picks) from 2 orchards and immediately placed into 33F cold storage. Fruit was rated for soft scald weekly for 15 weeks.

Blush: In 2012 we started two trials (Fuji, Honeycrsip) featuring Blush, a novel product containing a farnesene analog. Applications were scheduled according to label recommendations, with 100gal of water and Roadrunner surfactant. An additional handgun trial in Honeycrisp investigated different timing scenarios. At harvest, common fruit maturity parameters and commercial color grades were determined.

RESULTS & DISCUSSION

Delayed sunburn: Granny Smith remains a stable variety for Washington growers, representing 10-13% of the total crop. Returns have been adequate, around $20 FOB. However, packouts suffer in the second part of the storage season because of the onset of delayed sunburn. Delayed sunburn is a physiological disorder that appears on apples after several months in storage. Typically, 30-50% of

fruit are affected starting after 2 months in RA or CA storage. To date there is no known control measurement in the storage environment.

Delayed sunburn can be classified into two types: a) previously undamaged fruit, or b) fruit with visible sun damage at harvest.
When placing clean fruit in 9 months CA storage, no postharvest treatment tested prevented delayed sunburn development. Fruit position in the canopy significantly affects delayed sunburn severity. More fruit showed symptoms when grown on the west side of the tree, or in the lower parts of the canopy (4-5ft.) of the v-trellis (Table 2).

Table 1: Delayed sunburn development of clean Granny Smith apples after 9 months in storage.

	Storage	Delayed Sunburn	Y1	Y2	Y3	Tan	Black
		(%)	(%)	(%)	(%)	(%)	(%)
Raynox	CA	20 ns	19 ns	2 ab	0	0	0
Raynox	CA + 1-MCP	19	16	4 ab	0	0	0
Control	CA	26	26	0 b	0	0	0
Control	CA + 1-MCP	18	23	0 b	0	0	0

Table 2: Delayed sunburn development of clean Granny Smith apples after 9 months in CA storage.

	CLEAN (%)	Y1 (%)	Y2 (%)	Y3 (%)	Tan (%)	Black (%)
Raynox	69 ns	22 ns	7 ns	1 ns	0 ns	0 ns
Control	62 ns	32	6	0	0	0
		FACTORIAL ANALYSES				
East[z]	72 a	23 b	5 ns	0 ns	0 ns	0 ns
West	60 b	30 a	9	1	0	0
Upper [y]	70 a	22 b	7 ns	1 ns	0 ns	0 ns
Lower	62 b	31 a	6	1	0	0

[z] refers to east and west side of trellis; [y] refers to position of fruit within trellis: upper = 8-9 ft., lower = 5-6 ft.

The season long use of shade cloth prevented sunburn at harvest (data not shown). When placing clean fruit in CA storage, fruit remained clean for 8 months, when 16% delayed sunburn appeared (Figure 1). Clean fruit harvested from trees treated with a protective film, developed 38-48% delayed sunburn during the storage period, starting at 2 months of storage, (Figure 1). The economic impact of this disorder can be estimated as follows: at 50 bins/acre with 30% loss in 9 month CA, FOB prices per bushel of $20 = loss of 7.2 packs/bin or $168/bin for total loss/acre of : **$ 8,400.** This is a conservative number, since yields of commercially viable Granny Smith blocks are typically higher.

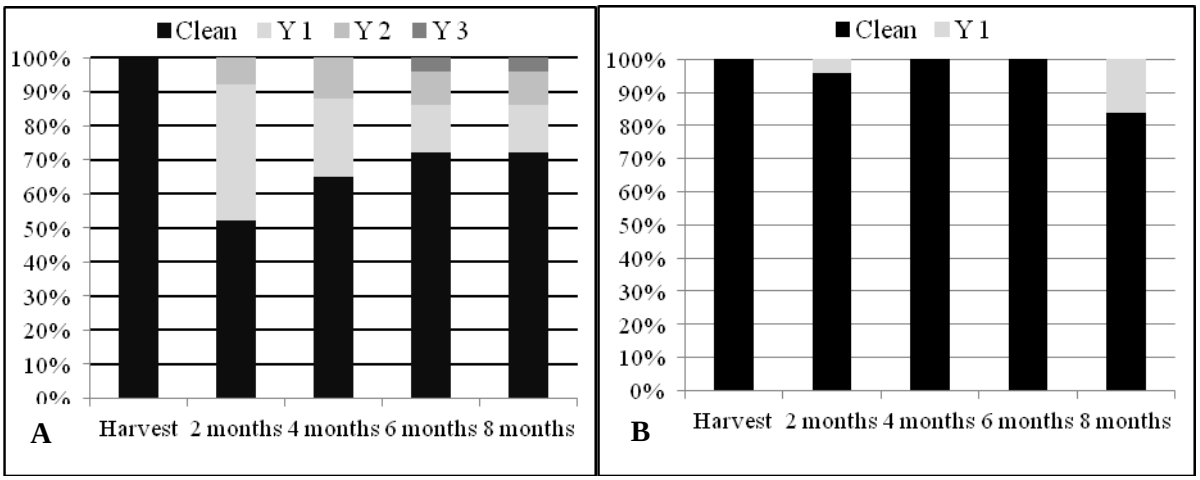
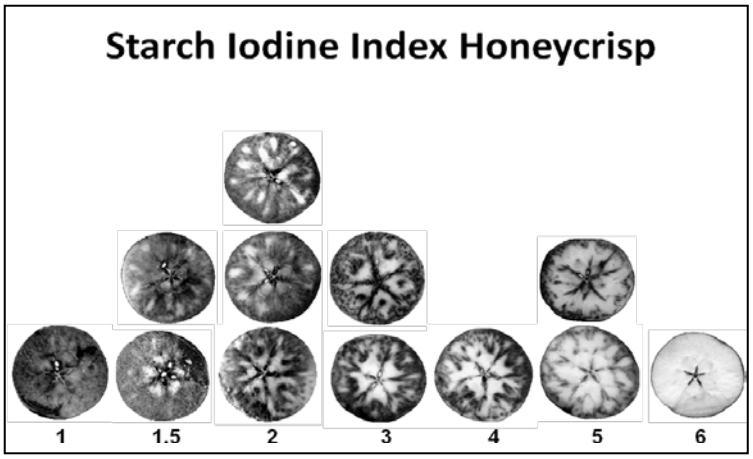


Figure 1: Development of delayed sunburn in Granny Smith apples during CA storage. A = protective film, B = shade cloth

Honeycrisp starch scale:



The scale was developed with pictures taken over a two year period and is available to download in color or black and white from: www.treefruitresearch.com

Figure 2: Starch Iodine Index for Honeycrisp. WTFRC 2012.

Honeycrisp soft scald development: In 1999, Honeycrisp was first planted in Washington State, USA. Growth since then has been exponential, with 9,100 acres planted in 2011. Having strong demand helped move the crop within three months during the first decade, well before major storage problems may occur. However, with the increasing volume, more fruit had to be kept until after January 1st. For example, by January 1, 2012 31% of the crop remained in storage, according to the Washington Growers Clearing House. Prices for Honeycrisp are higher towards the end of the storage season, but long term storage remains a gamble, since packouts can suffer dramatically due to increasing incidences of physiological disorders such as bitter pit and soft scald.

Soft scald is a physiological disorder of apple induced by cold temperatures in storage and not visible at harvest. Apple fruit skin shows tan to dark brown bands on the skin with very distinct margins. In severe cases affected areas are sunken and internal tissue is affected. Symptoms can appear after two weeks in cold storage and are typically fully expressed after 2-3 months in storage (Pictures shown on poster). Affected areas are often invaded by fungus and then black. May or may not extend into the flesh and can be firm to touch, if area is small. Misidentification is common, because early symptoms

of soft scald are very similar to shallow bruising but can be distinguished by cutting the fruit. Bruises will exhibit damaged tissue underneath the browning, while soft scald in early stages is superficial. Soft scald and fungal infections can also look similar.

Blush: A new product (Blush) manufactured by Fine was available for testing under an experimental use permit in Washington in 2012. WTFRC set-up several experiments in blocks with a known history of lack of red color development. No treatment significantly increased the amount of fruit in the premium commercial color grade (Table 3). However, for optimum product performance, slow drying conditions are needed. Applications were made in the evening, when temperatures dropped to 68◦F, but orchard humidity was low. We are planning to repeat the experiment in 2013, with adjustments to the application timing (i.e. closer to commercial harvest).

Table 3: Commercial color grade of Fuji and Honeycrisp apples at harvest as influenced by Blush application. WTFRC 2012.

TREATMENT	PICKING SEQUENCE	PREMIUM (%)	2nd GRADE (%)	3rd GRADE (%)	4th GRADE (%)
Auvil Early Fuji / Mark - Vantage[1]					
Shade net + Blush	1st pick	6 b	32 ns	62 a	0 ns
Shade net Control		10 ab	57	33 ab	0
Blush	1st pick	26 ns	52	22 b	0
Control		25	56	19 b	0
Honeycrisp / Bud. 9 - Gleed[2]					
Blush early	1st pick	14 ns	49 ns	37 ns	0 ns
Blush late		18	66	16	0
Blush early and late		11	46	43	0
Control		8	52	40	0
Blush early	2nd pick	16 ns	57 ns	27 ns	0 ns
Blush late		13	58	29	0
Blush early and late		16	55	29	0
Control		15	45	40	0
Blush early	3rd pick	26 ns	60 ns	13 ns	0 ns
Blush late		26	62	11	0
Blush early and late		24	62	14	0
Control		30	53	17	0

[1]Application dates: 8-20, 8-30; harvest date: 9-10
[2]Appliaction dates: 8-21, 9-4; harvest dates: 9-13, 9-19, 9-29

Collaborative research
We continue to build productive and dynamic research and outreach partnerships with a number of cooperators on projects relevant to pre-and postharvest fruit quality management (Table 4).

Table 4. 2012 WTFRC collaborations on pre- and post- harvest fruit quality management projects.

COLLABORATOR(S)	PROJECT	COMMENTS
Rudell	Disorder toolbox	Cooperator on SCRI project
NSure*	Honeycrisp disorder ID	WA field testing
Killinger	Packingline microb. safety	Field support (see AP-09-906)
Killinger	Overhead cooling	Filed support (see AP-12-108)
Pleus	Apple microbial risk factors	Cooperator on CPS/WTFRC project
Brunner, MSU	Solid set spray system	Cooperator on SCRI project
Evans/Auvil	WSU Breeding: P3	Storage evaluation (see Auvil/Evans cont.)
Gallardo	RosBREED	Cooperator taste panel

*project costs covered by companies

CONTINUING PROJECT REPORT **YEAR**: 1 of 3
WTFRC Project Number: AP-12-105A

Project Title: Implementation and evaluation of apple pollen tube growth models

PI:	Dr. Keith S. Yoder	**Co-PI (2):**	Dr. Greg M. Peck
Organization:	Virginia Tech	**Organization:**	Virginia Tech
Telephone:	(540)-869-2560 X21	**Telephone:**	(540)-869-2560 X19
E**-mail**:	ksyoder@vt.edu	**E-mail**:	greg.peck@vt.edu
Address:	595 Laurel Grove Rd.	**Address:**	595 Laurel Grove Rd.
Address 1:	Va. Tech AHS-AREC	**Address 1:**	Va. Tech AHS-AREC
City:	Winchester	**City:**	Winchester
State/Zip:	VA 22602	**State/Zip:**	VA 22602

Co-PI:	Dr. Gerrit Hoogenboom	**Co-PI**:	Dr. Melba Salazar
Organization:	Washington State University	**Organization**:	Washington State University
Telephone:	509-786-9371	**Telephone**:	509-786-9281
Email:	gerrit.hoogenboom@wsu.edu	**Email**:	m.salazar-gutierrez@wsu.edu
Address:	Washington State University	**Address:**	Washington State University
Address 2:	24106 North Bunn Road	**Address 2:**	24106 North Bunn Road
City:	Prosser	**City:**	Prosser
State/Zip:	WA 99350-8694	**State/Zip:**	WA 99350-8694

Cooperators: Leon Combs, Research Specialist, Va. Tech AHS-AREC; Winchester, VA; e-mail: lecombs@vt.edu
Sean Hill, Appl. Systems Analyst/Dev., AgWeatherNet, Washington State Univ., Prosser, WA; e-mail: sehill@wsu.edu
Tory Schmidt, Washington Tree Fruit Research Commission, Wenatchee, WA

Total Project Request: Year 1: $92,983 **Year 2: $83,343** **Year 3:** $86,688

Other funding Sources

Indirect support through the existing infrastructure of AgWeatherNet and its network of 137 weather stations.

WTFRC Collaborative expenses:

Item	2012	2013	2014
Total	$21,000	**$21,000**	$21,000

Note: Virginia Tech and Washington State University are submitting separate budgets as collaborative institutions.

Budget 1

Organization Name: Virginia Polytechnic Institute and State University (Va. Tech)
Contract Administrator: Sarah Lawrence
Telephone: 540-231-9393 **Email address:** sarah.lawrence@vt.edu

Item	2012	2013	2014
Salaries*	35,762	37,192	38,680
Benefits	10,282	10,693	11,121
Equipment (laptop-field work)	1,500		
Supplies (lab &field)	1,500	1,500	1,500
Travel (to Wash. St. orchards)	5,000	6,000	6,500
Contractual services & repairs	1,250	1,250	1,250
Total	$55,294	**$56,635**	$59,051

***Note:** Salary for Research Specialist Leon Combs.
Virginia Polytechnic Institute and State University (Va. Tech)

Budget 2

Organization Name: ARC-WSU **Contract Administrator: Carrie Johnson**
Telephone: 509-335-4564 **Email address: carriej@wsu.edu**

Item	2012	2013	2014
Salaries	24,699	16,674	17,341
Benefits	9,490	6,534	6,796
Wages			
Benefits			
Equipment			
Supplies	1,000	1,000	1,000
Travel	2,500	2,500	2,500
Miscellaneous			
Total	$37,689	**$26,708**	$27,637

Footnotes: Partial salary support for Research Associate (Dr. Melba Salazar) and for Application Development Programmer (Mr. Sean Hill).

I. Objectives

Our overall goal for 2012-14 is to collaborate with Washington State University and Washington Tree Fruit Research Commission to validate and implement a computer-generated pollen tube growth model for the major commercial apple cultivars on AgWeatherNet (www.weather.wsu.edu).

The specific objectives for 2013 are:

1) Complete model parameters for Honeycrisp (Virginia Tech).
2) Guide collaborative effort to validate model program and incorporate into AgWeatherNet site (WSU, WTFRC, and Virginia Tech).
3) Continue to improve the model interface based on user feedback (WSU, WTFRC, and Virginia Tech).
4) Continue to provide training to cooperators to recognize desirable amount of king bloom open to "start the model clock" (Virginia Tech, WSU, and WTFRC).
5) Continue field beta-testing of models for Gala, Fuji, Golden Delicious and Cripps Pink and begin field beta-testing on Honeycrisp (Virginia Tech and WTFRC).
6) Add plantings of Brookfield Gala, September Wonder Fuji, and Braeburn for future temperature testing (Virginia Tech).
7) Further develop reliable techniques for the study of a range of constant and variable temperatures and light conditions on pollen germination and tube growth (Virginia Tech).

II. Significant Findings

- Working with 60 test sites in Washington State, we are evaluating the pollen tube growth model as a precision bloom-thinning tool.
- Developing cultivar-specific equations for pollen tube growth and interfacing these models with real-time and forecasted weather data on the AgWeatherNet website.
- Creating web-based interface to make these models user friendly and the output results easy to understand.
- The AgWeatherNet interface allows for site and cultivar specific information to be generated.
- By using forecast data through the AgWeatherNet site, pollen tube growth is projected 48 hours into the future, which allows growers to more easily schedule bloom thinning sprays in advance.
- Microscopic evaluation of the model in the laboratory includes sampling flowers from the field to determine the percent of flowers that have been fertilized.
- Comparing average style length determined in the field and in the laboratory is an integral part of evaluating and refining the models to actual field conditions.
- Data results to date show that the pollen tube growth model is helping growers achieve their targeted crop load.

III. Methods for 2012

Winter-Spring, 2012: Translate and convert the currently available spreadsheet equations that have been developed for pollen tube growth for selected cultivars into models that are scientifically robust. Design a desirable interface for pollen tube growth model on

AgWeatherNet; establish flower sampling parameters and intervals needed to validate the model for Gala, Golden Delicious, Fuji and Cripps Pink; recruit potential 2012 test site cooperators.

Early Spring, 2012: Conduct in-depth training sessions for beta-testers on how to use the AgWeatherNet website models.

Bloom Period, 2012: As bloom approaches, adjust site/cultivar selection as necessary to facilitate optimal accomplishment of test pollination/bloom thinning and sampling from preferred cultivars within about one week near early to mid bloom; ship refrigerated samples to Virginia Tech for microscopic evaluation. Conduct specific Washington field tests regarding pollen tube growth in hand-pollinated flowers.

Spring-Summer, 2012: Examine samples in laboratory at Winchester, VA; compare results for pollen tube progression at selected sites, based on local temperatures during bloom, to predicted values; solicit beta-tester feedback on functional aspects of AgWeatherNet interface.

Fall, 2012: Report beta-tester comments/suggestions on functional aspects of AgWeatherNet interface; accuracy of test results, consider need for follow-up testing and fine-tuning.

Fall-Winter, 2012: Discussion of functional aspects of model on AgWeatherNet; consider needs for further validation with Gala, Golden Delicious, Fuji and Cripps Pink; set cultivar and validation priorities for 2013.

IV. Results and Discussion

Properly timed bloom thinning gives the grower the optimum advantage for producing the best quality fruit. Understanding the progression of pollen tube growth after pollination is critical in applying bloom thinners at the proper time. In addition to optimal sizing benefits, crop loads not sufficiently thinned could result in trees being thrown into biennial bearing with little or no crop in the 'off' year. The primary focus is to evaluate the pollen tube growth model for the wide range of growing conditions in Washington State. Real-time weather station data specific to that growing region will be downloaded to the AgWeatherNet model interface for program assimilation.

Beta-testing of the model this year has helped growers evaluate the program and thinning success. Their suggestions on improvements and modifications have helped refine the model into a more grower-friendly tool in just the first year. The following remarks and observations were made by beta-testers using the model this year:

Darin B. Case
Dovex Fruit Company, Orchard General Manager

> *Much easier to work with on forecasting and as we discussed before, a great tool to integrate into our program. Now we just need to perfect how we use it annually. Remember thinning is more of an art than science! But your model is getting closer.*

Harold Ostenson
Tree Fruit Consulting

> *I think the industry is 'doing better' using the model for bloom thinning material application timing. For the first time, 'they' are actually measuring styles in most cases and have a better understanding of timing. It is a step forward, no question.*

Harold Schell, Horticultural Manager
Chelan Fruit Company

> *I found the AgWeatherNet site to be very helpful and useful. I ended up using that site because of not getting the data logger up and going in time in my own orchard. It was a great help and would be of great usefulness to others I'm sure. All in all I was quite pleased and had the opportunity to show many of our growers. The two brothers that lease my place were so impressed that they are going to the model on all of their ranches. They farm close to 400 acres.*

Approximately 60 orchard test sites made up the beta-test field in 2012 involving several hundred acres of apples. Training sessions on using the AgWeatherNet pollen tube growth modeling program were held in early 2012 at Naches, WA with 21 attendees and an additional training session was conducted in Chelan, WA with 12 attendees for that meeting. Additional testing in 2013-14 will further improve and validate the effectiveness of this bloom thinning aide for growers. Accumulating data shown in the following graphics and tables show results from some beta-test sites for the 2012 growing season. Additionally shown below are data graphs and tables generated from testing done this year and snapshots of images generated by the AgWeatherNet interface showing data generated features of the program (Figs. 1-3).

In addition to the implementation of the pollen tube growth models on the AgWeatherNet for beta-testing, other testing continued to validate and expand the effectiveness of the modeling program. These tests included field tests in selected Washington orchards comparing weather station temperature data used by the AgWeatherNet interface to actual on-site temperature data-loggers (Fig.4). This data will help determine variations in temperature at orchard sites versus actual location of nearest AgWeatherNet weather station. Differences in projected pollen tube growth among locations probably reflect differences in elevations or distance from AgWeatherNet station for the test sites. If large variations are found, then possible implementation of some link to allow site specific temperature input may be added to the AgWeatherNet pollen model site. Continued sampling of style length on-site versus microscopic measuring is needed to verify current field sampling and measuring procedures. Current procedures seem appropriate with growers' field measurements within one standard deviation of the more precise lab measurements (Fig.5).

Tracking actual harvest totals versus desired cropping is needed to verify the models effectiveness as shown in Table 1. Comparing desired crop load with actual harvest data demonstrates either understanding of beta-testers in implementation or need for further training in initiation of the modeling program at the proper time.

Controlled growth chamber testing at the Virginia Tech research facility has continued on adding more cultivars to the modeling program. Current on-going tests include Honeycrisp and Red Delicious, with implementation of the Honeycrisp model tentatively scheduled for access to beta-testing on the AgWeatherNet pollen model site for the 2013 growing season. Additional growth chamber testing in 2013 will begin on Granny Smith.

We would like to thank the Washington Tree Fruit Research Commission for its continued support of this project. We would also like to thank the Washington Tree Fruit Research Commission staff, particularly Tory Schmidt, whose help on the project was an essential part of the project. Lastly, we would like to thank the beta-testers, growers, and others who are providing critical feedback on the pollen tube growth model.

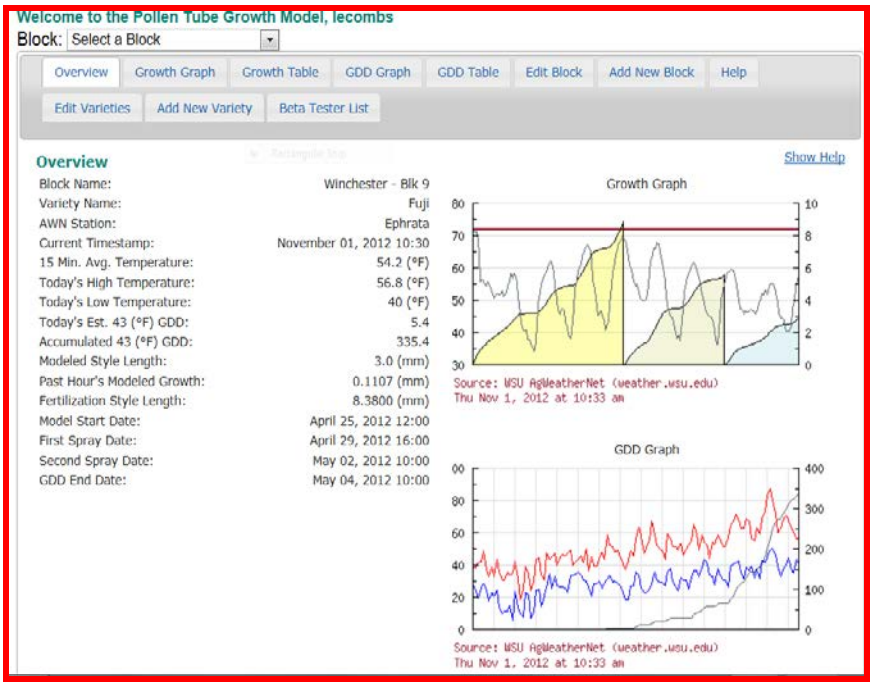


Figure 1. AgWeatherNet-generated overview of graphics and data input.

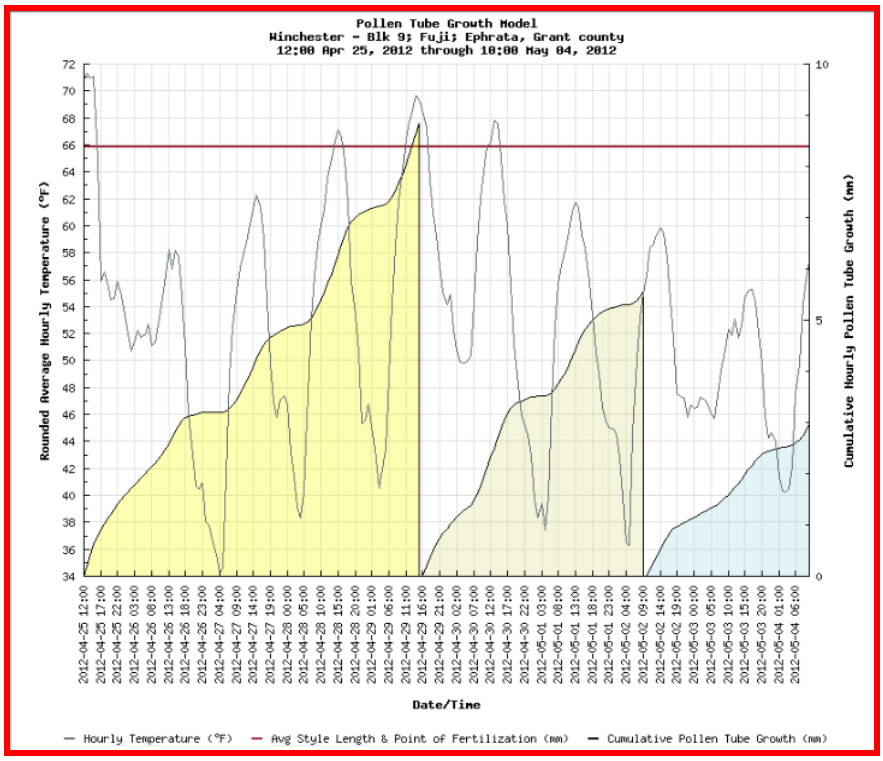


Figure 2. AgWeatherNet graphic showing application timing of bloom thinning sprays applied by beta-tester.

Pollen Tube Growth Model Table

Show Help

Date/Time	Air Temp (°F)	Hourly Growth (mm)	Accumulated Growth (mm)	% of Target	Spray Applied?
2012-04-25 00:00	53	0.08	9.83	128	
2012-04-24 23:00	52	0.07	9.76	127	
2012-04-24 22:00	55	0.09	9.68	126	
2012-04-24 21:00	55	0.09	9.59	125	
2012-04-24 20:00	57	0.11	9.50	124	
2012-04-24 19:00	59	0.13	9.39	122	
2012-04-24 18:00	62	0.16	9.26	121	
2012-04-24 17:00	66	0.19	9.10	119	
2012-04-24 16:00	68	0.20	8.91	116	
2012-04-24 15:00	70	0.20	8.72	114	
2012-04-24 14:00	72	0.21	8.52	111	
2012-04-24 13:00	73	0.21	8.31	108	
2012-04-24 12:00	73	0.21	8.09	105	
2012-04-24 11:00	72	0.21	7.88	103	
2012-04-24 10:00	72	0.21	7.67	100	
2012-04-24 09:00	70	0.20	7.46	97	
2012-04-24 08:00	66	0.19	7.26	94	
2012-04-24 07:00	62	0.16	7.07	92	
2012-04-24 06:00	58	0.12	6.91	90	
2012-04-24 05:00	58	0.12	6.79	88	
2012-04-24 04:00	57	0.11	6.67	87	
2012-04-24 03:00	57	0.11	6.56	85	
2012-04-24 02:00	60	0.14	6.45	84	
2012-04-24 01:00	60	0.14	6.31	82	
2012-04-24 00:00	63	0.17	6.17	80	
2012-04-23 23:00	65	0.18	6.01	78	
2012-04-23 22:00	66	0.19	5.82	76	
2012-04-23 21:00	68	0.20	5.64	73	
2012-04-23 20:00	67	0.19	5.44	71	
2012-04-23 19:00	69	0.20	5.25	68	
2012-04-23 18:00	78	0.22	5.05	66	
2012-04-23 17:00	82	0.22	4.83	63	
2012-04-23 16:00	85	0.22	4.61	60	
2012-04-23 15:00	83	0.22	4.39	57	
2012-04-23 14:00	82	0.22	4.17	54	

Figure 3. AgWeatherNet temperature data table for tracking pollen tube growth.

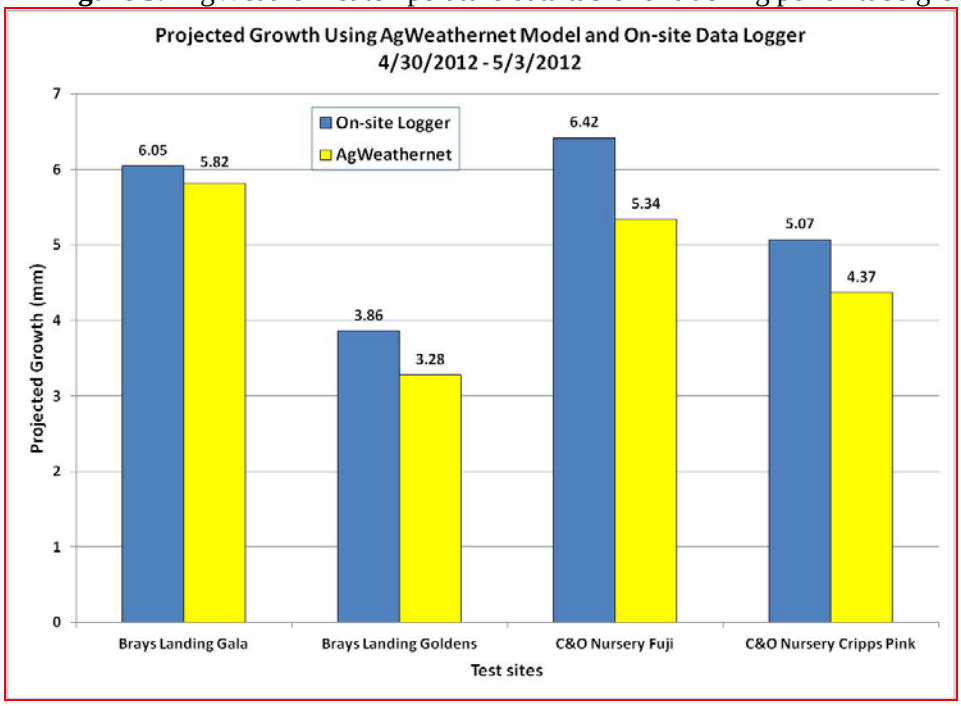


Figure 4. Field test comparing on-site temperature data logger vs AgWeatherNet weather station input.

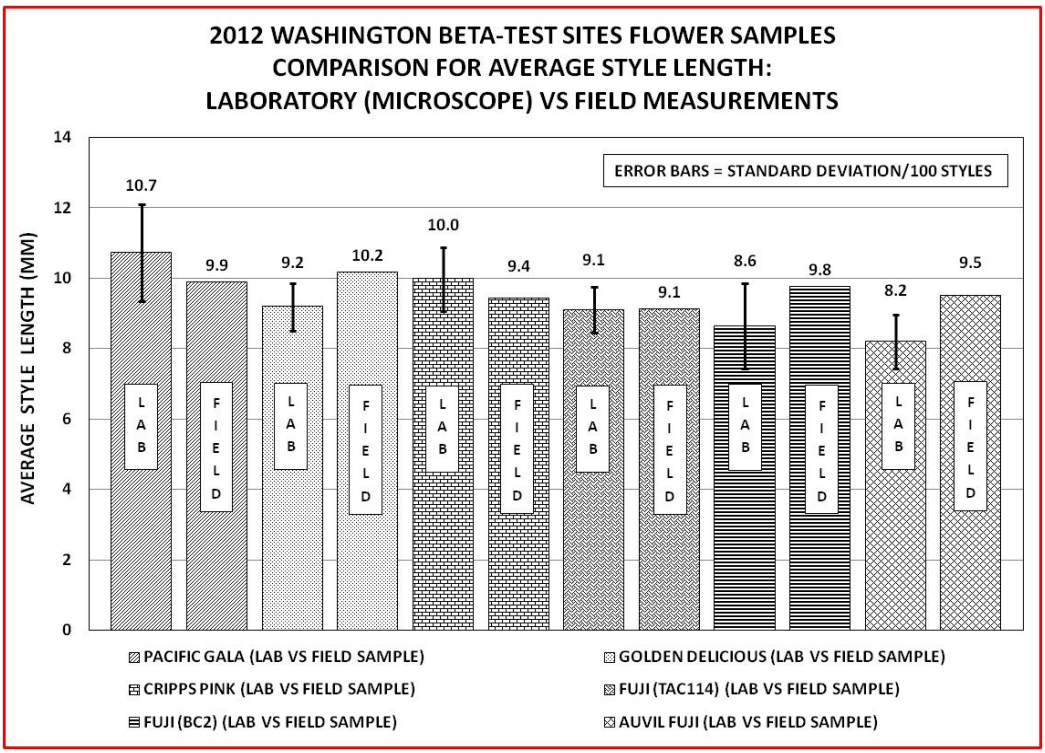


Figure 5. Comparison of average style lengths measured in the field vs microscopic measuring in lab.

POLLEN TUBE MODEL HARVEST DATA FOR BETA-TEST SITES IN QUINCY, WA (2012)

CULTIVAR	STRAIN	DESIRED 2012 PRODUCTION / ACRE	2012 PRODUCTION / ACRE	% DESIRED 2012 PRODUCTION
Gala	Pacific	50	55.7	111
Fuji	Nagafu 6	35	23.5	67
Golden Delicious	Standard	60	61.8	103
Golden Delicious	Standard	57	50.3	88
Fuji	TAC 114	35	27.4	78
Fuji	TAC 114	35	22.4	64
Fuji	Early Fuji	40	38.7	97
Cripps Pink	Pink Lady	45	40.3	90
Gala	Pacific	45	44.0	98
Golden Delicious	Smoothee	55	32.1	58

TABLE 1

Table 1. Harvest totals for 2012 comparing desired crop load vs actual harvest totals at beta-test sites in Quincy, WA.

CONTINUING PROJECT REPORT **YEAR**: 1 of 3
WTFRC Project Number: AP-12-104

Project Title: Development of apple bloom phenology and fruit growth models

PI: Gerrit Hoogenboom
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Co-PI(3): Tory Schmidt
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Co-PI (4): Nairanjana Dasgupta
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Address: Department of Statistics
Address 2: Neill 103
City/State/Zip: Pullman, WA 99164

Cooperators: Karen Lewis (WSU-Extension), Felipe Castillo (WTFRC)

Total Project Request: **Year 1:** $70,000 **Year 2: $82,500** **Year 3:** $85,000

Other funding sources

Indirect support through the existing infrastructure of AgWeatheNet and its network of 137 weather stations.

WTFRC Collaborative expenses:

Item	2012	2013	2014
Salaries	3,000	3,500	4,000
Benefits	1,200	1,400	1,600
Wages[1]	7,500	7,500	*0 or 7,500
Benefits			
RCA Room Rental			
Shipping			
Supplies			
Travel[2]	2,400	2,700	3,000
Plot Fees			
Miscellaneous			
Total	$14,100	**$15,100**	*$8,600 or $16,100

Footnotes: *Additional field data collected only if needed in 2014
[1] Labor calculated as 2 persons at $16.00/hr working 12 hrs per week for 13 weeks during the growth season.
[2] In-state travel to research plots

Budget

Organization Name: ARC-WSU **Contract Administrator:** Carrie Johnston

Telephone: 509-335-4564 **Email address:** carriej@wsu.edu

Item	2012	2013	2014
Salaries	53,936	65,536	67,496
Benefits	12,564	13,464	14,004
Wages			
Benefits			
Equipment			
Supplies	1,000	1,000	1,000
Travel	2,500	2,500	2,500
Miscellaneous			
Plot Fees	0	0	0
Total	$70,000	**$82,500**	$85,000

Footnotes: The budget that is requested through this proposal includes partial support for a Research Associate (Melba Salazar) who will be responsible for the overall evaluation and implementation of the various growing degree models that are applicable for conditions in the Pacific Northwest and partial support for an Application Programmer (Sean Hill) for integration of the model on the web portal of AgWeatherNet (www.weather.wsu.edu). We also have budgeted for a Graduate Student (to be hired) who will be responsible for the development of a physiological fruit growth model. The proposal includes a request for a computer for the graduate student during the first year of the project. Additional budget items include operating expenses for computer software and related costs and travel to participate in field data collection. Finally, this proposal includes support for Professor Dasgupta in the Department of Statistics to complete her statistical model development and evaluation (objective 2).

OBJECTIVES

1. Continue data collection on bloom phenology and fruit growth for selected sites and cultivars to enhance model accuracy and vigor. (Schmidt in collaboration with Castillo)
2. Continue refinement of statistical models for bloom phenology and fruit growth. (Dasgupta)
3. Develop physiological-based models for bloom phenology and fruit growth of apples. (Hoogenboom, Salazar)
4. Implement and evaluate models as decision support aids on the AgWeatherNet portal using industry beta-testers. (Hoogenboom, Salazar and Dasgupta in collaboration with Lewis)
5. Improve model/portal user interface based on feedback from beta-testers and other stakeholders. (Hoogenboom, Salazar in collaboration with Lewis)

Timetable for Project

Activities	**2013**				**2013-2014**				**2014-2015**			
1. Experimental data collection		x	x			x	x			x	x	
2. Statistical model development and evaluation	x	x	x	x	x	x	x	x	x	x		
3. Physiological model development and evaluation	x	x	x	x	x	x	x	x	x	x	x	x
4. Web-based user interface development			x	x	x	x	x	x				
5. Web-based user interface evaluation by WSU Extension and stakeholders; final implementation						x	x	x	x	x	x	x

METHODS

1. Data collection

WTFRC staff will continue with the collection of bloom phenology and fruit growth data from established sites to augment data sets from the previous project.

2. Continue refinement of statistical models for bloom phenology and fruit growth

For the growth models, data have been compiled for Gala for 2010 and 2011, while for Red Delicious and Cripps Pink data have been compiled for 2010. For the bloom models, data have been compiled for 2010 and an ordinal logit model has been used to fit the data. All data for phenology, growth and temperature will be compiled for 2011. For the growth model the data for 2010 and 2011 have to be combined and new parameters have to be estimated. For the bloom model similar procedures will be followed.

Following a successful development of both the statistical bloom phenology model and the statistical fruit growth model, they will be evaluated with the new data that will be collected during the 2012 and 2013 growing seasons.

3. Develop physiological models for bloom phenology and fruit growth

Physiological time will be used as input of the model for the different phenological stages for apple, referred to as Growing Degree Days (GDD) or Thermal time. A comparison among three of the most traditionally methods for GDD accumulation is planned. The requirements for the different phenological stages of the most important apple cultivars using hourly temperature records from the AgWeatherNet will be summarized. An evaluation is planning to determine the most accurate model using historical observed dates under different environmental conditions. The performance of the model will be compared using the weather data collected with the Hobo data loggers that have been part of the data collected by WTFRC.

4. Implement and evaluate models as decision support aids on the AgWeatherNet portal

To assist the growers for making decisions, an information delivery system and media tool will be posted in the web page using the models developed. This tool will provide, in an easy and user-friendly way, thermal time, cumulative chilling, and cumulative degree hours in real-time (current) for different environmental conditions where local weather data are available through tables and graphs as well as information about the current phenological and development stages and the climatic requirements to complete the next stage.

Alerts to the growers will be generated when the crop can be at risk due to the actual temperatures in excess of the threshold temperatures. The system will be available through a link created on the AgWeatherNet web portal and other web portals where information for apples is provided, including the Decision Aid System (DAS).

5. Improve model/portal user interface and release for general use

The overall goal is to develop a web portal that will provide a guideline and advisory for the growers who are monitoring their individual apple orchards in terms of weather conditions and weather predictions. Closely work with WSU Extension and industry representatives as beta testers during the second and third year of this project is planned to try to include the comments to improve the tool and decision aid to the benefit of the local apple growers.

RESULTS & DISCUSSION

1. Data collection

Observations of bloom phenology were recorded in 2012 by WTFRC internal staff every Monday, Wednesday, and Friday in 29 blocks clustered around 10 location nodes. Current varieties include Red Delicious, Cripps Pink and Gala. WTFRC staff also collected fruit size data starting at petal fall until final harvest with a brief break during thinning.

2. Continue refinement of statistical models for bloom phenology and fruit growth

For the statistical growth models, we obtained the following potential explanatory variables were: GDD, Julian date, DAFB, Cum Av Temp, Cum Av Soil Temp, Cum Av Dewpoint, Cum Av RH, Cum Min, Cum Max., Daily Min and Max and Solar Radiation. We studied the correlations of the above list with Diameter, at first for the variables based on time: Julian Date, DAFB and GDD. For the cultivar Gala we found that the diameter was correlated to all three but mostly to DAFB (r=0.928), while Julian Date was 0.923 and GDD was .847. As these are highly correlated it makes sense to have only one of these in our model. We then studied the correlations among diameter and the weather variables. These are as follows:

CumAvgRH	CumAvgTemp	CumMIN	CumMAX	CumAvgDewPt	CumAvgSoilTemp
-0.59211	0.73947	-0.44027	0.79668	0.51114	0.70750

It is evident that all these are fairly good predictors of diameter with Cumulative Average Temperature (CumAvgTemp) having the highest correlation .73947. It makes sense that both Cumulative Minimum temperature and Cum Average Relative Humidity (CumAvgRH) are negatively associated with diameter.

We used stepwise method in SAS to look at model selections for each cultivar. In each case, we report the best model. If GDD was not in our best model we looked at the best model with GDD in it and commented upon it. We did account for multi-collinearity among the weather variables and for each cultivar we report the two models and the corresponding R-squares. We report the data for 2010 as an illustration.

Gala 2010:
Model: Diameter = 0.89834 + 0.01918 DAFB - 0.00979 CumAvgRH -0.00905 CumMin + 0.00764 CumAvgDewPt

This model has a R-square of 89.29%

Using the same logic as above the best model for Red Delicious is given as:

Model: Diameter = 2.70984 + .00055 GDD - .0119 CumAvgRH - .0209 CumMin + .0043 CumAvgDewPt.

This model contains GDD and the R-square is 86%.

Using the same logic as above the best model for Cripps Pink is given as:

Model: D iameter = 1.618 + .0121 DAFB - .0336 CumAvgRH - .00813 CumMin + .08842 CumAvgDewPt - .0444 CumAvg SoilTemp + .00564 CurrentDewPoint

With a R-square of 89.9%. The model with GDD has a R-square of 82%.

It is clear that all three cultivars have the following variables in common: GDD/DAFB, cumAvgRH, CumMin, and CumAvgDewPt. Cripps Pink also has CumAvgSoilTemp and the current Dew Point in the model. The R-squares of these models range from 86% to 90% which is very promising, given the noisy nature of the data and the fact that location was not used in the models.

We have similar results for the 2011 data. For the growth data we are incorporating the weather variables and running ordinal logit models. Our results using cross validation are promising with r-squares over 90% for all three cultivars.

3. Develop physiological models for bloom phenology and fruit growth
The dynamics of the different phenological stages was analyzed for each location and each cultivar an example for year 2012 for Prosser and Wenatchee is presented (Figure 1).The analysis shows differences between locations among cultivars for the different phenological stages in terms of duration.

The duration in Growing Degree Days (GDD) was determined for Gala, Red Delicious and Cripps Pink for each season and from petal fall to harvest, using a base temperature of 43°F. An example for the two locations Prosser and Wenatchee is presented (Table 1). In general differences among cultivars were found at each location. Gala was the cultivar that needs to accumulate less GDD (2813) compared with Red delicious (3443) and Cripps Pink (3404) in Prosser. Similar trend was observed for the last four years of observations (2009-2012) in Wenatchee, the average of the GDD accumulation for Gala was (2539) followed by Red Delicious (3106) and Cripps Pink (3514).

Table 1. Growing degree days (GDD) using a base temperature of 43 ^{o}F starting on Petal Fall to Harvest for Prosser and Wenatchee.

Weather Station	Cultivar	2009	2010	2011	2012	Average
		Prosser				
Mabton East	Gala		2628	2936	2875	2813
	Red Delicious		3167	3645	3516	3443
	Cripps Pink	3891	3466	2936	3811	3526
		Wenatchee				
East Wenatchee	Gala	1871	2623	2637	3026	2539
	Red Delicious	2360	3094	3316	3655	3106
	Cripps Pink	3028	3792	3570	3667	3514

Fruit growth was observed in 10 locations during 2011 and 2012 (Figure 2). In General for all locations Red Delicious and Gala had significantly larger diameters than Cripps Pink, the final size of the fruit varied from year to year and location to location. Cultivar differences in fruit diameter reflect differences in mean fruit diameter as well as fruit growth period. According with the data analyzed for two locations Prosser and Wenatchee, the diameter of the fruits was bigger in Prosser during two consecutive seasons 2011 and 2012. Gala had bigger fruits in Prosser while Cripps Pink had bigger fruits in Wenatchee. Red delicious behaves different in both seasons and in both locations.

4 Implement and evaluate models as decision support aids on the AgWeatherNet portal
No activity to report

5 Improve model/portal user interface and release for general use
No activity to report

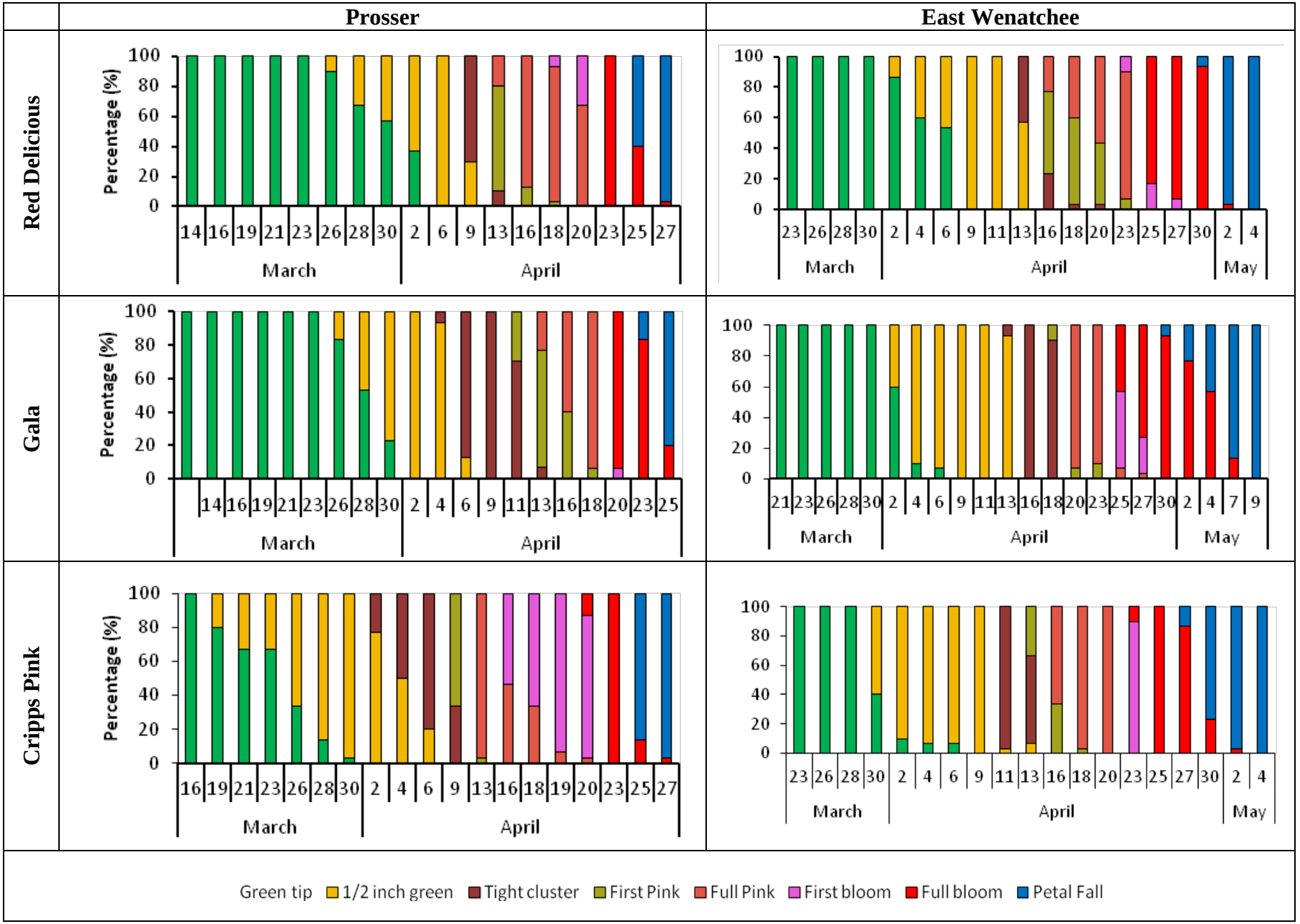

Figure 1. Progression of the Phenological stages for apples in Prosser and East Wenatchee, for Red Delicious, Gala and Cripps Pink cultivars during 2012 growing season.

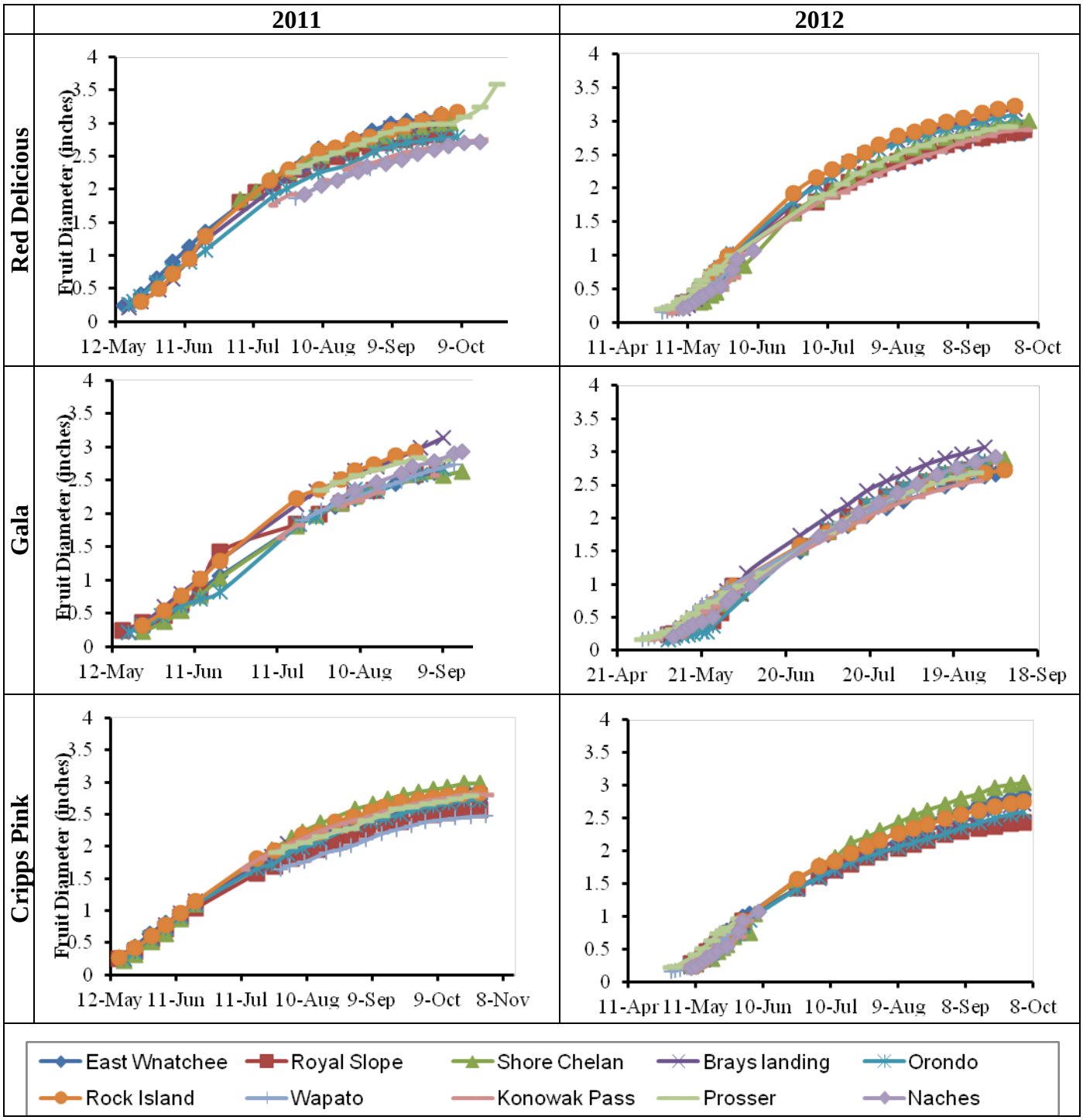

Figure 2. Seasonal changes in fruit diameter of three apple cultivars: Red Delicious, Gala and Cripps Pink in 10 locations, during the growing seasons of 2011 and 2012.

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

Project Title: Crop load and canopy management of apple

PI: Tory Schmidt
Organization: WTFRC
Telephone/email: (509) 665-8271 tory@treefruitresearch.com
Address: 1719 Springwater Ave.
City: Wenatchee
State/Province/Zip WA 98801

Cooperators: Jim McFerson, Ines Hanrahan, Felipe Castillo, Tom Auvil - WTFRC

Budget 1:
Organization Name: WTFRC **Contract Administrator:** Kathy Coffey
Telephone: (509) 665-8271 **Email address:** kathy@treefruitresearch.com

Year	2011	2012	2013
Salaries	16,000	16,000	16,000
Benefits	4,500	4,500	4,500
Wages	12,000	25,000	25,000
Benefits	3,800	8,000	8,000
Equipment			
Supplies	1,000	1,000	1,000
Travel	2,000	2,500	2,500
Stemilt lab fees	2,000	2,000	2,000
Statistical consulting	0	1,000	1,000
Total gross costs	41,300	60,000	60,000
Reimbursements	(20,000)	(53,000)	?
Total net costs	$21,300	$7,000	

Footnotes: Supply costs primarily covered by private industry cooperators
Travel includes fuel costs for driving to trial sites
Stemilt lab fees for use of single lane Aweta color grader
Statistical consulting for analysis of tree-to-tree variability for long-term cropping study on WSU Sunrise Granny Smiths

NOTE: Budget for informational purposes only; research is funded through WTFRC internal program

OBJECTIVES:

1) Continue screening new chemical and mechanical thinning technologies for apple
2) Develop practical PGR programs to manipulate floral initiation and promote annual bearing
3) Investigate horticultural effects of synthetic materials deployed as reflective ground covers or overhead shade/wind/bird protection
4) Profile natural tree-to-tree variation in long-term cropping patterns in a newly planted apple block
5) Expand collaborative efforts with other research programs working on crop load and canopy management

2012 HIGHLIGHTS:

No chemical bloom thinning program reduced fruit set or increased fruit size of Red Delicious or Fuji (Table 2)

Oil + lime sulfur programs remain the best option for chemical thinning, but endothall (ThinRite) has been effective in recent trials and may provide a viable alternative when registered (Table 3)

Most postbloom thinning treatments were effective in 2012 on Gala and Honeycrisp (Table 4); relative contributions of calcium phosphite added to tank mixes are not immediately clear

Metamitron shows potential as a postbloom thinner in WA (Table 4), but extensive testing is needed and potential path to registration is challenging

Crops may be effectively thinned chemically without use of carbaryl; BA + NAA programs demonstrate results equal or superior to those using carbaryl (Tables 4, 5)

Several years of results demonstrating reduced flowering from 4 applications of modest rates of GA_3 (Table 6) suggest potentially commercially viable programs to mitigate biennial bearing

4 replicated overhead netting pods successfully established on Granny Smith at WSU Sunrise orchard in preparation for new tree and fruit physiology studies to launch in 2013

BACKGROUND:

After years of robust efforts to evaluate various aspects of bloom and postbloom chemical thinning programs, our current focus is to screen new chemistries and provide collaborative support for external research programs working on crop load and canopy management. We continue to evaluate the relative success of chemical and mechanical thinning programs through three measurable targets which are directly tied to a grower's economic bottom line:

1. Reduction of green fruitlet hand-thinning
2. Improved fruit size and quality
3. Increased return bloom/annual bearing

The degrees to which our chemical thinning programs achieve each of these goals are reflected in our data labeled fruitlets/100 floral clusters, harvest fruit size, and percent return bloom, respectively.

Programs in 2012 are reflected in Table 1; in those which show a range of possible rates, higher concentrations are typically reserved for cultivars known to be difficult to thin, such as Fuji and Golden Delicious. In most cases, additional chemical thinning treatments were left to the discretion of individual grower-cooperators, provided that each experimental plot receives the same programs.

Table 1. Chemical thinning programs evaluated. WTFRC 2012.

BLOOM THINNERS (applied 100 gal water/A at 20% & 80% bloom)
2% Crocker's Fish Oil (CFO) + 2-3% Lime Sulfur (LS)
24 oz ThinRite (TR)/A
24 oz ThinRite /A + 2% CFO/A
24 oz ThinRite /A + 1% ATS/A
24 oz ThinRite /A + 1% ATS + 2% CFO/A

POSTBLOOM THINNERS (applied 100 gal water/A at PF & 10mm)
32 oz Carbaryl 4L + 6 oz Fruitone (NAA)/A
32 oz Carbaryl 4L + 128 oz benzyladenine (BA)/A
64-128 oz Sysstem-Cal (SC) + 32 oz Carbaryl 4L + 128 oz BA/A
128 oz BA/A
64-128 oz Sysstem-Cal + 128 oz BA/A

BLOOM THINNING:
Results from both 2012 bloom thinning trials were generally disappointing, with no treatments providing significant reductions in fruit set or increases in fruit size, including an industry standard program of oil + lime sulfur (Table 2). In fact, the clearest effect in both trials was a reduction in fruit size by a tank mix of ThinRite, ATS, and Crocker's Fish Oil. Apparently, stress to treated trees from this aggressive combination was sufficient to impair early fruitlet growth, but not adequate to significantly reduce fruit set.

Table 2. Crop load and fruit quality effects of bloom thinning programs. WTFRC 2012.

Treatment	Fruitlets/100 floral clusters	Blanked spurs	Singled spurs	Harvest fruit weight	Relative box size	Russeted fruit
		%	%	g		%
Fuji/Bud.9 - Orondo						
CFO + Lime sulfur	73 ns	49 ns	35 ns	207 a	88	1 ns
ThinRite	72	49	35	204 a	9	0
ThinRite + ATS	72	49	35	197 a	92	0
ThinRite + ATS + CFO	66	54	32	141 b	129	1
ThinRite + CFO	75	49	33	213 a	85	1
Control	79	49	30	213 a	85	2
Red Del./M.9 - Royal Slope						
CFO + Lime sulfur	90 ns	28 ns	55 ns	204 a	89	24 ns
ThinRite	85	30	56	189 ab	96	17
ThinRite + ATS	84	34	48	186 ab	98	10
ThinRite + ATS + CFO	76	36	51	172 b	106	20
ThinRite + CFO	78	34	54	188 ab	97	9
Control	90	31	48	187 ab	97	9

Despite poor results this year, we remain confident that ThinRite can become a valuable tool for chemical thinning of apple. Table 3 summarizes results from all apple bloom thinning trials conducted by the WTFRC since 1999, and demonstrates that ThinRite has historically outperformed ATS as a bloom thinner in our trials with respect to reducing fruit set. Unfortunately, we have yet to observe significant benefits in fruit size or return bloom with ThinRite as a stand-alone product.

According to the registrant (United Phosphorus Inc.), ThinRite should be fully registered as a bloom thinner and available for commercial use for the 2014 thinning season.

Table 3. Incidence and percentage of results significantly superior to untreated control. Apple chemical bloom thinning trials. WTFRC 1999-2012.

Treatment	Fruitlets/100 blossom clusters	Harvested fruit size	Return bloom[1,2]
ATS	15 / 57 (26%)	10 / 60 (17%)	4 / 52 (8%)
NC99	15 / 32 (47%)	7 / 34 (21%)	2 / 28 (7%)
Lime sulfur	25 / 54 (46%)	12 / 48 (25%)	9 / 47 (19%)
CFO + LS	62 / 110 (56%)	27 / 101 (27%)	21 / 98 (21%)
JMS + LS	14 / 24 (58%)	8 / 23 (35%)	4 / 22 (18%)
WES + LS	14 / 27 (52%)	4 / 26 (15%)	4 / 26 (15%)
ThinRite	7 / 20 (35%)	0 / 21 (0%)	0 / 8

[1]Does not include data from 2012 trials.

[2] (no. blossom clusters year 2/sample area) / (no. blossom clusters year 1/sample area)

POSTBLOOM THINNING:

While we remain convinced that chemical bloom thinning is more consistently effective than postbloom thinning in achieving our three goals for chemical thinning (Tables 3, 5), our 2012 results demonstrate an exception to that rule in that we saw more successful outcomes in our postbloom thinning trials (Table 4) than our bloom thinning trials (Table 2). Indeed, weather conditions were generally favorable for effective thinning at both postbloom trial sites, with temperatures in the 70s and 80s during and for several days after treatment applications. A primary focus of this year's trials was to evaluate the addition of a phosphite material (Sysstem-Cal) to standard thinning agents. Most thinning treatments were effective in both trial sites whether or not Sysstem-Cal was in the spray tank, making it difficult to assert any claims as to its specific contributions to the results. Fruit samples are currently in storage for several of these treatments and we look forward to assessing whether potentially elevated calcium levels from the product might improve fruit quality.

Our ability to continue evaluation of metamitron as a postbloom thinner was limited by availability of product. This sugar beet herbicide has shown great promise in trials across Europe and is nearing registration as a postbloom thinner of apple in several EU countries. Preliminary results in the US show good product efficacy in the humid, lower light conditions of North Carolina and Massachusetts, but were unable to see the same in local 2011 WTFRC trials. This year, however, we had more favorable results in Gala with higher rates (Table 4) and believe the product has great potential for our industry if we are able to determine how aggressively to use it in our conditions. Unfortunately, the owner of the chemistry, Makhteshim Agan North America, has been unwilling to support investigation of their product as a potential chemical thinner and we continue to have great difficulty acquiring product for further testing.

Table 4. Crop load and fruit quality effects of postbloom thinning programs. WTFRC 2012.

Treatment	Fruitlets/100 floral clusters	Blanked spurs	Singled spurs	Harvest fruit weight	Relative box size	Russeted fruit
		%	%	g		%
Honeycrisp/M.26 - Gleed						
Exilis	89 ab	38 ab	40 ns	184 ns	99	12 ab
Exilis + Carbaryl	94 ab	32 ab	47	189	96	15 ab
Exilis + Fruitone L	97 ab	35 ab	40	194	94	15 ab

SC 128 oz + Exilis	107 a	29 b	43	185	98	11 b
SC 128oz +Exilis+Carbaryl	83 b	41 a	40	180	101	25 a
SC 64oz + Exilis	90 ab	37 ab	41	192	95	15 ab
SC 64oz+Exilis+Carbaryl	92 ab	35 ab	44	182	100	12 ab
Control	95 ab	35 ab	42	173	105	17 ab
Gala/M.9 – Beebe Bridge						
MaxCel + Carbaryl 4L	74 abc	45 ab	40 ns	197 ns	92	34 ns
MaxCel + Fruitone L	55 c	55 a	37	196	93	36
Carbaryl 4L + Fruitone L	67 abc	48 ab	39	186	98	26
Metamitron + Regulaid	78 ab	46 ab	34	190	96	19
Metamitron 400ppm	66 bc	49 ab	39	193	94	24
Metamitron 600ppm	67 abc	50 ab	35	203	89	34
SC 128oz+MaxCel+Carbaryl	76 abc	46 ab	37	185	98	25
SC 64oz+MaxCel+Carbaryl	65 bc	50 ab	38	186	98	35
SC + MaxCel	71 abc	48 ab	34	185	98	26
Control	90 a	38 b	40	182	100	31

Our 2012 results demonstrate once again the benefits of thinning with tank mixes of BA and NAA products (Table 4). These continue to be our most effective postbloom thinning programs (Table 5) and we feel increasingly confident that industry will still be able to effectively thin their crops chemically if and when carbaryl ever loses it registration.

Table 5. Incidence and percentage of results significantly superior to untreated control. Apple chemical postbloom thinning trials. WTFRC 2002-2012.

Treatment	Fruitlets/100 blossom clusters	Harvested fruit size	Return bloom[1,2]
BA	2 / 19 (11%)	0 / 20 (0%)	0 / 19 (0%)
Carb + BA	30 / 82 (37%)	10 / 81 (12%)	11 / 76 (14%)
Carb + NAA	13 / 55 (24%)	9 / 55 (16%)	5 / 53 (9%)
BA + NAA	8 / 19 (42%)	5 / 19 (26%)	2 / 14 (14%)
Carb + NAA + Ethephon	0 / 5	0 / 5	2 / 5
Carb + NAA + BA	0 / 8	0 / 8	3 / 8

[1]Does not include data from 2012 trials.

[2] (no. blossom clusters year 2/sample area) / (no. blossom clusters year 1/sample area)

RETURN BLOOM PROGRAMS:

The major hallmark of our results with gibberellic acid (GA) trials to inhibit return bloom as a means to combat biennial bearing has been variability, both from trial-to-trial and from tree-to-tree within the same plot. Nonetheless, we have generated enough positive outcomes with multiple applications of relatively modest concentrations (100-200 ppm) of GA_3 to have some confidence that such programs could be commercially viable. Table 6 describes results from treatments made in 2011, where we found significant reductions in 2012 bloom in three of six possible trial sites. When pooled with results from all previous trials, those treatments have produced statistically significant reductions in return bloom in 6 of 15 possible cases since 2009; while a 40% success rate is less than ideal, it is in fact comparable to the success rates of our best chemical thinning programs (Tables 3, 5) and merits consideration by registrants of GA_3 products for possible label amendments to accommodate

such use patterns. Our efforts to encourage representatives from Valent and Fine to make such changes to the labels for ProGibb and Falgro (respectively) are ongoing.

Table 6. Key effects of WTFRC 2011 GA_3 return bloom trials.

GA_3 concentration	2011 shoot length	2011 fruit weight	2012 return bloom
ppm	cm	g	flower clusters/cm^2 LCSA
Golden Del./M.7A – Pasco			
4 x 100	44.8 a	233 ns	1.5 b
4 x 200	46.4 a	227	1.6 b
Control	41.6 b	230	2.4 a
Fuji/M.26 – Burbank			
4 x 100	19.3 b	202 ns	5.6 b
4 x 200	20.9 ab	217	6.0 ab
Control	21.5 a	213	7.6 a
Fuji/M.9 – Burbank			
4 x 100	16.3 a	205 ab	6.7 b
4 x 200	14.2 b	208 a	5.3 b
Control	14.0 b	193 b	8.4 a
Fuji/M.26 – Benton City			
4 x 100	52.2 ns	238 ns	5.1 ns
4 x 200	54.7	233	4.4
Control	52.3	225	4.5
Fuji/M.26 – Royal Slope			
4 x 50	45.1 ns	232 ns	2.0 ns
4 x 100	44.9	219	2.1
4 x 200	45.2	234	2.1
Control	42.1	230	1.8
Red Del./MM.106 – Royal Slope			
4 x 50	33.6 a	196 ns	4.4 ns
4 x 100	30.5 ab	204	4.4
4 x 200	27.3 b	197	4.7
Control	29.2 ab	192	4.2

PROTECTIVE MATERIAL TRIALS:

2012 marked a transitional year for our horticultural fabric program, as we concluded our work with reflective ground cloths and prepared for upcoming studies with overhead and draping fabrics designed to protect trees and fruit from stresses associated with excess heat, light, and wind. Working with Extenday® USA, we helped construct a prototype 40’ x 60’ pod that fully encloses 72 Granny Smith trees at the WSU Sunrise orchard near Rock Island in 2011. In 2012, three more replicated pods were constructed in the same block (Figure 1) in preparation for formal studies to begin in 2013. Protocols for these studies are still being developed, but should include focus on tree yields and fruit quality. Additionally, we made preliminary installations of drape netting directly on top of trees in the same block (Figure 2). These plots were established too late in the summer to fairly assess effects on fruit sunburn, but will help lay the groundwork for expanded full season testing of similar netting

in 2013. Finally, this year we hope to establish overhead netting plots with no side walls in the same Granny Smith block, as well as 1-2 commercial Honeycrisp orchards to evaluate effects on sunburn and potential protection against hail. Extenday® representatives estimate that various types of protective nets with 10 year life spans can be installed for $600-9000 per acre; clearly, some systems in that spectrum would offer greater protection against sunburn, hail, bird damage, etc., and we plan to maintain focus on the economics of such technologies as we proceed.

Figure 1. Completed overhead netting pods at WSU Sunrise orchard. August 2012.

Figure 2. Installed drape shade netting at WSU Sunrise orchard. August 2012.

RELATED PROJECT UPDATES:

- **Pollen tube growth model (Yoder, Hoogenboom):** positive feedback from beta testers indicates model can improve precision of bloom thinner application timing; initial integration into WSU AgWeatherNet very successful. See project report AP-12-105A for more details
- **Bloom phenology and fruit growth models (Hoogenboom, Dasgupta):** preliminary models show promise as tools to help growers predict bloom development and fruit size. See project report AP-12-104 for more details
- **Solid Set Canopy Delivery System (Brunner, Whiting):** postbloom thinners delivered by SSCDS reduced fruit set in Gala and Fuji in 2012, but not as effectively as concurrent applications by airblast sprayer; ongoing modifications to system should improve its efficacy
- **Mechanized thinning (Lewis):** ongoing studies demonstrate effective thinning with tractor mounted and handheld string thinners, but concerns over leaf and spur damage remain; current project at WSU Sunrise is assessing long term cropping effects of thinning Granny Smith with Darwin vs. chemical thinners over several seasons
- **Vegetative growth management (internal):** 2012 trial in Fuji demonstrates a new product currently being developed is as effective as Apogee in suppressing shoot extension
- **Variability in tree-to-tree cropping and growth (internal):** data collection is ongoing on 100 Granny Smith trees at WSU Sunrise orchard; preliminary data is currently being examined by WSU statisticians for potential analytic methods

CONTINUING PROJECT REPORT **YEAR**: 2 of 2
WTFRC Project Number:

Project Title: 3D machine vision for improved apple crop load estimation

PI: Manoj Karkee
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Address 2: P.O. Box 37
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Cooperators: None

Total Project Request: **Year 1**: $33,104 **Year 2**: $34,402

Other Funding Sources: None

Budget 1
Organization Name: WSU **Contract Administrator:** Carrie Johnston
Telephone: 509.335.4564 **Email address:** carriej@wsu.edu

Item	Current Year	
Salaries[1]	$23,817	
Benefits[1]	$1,893	
Wages[2]	$6,515	
Benefits[2]	$625	
Equipment		
Supplies	$1,000	
Travel[4]	$552	
Plot Fees		
Miscellaneous		
Total	$34,402	

Footnotes:
[1] Salary and benefit for a graduate student
[2] Wages and benefits for hourly help to fabricate sensor platform and collect field data
[3] Cost to purchase materials and build a sensor platform
[4]Travel cost for field data collection and testing

OBJECTIVES

The following were the specific objectives of this project.

1. Develop a sensor system with 3D and color vision cameras for imaging apple trees from both sides of the tree canopy
2. Develop an image processing technique to create 3D maps of the fruit and estimate apple crop-load
3. Evaluate and improve the accuracy of crop-load estimation

SIGNIFICANT FINDINGS

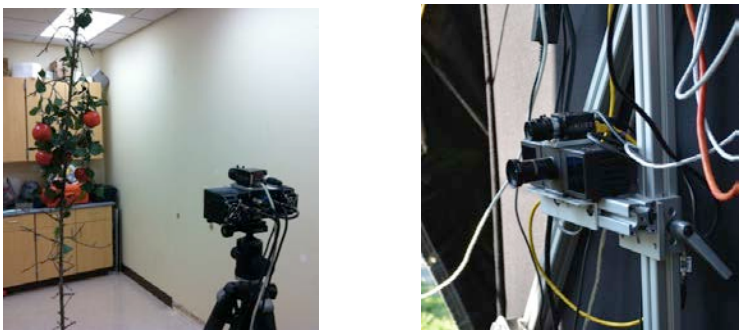
- Visibility of apples increased substantially when images were taken from both sides of the tree canopy.
- The mapping algorithm developed in the laboratory setting showed promise for reducing repeat counting by co-registering of 3D images taken from both sides of the tree canopy.
- Over-the-row sensor platform with a tunnel structure minimized variability in lighting condition and background, which helped improve image processing techniques for fruit identification and mapping.

METHODS

To reduce occlusion caused by nearby fruit, leaves and branches, images of apple trees were taken from both sides of the tree canopy. Because some apples were visible from both sides of the canopy, a single apple could be counted twice. A 3D camera was incorporated into the system to minimize recounting of the same apple by measuring the position of the apples on the tree. In the following paragraphs, we describe the sensors, platform, and algorithms we have been developing to capture and analyze images for improved crop-load estimation.

Sensors and Calibration:

The sensor system consists of a color camera and a 3D camera (Fig. 1). A Prosilica camera (GigE 1290c, Allied Vision Technologies, Stadtroda, Germany) was used to capture color images of apple trees with fruit. A PMD camera (CamCube 3.0, PMD Technologies, Siegen, Germany) was used to take 3D images. These 3D images are used in conjunction with the color images to minimize repeatcounting of apples.

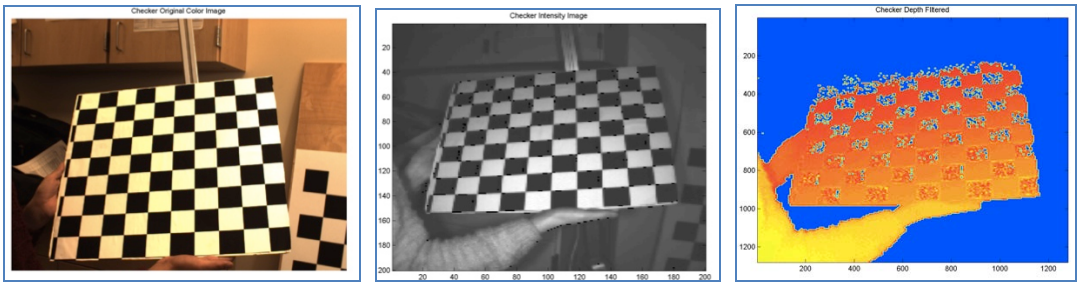


a) **b)**

Fig. 1: Sensors used for image acquisition in laboratory setup (a), and in field tests (b). Prosilica GigE 1290c color camera is on the top of the camera mount and PMD CamCube 3D camera is on the bottom.

A checkerboard-based camera calibration technique was used to identify intrinsic and extrinsic parameters of the color camera and 3D camera. A checkerboard was placed in front of the imaging system in such a way that it appeared within the imaging field-of-view of both cameras. The image from the color camera (Fig 2a) and the intensity image (Fig 2c) obtained from the 3D camera were used to calibrate for intrinsic and extrinsic camera parameters. The extrinsic parameter gives the

relative position of two cameras. Using these parameters, the 3D coordinates from the 3D camera (Fig 2c) were projected onto the image plane of the color camera to obtain distance-mapped color images.



a) **b)** **c)**

Fig. 2: Checker board-based camera calibration; a) original color image of checker board, b) distance image of the board, and c) intensity image of the board

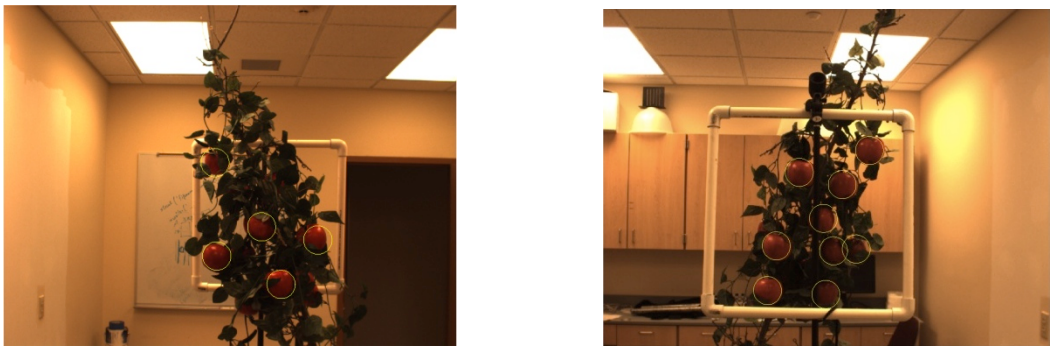
3D Mapping Algorithm Development

Images captured from both sides of a tree canopy in 2011 and 2012 have shown substantial increase in apple visibility. However, it is also evident that some apples are visible from both sides (Fig. 3), thus requiring 3D mapping of apples to avoid duplicate counting.

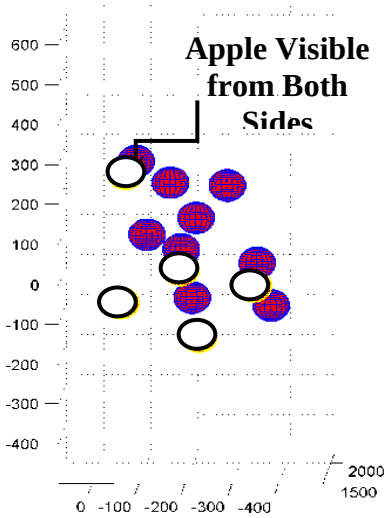
a) b)

Fig 3: a) and b) are the images captured from two opposite sides of an example tree canopy. Apples visible from both sides are circled.

An algorithm was developed using a laboratory set-up to register images captured from both sides. Color and 3D images of a model of an apple tree (a real, dead tree with fake leaves and fruit on it; Fig. 4a) were captured. The 3D coordinates of objects in the field-of-view were transformed from the 3D camera coordinate system to project on the imaging plane of the color camera so as to obtain a distance-mapped color image. Each pixel in this distance-mapped color image included color information with the corresponding 3D location information. The center of apples visible from each side of the canopy was located as shown in Fig. 4a and 4b. 3D locations of the four corners of the reference frame (GI pipe square in Fig. 4a, and 4b) were used to obtain the rigid transformation between these two camera positions. Using the rigid transformation, all the corresponding apple locations from one side of the canopy were transformed to the coordinates on the other side. Fig. 4(c) shows 3D locations of apples viewed corresponding to Fig. 4a (hollow circles) and Fig. 4b (solid circles) respectively. The apples visible from both sides of the canopy can be seen overlapping each other. Apples within a distance of less than the diameter of corresponding apples were considered to be the same apple mapped from the opposite sides.



a) b)



c)

Fig. 4: a) and b) Color images from front and back side of the tree; c) 3D-mapped apples of corresponding color images in (4a) as hollow circles and (4b) as solid circles (all axes in millimeters).

Platform Modification and Data Collection:

Field data was collected in the 2011 harvest season with the first prototype of the over-the-row sensor platform. An improved sensor platform was designed and fabricated in 2012 (Fig. 5) based on knowledge learned in 2011. The new platform was lighter and more robust than the earlier platform. A fixed platform width was used in the new design to reduce unnecessary degrees of freedom. The platform included a sliding mechanism for convenient mounting and positioning of cameras. The images acquired during daylight in 2011 harvest season were affected by variation in lighting conditions such as presence of direct sunlight and shadows. To eliminate variations in lighting conditions, a tunnel structure was added to block direct sunlight in the tree canopy during imaging. An artificial lighting system was integrated to create a controlled lighting environment while taking images. The artificial lighting system also added capability for nighttime operation (Fig. 5 (b) and (c)). Data collection in commercial orchards with the improved platform began the week of September 24th and continued until the last week of October. We collected daylight images of Jazz apple trees in tall spindle architecture (row spacing 9'0" and inter-plant spacing 3'10") in Prosser, WA (Yakima Valley Orchards) (Fig. 5(a)). The night-time images in Jazz and Fuji apple trees were collected in Prosser and Grandview, WA (commercial orchards of Allan Bros., Inc.). Human manual counts included number of apples visible from each side of the tree canopy, number of apples visible from both sides of the canopy (repeat counts) and total number of apples.

a)

b)

c)

Fig. 5: a) A tall spindle commercial orchard of Allan Bros., Inc. in Prosser, b) and c) new over-the-row platform taking images of Jazz apples during daylight and night-time.

Apple Identification Algorithm Development:

An algorithm was developed to identify and count apples from images captured in commercial orchards (Fig. 6). To minimize the counting error due to clustering of apples, the ratio of major axis and minor axis of identified apples was determined. If the ratio was greater than three, it was assumed that the identified object is a cluster of two apples. Currently, it is assumed that a cluster does not include more than two apples. This limitation will be addressed in the remaining period of the project. The apple count estimated from both sides of the tree canopy was added to obtain the total number of apples. Finally, the count of apples visible from both sides was obtained (so far manually) from the images and was subtracted from the total count. Application of 3D mapping technique for automatic removal of duplicate counting and for apple sizing will also be addressed in the remaining period of this project.

Fig. 6: Elliptical shapes indicate apples identified from side A (a) and side B (b) of a sample tree canopy of Fuji apples In Grandview, WA

RESULTS AND DISCUSSIONS

The improved sensor platform increased the efficiency of data collection in the field. It was easier to move in the orchards since it was lighter and more robust. The new sliding mechanism improved camera mobility. Images could be taken from different heights to ensure proper overlapping between images. The tunnel helped to reduce variability in lighting conditions. Images taken in a controlled lighting environment will make image processing much easier. Images taken at nighttime with LED lights showed promise for night time operation of the system.

Manual counting of apples revealed that the visibility of apples increased substantially when dual-sided canopy imaging was used (Table 1). Average visibility of apples was 60-70% when imaged from one side, which increased to more than 95% (Table1) when imaged from two opposite sides.

The apple identification algorithm was applied to day and night-time images. The algorithm was able to identify and count the number of apples in the image. Preliminary results showed root mean square error (RMSE) of 15.4% on identifying the apples from image analysis.

Table 1: Visibility of Apples

S.N	Apple count from Image (Number)				Field Count	Visibility		
	Side A	Side B	Duplicate	Total		Side A	Side B	Total
1	64	57	18	103	106	61	54	98
2	55	53	20	88	75	74	71	118
3	52	68	39	81	90	58	76	90
4	40	53	26	67	94	43	56	71
5	50	69	25	94	92	54	75	102
6	45	43	28	60	84	54	51	71
7	43	63	22	84	115	37	55	73
8	35	24	15	44	40	88	60	110
10	47	32	19	60	50	95	65	121
11	24	23	14	33	31	77	74	106
12	38	36	20	54	54	71	67	101
13	62	52	34	80	90	69	58	89
14	60	62	38	84	100	60	62	84
15	38	31	18	51	44	86	70	116
16	46	72	35	83	73	63	99	114
17	64	57	43	78	78	83	74	101
18	75	62	34	103	107	70	58	96
19	72	83	32	123	130	56	64	95

The algorithm developed in 2012 to co-register 3D and color images as well as 3D images from both sides of a canopy was able to register images captured in the laboratory setup. Results from the laboratory tests showed that duplicate counts of apples can be avoided by using distance between apples presented in a co-registered 3D map (Fig.7). The algorithm has shown promise for application to field data.

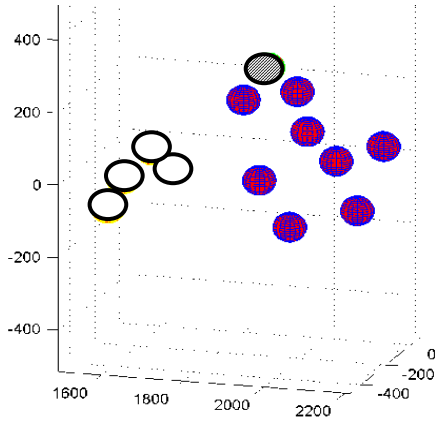


Fig 7: 3D mapped apples visible from front (hollow circles), back (solid circles) and both (hashed circles) sides (axes in millimeters).

In 2013, we will focus on improving and applying image processing and 3D mapping technique to the dataset collected in commercial orchards. A new set of images will be collected in 2013 using the improved sensor platform developed in 2012. We will also develop techniques to obtain position and orientation of the cameras on one side relative to the cameras on the other side of a canopy so that the accuracy of 3D mapping can be improved. Geometric information of the over-the-row sensor platform and orientation sensors (if necessary) will be used to obtain relative position and orientation information.

CONTINUING PROJECT REPORT **YEAR**: 1 of 1
WTFRC Project Number: TR-12-103

Project Title: Design and development of apple harvesting techniques (approved through technology committee)

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Co-PI (4): Mark De Kleine
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City/State/Zip: Prosser, WA 99350

Cooperators: None

Total Project Request: **Year 1:** $53,395

Percentage time per crop: Apple: 100%

Other funding sources

Agency Name: **NONE**

Budget 1
Organization Name: WSU
Telephone: 509.335.4564
Contract Administrator: Carrie Johnston
Email address: carriej@wsu.edu

Item	2012
Salaries[1]	$30,534
Benefits[1]	$1,997
Wages[2]	$6,240
Benefits	$624
Equipment	
Supplies[3]	$10,000
Travel[4]	$4,000
Plot Fees	
Miscellaneous	
Total	**$53,395**

Footnotes:
[1] Salary and benefit for a graduate student
[2] Wages and benefits for hourly help to fabricate sensor platform and collect field data
[3] Cost to purchase materials and build sensor platforms
[4]Travel cost for field data collection and testing

Objectives

Our long term goal is to improve the sustainability and productivity of tree fruit production through reduced labor use and associated risks and costs. Originally, this project was proposed for three years with the following specific objectives.

1. Design and develop two prototypes for semi-automated apple harvesting techniques.
2. Characterize the efficiencies of harvesting in two variations of fruiting wall architectures.

However, the project was funded for only the first year to demonstrate the feasibility of the concept. The scope for the first year for the project involves prototype development and preliminary evaluation in lab and field environment.

Significant Findings

- Vertical twisting with compressive pressure can remove apples from a spur.
- Damage can occur if the apple is rolled against the limb.
- Fruit removal classified as 'stem intact- no spur', is significantly dependent on rotational direction and cultivar.

Methods

Two methods for apple removal are being investigated and evaluated based on fruit removal effectiveness and fruit and spur damage. The preliminary results have been used to modify the apple harvesting system design. The two methods focus on twisting apples in vertical and horizontal directions.

Fabrication and initial testing with the first proof-of-concept prototype was completed in fall 2012. The prototype consisted of two six inch rubber tires mounted to two Drillmaster 18V, 3/8" electric hand drills, as shown in Figure 8.

Figure 8 First apple twisting prototype (hand-held) built with rubber tires mounted to electric motors.

Two input variables were defined as speed and rotational direction. Speed was divided into three levels: 240RPM, 420RPM, and 890RPM. Wheel rotational direction was either counter-clockwise (CCW) or clockwise (CW). Harvested apples were classified into three different categories: stem intact– no spur; stem not intact– no spur; stem intact– spur attached.

Five levels of wheel speed was used for testing: 1) Slow-Slow, 2) Slow-Medium, 3) Medium-Medium, 4) Medium-Fast, 5) Fast-Fast, corresponding to each wheel. Direction was classified into three levels: 1) CW-CW, 2) CW-CCW, 3) CCW-CCW, corresponding to each individual wheel.

Figure 9. Prototype harvester wheel placement on an apple.

The two wheels were place on either side of an apple as shown in Figure 9. Adequate pressure was applied so that the wheel does not slip on the apple skin. As the wheels spun, a twisting motion was applied to the apple about the stem abscission point.

Results and Discussion- Harvest

Fruit removal with stem intact–no spur ranged from 36% for 'Granny Smith' to 86% for 'Pacific Rose'. Apple cultivar had a significant effect on the fruit removal condition (p=0.000) at a 95% confidence level. Apples removed with stem not intact ranged from 8% on 'Pacific Rose' to 64% on 'Golden Delicious'. The highest percentage of apples removed with spurs attached was 10% in 'Jazz'. The direction of rotation had a significant effect on the fruit removal condition (p=0.000) at a 95% confidence level.

Table 2 Harvesting results for five cultivars of Washington apples.

	Jazz	Pacific Rose	Golden Delicious	Granny Smith	Jonagold
Stem Intact – No Spur	70%	86%	80%	36%	58%
Stem Not Intact – No Spur	20%	8%	18%	64%	40%
Stem Intact – Spur Attached	10%	6%	2%	0%	2%

The desired classification is stem intact – no spur. When variables were set at slow-slow and CW-CCW, 100% of removed apples, across all cultivars, did not meet this classification. When the wheel speed changed to medium-medium, at CW-CCW, 89% of the removed apples did not meet this classification. Practically speaking, when the rotation of the wheel is opposing (CW-CCW), the apple

is pulled away from the branch rather than twisted. For opposing rotational direction and speeds set at either slow-medium or medium-fast, 33% and 56% of apples were not classified as stem intact– no spur, respectively. Although the same principle of pulling rather than twisting holds true, the slight variation in speed tended to apply a minor twist or rotation. This potentially helped increase the percentage of stem intact– no spur classified apples.

The highest rate of stem intact– no spur for all varieties occurred at CW-CW direction for slow-medium and medium-medium speeds. Fruit removal at these speeds resulted in 89% of the apples being categorized as stem intact– no spur. For 'Golden Delicious', 100% of the harvested apples that were categorized as stem not intact– no spur or stem intact– with spur attached occurred when the speed of the wheels were equal to each other. For 'Granny Smith' and 'Jonagold' cultivars, 66% and 67% of the harvested apples that were categorized as stem not intact– no spur or stem intact– with spur attached occurred when the speed of the wheels were equal to each other.

Discussion- Punctures

Initial tests, on 'Gala' apples, show that twisting in a vertical direction has the potential to remove fruit from limbs. It was also observed that rolling an apple across a branch can cause damage to the apple. More specifically, uncontrolled rolls across a limb can puncture the fruit. Based on this observation, a separation barrier was fabricated to facilitate a controllable shoulder to roll the apple on. A simple wireframe structure separated the apples from the limbs (Figure 10).

In efforts to reduce the number of stem pulls, razor blades were mounted to the separation barrier to slice or cut the stem entirely. This concept deviates slightly from the initial proposal but collectively focuses on the overall objective of fruit removal. Tests will be continued during the 2013 apple harvest season. Additional adjustments in the structural design of the harvesting system are expected throughout the remainder of the project. We do expect that stem pulls will continue to be a challenge but we will investigate different ways to minimize them as we develop and evaluate improved prototypes.

Figure 10 Wireframe separation barrier used for puncture reduction testing.

The remainder of year 1 will focus on the improvement and scaling of the harvesting prototype for trellised orchards trained to both random and formal architectures. During the winter season, the focus will be on developing a multiple wheel structure, and the addition of the bioyield pressure applicator (Fig. 5). It is expected that phase II of the prototype harvester will be completed by early summer 2013.

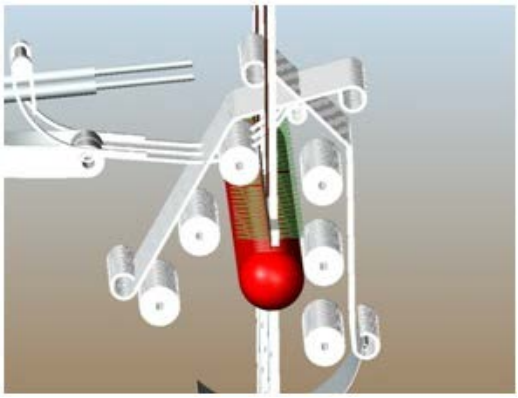

Figure 5 Phase II of the prototype apple harvester.

Conclusions

The initial results from year 1 show a successful fruit removal technique applicable to trellised apples. Signs of branch punctures were visible on some of the apples. A method to seclude the apple from any branch or ensure that no branches can be pressed between the wheel and the apple skin was attempted. Although no initial measurements were made to classify the varying degrees of this type of damage, it will be persistently examined in year 2 and 3*. Apples located on short spurs tended to be removed easier than apples growing on long flexible branches. apples located on long branches moved more freely and this flexibility reduced the twisting, or torque, that was ultimately transferred to the apple and stem resulting in less effective removal rates. Horticulture and genetics or phenotype can play an important role in aiding the fruit removal technique described in the above research.

Table 3 shows the project timeline for the originally proposed duration of the project. Year one focuses mainly on hand harvest evaluation, prototype design and evaluations. All of these tasks are currently being carried out and will be continued through the 2013 harvest season. Prototype end effector design and improvement will continue through the winter. Complete evaluation of the prototype will be during the 2013 and 2014 harvest seasons. Grower feedback, suggestions, and evaluation will continue to occur in informal interviews and a symposium during the winter. It is noted that the activities proposed for Year 2 and Year 3 are contingent upon our success on securing further funding for the project.

Table 3 Project timeline for years 1 through 3*

	Year 1	Year 2	Year 3
Grower Input			
Grower Feedback			
Grower Evaluation			
Hand Harvest Evaluation			
Prototype End Effector Design			
End Effector Phase 2			
Lab and Field Evaluations			
Preliminary Economic Evaluation			
Machine Integration and Demonstration			

**Note:* *Activities in Year 2 and Year 3 are contingent upon our success on securing further funding for this project.*