FINAL PROJECT REPORT

Project Title: Targeting the ethylene biosynthetic pathway to improve cherry quality

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Other funding sources

Agency Name: USDA-CSREES Specialty Crop Research Initiative
Amount awarded: $3.8 mil plus equal matching, Sep 2009 – Aug 2013
Notes: "A total systems approach to developing a sustainable, stem-free sweet cherry production, processing and marketing system". PI: Whiting. Co-PIs include Oraguzie and Dhingra. Develops genomics knowledge on cherry abscission for amenability to mechanical harvesting, with ethylene physiological analyses of local cultivars.

Total Project Funding: $56,000

Budget History:

Item	**Year 1: 2010**	**Year 2: 2011**	
Salary	5,312	5,524	
Benefits	2,019	2,099	
Wages	4,959	5,157	
Benefits	471	490	
Equipment			
Supplies	10,739	10,230	
Travel	4,500	4,500	
Miscellaneous			
Total	28,000	28,000	

RECAP ORIGINAL OBJECTIVES

Our overall goal is to improve the storage and shelf life of sweet cherry cultivars through an understanding of the ethylene genetic control in this apparently non-climacteric crop. Specific objectives were to:

1. Characterize differences in the ethylene biosynthetic pathway between sweet cherry and peach—*why do they ripen differently?*
2. Identify ethylene physiology categories of cherry cultivars that simplify development of strategies to extend market life—*does increased synthesis and/or sensitivity to ethylene determine ultimate postharvest response?*
3. Provide specific selection criteria in breeding for improved market life based on ethylene biosynthesis and response—*can a simple high-throughput physiological or genetic assay help predict market life?*

SIGNIFICANT FINDINGS

- Sweet cherry is confirmed as a non-climacteric fruit.
- Mature ripe sweet cherry fruit did not respond to ethylene treatment by any changes in respiration or ethylene production.
- An early peak in ethylene production in tiny fruitlets subsided well before, just before, or soon after color break (depending on the cultivar) and became undetectable upon further maturation.
- Differences in several *ACS* genes were observed among three cherry cultivars over six sampling times throughout fruit development
- Activity of ACO (the "ethylene forming enzyme") in sweet cherry fruit declined to zero with advancing fruit development. In stark contrast, ACO activity in peach was high in early season fruit, leveled off, then had a medium activity in mature ripe fruit.
- ACO activity patterns for nine sweet cherry cultivars throughout fruit development showed several patterns. However, no significant associations of these patterns with fruit quality traits were detected, including after a 1-week storage regime.
- The lack of endogenous ethylene production or ethylene response indicates that there are no off-tree quality improvements to be realized for sweet cherry fruit beyond color break.
- These results lead us to believe that the difference between cherry and its climacteric relatives is due to synthesis over-regulation and/or is in the ethylene signal response pathway such as the absence of critical response elements.

RESULTS & DISCUSSION

Sweet cherry and peach fruit were compared for their ethylene physiology and responses, which determined that sweet cherry is truly non-climacteric. Next, components of ethylene biosynthetic machinery were examined to narrow down the missing component in sweet cherry compared to its classically climacteric relative, peach. In cherry, *ACS* gene expression was detected in developing

fruit, but ACO activity beyond color break was low to zero. Therefore, ethylene production machinery appears functional until color break. Different patterns of ACO activity were observed among diverse cultivars, but no fruit quality differences were found to be associated.

Fruit sampling, respiration, and endogenous ethylene production: Cherry fruit were collected from WSU, IAREC, Prosser twice per week beginning at full bloom (April 2010) until the commercial harvest (July 2010). Three cultivars 'Bing', 'Chelan' and 'Skeena' with differential abscission characteristics, as abscission is related to ethylene levels, were included in the present study. 'Early Elberta' peach was harvested from a nearby commercial orchard for a comparative fruit during the 2010 season. During the 2011 growing season, cherry fruit of 9 varieties, including 'Bing', 'Chelan', and 'Skeena' were harvested weekly at WSU, IAREC, Prosser. 'Early Elberta' peach was harvested from the same orchard as in 2010.

In contrast with climacteric fruits in general, immature (green) stages of sweet cherry fruit were associated with higher rates of respiration and low ethylene production. Respiration and ethylene production declined with fruit development. Ethylene levels were undetectable following color break. In the climacteric peach fruit studied, fruit in early developmental stages exhibited a high respiration rate with no ethylene production. As peach fruit developed to maturity and beyond, both respiration and ethylene increased concomitantly.

Response to added ethylene: Exogenous ethylene was treated on early season 'Bing' cherries at 75 DAFB, pre-climacteric 'Early Elberta' peach at 131 DAFB, and post-climacteric 'Early Elberta' peach at 146 DAFB. Etherel (Sigma Aldrich, St. Louis, MO) at 21% AI was diluted to a concentration of 250 ppm. The solution was mixed with ddH_2O and then titrated with 10 M KOH to a pH of 6.0. At this pH the solution releases ethylene gas. Individual dilutions were made to achieve 125 ppm and 12.5 ppm. Each of the ethylene concentrations were placed into 50 ml beakers with a 20 ml etherel solution and set into 6.0L respiration chambers holding the fruit. The final concentrations provided 1.7 ppm, 0.7 ppm, and 0.11 ppm in a constant flow system of 100 ml/min air. The ethylene and CO_2 levels were monitored by gas chromatography as specified in year 1 of our report as (n=3). Each of the chambers receiving ethylene was quantitatively reported by subtracting the etherel supplied ethylene, from each data point and reported a ul/Kg/Hr. Ethylene was reported for pre-climacteric fruit treatments.

To study the non-climacteric nature of cherry fruit in relationship to the climacteric nature of peach, applications of continuous ethylene were established (Fig. 1). At the onset of color break, 3 concentrations of ethylene were treated to freshly harvested cherry fruit at 58 DAFB. Respiration rate and ethylene production are shown if figures 5A and 5B. All 3 concentrations of ethylene induced a higher respiration rate on fresh cherry after 3 days at 66^{o}F (Fig. 1A). Similarly, pre-climacteric peach (131 DAFB) responded to ethylene treatments as the respiration rates all increased due to ethylene concentration within 1 day at 66^{o}F (Fig. 1C). The ethylene production from early season cherry fruit was zero over 5.5 days and no ethylene was produced due to ethylene treatment (Fig. 1B). Similarly, the pre-climacteric peach showed nearly zero levels of ethylene for the control and all ethylene treatments (Fig. 1D). It has been documented that in non-climacteric fruit and the pre-climacteric state of a climacteric fruit, a dose of continuous ethylene treatment triggers increased respiration (Kays, 2005). Upon removal of the exogenous ethylene and the respiration rate will resume to normal control levels.

Upon treatment of 'Early Elberta' peach in the climacteric state (146 DAFB) the dose of ethylene had very low effects on stimulating respiration up to 14 days, however, the lowest doses stimulated respiration greater than the higher doses (Fig. 1E). Similar effects on ethylene production were shown up to day 11 at which time the lowest ethylene does (0.11 ppm) slightly stimulated ethylene in climacteric peach (Fig. 1F).

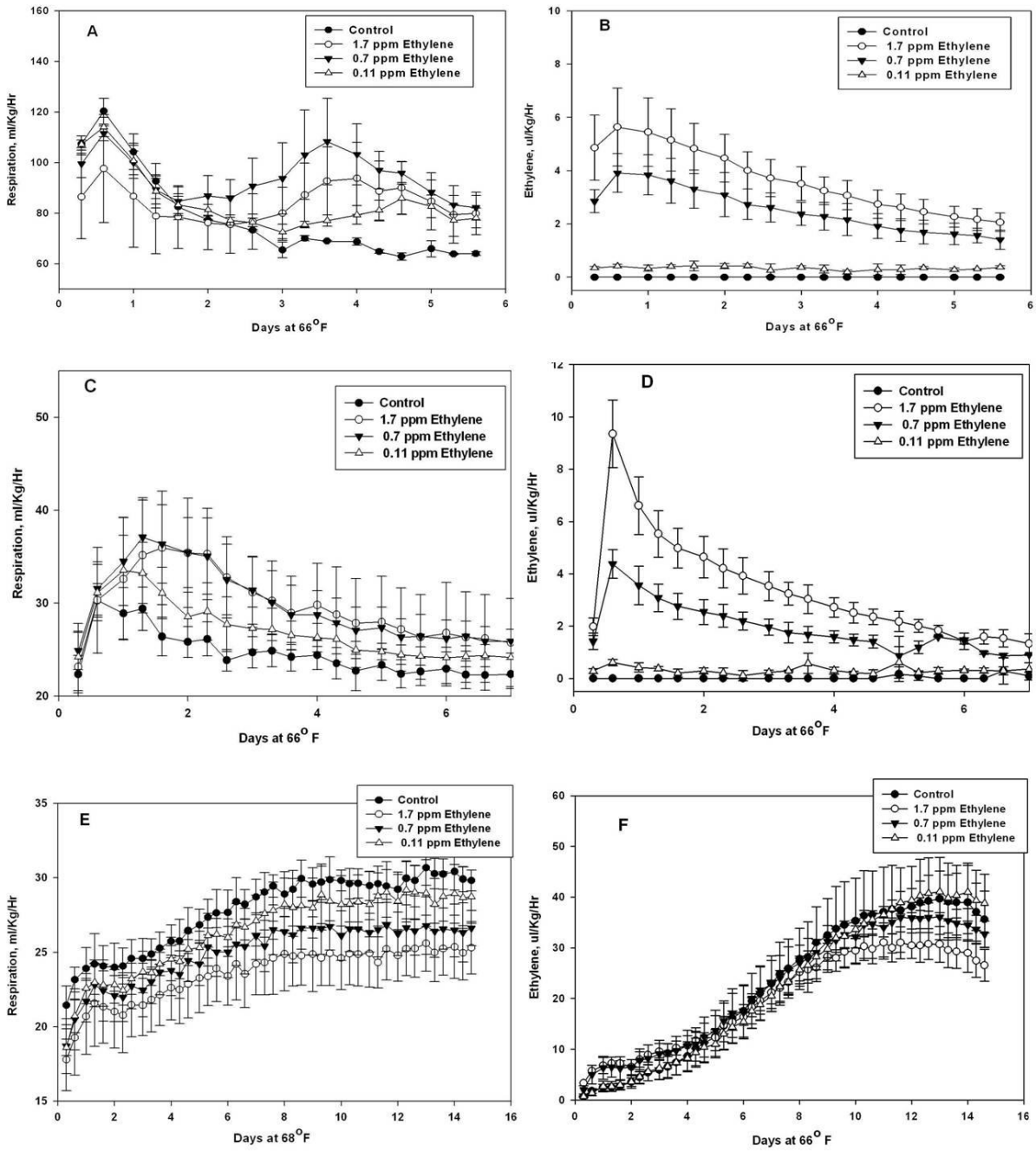


Figure 1. Respiration rates and ethylene production monitored on 'Bing' sweet cherries (A-B), pre-climacteric peach (C-D), and post-climacteric peach (E-F) with continuous ethylene treatment using ethephon solutions at 1.7 ppm, 0.7 ppm, and 0.11 ppm (n=3).

There are several reports as to the effects of dosing mature sweet cherries with ethylene, and monitoring respiration and ethylene production. (Li et al., 1994) applied ethephon to various stages of 'Bing' cherry maturities, but at the ripe state, the ethephon treated fruit did not change in respiration rate or ethylene production over the control after 8 days at 66°F. In fact ethylene levels were zero for the ethephon treated fruit. Gong, et al. (2002) showed similar results using mature commercial harvest 'Bing' sweet cherries. The ethylene exposure was applied at 80 ppm ethylene gas for 6 hours. The

ethylene production increase lasted 1 day, and the increase was extremely low at 0.02 umol/Kg/Hr (Gong et al., 2002). The control fruit produced zero ethylene in the experiment (Gong et al., 2002).

A research study where continuous ethylene gas was exposed to a climacteric fruit such as 'Anjou' pear over 4 harvest timings suggested similar results as shown by this study with 'Early Elberta' peach. Up to 5 concentrations of exogenous ethylene treatment onto 'Anjou' fruit in the pre-climacteric state showed increases in respiration over 18 days, and no increases in ethylene production until 6 days (Wang et al., 1972). Similar applications of exogenous ethylene onto mature stage IV fruit showed increases in respiration immediately up to 18 days and increases in ethylene at 4 days (Wang et al., 1972).

Testing if ethylene pathway genes are functional in cherry: The genes from several *Prunus* equivalent *Md-ACS1-5* sequences were analyzed for 'Chelan', 'Bing', and 'Skeena' in fruit from the 2010 season (Fig. 2). The *ACS 1* and *ACS 2* gene expression assays were not successful, perhaps indicating the absence of these genes. However, optimization did show success for gene expression products describing *ACS 3-5*, as well as the ribosomal *r18S* control (Fig. 2). *ACS 3-5* expression in 'Bing' samples was evident until at 56 DAFB, then again showed up at 76 DAFB (Fig. 2). 'Chelan' and 'Skeena' showed similar *ACS 3-5* gene expression patterns; however, signal disappearance occurred at a different stages than 'Bing' (Fig. 2). In a study detailing the level of *ACS* transcripts during a climacteric fruit development cycle, Yokotani et al. (2009) showed that *ACS 1, 2, 4,* and *6* have different levels of expression between early immature tomato stages, to mature green, and finally into the red ripe stages, similar to the variable levels we observed for cherry.

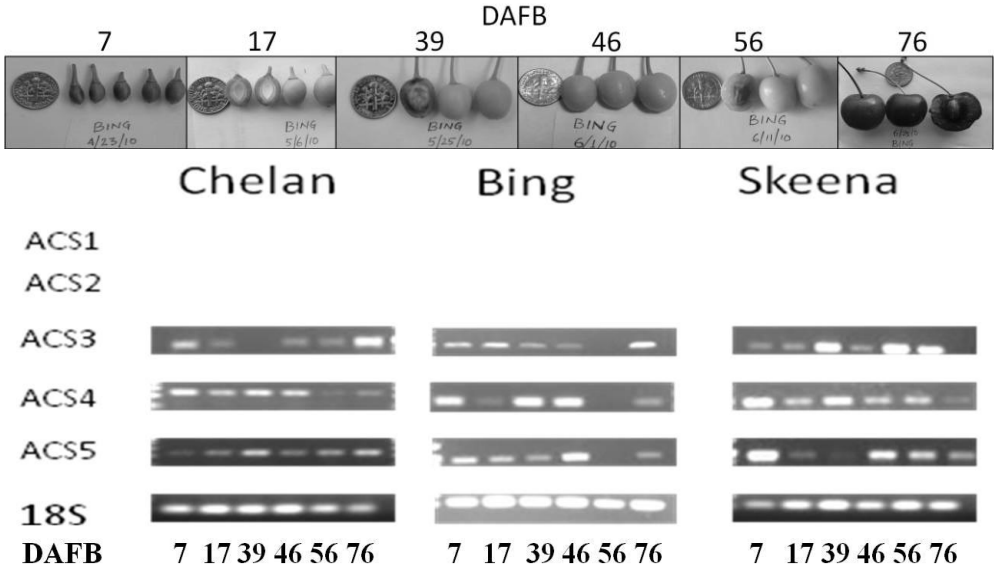


Figure 2. (A) Fruit development stages used for gene expression studies. (B) Semi-quantitative *ACS* gene expression for three sweet cherry cultivars.

Ethylene enzymes and products: The enzymatic regulation of ACC (1-aminocyclopropane-1-carboxylate) synthase (ACS) and ACC oxidase (ACO) during fruit growth and development of cherry were compared to that of peach. During the most recent growing season, the ACO disk assay was optimized to assure the ethylene produced is due to *in vivo* feeding with (+) or without (-) the addition of the enzyme's substrate, ACC. Other pathways exist whereby ethylene may be produced to due oxidation of ACC to ethylene by superoxide radical generation upon tissue wounding (Kumar and Knowles, 2003)**.** The use of *n*-propyl gallate under assay conditions was fed to 'Regina' cherry disks,

with and without ACC. Ethylene is produced under assay conditions, at nearly zero levels without ACC, and up to 12 nl/g/hr. with ACC, as well as with ACC and propyl gallate. The assay therefore is not limited by superoxide radical generation due to wounding as a mechanism to produce ethylene after 5-24 hours under *in vivo* conditions.

As described in year one of the study, by following ACO activity levels with and without ACC, varying peak levels of ACO activity were observed for ACO. Endogenous ACO activity, obtained without adding ACC, peaked for 'Chelan' at 35 days DAFB, at 45 DAFB for 'Bing', and 40 DAFB for 'Skeena' (Fig. 3). These peaks occurred near color break. ACO activity levels, with or without ACC, leveled off to zero near physiological and commercial maturity (Fig. 3). This result concurs well with whole fruit ethylene and respiration in cherry, where both level off to undetectable levels and very low levels, respectively, just before cherry harvest (data shown year 1).

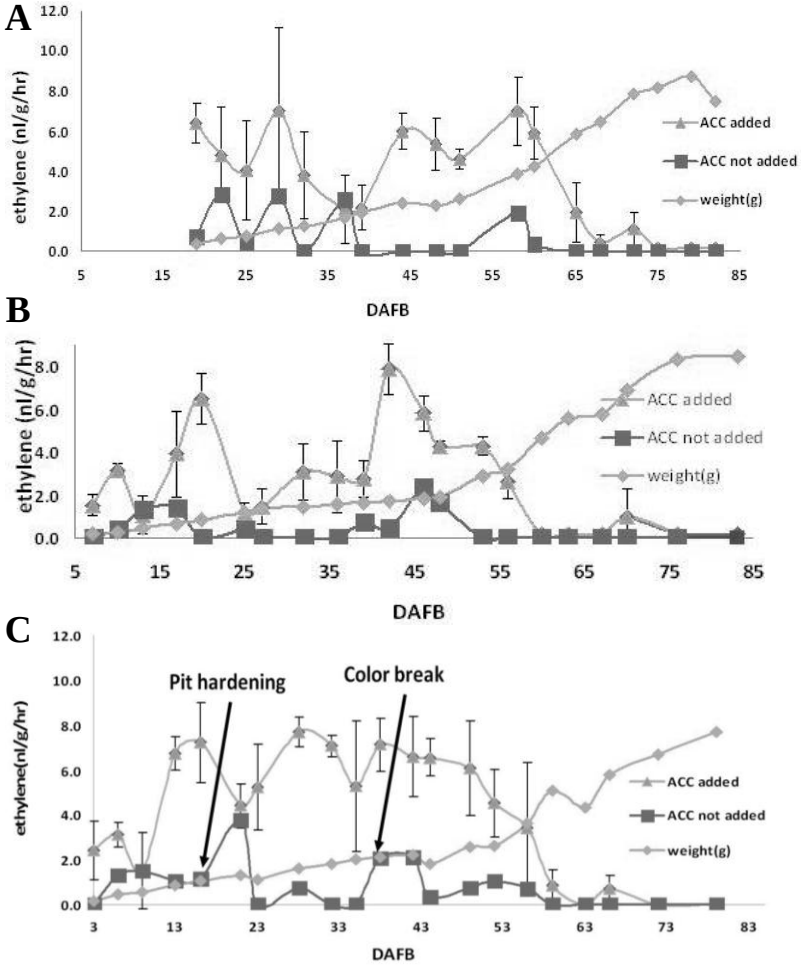


Figure 3. ACO enzyme activity and fruit weight changes during fruit development of three sweet cherry cultivars in 2010. (A) 'Chelan'. (B) 'Bing'. (C) 'Skeena'.

Peach was assayed at six points during fruit development in 2010 (Fig. 4A) and four points, plus an additional 146 DAFB ripening at 66°F for 23 days, in 2011 (Fig. 4B). The ACO level as +ACC changed from nearly zero at 113 DAFB to 10 nl/g/hr at 125 DAFB then leveled off to nearly 9 nl/g/hr at 146 DAFB (Fig. 4A). The ACO levels a –ACC data for 2010 showed an increase at 125 DAFB, and again a 146 DAFB, closely paralleling the +ACC data (Fig. 4A). The peach ACO activity as

+ACC had very high levels in 2011 at 110 DAFB, then leveled off to nearly 14 nl/g/hr at 146 DAFB. The ACO activity as –ACC did not parallel the +ACC enzyme activity. By maturity, the 146 DAFB fruit for 23 days at 66°F, the ACO as –ACC increased to 6 nl/g/hr (Fig. 4B). At this same time point the ACO as +ACC decreased to nearly 6 nl/g/hr (Fig. 4B). Although the ACO data as +ACC and as –ACC over two harvest seasons showed similarities, data at 146 DAFB, the climacteric state of peach was similar (Fig. 4A-B). The ACO as +ACC was the highest for both years at this time point (Fig. 4). This result was similarly shown by Zhang and Hui-Juan (2005) in peach as the ACC content, the *in vitro* ACO, and whole fruit ethylene increased at the mature ripe state. Additional work by Tonutti et al., (1996) showed *in vivo* levels of ACO increase along with ethylene generated from –ACC-spiked fruit disks, at the time of early ripening in three peach varieties.

The ACO enzyme activity as +ACC in three sweet cherry cultivars was zero at mature ripe (Fig. 3), therefore, a major difference to the peach ACO at mature ripe (Fig. 4). Several authors have described ACS activity, ACO activity, and ethylene levels in peach, but at this time no information exists for sweet cherry ACS or ACO levels through harvest. The ACO with and without ACC, whole fruit ethylene levels, and respiration comparisons between sweet cherry and peach over the growth and development period are the first such studies.

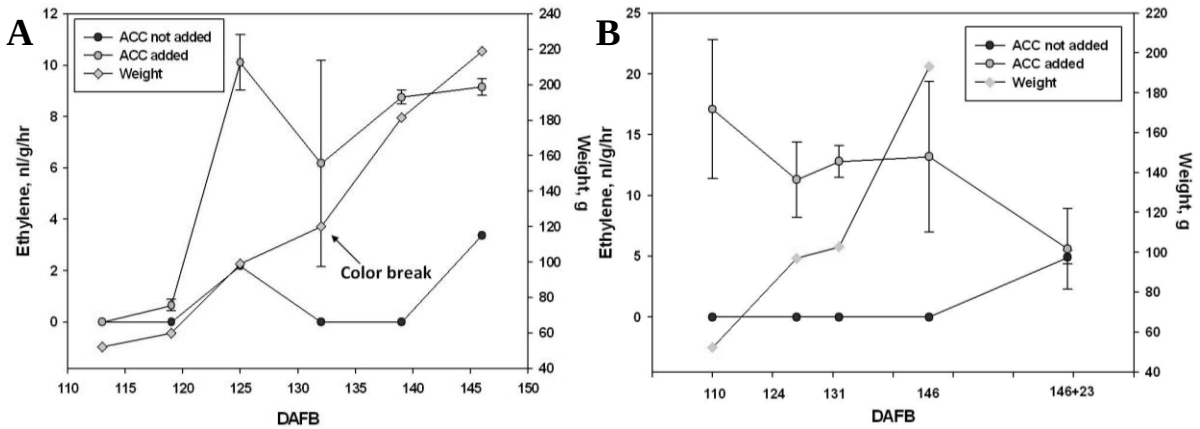


Figure 4. ACO enzyme activity in 'Early Elberta' peach in (A) 2010, and (B) 2011. Assay points in 2010 were measured on 3 samples for +ACC while –ACC was performed on 1 sample. Assay points in 2011 were for 3 samples for both +ACC and –ACC.

A second part of the study aimed at "physiological assays to assign cherry cultivars to ethylene physiology categories" consisted of measuring the ACO enzyme activity in 9 sweet cherry varieties from fruit set until harvest (stylized in Fig. 5, complete results in Fig. 6). The *in vivo* assay was performed similar to studies in year one for cherry and year one and two for peach (Fig. 3 and 4). The measurement of ACC as +ACC revealed many patterns among varieties. The latest season sweet cherry variety 'Regina' had the overall highest ACO activity at nearly 16.0 nl/g/hr (Fig. 5). Also, 'Regina' had the latest peak in ACO activity as +ACC ending at 64 DAFB (Fig. 5). 'Glacier' had the lowest ACO activity among all varieties (4.0 nl/g/hr), and 'Lapins' ACO activity ended the soonest after full bloom, at about 52 DAFB (Fig. 5). A unique observation of the ACO activity as +ACC was that the early color break point (highlighted in red) occurred before the last peak in ACO (Fig. 5). However, two varieties, 'Chelan' and 'Rainer' showed color break at the last peak in ACO activity as +ACC (Fig. 5). This result was noted in year one of the ACO study on three varieties, 'Chelan', 'Bing', and 'Skeena' (Fig. 1). The difference in year one being the peaks in ACO activity seemed to associate with –ACC fed assays. The current year data does not show appreciable peaks in ACO activity as –ACC near color break for any of the 9 varieties (Fig. 5).

In work closely related, various regulators and biochemicals were applied to sweet cherries at several maturities (Hartman, 1992). Ethephon does not stimulate anthocyanin synthesis, moreover, applications of ABA do stimulate anthocyanin production at 8 weeks after full bloom (Hartman, 1992).

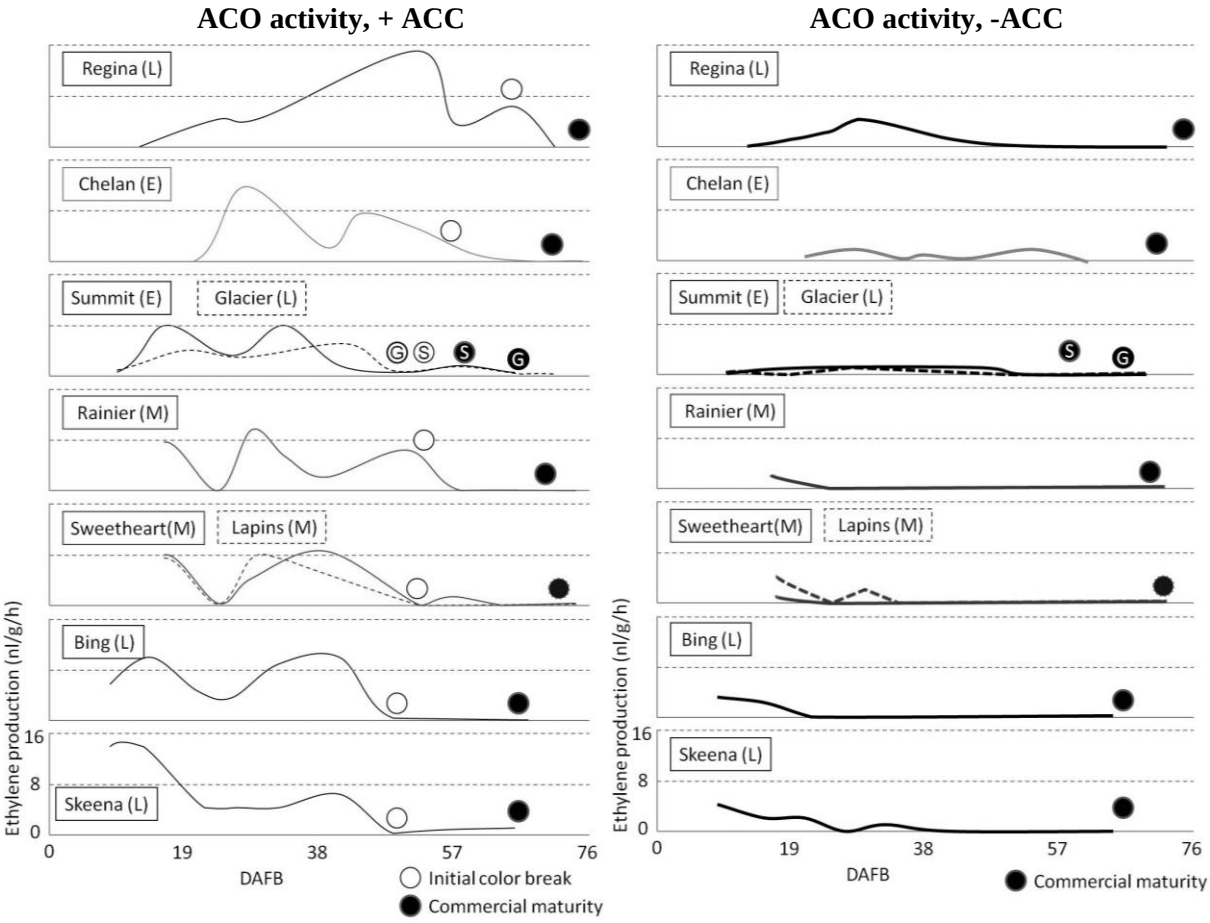
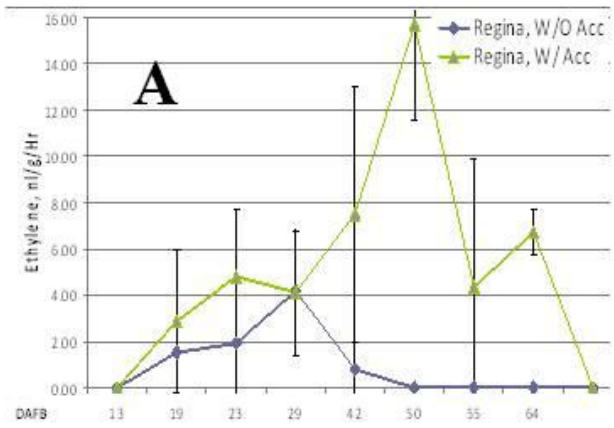
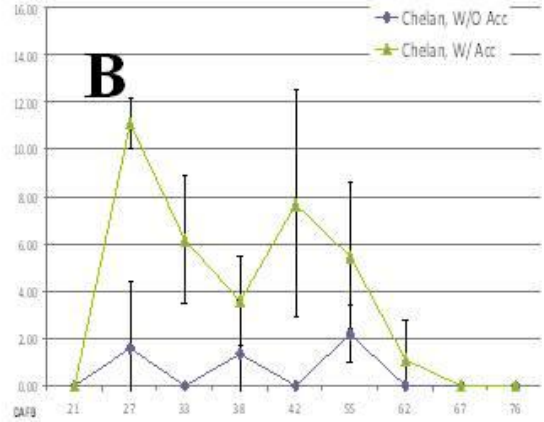
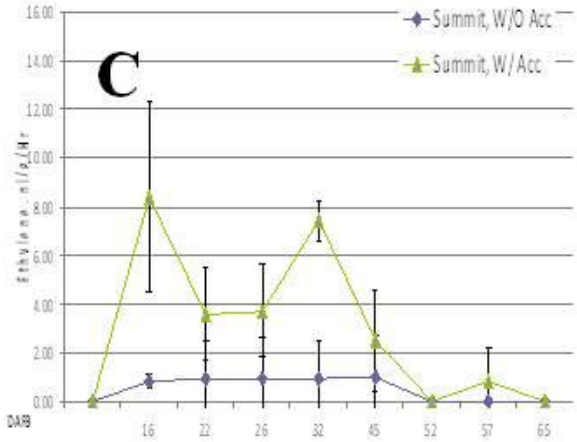


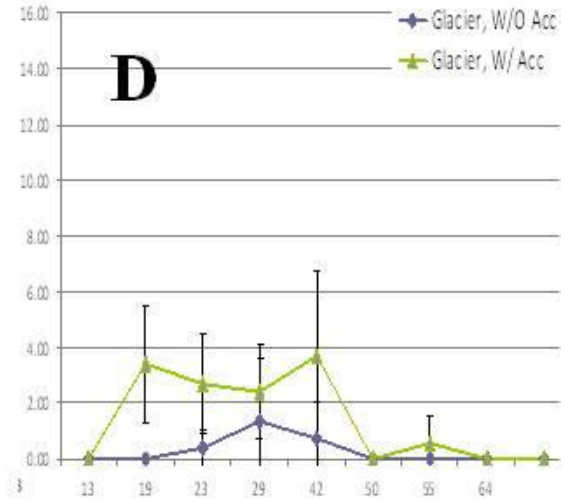
Figure 5. Summary of patterns of ACO enzyme activity from *in vivo* assays on nine sweet cherry cultivars through 76 days after full bloom (DAFB) during the 2011 growing season. ACO assays are displayed as +ACC (addition of the enzyme substrate, ACC) or –ACC (no ACC added).

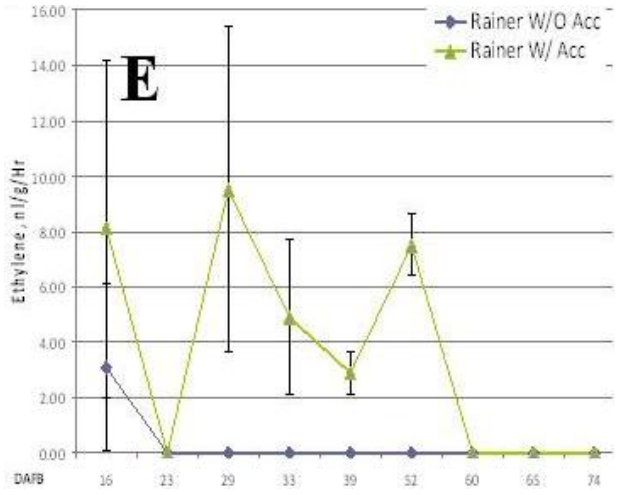
Figure 6 (next pages). ACO activity from *in vivo* assays in sweet cherry fruit, (A) 'Regina', (B) 'Chelan', (C) 'Summit', (D) 'Glacier', (E) 'Rainer', (F) 'Sweetheart', (G) 'Lapins', (H) 'Bing', and (I) 'Skeena' (n=3 for each time point, +ACC or –ACC).

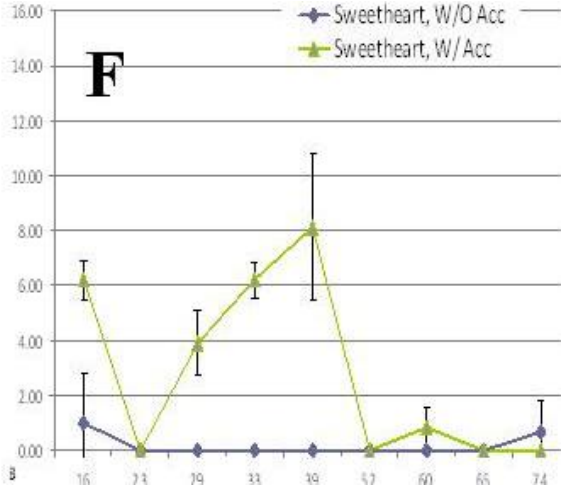

A
Regina, W/O Acc
Regina, W/ Acc
Ethylene, nl/g/Hr
DAFB

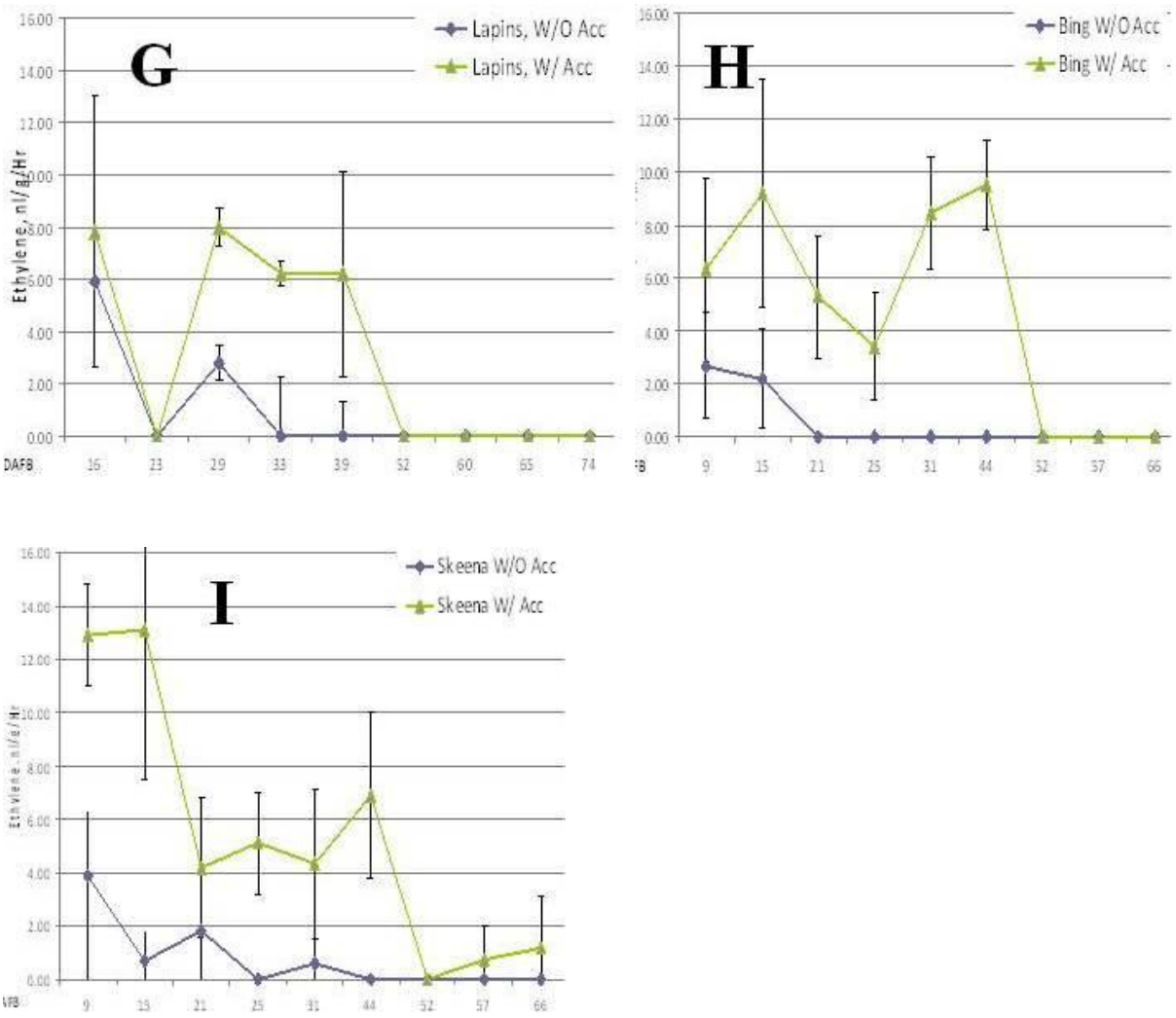

B
Chelan, W/O Acc
Chelan, W/ Acc
DAFB


C
Summit, W/O Acc
Summit, W/ Acc
DAFB


D
Glacier, W/O Acc
Glacier, W/ Acc


E
Rainer W/O Acc
Rainer W/ Acc
Ethylene, nl/g/Hr
DAFB


F
Sweetheart, W/O Acc
Sweetheart, W/ Acc



Fruit quality evaluation and association with ethylene physiology categories*:* Fruit quality was determined for nine sweet cherry cultivars grown at IAREC in Prosser, WA, at 1 day after harvest (at commercial maturity) and after a 7-day storage regime of 32^{o}F, 95% RH. Fruit weight at day 1 varied among cultivars between 7.0 and 11.2 grams (data not shown). Firmness (deflection force) increased between day 1 and day 7 for all cultivars except 'Sweetheart' (Table 1). Sweetness (^{o}Brix) increased between day 1 and 7 for 'Regina', 'Chelan', 'Sweetheart, and 'Skeena', and slightly decreased for the other five cultivars (Table 1). Acidity (% TA) decreased for all varieties, except for 'Skeena' which had no change (Table 1). Change in skin color (a+ and Hue) between day 1 and day 7 was variable among cultivars, tending to increase toward redness for 'Lapins' and 'Skeena' and losing redness for 'Chelan', 'Glacier', and 'Rainer' (Table 1). We were not able to find in the literature a more detailed dataset as this on comparative fruit quality after storage for PNW sweet cherry cultivars. Cultivars were grouped in various ways according to ACO activity patterns (Fig. 5), but no consistent statistical differences in any fruit quality attribute was found among groups. Therefore, although ACO activity during sweet cherry fruit development appears to vary among cultivars, these differences do not appear to be associated with differences in fruit quality at harvest or after a week of storage.

Table 1. Fruit quality for nine sweet cherry cultivars evaluated in 2011 at one day after harvest and after 7 days of cold storage. Presented are average values for 10-15 fruit, except for acidity which was an average of 4 readings from 7-8 fruit per sample.

Cultivar	Harvest date	Harvest weight	Firmness (g/mm)		Sweetness (^{o}Brix)		Acidity (%TA)		Color (a+)	
			day1	change	day1	change	day1	change	day1	change
Bing	7 Jul	7.4 g	356	+49	18.4	-0.7	1.16	-0.05	30.2	+1.3
Chelan	28 Jun	7.0 g	443	+71	19.9	+0.2	1.25	-0.08	22.3	-6.5
Glacier	7 Jul	10.3 g	397	+19	23.0	-1.8	1.32	-0.08	25.3	-1.6
Lapins	7 Jul	9.2 g	337	+56	16.5	-0.5	1.01	-0.10	27.9	+6.9
Rainier	7 Jul	10.1 g	298	+103	19.3	-1.9	1.14	-0.06	39.5	-8.6
Regina	21 Jul	10.3 g	425	+2	18.3	+0.4	1.13	-0.11	21.3	+2.1
Skeena	7 Jul	11.2 g	463	+21	17.5	+0.2	1.24	0.00	32.7	+7.8
Summit	28 Jun	9.2 g	394	+16	12.1	-0.5	0.83	-0.08	48.2	+2.4
S'heart	7 Jul	9.3 g	475	-15	14.7	+0.5	1.52	-0.30	43.9	+1.5

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EXECUTIVE SUMMARY

Our overall goal is to improve market life of sweet cherry cultivars through an understanding of the ethylene genetic control in this non-climacteric crop. Specific objectives were to:

1. Characterize differences in the ethylene biosynthetic pathway between sweet cherry and peach—*why do they ripen differently?*
2. Identify ethylene physiology categories of cherry cultivars that simplify development of strategies to extend market life—*does increased synthesis and/or sensitivity to ethylene determine ultimate postharvest response?*
3. Provide specific selection criteria in breeding for improved market life based on ethylene biosynthesis and response—*can a simple high-throughput physiological or genetic assay help predict market life?*

Answers to these above questions are:

1. *Differences between non-climacteric sweet cherry and its climacteric relative peach are either in ethylene synthesis over-regulation and/or in the ethylene signal response pathway such as the absence of critical response element.*
2. *Probably not.*
3. *Possibly, but we haven't found it yet.*

Significant findings and conclusions:

- Sweet cherry is confirmed as a non-climacteric fruit.
- Mature ripe sweet cherry fruit did not respond to ethylene treatment by any changes in respiration or ethylene production.
- An early peak in ethylene production in tiny fruitlets subsided well before, just before, or soon after color break (depending on the cultivar) and became undetectable upon further maturation.
- Differences in several *ACS* genes were observed among three cherry cultivars over six sampling times throughout fruit development
- Activity of ACO (the "ethylene forming enzyme") in sweet cherry fruit declined to zero with advancing fruit development. In stark contrast, ACO activity in peach was high in early season fruit, leveled off, then had a medium activity in mature ripe fruit.
- ACO activity patterns for nine sweet cherry cultivars throughout fruit development showed several patterns. However, no significant associations of these patterns with fruit quality traits were detected, including after a 1-week storage regime.
- The lack of endogenous ethylene production or ethylene response indicates that there are no off-tree quality improvements to be realized for sweet cherry fruit beyond color break.
- These results lead us to believe that the difference between cherry and its climacteric relatives is due to synthesis over-regulation and/or is in the ethylene signal response pathway such as the absence of critical response elements.

Future directions:

- Systematically examine gene expression for all members of the ethylene biosynthetic and signal transduction pathway to identify critical differences between sweet cherry and peach, and key differences among sweet cherry cultivars. Examine whether differences in gene expression patterns among cultivars are associated with differences in market life.
- Comprehensively evaluate many cultivars across the season for their fruit quality attributes at harvest and over extended storage regimes and extended room temperature ripening. Use such a detailed market life performance dataset to better understand market life differences among cultivars. Such a dataset can also be used to seek associations with gene expression differences in the ethylene pathway or other likely fruit quality biochemical pathways.