FINAL PROJECT REPORT

Project Title: Physiological genomics of 1-MCP use in pear

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Total Project Funding: $99,180 (3 years)

Budget History:

Item	2012	2013	2014
Salaries[1]		32736	34045
Benefits		2156	2243
Wages			
Benefits			
Equipment			
Supplies[2]	8500	7500	7500
Travel[3]	2500	1000	1000
Miscellaneous			
Plot Fees			
Total	$11,000	$43,392	$44,788

Footnotes: [1] Salaries for agriculture research assistant for performing physiological and genomic profiling and all molecular work. The increase in salaries for year three reflects a 4 % rate increase.
[2] Supplies includes monies for ethylene gas, compressed air, two proprietary chemistries, fruit sampling, RNA isolation, quantitative reverse transcription PCR and consumables.
[3]Travel includes monies for fruit pick up at BlueStar and AgroFresh.

This project addresses the US Pear Industry's topmost priority of ensuring uniform fruit quality to enhance per capita consumption.

OBJECTIVES

The aim of this project was to gain an understanding of pear genetic responses to 1-MCP treatment, and test chemical approaches to obtain optimal fruit quality in response to 1-MCP treatment through the following objectives.

1. (Years 1 and 2) Test the activity of 35 genes seen to be activated in relation to the proposed cold-induced ripening master-switch gene in D'Anjou and Bartlett fruit.

As part of objective 1, we studied how the ripening-related genes behave in relation to each other and the proposed cold-induced ripening master-switch gene before, during and after 1-MCP treatment, under 3 storage temperatures. Further, we compared the activity of the selected subset of genes between two different varieties to correlate them with the fruit ripening phenotype. From this work, we identified potential genes and associated metabolic pathways which become non-functional in response to 1-MCP treatment making the fruit unsuitable for ripening.

2. (Years 2 and 3) Establish a relationship between gene pathway activity, fruit ripening phenotypes, and chemical approaches to address controlled initiation of pear ripening in 1-MCP treated fruit.

Based on the identification of potential pathways from the results obtained as part of Objective 1, we identified several candidate ripening compounds that could potentially reactivate the process of ripening in 1-MCP treated D'Anjou and Bartlett fruit. The analyses for reactivation of ripening with the chemical compounds involved monitoring of fruit quality, ethylene and respiration rates. With the chemical approach we have demonstrated reactivation of ripening in 1-MCP treated fruit. Comprehensive analysis of gene activity, fruit quality, and ethylene release and respiration rates in Bartlett and D'Anjou varieties revealed the differences in response to 1-MCP treatment.

All the experiments were performed with commercial grade fruit obtained in 2012 and 2013 from project cooperator Blue Star Growers (Bob Gix). D'Anjou and Bartlett fruit were harvested at commercial maturity and treated with 1-MCP at the current industry recommended rates of 100 and 300 ppb respectively.

SIGNIFICANT FINDINGS

- Based on the analysis of the activity of several genes related to metabolic pathways that facilitate ripening, specific pathways were identified that are blocked in unconditioned Bartlett and D'Anjou fruit treated with 1-MCP.

- A total of nine potential ripening compounds have been identified. Some of these 'ripening compounds' or RCs that were tested in this project have been shown to reverse the effect of 1-MCP in Bartlett and D'Anjou.

- The optimal concentration of RCs tested, duration of treatment and type of response elicited from the fruit for each RC were established.

- In experiments with three RCs, reactivation of ripening as measured by release of ethylene or carbon dioxide in 1-MCP treated Bartlett and D'Anjou fruit met or exceeded the proposed increase of 20% compared to the control.

- Interestingly, it was observed that RCs not only reversed the impact of 1-MCP, they also induced ripening in non 1-MCP treated control fruit that had not met the chilling requirement. Ripening response was consistently obtained in both varieties tested in a dose-dependent manner, allowing reliable timed induction of ripening.

- While all the experiments done in this project were performed by soaking the fruit in RC solutions for 24 hours, a few recent experiments were successfully performed by vaporizing one of the RCs. This is expected to open up avenues for application of RCs on fruit in bins via fogging.

RESULTS & DISCUSSION

Pre-harvest and post-harvest ethylene perception or production blocking chemicals are desirable products to regulate fruit production and postharvest storage. By inhibiting ethylene sensitivity, ethylene-responsive processes are slowed down in treated fruit. The responses include ethylene biosynthesis, fruit quality development and respiration. A complete fundamental understanding of enhanced sensitivity to 1-MCP treatment and variable recovery is yet to be developed in pears, although physiological approaches for postharvest management for non-1MCP treated fruit have been developed (Chiriboga et al., 2011; Chiriboga et al. 2014). Traditionally, respiration rates are considered to be tightly coupled to ethylene-pathway activity in climacteric fruits. Results from work performed in this project have identified additional factors that exert influence on respiration despite 1-MCP induced reduction in ethylene-sensitivity. Recently, similar results have been reported in 1-MCP treated tomato (Xu et al., 2012), suggesting that genetic components of respiration-related pathways may not be completely coupled to ethylene pathway activity in climacteric fruit. Inversely, chemical inhibition of this pathway has been shown to suppress the respiratory climacteric and alternative oxidase (AOX) pathway activity in tomato (Xu et al., 2011).

Respiration rate of the fruit closely correlates to the development of desirable fruit quality and thus marketability. In addition to 1-MCP products, controlled atmosphere storage is also used to slow down the respiration rates and delay ripening. However, total respiratory activity is a cumulative result of multiple inputs where metabolites and energy are utilized in different ways. Cytochrome (CYT) and alternative oxidase (AOX) pathways are two different avenues by which energy balance in achieved. Besides some other genes, an increased abundance of alternative oxidase genes was observed in Bartlett pear in response to conditioning treatments (Figure 1). In Bartlett fruit, this correlated to an approximate 4-fold increase in response to cold-exposure relative to unconditioned controls. A similar increase was not observed in D'Anjou samples, suggesting either a variably expressed cold-responsive pathway that may influence pears ripening profile or another AOX allele may be active that was not sampled. Generally, AOX activity serves to maintain oxidative and biochemical conditions needed to drive energy production in plant cells. Alternative oxidase activity is associated with ripening in fruits (Perotti et al., 2014; Huang et al., 2013), and various stresses in plant tissues (Vanlerberghe, 2013). This was one of the several genes that demonstrated differential expression in conditioned vs. non-conditioned controls.

A set of chemical compounds was identified which were selected as potential candidates that could activate blocked pathways. The impact of increased respiratory activity from these compounds was thought to increase ethylene production. Stimulating respiration in 1-MCP treated pear can enable the

reactivation of ripening at will. In this role, RCs could serve as a companion product to existing postharvest strategies utilizing 1-MCP products.

Sample fruit were obtained from Blue Star Growers (Cashmere, WA) from multiple grower lots harvested at commercial maturity. After initial room-temperature aqueous drenching of fresh whole 1-MCP treated Bartlett and D'Anjou fruit (at 300 and 100 ppb, respectively) for 12-24 hours (2012 and 2013 season fruit) significant stimulation of respiration, ethylene production and flesh softening was observed. Between 2012 and 2013 growing seasons, 20 experiments were performed using 9 RCs. Experiments with some RCs were not pursued due to high costs and safety concerns. Results indicate significant stimulation of respiration, ethylene production and fruits' softening in 6 experiments using 4 compounds (Table 1). Respiration was stimulated to or beyond the 20% threshold established for an effective RC (Figure 2). In summary, significant stimulation of respiration and ethylene production was observed in 12 experiments using 8 RCs in 1-MCP treated fruit. Fruit softening was accelerated in 10 experiments using 6 RCs. Additional RCs stimulating all parameters measured produced significant tissue damage to sample fruit.

Notably, not all RCs tested produced significant stimulation of these parameters together. For example, RC1 elicited stimulation of respiration and ethylene production in 2013 season 1-MCP treated D'Anjou, but failed to accelerate fruit softening. Similar results were observed in the 2013 trial of RC2 on 1-MCP treated Bartlett. While fruit softening was accelerated in these samples, analysis of available data indicated a lack of stimulation of respiration and ethylene production. However, data logging system failures likely impacted these results. Despite equipment failures, observed stimulation of 1 or more (but not all concurrently) parameters measured in this study supports that idea that respiration, ethylene production, softening and ripening-associated processes are influenced by more than just ethylene. Further, these results support findings from prior studies in other crop systems (Xiao et al., 2010; Pastore et al., 2001), suggesting observed responses from this work may not be specific to 1-MCP treated pear. Overall RC1, RC2 and RC3 significantly accelerated respiration, ethylene production and softening in 1-MCP treated Bartlett and/or D'Anjou sample fruit at concentrations less than 1.0 mM without undesirable tissue damage or loss of soluble solid content. Among all experiments conducted in this study, soluble solid content was significantly increased in 2012 Bartlett (1-MCP treated and untreated) only in response to RC2. The unaffected soluble solid content may indicate that accelerated respiration in response to RC treatments may result in an increased sugar and organic acid consumption to drive energy production, and other ripening-related processes. Together, these findings demonstrate that at effective concentrations, RCs can accelerate ripening in 1-MCP treated pears without sacrificing quality or marketability of the fruit.

As an indicator of ripening processes in pear, ethylene biosynthesis was measured in 1-MCP treated and untreated control pears after exposure to RCs. Extensive physiological studies have demonstrated the putative need for ethylene pathway activity for induction of many ripening-associated processes and fruit quality development (Villalobos-Acuna and Mitcham, 2008). European and Chinese white pears are unique among climacteric fruits, requiring variable conditioning treatments to gain ripening capacity and marketability. Without this conditioning, ethylene production is negligible in pears.

Current experimental equipment and infrastructure limited the number of samples that could be tested concurrently. Thus, sample fruit used in each experiment experienced variable amounts of storage at 2°C storage prior to RC exposure and subsequent monitoring (Table 2). Despite this, results of RC2 and RC3 experiments were obtained using Bartlett and D'Anjou fruit held in storage for a maximum of 1 month. Without 1-MCP treatment, conditioning treatments at this storage temperature span 4-8 weeks (in D'Anjou). These results indicate that RC responses observed in these experiments may also mitigate the cold conditioning requirements. By accelerating ripening-related processes in preclimacteric pears which have not received sufficient conditioning, RC responses may be further

leveraged to better control onset of ripening. Further controlled studies are needed to assess the impact of these RCs in unconditioned fruit. Observed stimulation of respiration, ethylene production and softening in both 1-MCP treated and partially conditioned pear supports the model of RC responses being independent of ethylene pathway activity. Together, these responses can allow for expansion of fresh pear market in which maintenance of optimal fruit quality can be managed over greater distances and timeframes from distribution hubs.

While effective, 24 hour aqueous drenching applications of RCs present challenges for commercial application. To identify minimum exposure periods required to induce significant responses, we tested alternative delivery methods and reduced exposure times. An experiment was conducted with 1-MCP treated D'Anjou pears from the 2012 growing season using two effective RCs identified from prior studies. Instead of an aqueous 24 hour exposure to RC2 (Table 1), sample fruit were exposed for 12 hours using identical experimental concentrations. In both the 24 and 12 hour exposure experiments, sample fruit were held in storage for up to 4 weeks at 2°C prior to RC2 exposure. Results in D'Anjou from 12 hour exposure to RC2 indicated significant stimulation of fruit respiration and softening, but not ethylene production. In Bartlett samples exposed for 12 hours neither respiration and ethylene production nor fruit softening was stimulated. While further experimentation is needed to clarify the role of fruit maturity at harvest, annual fluctuations, and length in storage prior to RC2 exposure, shorter duration of exposure was not reliable. As a result of these findings and prior observations of peel tissue damage upon exposure to higher RC2 concentrations (Figure 3), further experiments with shorter exposure times were not conducted. Interestingly, we performed one experiment recently where RC2 can be vaporized and applied to the fruit directly opening up the possibility of applying the product via fogging and estimating the duration of exposure.

To determine if ripening-responses could be further enhanced, additional combination RC experiments were conducted. RC3 was included in combination experiments as it known to induce oxidative stress in exposed tissues. RC4 was included in combination treatments as it is a known precursor to auxin production, a known physiological cue to ripening competency in climacteric fruits. Two combination experiments were conducted using RC2 and RC4. Interestingly inclusion of higher RC4 concentrations with RC2 failed to elicit accelerated ethylene production in 1-MCP Bartlett samples. Similarly, softening was not stimulated in Bartlett samples exposed to lower RC4 concentrations. These results failed to demonstrate a significant gain over RC2 treatments, indicating RC4 may not offer desirable ripening responses in 1-MCP treated pears. RC3 produced unacceptable peel tissue damage and necrosis in all experimental levels.

An additional experiment (Table 1) was conducted using a reduced exposure time to gaseous RC1. An initial experiment using 96 hour exposure of 10 ppm RC1 (mixed in compressed atmospheric air) indicated significant stimulation of 1-MCP treated Bartlett respiration and ethylene production. Only fruit respiration was stimulated with fruit failing to soften much at all over the course of the experiment, relative to 1-MCP untreated controls.

To apply RCs commercially, gaseous or misting applications need to be explored. Development of fogging applications with the use of adjuvants or penetrants (including dilute ethanol) is expected to enhance penetration of active compounds into fruits' interior, increasing efficacy. While the objectives of this project have been met, further work is needed to optimize RC delivery protocols. To best address these needs, reliable, accurate high-throughput experimental capacity is needed to best ensure RC experiments are performed on sample fruit from equal physiological states. Results from this work offer an expanded understanding of regulation of ripening and 1-MCP responses in pears. To disseminate this, a manuscript describing this work and its impacts has been submitted to Nature Horticulture and an article in Good Fruit Grower is forthcoming.

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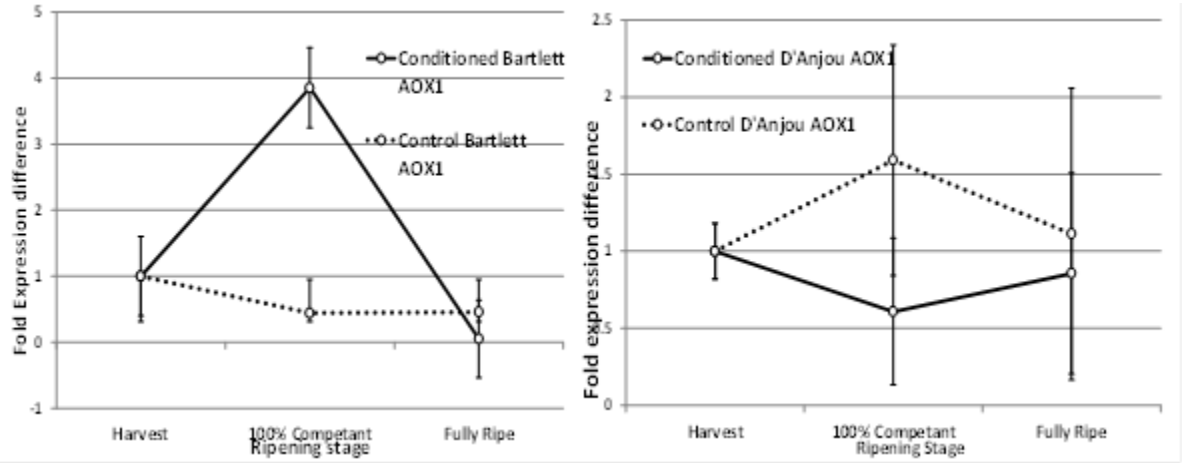

Figure 1. Relative gene abundance of alternative oxidase genes in Bartlett (left) peel and D'Anjou (right) peel tissues at harvest, fully conditioned, and fully ripened stages of postharvest management. Error bars represent standard deviation from the mean among 3 biological replicates. Note the nearly 4-fold increase in expression of AOX1 in Bartlett.

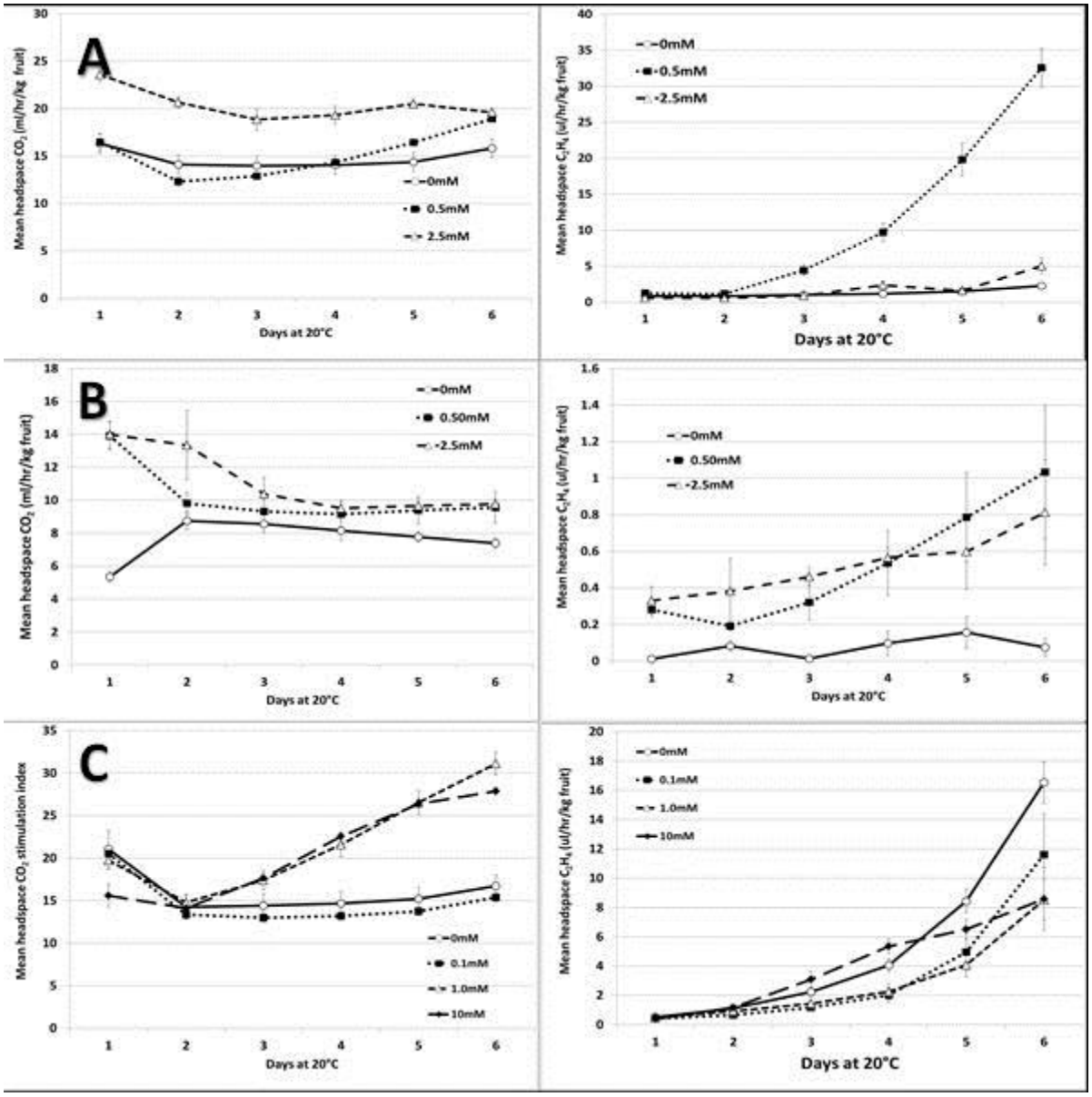

Figure 2. Mean respiration (left), and ethylene production (right) from sample pears in response to RC-1(A-Bartlett), RC-2 (B-D'Anjou) and RC-3 (C-Bartlett) in 1-MCP treated pears. Error bars represent standard error from the mean among 4 biological replicates.

Figure 3. Peel tissue damage and necrosis in 1-MCP treated Bartlett pears from highest level of RC2. Image of RC2 treated fruit was taken 6 days after 24 hour treatment.

Table 1. Significance of primary treatment factors (level of ripening compound, 1-MCP treatment and phenology) shown from 2-factor ANOVA and 3-factor ANOVA analysis in CO_2, ethylene evolution and fruit firmness (respectively). *- significant at α=0.05. **- significant at α=0.01. NS- no significance. !- indicates data was log transformed to meet normality assumptions of the ANOVA test.

Ripening compound	*Year*	*Cultivar*	*Hours exposure*	*Respiration*	*Ethylene production*	*Firmness*
RC1	2012	Bartlett	24	**	*!	**
RC1	2012	Bartlett	24†	*	NS	NS
RC1	2012	Bartlett	96†	**	**	NS
RC1	2012	D'Anjou	24	**	NS	**
RC3	2013	Bartlett	24	*	**	**
RC2	2012	Bartlett	24	**!	**	**
RC2	2012	D'Anjou	24	**	NS!!	NS
RC2	2013	Bartlett	12	NS	NS	NS
RC2	2013	Bartlett	24	NS!!	NS!!	**
RC2	2013	D'Anjou	12	**	NS	**
RC2	2013	D'Anjou	24	**	**	NS
RC2+RC4	2013	Bartlett	24	**	**	NS
RC2+ RC4	2013	Bartlett	24‡	*	NS	**
RC2+RC3	2013	D'Anjou	24	**	**	**
RC3	2013	Bartlett	24	**	**	**
RC3	2013	D'Anjou	24	**	**	NS
RC5	2013	Bartlett	24	NS	NS	NS
RC6	2013	D'Anjou	24	**	**	NS
RC7	2013	D'Anjou	24	*!	**!	NS
RC3	2013	Bartlett	24	**	*!	**

Table 2. Bartlett and D'Anjou mean maturity and soluble solid content at harvest, harvest dates, sample collection dates and experimental start dates from the 2012 and 2013 growing season. Upon harvest, fruit were placed in short-term storage at 5°C until receipt. Upon receipt, samples were placed into 2°C until experiment start date.

RC	*1-MCP treatment*	*Grower numbers*	*Pear cultivar*	*Harvest date*	*Maturity at harvest*	*Soluble solids at harvest*	*Sample collection date*	*Experiment start date*
RC1	+	1172	Bartlett	9/07/2012	16.96	12.8	9/13/2012	11/07/2012
RC1	-	740	Bartlett	9/02/2012	17.51	12.4	9/13/2012	11/07/2012
RC1	+	1172	Bartlett	9/07/2012	16.96	12.8	9/13/2012	12/20/2012
RC1	-	740	Bartlett	9/02/2012	17.51	12.4	9/13/2012	12/20/2012
RC1	+	1172	Bartlett	9/07/2012	16.96	12.8	9/13/2012	12/20/2012

RC1	-	740	Bartlett	9/02/2012	17.51	12.4	9/13/2012	12/20/2012
RC1	+	581	D'Anjou	9/23/2012	15.28	13.1	9/28/2012	10/16/2012
RC1	-	886	D'Anjou	9/19/2012	15.64	12.6	9/28/2012	10/16/2012
RC2	-	451	Bartlett	8/17/2013	17.94	11.9	9/11/2013	9/20/2013
RC2	+	581	Bartlett	9/23/2012	15.28	13.1	9/28/2012	10/31/2012
RC2	-	886	Bartlett	9/19/2012	15.64	12.6	9/28/2012	10/31/2012
RC2	+	581	D'Anjou	9/23/2012	15.28	13.1	9/28/2012	10/16/2012
RC2	-	886	D'Anjou	9/19/2012	15.64	12.6	9/28/2012	10/16/2012
RC2	+	1001, 591	Bartlett	8/17/2013	18.12	12.6	9/11/2013	10/20/2013
RC2	-	451	Bartlett	8/17/2013	17.94	11.9	9/11/2013	10/20/2013
RC2	+	23, 172	D'Anjou	9/14/2013	14.07	13.1	10/03/2013	10/31/2013
RC2	-	2128, 192	D'Anjou	9/10/2013	13.18	13.1	10/03/2013	10/31/2013
RC2	+	23, 172	D'Anjou	9/14/2013	14.07	13.1	10/03/2013	10/07/2013
RC2	-	2128, 192	D'Anjou	9/10/2013	13.18	13.1	10/03/2013	10/07/2013
RC2	+	1001, 591	Bartlett	8/17/2013	18.12	12.6	9/11/2013	11/07/2013
RC2	-	451	Bartlett	8/17/2013	17.94	11.9	9/11/2013	11/07/2013
RC3	+	1001, 591	Bartlett	8/17/2013	18.12	12.6	9/11/2013	9/13/2013
RC3	-	451	Bartlett	8/17/2013	17.94	11.9	9/11/2013	9/13/2013

EXECUTIVE SUMMARY

Variable ripening after 1-MCP application along with the conditioning requirements have hindered the adoption of 1-MCP use in pears. Therefore, strategies to reverse 1-MCP responses may allow for predictable ripening and control of fruit quality.

Analysis of 35 important genes related to 8 different ripening related pathways resulted in the identification of specific pathways that were blocked in unconditioned Bartlett and D'Anjou fruit treated with 1-MCP. A total of nine potential ripening compounds were identified and 3 have been shown to have the potential to reactivate the blocked pathways. It was interesting to find out that RCs not only reversed the impact of 1-MCP, they also induced ripening in non 1-MCP treated control fruit that had not met the chilling requirement.

One of the limitations of the experiment was that the RCs were applied by soaking the fruit in RC solutions for 24 hours. However, we have made a headway to apply the RCs via fogging. In one of the recent experiments RC2 was applied by vaporization. This is expected to open up avenues for application of RCs on fruit in bins via fogging.

This project demonstrated that the development of short term solutions for tree fruit improvement is feasible when a problem is addressed by using physiology-guided gene activity studies. We demonstrated that Bartlett and D'Anjou pear respiration can be stimulated by ripening compounds to reverse the impact of 1-MCP.

In summary, this project have identified a novel means of stimulating ripening processes in 1-MCP treated Bartlett and D'Anjou whole fruit. We are proceeding to continue the work with RC2 since it can be applied via vaporization and is stable at room temperature. RC2 is currently approved for use in the food chain through the FDA. RC2 exposure can be used to antagonize 1-MCP responses in fresh pear tissues and can be further developed as a companion product to 1-MCP in the postharvest management chain. These responses may be particularly effective in application to sliced pear products, where the increased flesh surface area can rapidly absorb the active compound. The impacts of this expanded toolset in postharvest management of fresh pear tissues will enable leveraging of the benefits of 1-MCP use, while mitigating its drawbacks of variable, unreliable recovery periods. Together this can allow for increased inventories, distribution and potential sales of pear products in expanding markets.