MECHANISMS OF INJURY TO ‘HONEYCRISP’ APPLE UNDER CONTROLLED ATMOSPHERE (CA) STORAGE CONDITIONS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Horticulture—Doctor of Philosophy

2018

ABSTRACT

MECHANISMS OF INJURY TO ‘HONEYCRISP’ APPLE UNDER CONTROLLED ATMOSPHERE (CA) STORAGE CONDITIONS

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Controlled atmosphere (CA) storage is used to maintain the fresh quality of most commercial varieties of apples in the US. CA storage units are typically operated at O_2 levels below 3 kPa and CO_2 levels between 1 and 5 kPa. However, 'Honeycrisp' fruit is very sensitive to standard CA conditions, which can cause jagged-edged brown lesions in the fruit cortex and lens-shaped voids. The brown lesions develop rapidly, maximizing within the first 1.5 months of CA storage, and the voids develop more slowly, increasing in frequency with storage time. We found that the severity of CA-injury rose with increasing CO_2 concentrations. The fruit treated with the antioxidant diphenylamine (DPA) before CA storage experienced minimal CA injury. The damage caused by elevated CO_2, in combination with 3 kPa O_2, induced the formation of fermentative volatiles ethanol, ethyl acetate, and acetaldehyde. Our data suggested that the fermentative volatiles do not cause damage, but rather they are the result of the damage caused by CA conditions. The injury was found to be associated with changes in cellular metabolites associated with energy interconversion and reducing potential. CA injury was associated with a shortage of reducing agents in ‘Honeycrisp’ apple. The data suggest that the tissue does not have enough adenylate energy charge (AEC) for cellular survival and sufficient antioxidants for scavenging oxygen free radicals that accumulate under CO_2 stress. Consequently, cell death follows, leading to the browning symptoms and lens-shaped voids of CA injury.

ACKNOWLEDGMENTS

I deeply thank my professor and advisor Dr. Randolph Beaudry for his amazing academic training and financial support throughout my studies in postharvest physiology. Dr. Beaudry gave me the opportunity to present my research in top scientific meetings. I would like to thank my committee members: Drs. Daniel Jones, Wayne Loescher, and Muraleedharan Nair, who always gave me great advice and deepest support whenever I was faced with difficulty in my research.

Special thanks to the apple industry in Michigan which gave financial support to me doing research and the apple growers in Michigan who supplied apples for my research.

Special thanks to my wonderful and nice lab mates Dr. Nihad, Ben (especially for setting up experiments), Phil (especially for dissertation revision), Renato (especially for statistical issues), Rosella, George, Patrick (especially for HPLC techniques), Zafri, and Sangram and the very nice undergrad students Laura, Emilie, Noah, Denny, Tye, Emily, Dan, and Matt who helped me and shared cheerfulness and troubles with me during my study at postharvest lab in MSU. I would like to thank Ms. Sue Hammer, who always ordered liquid nitrogen quickly when I am in need (I used liquid nitrogen a lot!). Special thanks to Dr. Jesse Traub who instructed me very carefully in using the freeze dry machine in Loescher lab, which was very important for my research. He even came to the lab when I had troubles with the machine.

Special thanks to Ms. Lijun Chen, the most careful technician I have ever known. She, together with Dr. Daniel Jones and Dr. Scott Smith, helped me a lot in successfully detecting key metabolites in apples. I enjoyed working at RTSF Mass Spectrometry and Metabolomics Core at Michigan State University at which I spent most of my days and nights when analyzing samples.

The technicians were all so nice and supplied me with equipment, chemicals, and new columns when I was in need.

I would like to thank professors, staff, and friends in the Department of Horticulture who supported me during my programs. Special thanks to Sherry who is a wonderful administrative staff member of the Department. Thanks to friends in HOGS with their kind support and friendship, especially to Qingwu Meng, Yujin Park, and Ben Mansfeld for their precious time helping me with technical issues, presentation improvements and dissertation formatting.

Thanks to my Vietnamese friends in the Association of Vietnamese Students and Visiting Scholars at Michigan State University who considered me as a big sister and respected me always and helped me in this big family.

Thanks to Ms. Carolyn White together with her Lansing Mediation groups who trained me how to do meditation, which helped me to release stressful moments in study. I enjoyed peaceful moments in walking mediation, sitting meditation and conversations every Wednesday night with them. And special thanks to Ms. Joette Laseur, Mr. Gerrit Laseur, and Ms. Hang Nguyen for their love and support to me and my son during my study. I show my respects and thanks to Becky, my advisor's wife for her nice gifts and cakes.

I would like to show my gratitude to MSU Student Parent Resource Center board members Lori, Laraine, Kim with their wonderful activities and financial support for parent students like me. Thanks to all teachers at ESL & Friends Free English Classes (especially to John, Bonnie, and Rick) for their English lessons.

A deeply special love and thanks to my Dad, who has devoted almost his life as a leader in agriculture to improve agriculture in Binh Thuan province and nourished my ambition to study in higher education to help farmers.

Thanks to the Vietnamese farmers who I worked with when I was a researcher. They taught me how to drink rice wine and their hardship was the main motivation for which I left my peaceful and leisured life and good job in Vietnam to come back to the research environment and try to find a way to help them improve their life.

I would like to show my deep gratitude to my advisor for my undergrad thesis and Master thesis, Professor Mai Tran Ngoc Tieng, who got her Ph.D. degree at Purdue University in 1962. She was the most famous scientist of Plant Physiology in Vietnam, extremely strict, but trained me from a "baby-doll" girl to be a more determined, thoughtful, self-disciplined, self-respected, and logically-thinking person. These characteristics are very helpful for my career life and especially my studies in the US.

My amazing husband for his sacrifice, responsibility, and unconditional love for me. My warm-hearted and very independent son for his great support in doing research on apples in the postharvest lab at night. He even helped to do housework for me and even did massage for me when I felt pain in my body.

My mother, my brother, my colleagues, my relatives, and my friends who always sent regards and motivation to cheer me up when my mood was very down while living in the U.S alone.

Vietnam Education Foundation (VEF) fellowship sponsored by the U.S. government, the toughest fellowship but it is the only one available at the time I applied which accepted candidates who were doing administrative work.

And …thanks to the apple scent in the hallway of the postharvest lab which seemed to condense at night …

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KEY TO ABBREVIATIONS

1-MCP	1-methylcyclopropene
Acetyl CoA	Acetyl coenzyme A
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
Asc	Ascorbic acid
ATP	Adenosine triphosphate
CA	Control atmosphere
CoA	Coenzyme A
DPA	Diphenylamine drench
GSH	Glutathione
GSSG	Glutathione disulfide
NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
$NADP^+$	Oxidized nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NASS	National Agricultural Statistics Service
PEP	Phosphoenolpyruvate
RA	Refrigerated atmosphere
SA	Succinic acid
UDP-G	Uridine diphosphate glucose

CHAPTER 1.

INTRODUCTION

1.1 Issues of ‘Honeycrisp’ apple under CA storage

Since its release in 1991 from the Minnesota Agricultural Experiment Station's Horticultural Research Center (Bedford, 2001; Luby and Bedford, 1992), the ‘Honeycrisp’ apple (*Malus x domestica*) cultivar has been widespread and become one of the most valuable cultivar grown the United States (National Agricultural Statistics Service, 2011, 2012, 2015). A reconstructed pedigree for ‘Honeycrisp’ based on haplotype analysis using SPN data proved that the cultivar was bred between ‘Keepsake’ parent and previously unreported parent ‘MN1627’ (Howard et al., 2017). ‘Honeycrisp’ apple now occupies a significant share of the apple market in the U.S. since the cultivar has become a favorite fruit of consumers because of its crisp texture and unique flavor (Abad-Santos, 2017; Yue and Tong, 2001). Due to a dramatic and continues increase in planting area of this cultivar, it is very necessary to extend the marketing season for the apple. Thus, long-term storage for marketing season is needed (Beaudry et al., 2014; Watkins and Rosenberger, 2000).

Controlled atmosphere technology functions as a supplement to cold temperature storage to prolong storage life of horticultural crops after harvest. CA storage includes an increased CO_2 concentration (usually in the range of 2% to 30%) and/or decreased O_2 concentration (usually in the range of 0.5% to 14%) (Gormley, 1985). However, very low O_2 and/or very high CO_2 concentrations can induce the development of physiological disorders in many apple cultivars. Low O_2 or high CO_2 alone or a combination of both gases caused CA storage injury (CA injury) (Pierson et al., 1971).

Since the 1960s, CA storage has been developed and applied to most commercial varieties of apples (Golding and Jobling, 2012) including ‘Honeycrisp’ in the US and in major apple production areas around the world. Unfortunately, however, 'Honeycrisp' has a high

sensitivity to low storage temperatures and low O_2, high CO_2 atmospheres, leading to storage disorders. Therefore, it is challenging in storing the fruit for a long-tern. Soggy breakdown and soft scald which are classified as typical low temperature disorders (i.e. the chilling injury symptoms) (Beaudry and Contreras, 2009; Brook and Harley, 1934; Plagge and Maney, 1928; Ramsey et al., 1917; Watkins and Rosenberger, 2000; Watkins et al., 2004, 2005). Common symptoms of CA injury include internal browning and lens-shaped voids in the flesh. In particular, CA-related injury causes jagged-edged brown lesions in the apple cortex which may extend to the core (Beaudry and Contreras, 2009). Testing disorder incidence on 'Honeycrisp' under different CA conditions with varied combinations of O_2/CO_2 partial pressures (kPa): 1/0, 3/0, 1/3, 3/3, 21/3, 21/0 (air) showed that the symptom was caused by elevated CO_2 levels and was exacerbated by reduced O_2 levels (Contreras et al., 2014).

CA storage recommendations for 'Honeycrisp' apple in Michigan, New York, Minnesota, Nova Scotia, and Ontario are being developed (Beaudry and Contreras, 2009; Beaudry et al., 2014; Contreras et al., 2014; DeEll and Ehsani-Moghaddam, 2012; DeLong et al., 2004a; Leisso et al., 2017; Watkins and Nock, 2012b, 2012a). Preconditioning (3, 10, and 20 °C for 5 days), diphenylamine drench (DPA, an antioxidant; 1000 $\mu L \cdot L^{-1}$), or 1-methylcyclopropene (1-MCP, an ethylene action inhibitor; 1 $\mu L \cdot L^{-1}$) were applied to 'Honeycrisp' before CA storage under O_2/CO_2 partial pressures (kPa) of 3/0 and 3/3 for preconditioning and DPA and 21/0 for 1-MCP (Contreras et al., 2014). Preconditioning and DPA drench before any preconditioning treatments almost eliminated CA injury. Additionally, 1-MCP before air storage was found to not to cause deleterious effects on the fruits (Contreras et al., 2014). Additional work revealed that conditioning at higher temperatures for shorter periods of time could also effectively suppress CA injury. Fruits can be preconditioned 3 days at 20 to 25 °C before CA storage at 3

kPa O_2 plus 3 kPa CO_2 at 3 °C (Beaudry et al., 2014). However, since this result was based on limited data, it needs to be additionally tested. So far, there has been no safe recommendation emerging from most 'Honeycrisp' production areas because the effects of these applications have not been consistent (Watkins and Nock, 2012a). Moreover, the mechanisms causing injury to 'Honeycrisp' under CA conditions are not understood. This knowledge may be helpful in finding ways to eliminate CA injury to 'Honeycrisp'.

1.2 Possible causes of CA injury

1.2.1 Cellular energy state shortage

Adenosine 5'-triphosphate (ATP) is the principal molecule for storing and transferring energy in cells. It is considered as the energy currency of the cell because it can be "spent" so that chemical reactions can occur. The adenylate energy charge (AEC) is one way of describing the energy status of a cell. AEC value is equal to [ATP] + 0.5 [ADP])/ ([ATP] + [ADP] + [AMP]) which "represents the relative saturation of the adenylate pool in phosphor anhydride bonds" (Atkinson, 1977). Energy status helps maintain the integrity of cell membranes because adenylate nucleotides play a vital role in the biosynthesis of fatty acids of membrane lipids (Saquet et al., 2003).

At harvest, the fruit respires to breakdown energy-containing compounds and synthesizes ATP for its continual survival (Nelson and Cox, 2013; Taiz and Zieger, 2010). In cytosol, one glucose molecule splits into two pyruvate molecules (from glycolysis). Under normal aerobic respiration, pyruvate is transported into the mitochondrial matrix, decarboxylated, and dehydrogenized to acetyl CoA which is the first substrate in the Krebs cycle. NADH and $FADH_2$ regenerated in Krebs cycle will supply hydrogen to hydrogen carriers and electrons to electron carriers to makes energy available for the synthesis of ATP from ADP and Pi by creating a

proton gradient across the inner mitochondrial membrane. In summary, one glucose molecule makes 36 ATP molecules under aerobic respiration (Taiz and Zeiger, 2010). The impact of higher aerobic respiratory activity can be seen in avocado where ATP levels rose in accordance with the rate of CO_2 production and then declined during storage (Bennett et al, 1987). When the cell limits or lacks oxygen for cytochrome c, the Krebs cycle is hindered. Instead, ATP is synthesized via anaerobic respiration. During anaerobic respiration, one glucose molecule produces only two ATP molecules (from glycolysis). Concurrently, pyruvate from glycolysis is decarboxylated to acetaldehyde which is reduced to ethanol molecules (Nelson and Cox, 2004) (Taiz and Zeiger, 2010).

After harvest, fruit ripens, senesces, and dies. ATP levels are affected by both ripening and senescence. Fresh fruit ripening is an irreversible programmed cell death process of which characteristics have been investigated recently on over-ripening banana (Ramírez-Sánchez et al., 2018). ATP levels declined significantly during senescence and exogenous ATP application reduced browning of litchi skin and delayed senescence of cut carnation flowers (Song et al., 2006b) (Song et al., 2008)(Wang et al., 2013). ATP levels decreased when the apple and pear stored at elevated CO_2 and low O_2 and exposed to CA injury (Saquet et al., 2000).

Inhibition of activities of some enzymes in glycolysis and Krebs cycle under CA condition resulted in a decrease in aerobic respiration rate (Kader, 1989), which can hinder ATP synthesis (Ke et al., 1993). CA has been suggested to cause local ATP deficiency in ‘Kanzi’, ‘Jonagold’ and ‘Braeburn’ apples (Ho et al., 2013a), 'Bartlett' pear (Nanos and Kader, 1993), ‘Conference' pears and ‘Jonagold' apples (Saquet et al., 2000). When ATP levels fall below a critical level, it would probably no longer be sufficient to support life for tulip petals in senescence stage (Azad et al., 2008) and may, therefore, cause cell death that horticulturalists

refer to as disorders which incidence of the severe disorders increased. (Saquet et al., 2000). However, low ATP is may not always cause disorders; under anoxia (0 kPa O_2 with or without CO_2) pears show no damage despite very low ATP levels in the tissue (Veltman and Peppelenbos, 2003).

1.2.2 Fermentative toxicity

Fermentative metabolism does not typically happen under standard CA conditions (Ke et al., 1993). Under severe hypoxic conditions, however, fruit respiratory metabolism will switch to fermentation (Ke et al., 1993). Ethanol and acetaldehyde increased in avocado, pears, lettuce and strawberry (Fernández-Trujillo et al., 1999; Ke et al., 1995; Watkins et al., 1999) under low O_2 (0.25 kPa) and high CO_2 (20 – 80 kPa). Ethanol, acetaldehyde, and methyl esters levels had been increased in 'Conference' pears (Saquet and Streif, 2006), 'Fuji' apples (Lumpkin et al., 2015), and 'Jonagold' apple (Saquet and Streif, 2008) under lower CO_2 concentrations (6 kPa) in combination with 0.5 kPa O_2. It is still unknown if fermentative metabolism is a cause or a result of internal disorders in pome fruit, although a correlation between the browning rate and fermentative volatile level was demonstrated (Lee et al., 2012; Pintó et al., 2001; Volz et al., 1998). In addition, Fernandez-Trujillo et al. (2001) suggested that accumulation of the fermentative volatiles resulted from internal browning of apples. The link between cellular damage and the synthesis of fermentative volatiles may be common in the plant world. There was an accumulation of acetaldehyde and ethanol in red pine and paper birch trees which exposed to stressful conditions such as ozone, sulfur dioxide, freezing temperature, and drought (Kimmerer and Kozlowski, 1982).

1.2.3 Metabolic dysfunction

High concentrations of CO_2 in CA conditions influence carbohydrate metabolic pathways. 10% CO_2 caused an increase in fructose-6-phosphate and a decrease in fructose-1, 6-diphosphate in 'Bartlett' pear (Kerbel et al., 1988). CA conditions also interfered with normal metabolisms of the TCA cycle, causing increases in alanine, galactose, mannitol, sorbitol, and xylose and decreases in malic acid and sucrose (Hatoum et al., 2014). Vandendriessche et al. (2013) found that there is an increase in alanine in 'Braeburn' apple. Alanine accumulation might be the result of, not the cause for, 'Braeburn' cell death (Hatoum et al., 2014).

Accumulated galactose in 'Braeburn' at very early CA storage did not link with senescence, but with cell wall dysfunction, resulting in browning in the cortex (Hatoum et al., 2014). Sorbitol, an indicator of disturbed metabolism, and mannitol, a protector against oxidative damage, accumulated in damaged/brown 'Braeburn' inner cortex (Hatoum et al., 2014). An exogenous application of a high concentration of succinate application on apple peels caused browning of the tissue (Hulme, 1964; Neal and Hulme, 2018). When apple fruit were stored under very high CO_2 level (20%), succinic dehydrogenase activity was obstructed, causing an increase in succinic acid to a level that becomes poisonous to fruit tissues (Fernández-Trujillo et al., 2001; Hatoum et al., 2014; Hulme, 1956). Succinate accumulation, however, has not always been found to be directly related to CO_2 injury (Fernández-Trujillo et al., 2001). To sum up, CA conditions alter carbohydrate metabolism, but it is unknown if the metabolites have direct or indirect effects on fruit damage.

1.2.4 Reactive oxygen species (ROS)

ROS can be destructive or act as signaling molecules to plant cells, depending on their levels. ROS in plants are naturally produced from the electron transport chains of photosynthesis

and respiration. When ROS are maintained under conditions of homeostasis, they will be an effective secondary messenger to help plant cells tolerate environmental stresses such as to low O_2, elevated CO_2, mechanical injury, pathogens, drought, too high or too low temperature (Chomkitichai et al., 2014). Extracellular ATP (eATP), one of the damage-associated molecular patterns (DAMPs) of plants to activate plant defense responses (Martínez-Reyes and Cuezva, 2014), induces an accumulation of ROS by triggering activation of Ca^{2+} and NADPH oxidase in cytosol. However, when ROS level exceeds a threshold of defense mechanisms, it causes "oxidative stress" and eventual death of the cells (Saed-Moucheshi et al., 2014).

1.2.5 Cellular membrane damage

Under oxidative stress, cells can undergo lipid peroxidation, causing alterations in cellular membrane properties, ion leakage, and cellular decompartmentation (Chomkitichai et al., 2014). Proteins, nucleic acids, and enzymes are also damaged by ROS (Chomkitichai et al., 2014). In a review of (Maragoni et al., 1996), phospholipases and lipoxygenases cause loss of function of cellular membrane damage because they change membrane lipid and protein properties. Changes in the expression of genes involved in fatty acid oxidation and cell wall loosening of 'Braeburn' which exposed to browning incidence under CA condition (3 kPa O_2 + 0.7 kPa CO_2) (Mellidou et al., 2014).

Following cellular decompartmentation, phenolics from the vacuole will be oxidized by polyphenol oxidase (PPO) and/or peroxidase (POD) to o-quinones. The accumulation of melanins derived from such quinones results in browning in the litchi fruit skin (Chomkitichai et al., 2014). Cellular membrane damage affected by stresses during CA storage is the main reason for internal browning in pear fruit in the review of (Franck et al., 2007).

1.2.6 Insufficiency in NADH, NADPH, and antioxidants

1.2.6.1. NADH and NADPH

The nucleotides NADH and NADPH [collectively, NAD(P)H] comprise redox energy currency. Glutathione (GSH), a non-enzymatic antioxidant, accumulates in its reduced form when receiving electrons from NAD(P)H via the ascorbate-glutathione cycle. At the same time, the cycle also produces ascorbic acid (Asc), a non-enzymatic antioxidant (Noctor and Foyer, 1998). There are many studies of the roles of NAD(P)H on redox balance in plant cells under osmotic, drought, and pathological stresses. However, its roles in fruits under CA storage has not been much investigated. Under CA conditions, NAD(P)H levels increased in avocado (Ke et al., 1995), 'Conference' pears and 'Jonagold' apples (Saquet et al., 2000). The studies so far have not demonstrated a clear relationship between NAD(P)H pools and stresses caused by CA conditions on fruits.

1.2.6.2. Antioxidants

The antioxidative system in plant cells provides essential protection against oxidative damage in scavenging or detoxification of surplus ROS. There are two kinds of antioxidant: enzymatic antioxidants and nonenzymatic antioxidants. Two vital non-enzymatic antioxidants in plants are ascorbate (Asc) and glutathione (GSH), which are the most abundant low molecular weight antioxidants in cells. They join in the ascorbate-glutathione cycle to reduce H_2O_2 to H_2O (Noctor and Foyer, 1998; Sharma et al., 2012). Asc is considered the most powerful plant antioxidant. If Asc level is below a threshold to scavenge ROS, oxidative stress can damage membranes and cellular constituents, and cause browning in fruits (Veltman et al., 2000). Pome fruits held in CA conditions had decreased Asc, which was associated with the occurrence of browning disorders (Haffner et al., 1997; Veltman et al., 2000, 2003). GSH is another important

antioxidant. In addition to regeneration of Asc via Asc-GSH cycle. GSH can directly eliminate $O_2^{\bullet-}$, $^{\bullet}OH$, and H_2O_2. Under oxidative stresses, glutathione accumulation was dramatically induced (Noctor and Foyer, 1998). Regeneration of GSH did not happen in strawberries under 20 kPa O_2 whether the CO_2 levels: were 40 or 0 kPa (Blanch et al., 2013).

1.2.6.3. Diphenylamine (DPA) and its role as an antioxidant

Due to its antioxidant function, DPA could reduce oxidation of the sesquiterpene α-farnesene, resulting in eliminating superficial scald on apple peel of 'Granny Smith' and 'Crofton' (Huelin and Coggiola, 1970), of 'Cortland' apple (Mir and Beaudry, 1999).

DPA and its hydroxylated derivatives (2-, 3- and 4-hydroxydiphenylamines) also prevented internal browning on 'Braeburn' apples under CA conditions (Lee et al., 2012; Mattheis and Rudell, 2008). DPA suppressed amino acid accumulation (Lee et al., 2012), which would have resulted from enhanced proteolysis during cell death (Muntz, 2007). Consequently, fermentative volatile production of ethyl esters using these amino acids as substrates did not increase in DPA treated 'Fuji' and 'Braeburn' fruit (Lee et al., 2012; Argenta et al., 2002). However, there were no significant differences in fermentative volatiles between DPA-treated and untreated 'Cortland' and 'Law Rome' exposed to 45 kPa CO_2 for 12 days (Fernández-Trujillo et al., 2001). In addition, it is suggested that DPA eliminated the toxic effects of high succinate levels for apples under CA conditions (Lee et al., 2012). In general, DPA application eliminated CA disorders of apples (Contreras et al., 2014; Lee et al., 2012; Mattheis and Rudell, 2008). However, the mechanism whereby DPA eliminates CA disorders in the fruit is still unknown.

1.3 Hypothesis of mechanisms causing CA injury in 'Honeycrisp' apple

We hypothesize that CA storage may cause a shortage of adenylate charge (AEC), redox energy compounds, and/or antioxidants in 'Honeycrisp' apple. We anticipate that the pools of these metabolites may not be sufficient for cellular survival under the stressful conditions of high CO_2 and low O_2 and the associated cell death leads to a loss in tissue compartmentation and browning of the affected tissues. We propose that diphenylamine (DPA), which protects the fruit from CO_2 damage, does so by maintaining Asc above a threshold level and succinic acid below a critical threshold in the fruit cells.

1.4 Objectives

To study mechanisms by which CA storage conditions cause physiological injury to 'Honeycrisp' apple fruit, we investigated:

1) CA conditions that cause physiological injury to 'Honeycrisp',
2) Metabolite pools of essential processes in tissues injured by CA conditions,
3) The role of the antioxidant DPA in suppressing changes in metabolite pools,
4) The role of the preconditioning in suppressing changes in metabolite pools, and
5) Mode of actions causing cell death in cortical tissues under CA conditions.

1.5 Summary of research methodology

In Chapter 2, to find out how quickly CO_2 injury symptoms developed inside the apple cortex and the dose response of the injury to CO_2, CA conditions were established that would yield a range of injury symptoms. 'Honeycrisp' fruit were stored under 5 CO_2 levels (0, 1.5, 3, 5, 10, and 20 kPa) at 3 kPa O_2 at 3 °C. We also studied low O_2 levels (0.1 – 0.4 kPa) at 0 kPa CO_2 at 3 °C. In addition, the antioxidant diphenylamine (DPA) was used to suppress symptom development and the impact of this chemical control measure on the cellular metabolic pool and

in the presence of otherwise toxic CO_2 levels was evaluated. We evaluated the use of the ethylene action inhibitor 1- MCP and the use of preconditioning treatments on CA injury and fruit quality. Internal and external disorders of the treatments were analyzed during storage.

In Chapter 3, we analyzed the association of injury with emissions of fermentative volatiles (ethanol, acetaldehyde, and ethyl acetate).

In Chapter 4, we studied the impact of CO_2, DPA, and preconditioning on 15 important metabolites, using tissue samples collected before symptom development, at the onset of injury, at half maximal injury development, and at maximal injury development. The metabolites include adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), reduced nicotinamide adenine dinucleotide (NADH), oxidized nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), oxidized nicotinamide adenine dinucleotide phosphate (NADP), ascorbic acid (Asc), glutathione (GSH), glutathione disulfide (GSSG), coenzyme A (CoA), acetyl coenzyme A (acetyl CoA), succinic acid (SA), phosphoenolpyruvate (PEP), and uridine diphosphate glucose (UDP-G).

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CHAPTER 2.

INJURY OF ‘HONEYCRISP’ APPLES CAUSED BY CA STORAGE AND APPROACHES TO REDUCE THE INJURY

2.1 Introduction

2.1.1 CA injury

Since commercial production of ‘Honeycrisp’ apple (*Malus* × *domestica*) began in 1991, the cultivar has become a favorite for consumers because of its crisp texture and unique flavor. In the U.S. its growing area has increased in Michigan, New York, and Washington (National Agricultural Statistics Service, 2011, 2012, 2015). The demand for 'Honeycrisp' apples has led to the need to extend the marketing season. Long-term storage is needed for this apple to meet the demand. Low temperature storage and the use of controlled atmosphere (CA) storage are two technologies that normally prolong the storage life of apple fruit by apple industry. However, ‘Honeycrisp’ fruit is very sensitive to low temperature and CA conditions and can be severely damaged in storage (Beaudry and Contreras, 2009; Contreras et al., 2014; Watkins and Nock, 2012b).

Two common low temperature disorders in 'Honeycrisp' are soggy breakdown and soft scald (Beaudry and Contreras, 2009; Watkins and Rosenberger, 2000). The injury caused by CA conditions is called CA-related injury (i.e., CA injury), which, in some cases, is very similar in appearance to soggy breakdown. It is characterized by brown lesions/patches in the fruit cortex, often with irregular edges and sometimes with the inclusion of lens-shaped openings in the brown lesions (Beaudry and Contreras, 2009; Contreras et al., 2014). The symptom of CA injury has been described in detail by Beaudry et al. (2014) and can be distinguished to chilling injury in both appearances on the apple cortex and fermentative scent. CA injury is also considered as CO_2 injury since high CO_2 plays as a driving factor. and low O_2 combined in CA intensifies the symptom (Plagge, 1929).

2.1.2 Preconditioning treatment

"Delayed cooling" or "pre-storage conditioning" is another term of preconditioning. If 'Honeycrisp' apples was kept at a temperature about 10 – 15 °C higher than long-term storage temperature (i.e. about 3 °C) for several days before stored in CA room, soft scald and soggy breakdown symptoms reduced (Beaudry et al., 2010; DeLong et al., 2004b; Watkins and Nock, 2003; Watkins et al., 2004). These injuries can be reduced by storage at 3 °C and by conditioning the fruit by holding for several days at 10 to 20 °C prior to CA storage (Beaudry et al., 2010; Moran et al., 2010; Watkins and Nock, 2012a). Preconditioning was also applied on the fruit to reduce CA injury (Beaudry and Contreras, 2009; Beaudry et al., 2014; Contreras et al., 2014; Leisso et al., 2017; Moran et al., 2010; Watkins and Nock, 2012b). However, a repeated preconditioning experiment is necessary for preventing chilling injury and CA injury in the cultivar.

2.1.3 DPA treatment

Diphenylamine (DPA), an arylamine antioxidant, can be an important tool in preventing CA injury to 'Honeycrisp', a CA sensitive cultivar. DPA drench almost completely eliminated the disorder of the apples when stored at 3% O_2 and 3% CO_2 (Contreras et al., 2014). It also successfully eliminates superficial scald on the skin of 'Granny Smith' and 'Crofton' apple because it can suppress auto-oxidation of α-farnesene which is a causative agent of superficial scald because of its antioxidant properties (Huelin and Coggiola, 1970).

DPA (1000 $\mu L \cdot L^{-1}$) drench before any preconditioning treatments almost completely eliminated CA disorder of the 'Honeycrisp' apples under 3% O_2 and 3% CO_2 (Contreras et al., 2014). DPA and its derivatives also prevented internal browning on 'Braeburn' apples under CA conditions (Lee et al., 2012; Mattheis and Rudell, 2008). Our preliminary experiments showed

that diphenylamine (1000 $\mu L \cdot L^{-1}$ or ppm in 30s) could prevent CA injury in Honeycrisp' apple. We found that even 250 ppm DPA (1/4 label dosage) DPA was also effective at suppressing CA injury for the fruit stored in 5 kPa CO_2 (data not shown). We need to learn the limitations for DPA concentration needed to protect 'Honeycrisp' apples against CA injury for standard CA conditions.

2.1.4 1-MCP

DPA residues on fruit are not accepted in European Union countries even at very low doses (Calvo and Kupferman, 2012). Therefore, use of 1-MCP, an ethylene antagonist, can extend the storage life of apple fruit and might avoid CA injury caused by CA conditions because the fruit can be stored in air (21 kPa O_2 + 0 kPa CO_2). The application of the ethylene antagonist 1-methylcyclopropene (1-MCP) can extend the storage life of apple fruit. 1-MCP has been commercially applied in the apple industry since 2002 under the commercial name SmartFresh^{TM} (AgroFresh Inc., Spring House, PA, USA) (Beaudry and Watkins, 2003). The advantage of 1-MCP is that it can strongly and, apparently, permanently bind to ethylene receptors at very low concentrations (from 0.005 – 1 ppm), depending on the exposure durations of apples to the compound (Beaudry and Watkins, 2003; Sisler et al., 1996). With a single dose treatment of 1-MCP to 'Honeycrisp' apples before air storage, production of ethylene in the fruit dramatically reduced relative to the control (DeEll and Ehsani-Moghaddam, 2012; Watkins and Nock, 2012a). Consequently, fruit ripening and senescence were retarded (Watkins and Nock, 2012a).

Ethylene receptors, however, can continue to be produced in fruit tissues especially when the fruit is at climacteric (Nakatsuka et al., 1998). To maintain the effectiveness of 1-MCP in blocking ethylene receptors, multiple applications of the compound were applied to

‘Northern Spy’, ‘Empire’, and ‘McIntosh’ apples (DeEll et al., 2016), and ‘Redchief Delicious’ apples (Mir et al., 2001) and found to inhibit ripening significantly better than single applications. Similarly, continuous application of 1-MCP delays color development for tomatoes especially at breaker stage (Mir et al., 2004) and 2 doses of 1-MCP application on the apples helped maintain their firmness (DeEll et al., 2016).

A single dose treatment of 1-MCP has been applied to ‘Honeycrisp’ apples before air storage to reduce ethylene production (DeEll and Ehsani-Moghaddam, 2010; Watkins and Nock, 2012b). For further understanding role of 1- MCP in inhibiting ripening on the fruit, a multiple application need to be researched.

'Safe' recommendations for the CA storage of 'Honeycrisp' so far have not emerged because of inconsistent control of CA injury (Watkins and Nock, 2012b). To elucidate safe recommendations of using CA storage for this cultivar, we need to better understand the responses of 'Honeycrisp' fruit to CO_2 in the storage environment. Aims of this study were: 1) to know the dynamics of CA injury of ‘Honeycrisp’ apples in response to variations in CO_2 concentrations and 2) to improve current practices including DPA, preconditioning, and 1-MCP treatments. Experiments were focused on dose-response relationships between CO_2 concentration and DPA concentration, dose-response relationships between preconditioning temperature and preconditioning duration, and the capacity for use of 1-MCP to preserve fruit quality without the use of CA storage.

2.2 Materials and Methods

2.2.1 Plant material

‘Honeycrisp’ apples were harvested at commercial maturity (i.e., at the time of the primary harvest by the growers from whom the apples were sourced) in 2014, 2015, and 2016 in

Michigan. At each orchard, two 18-bushel bins of fruit were harvested in the morning. Fruit was transferred to 60 x 40 x 18 cm plastic crates (model 5000206, Twinpack B.V., Netherlands) and immediately transported to the Postharvest Physiology Laboratory at Michigan State University. At the onset of each experiment, 20 fruits from each orchard were used for maturity analysis on the day of harvest (day 0) and after one week at room temperature (day 7). Maturity indices included the fruit weight (kg), ethylene (ppm), background color (1 -5) and skin color (%), starch index (1-8) and total soluble solids (°Brix). Methods of measuring the indices were performed as previously described by Contreras et al. (2014).

2.2.2 Experiment 1. Effect of CO2 on the severity of CA injury of the fruit

The relationships between CO_2 concentration and the rate and severity of CA injury were determined over three years (2014 – 2016) using the protocol outlined in Fig. 2.1 and Table 2.1. The storage temperature of 3 °C was used to avoid/suppress chilling injury symptoms and thereby isolate CA injury symptoms. Similarly, the oxygen concentration was maintained at 3 kPa to reduce hypoxia-related fermentation and to determine only the effect of CO_2 on CA injury.

In 2014, fruit from five orchards were used. On the day of harvest, 30 crates (approximately 40 fruits per crate) of apples from each orchard were divided into six lots (i.e., five crates per lot). The five crates were placed into each of six CA chambers (Storage Control Systems, Sparta, MI) and held under CO_2/O_2 partial pressure (kPa) combinations of 0/3, 1.5/3, 3/3, 5/3, 10/3, and 20/3, respectively, at 3°C.

The atmospheres in CA chambers were monitored and regulated by an atmosphere control system (ICA 61 Laboratory System; International Controlled Atmosphere Ltd., Paddock Wood, U.K.). Chamber temperature (3 °C) was regulated by the cold room in which the

chambers were held. There were 13 sampling dates (day 7, 14, 21, 28, 35, 42, 49, 56, 70, 84, 112, 140, 168, and 240). At each sampling date, 10 – 20 fruits from each treatment from each orchard were taken out of the chamber to be assessed for incidence and severity of storage disorders (Fig. 2.1). Total storage duration was 6 months or until all sampled apples had been damaged depending on CO_2 treatments. A symptom of internal browning and the development of lens-shaped voids were evaluated as described by Beaudry and Contreas (2009).

In 2015, fruits from each orchard were put into 20-L buckets connected to the CA system described previously to obtain for 4 CO_2 levels (0, 5, 10, and 20 kPa) in combination with 3 kPa O_2 at 3 °C. There were two replicates for each CA condition.

In 2016, fruits were stored in two CO_2/O_2 combinations of (0/3 and 5/3) at 3 °C in CA chambers as previously described, using 2 chambers for each CO_2 concentration as replicates. Internal disorders included CA injury, CA injury index, lens-shaped voids, and senescent breakdown. External ones consisted of fruit bitter pit, decay, and soft scald. CA injury in the fruit cortex was categorized into four levels: 0 (0%); 1 (1 - 10%); 2 (10-25%); 3 (25 - 50%); 4 (> 50%) of browning area on the cut surface of each fruit (Table 2.1).

Each year, experiments consisted of completely random split-plot designs in which CO_2 was the whole plot treatment factor with two CA chambers (2014 and 2016) and two buckets (2015) as replicates. Storage day was a split-plot treatment factor. Since we used different fruit trays at each sampling time, this observational unit was treated as a random factor. All data for the variables of the experiments were subjected to test normality and assumptions for ANOVA using SAS 'Proc mixed' procedure (Version 9.4; SAS Institute Inc., Cary, NC). Mean separations are examined using Duncan's multiple range test and only differences significant at $P \leq 0.05$ are discussed

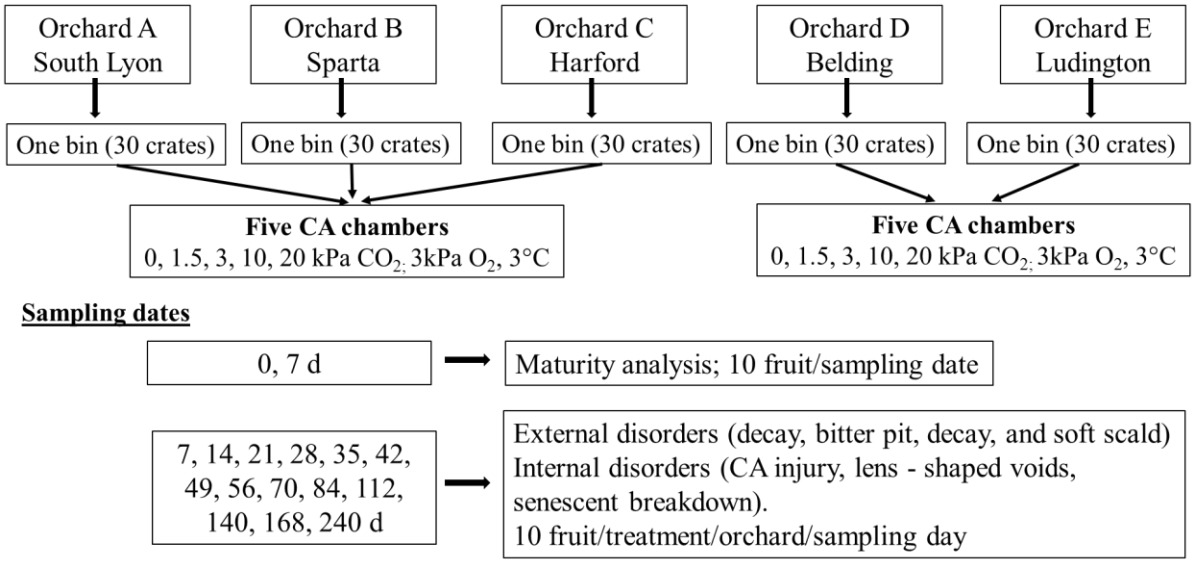

Figure 2. 1. Unbalanced completely random split-plot design using five partial pressures of CO_2 (0, 1.5, 3, 10, and 20 kPa) in combination with 3 kPa O_2 at 3 °C for 'Honeycrisp' apple from five commercial orchards in Michigan in 2014. Samples were used for analysis of external and internal disorders.

Table 2. 1. CO_2 concentrations applied, sampling dates, and storage disorders assessed for 'Honeycrisp' apple fruit in 2014 – 2016.

Year	CO_2 level (kPa)	Sampling dates (Days in CA)	Observations
2014	0, 1.5, 3, 10, 20 *	7, 14, 21, 28, 35, 42, 56, 70, 84, 112, 140, and 168	Internal disorders; External disorders
2015	0, 5, 10, 20	3, 7, 14, 21 and 42	Internal disorders
2016	0, 5	3, 7, 14, 21, 28, 35, 42, 56, and 140	Internal disorders

**5 kPa CO_2 was also applied but the CA system failed to maintain the desired atmosphere.*

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2.2.3 Experiment 2. Effect of DPA on CA injury of the fruit

The ability of DPA to suppress CO_2 injury across a range of CO_2 concentrations was evaluated. In 2014, fruit at commercial maturity from three orchards were harvested and handled as previously described. Fruit from each orchard were divided into 24 lots of approximately 40 fruit each and each lot was placed in a plastic crate. Apple fruit were treated with DPA (1000 ppm, drenched for 30 s and air dried) and 12 lots each were stored under 3 or 10 kPa CO_2 in combination 3 kPa O_2 at 3 °C (Fig. 2.2). One lot of fruit was evaluated for storage disorders after 7, 14, 21, 28, 35, 42, 56, 70, 84, 112, 140, and 168 d storage.

In 2015, fruit to more precisely understand the relationship between the concentrations of CO_2 and DPA on the development of injury symptoms, we tested a matrix of two factors: CO_2 levels (0, 5, 10, and 20 kPa) and DPA concentrations (1, 10, 50, 100, 250, and 1000ppm) (Fig. 2.3). at commercial maturity from five orchards were harvested and handled as previously described. Fruit from each orchard were divided into 72 lots of approximately 40 fruit each and each lot was placed in a plastic crate. Twelve lots (crates) were drenched in each of six DPA concentrations (1, 10, 50, 100, 250, and 1000 ppm a.i.) for 30 s. The fruit were dried for approximately two hours then stored in each of four CA chambers with CO_2 levels of 0, 5, 10, and 20 kPa and held at 3 °C. One lot of fruit from each treatment combination was evaluated for storage disorders after 42, 90, and 180 d storage.

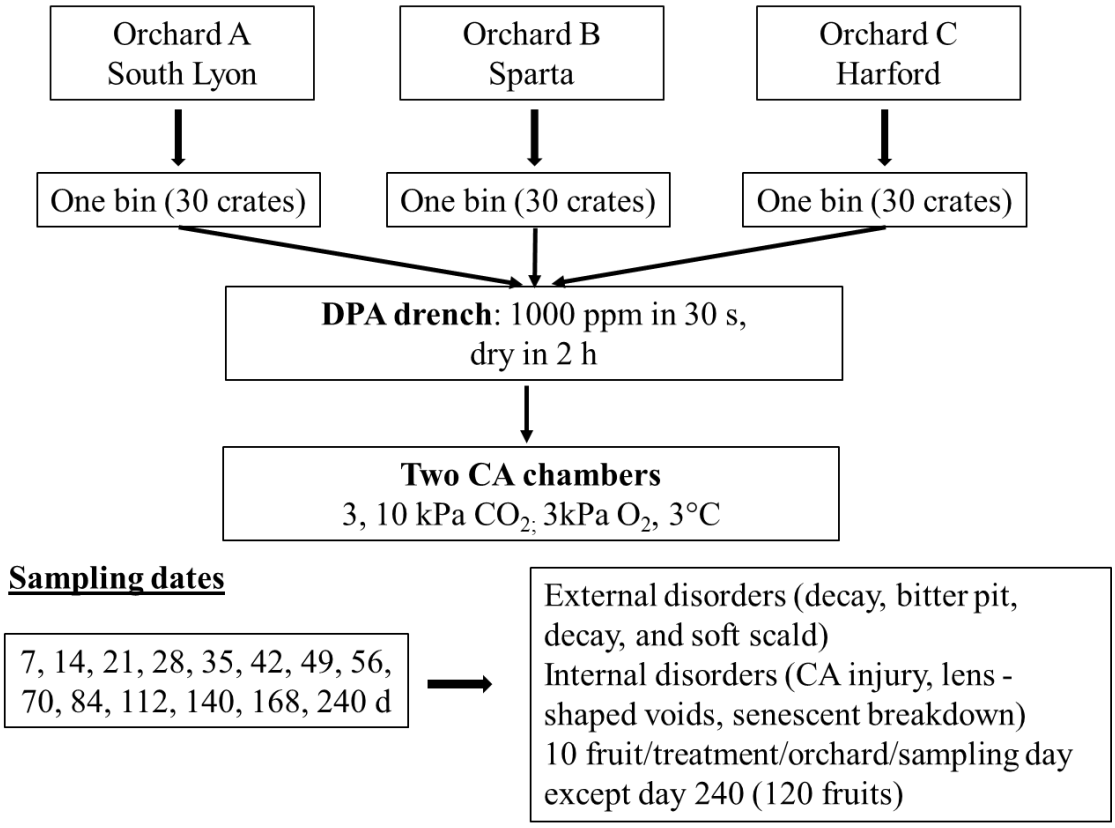


Figure 2. 2. Experiment design of the 'Honeycrisp' apple fruit harvested from three commercial orchards in Michigan in 2014, treated with DPA (1000 ppm, 30s), and then stored under 3 and 10 kPa CO_2 in combination 3kPa O_2 at 3 °C. Samples were used for analysis of external and internal disorders.

The ability for 1-MCP in air storage to suppress ripening was evaluated as a means of avoiding the use of CA storage and incurring injury due to the storage atmosphere. In 2015, in a preliminary experiment, we applied a single dose treatment of 1-MCP to ‘Honeycrisp’ apples before air storage; production of ethylene of the fruit was dramatically reduced relative to the control (data not shown) and the results were consistent with a previous study on the same cultivar (DeEll and Ehsani-Moghaddam, 2010; Watkins and Nock, 2012b). In 2016, to maintain the effectiveness of 1-MCP in blocking ethylene receptors, multiple applications of the compound were applied. The methods and results are presented in detail in a manuscript titled

"Response of air-stored 'Honeycrisp' apple fruit to repeated application of 1-MCP" for ISHS Postharvest Unlimited Conference 2017 in the appendix A of CHAPTER 2. Single (at harvest), double (at harvest and after 1.5 months) and triple (at harvest and after 1.5 and 3 months) applications of 1-MCP were given to 'Honeycrisp' apples harvested from four orchards across Michigan in 2016 before storing the fruit in air (21 kPa O_2 + 0 kPa CO_2) at 3 °C.

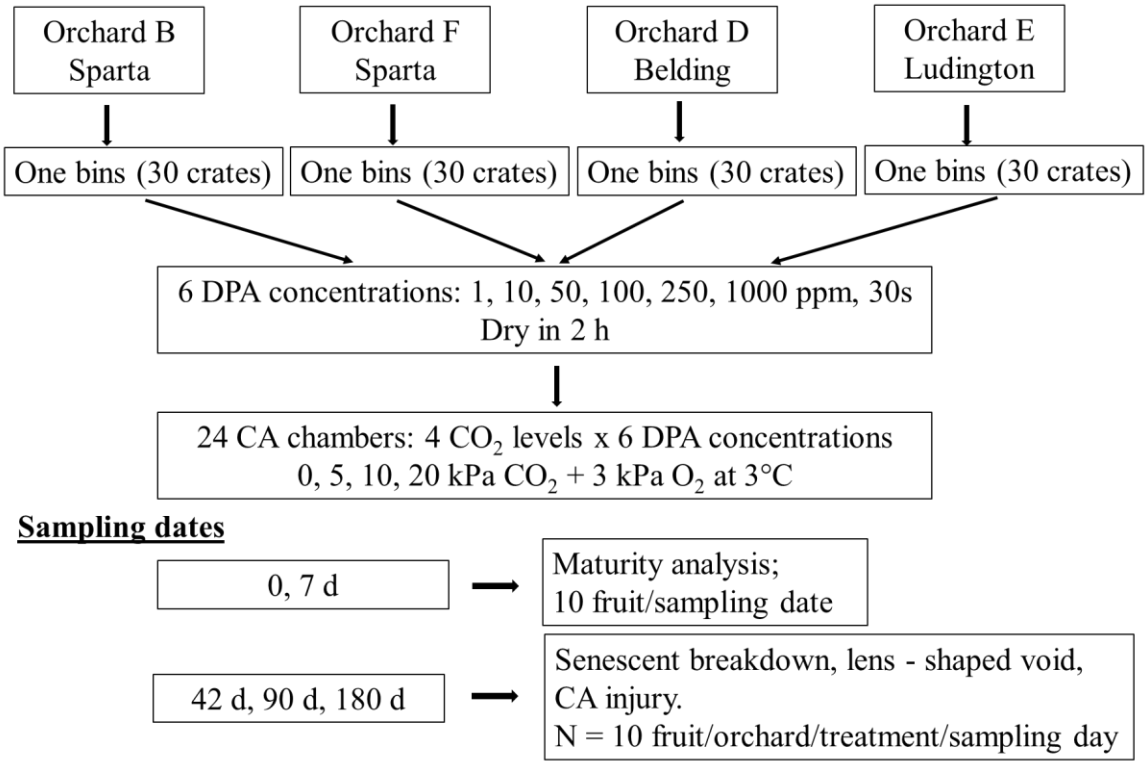


Figure 2. 3. Experimental design of 24 matrix treatments of two factors for the storage of 'Honeycrisp' apple fruit: 1) DPA concentrations (1, 10, 50, 100, 250, and 1000 ppm) and 2) CO_2 levels (0, 5, 10, and 20 kPa) for the fruit harvested from four commercial orchards in 2015.

2.2.4 Experiment 3. Using 1-MCP in air storage to substitute for CA storage

In 2017, fruit from two orchards were obtained at commercial maturity. Fruit were given 0, 1, 2, or 4 doses of 1-MCP (1 $\mu L \cdot L^{-1}$) while in air storage at 3 °C. The first dose was applied after 15 days storage with additional treatments at 15-day intervals, fruit receiving only 3 doses

were not evaluated; all were also given a fourth dose. Fruit were held in 0/21 and 3/3 combinations of O_2/CO_2 partial pressures (kPa) (Fig. 2.4). Ethylene, selected volatile esters (ethyl acetate, hexyl acetate, butyl acetate, butyl 2-methylbutanoate, and 2-methylbutyl acetate), and greasiness were used as measures of ripening behavior. These indices were measured after 1.5, 3, 4.5, 6, and 9 months of storage. The methods of analysis and quantification of the indices were the same as methods section which was presented in detail in the appendix A of CHAPTER 2.

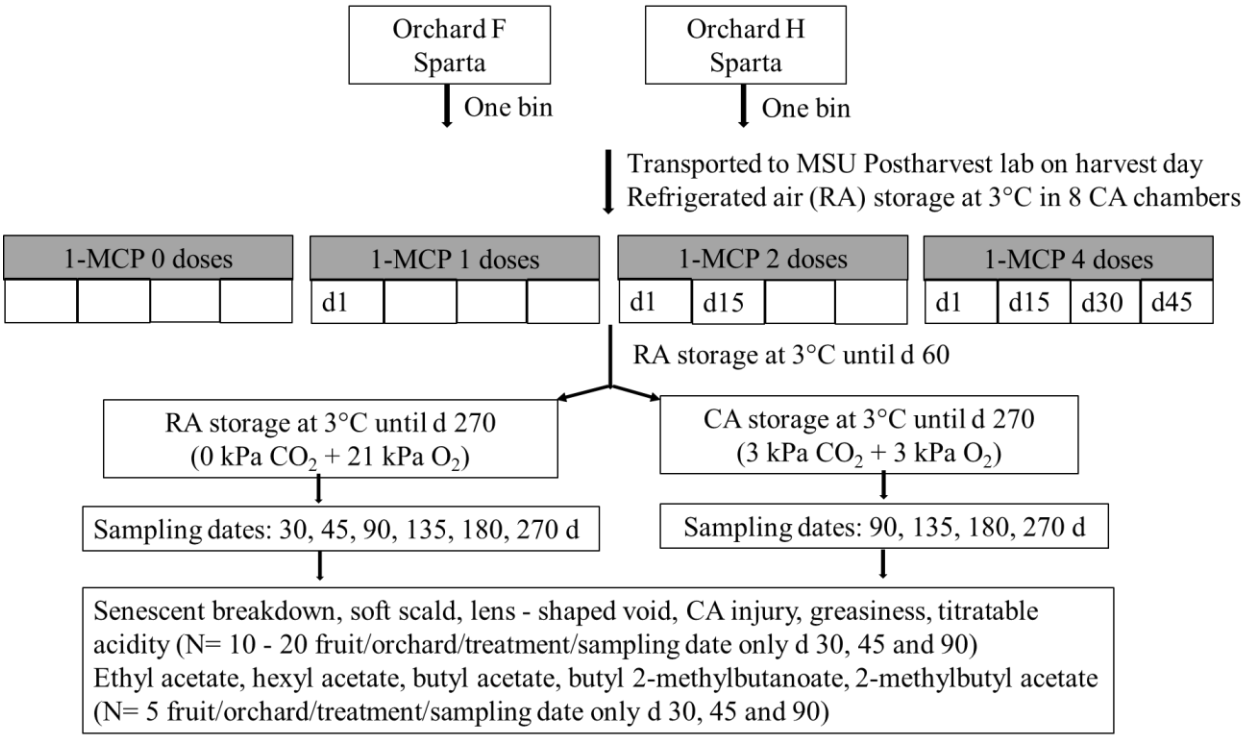


Figure 2. 4. Experimental design of eight matrix treatments of two factors: 1) 1-MCP application dose (0, 1, 2, 4 doses with 15-day interval each treatment); and 2) Atmosphere condition (Air: 21 kPa O_2+ 0 kPa CO_2 and CA: 3 kPa O_2+ 3 kPa CO_2) for the fruit harvested from two commercial orchards in 2017.

2.2.5 Experiment 4. Effect of preconditioning and the combination of preconditioning and DPA on CA injury of 'Honeycrisp' apple fruit

Eight matrix treatments of three factors: 1) DPA (0 and 1000 ppm), 2) preconditioning (0 and 5 days at 10°C), 3) CO_2 levels (0 and 5 kPa) were set up for fruit harvested from four commercial orchards across Michigan in 2016. The fruit was evaluated for disorders after 7, 14, 28, 56, and 120 days (Fig. 2.5).

In 2017, fruit from two orchards in Sparta, Michigan were treated with DPA, kept at the lab at two or five days at 20 °C, and then stored at two CA conditions (5 kPa or 3 kPa CO_2 + 3 kPa O_2) at 3 °C. After 4.5 and 9 months of storage, disorders were recorded (Fig. 2.6).

In the same year, we implemented a preconditioning experiment in the field for 0, 1, 3, 5, and 7 days and in the lab for five days at 20 °C (Figure 2.7) and stored at five CO_2 levels (0, 3, 5, 10, and 20 kPa CO_2) in CA conditions at 0 and 3 °C. Storage disorders, greasiness, and titratable acids were evaluated after 120 days of storage. In total, there were 30 treatments (6 preconditioning levels x 5 CO_2 levels) and two bins of apples were used as replicates. The greasiness, soft scald, and CA injury were tested after 4.5 months of storage.

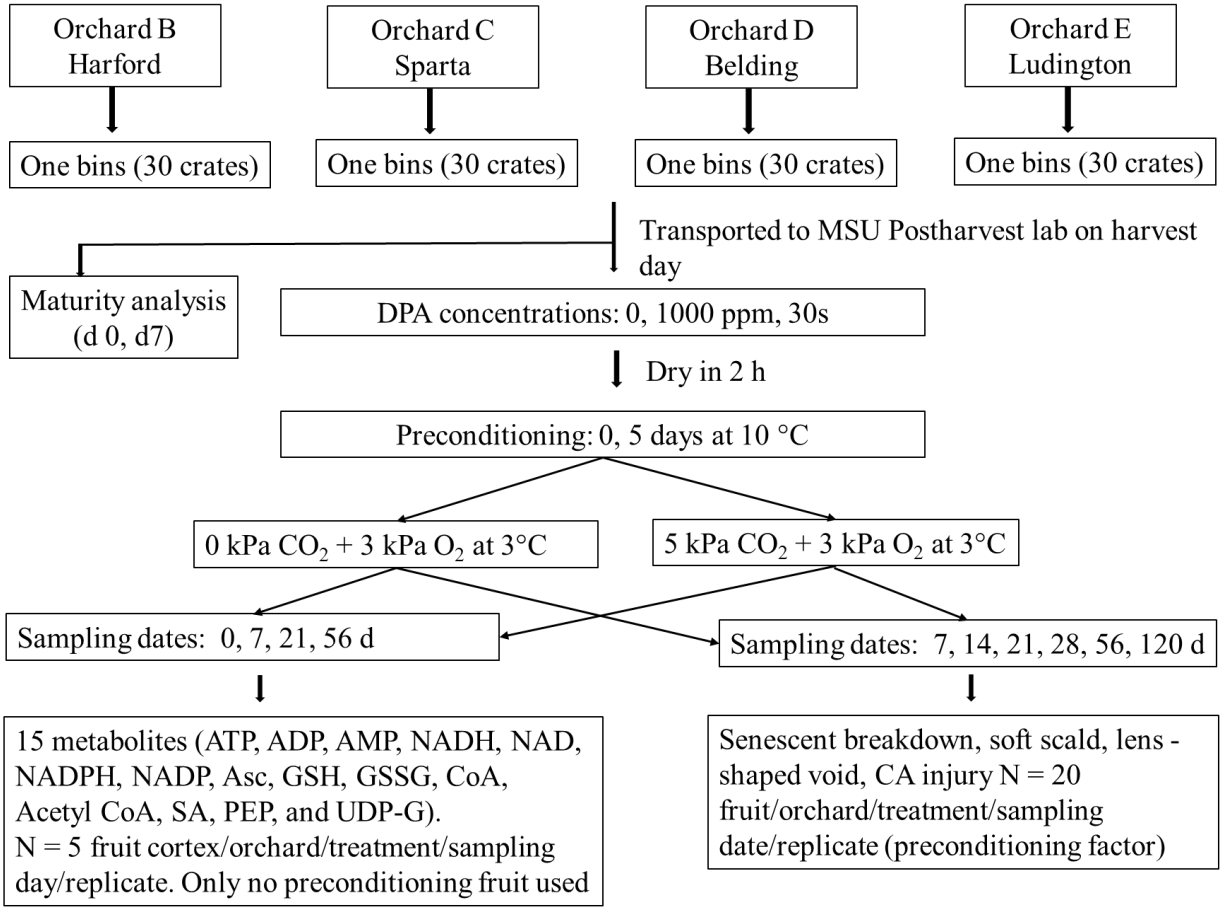


Figure 2. 5. Experimental design of a matrix of eight treatment combination s of three factors: 1) DPA (0 and 1000 ppm), 2) preconditioning (0 and 5 days at 10°C), 3) CO_2 levels (0 and 5 kPa) for the fruit harvested from four commercial orchards in 2016.

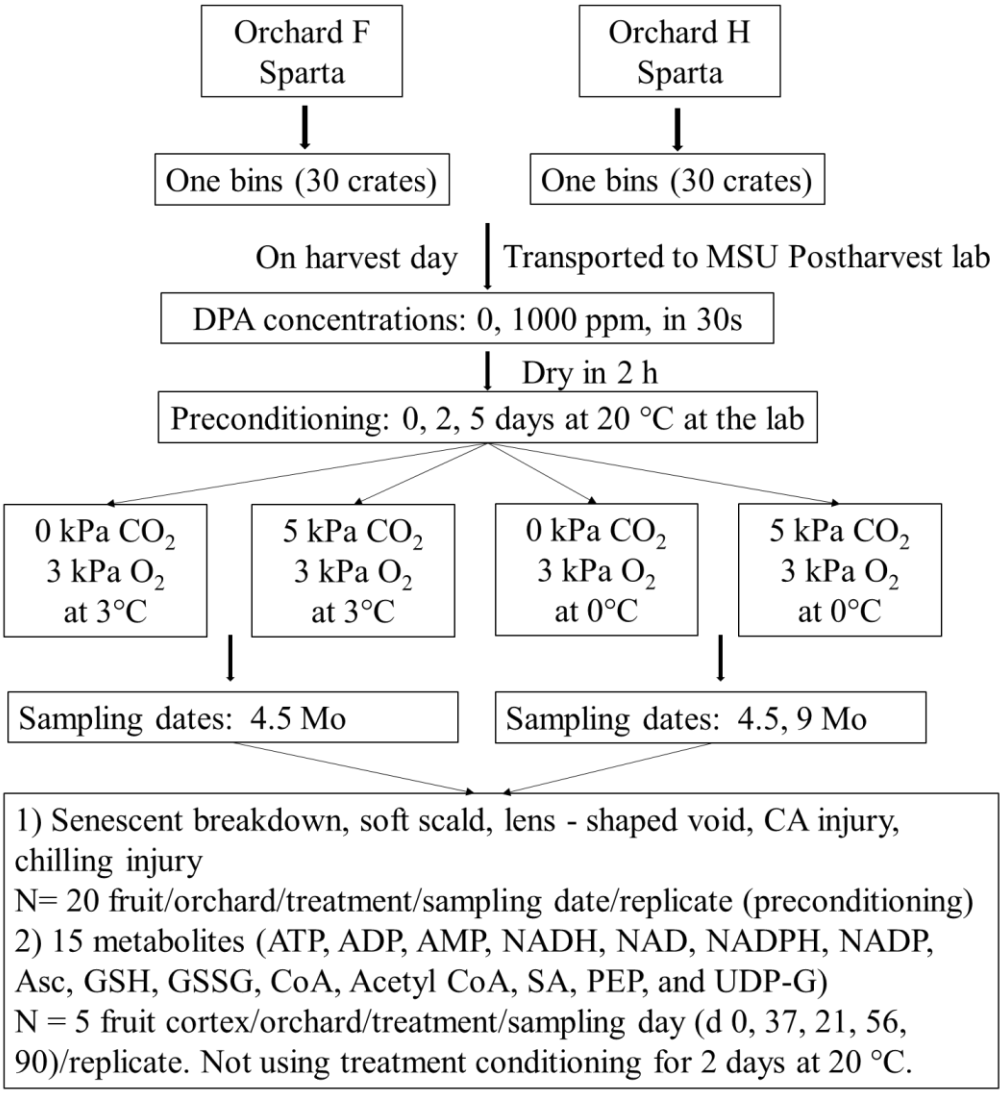


Figure 2. 6. Experimental design of 24 matrix treatments of four factors: 1) DPA levels (0, 1000 ppm), 2) preconditioning (0, 2, 5 days at 20 °C), 3) CO_2 levels (0, 5 kPa CO_2), and 4) storage temperatures (0, 3 °C) for the fruit harvested from two commercial orchards in 2017. Two crates of fruit for each treatment for precondition factor were used as replicates.

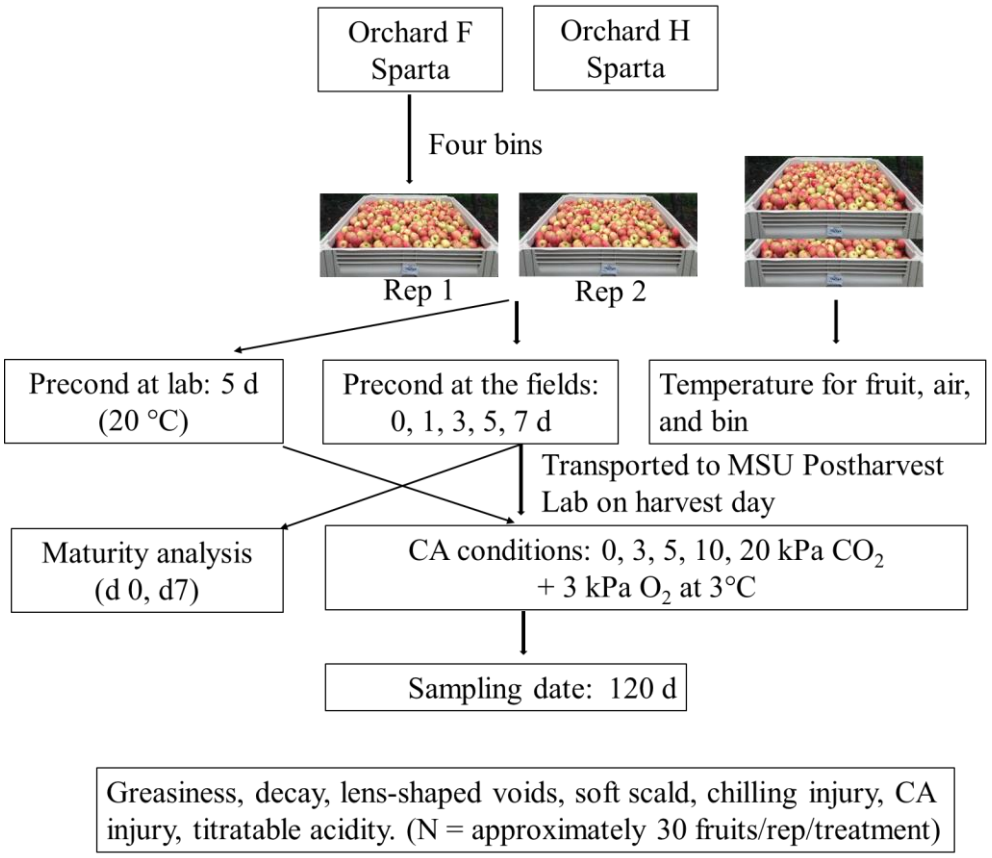


Figure 2. 7. Experimental design of 30 matrix treatments of two factors: 1) Preconditioning in the field for 0, 1, 3, 5, 7 days and at the lab for 5 days; and 2) CO_2 levels (0, 3, 5, 10, 20 kPa CO_2) for 'Honeycrisp' fruit harvested from two commercial orchards in 2017. Two crates of fruit for each treatment for precondition factor were used as replicates.

2.2.6 Experiment 5. Effect of hypoxia on CA injury of the fruit

In 2017, we performed an additional experiment to test the effect of low oxygen on CA injury using ‘Honeycrisp’ apples after 3 months of refrigerated air storage. The fruits were put into 20-L plastic buckets fitted with an airtight gasket-sealed lid (Gamma Plastics Company) and flushed with nitrogen gas at a flow rate 20 mL min^{-1} to achieve three oxygen levels 0.1, 0.2, and 0.4 kPa. Control fruits were from a CA chamber in which oxygen concentration was maintained

at 21 kPa. In each environment, the CO_2 partial pressure was 0 kPa and storage temperature was 3 °C.

2.3 Results and discussions

2.3.1 Experiment 1. Effect of CO2 on the severity of CA injury

In response to applied CO_2 during storage, jagged-edged brown lesions appeared in the central region of the 'Honeycrisp' apple cortex tissues within the first two months of storage (Fig. S-B2.2), consistent with damage reported by Contreras et al. (2014). Early in browning injury development, the 'Honeycrisp' apple injury symptoms were brown lesions in the cortex possessing white areas near the center of some of the lesions (Fig. 2.8).

The extent of CA injury of ‘Honeycrisp’ apples was positively correlated with CO_2 concentration and storage period (Fig. 2.8 and Fig. S-B2.2). The onset of injury development was most rapid for the 20 kPa CO_2 treatment where the injury was first noted after 7 days of storage (Fig. 2.8). In this treatment, 75% of the fruit under 20 kPa CO_2 were injured after 14 d, while no injury symptoms were noted for the 0 kPa CO_2 treatment (Fig. 2.8). 100% of the fruit treated with 20 kPa CO_2 were damaged after 28 d (Fig. 2.8). However, even at 0 kPa CO_2, the fruit also suffered CA injury incidence of up to 18 % (Fig. S-B2.2). The effect of CO_2 concentration on the severity level of CA injury was relatively consistent over the three years of the study (Fig. 2.9).

Figure 2. 8. ‘Honeycrisp’ apples from orchard A stored at 0 kPa CO_2 (left) and 20 kPa CO_2 (right) after 14 days.

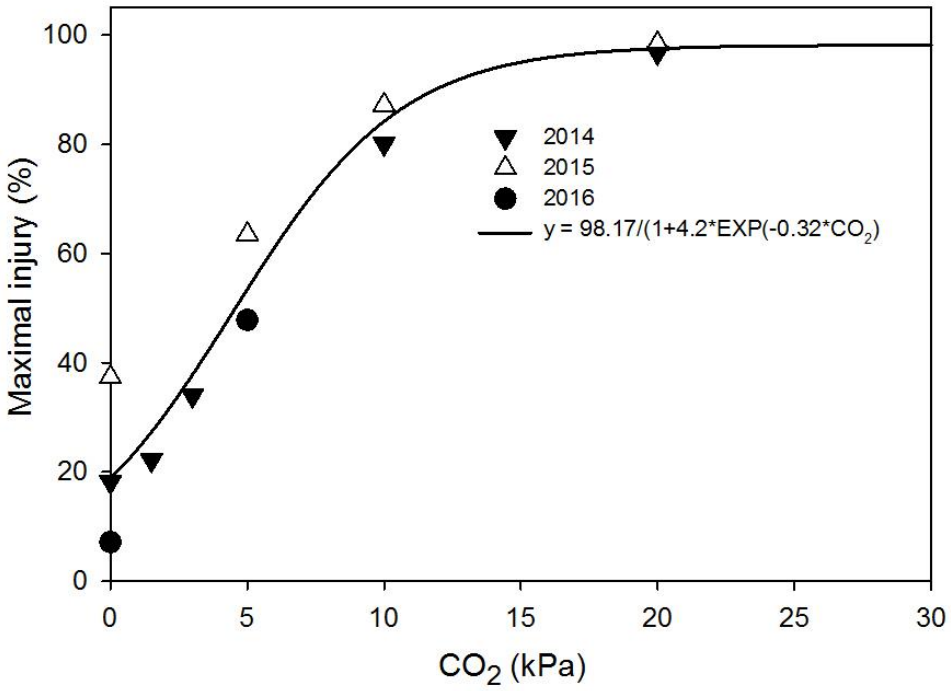


Figure 2. 9. The relationship between CO_2 concentration and maximal injury on ‘Honeycrisp’ apples based on data from 2014 -2016. The curve fit equation was made using Proc Univariate and 'Proc nlmixed' procedures in SAS 9.4 (AIC = 84.6), P< 0.0001.

The rate of the development of the CA injury was highly dependent on CO_2 concentration. Depending on CO_2 concentrations, the fruit reached half-maximal and maximal injury severity at different storage days (Table 2.2, Fig. S-B 2.2). The practical implication is that CA managers should check for CA injury symptom after two weeks of storage and decide whether to sell the fruit immediately in order to avoid excessive loss due to CA injury disorders.

Table 2. 2. The storage time for 'Honeycrisp' apples stored at different CO_2 levels needed to achieve half-maximal and maximal CA injury

CO_2 (kPa)	Half-maximal injury	Maximal injury
0	Week 4-5	Week 11-12
1.5	Week 4-5	Week 11-12
3	Week 3	Week 8
5	Week 3	Week 7
10	Week 2	Week 7
20	Week 2	Week 4

There was no significant difference in external disorders (bitter pit, decay, soft scald) and lens-shaped void incidence among CO_2 levels (0, 1.5, 3, 10 kPa) of the fruit that had received maximal injury (Fig. 2.10 - A2 to D2). 20 kPa CO_2 quickly caused 100% damage of the fruit at early storage. Therefore, we did not observe disorders other than bitter pit, which was noted after only one week of storage at all CO_2 treatment (Fig. 2.10 - B2).

Since there was no effect of CO_2 on these indices, we tested if they will change with storage time (from day 0 to 240) by using average data for each index of five CO_2 levels (0, 1.5, 3, 10, and 20 kPa) at each sampling date. Decay, soft scald and lens-shaped void incidences of the fruit significantly increased with storage time (Fig. 2.10 - A1, C1, and D1). However, maximum severity of the decay, soft scald, and lens-shaped cavity incidences were only about 10, 5, and 20%, respectively (Fig. 2.10 - A1, C1, and D1). Lens-shaped voids were formed after browning incidence occurred in the cortex. In this experiment, this symptom was first noted

when the fruit started to reach maximal CA injury, and eventually, it increased with storage time (R^2 = 0.83, Fig. 2.10-D1). Only approximately 2% of sampled fruit (i.e. 27 out of 1323 fruits) after 168 and 240 days of storage had senescent breakdown symptom (data not shown).

2.3.2 Experiment 2. Effect of DPA on CA injury of the fruit

In 2014, in response to 1 $g \cdot L^{-1}$ a.i DPA, the fruit showed no CA injury at any CO_2 level, compared to control fruit stored at 3 kPa and 10 kPa CO_2 which received 34% and 78% maximal damage, respectively (Fig. S-B2.2). We also observed that at 250 ppm DPA (1/4 label dosage) DPA was also effective at suppressing CA injury for the fruit stored in 5 kPa CO_2 (data not shown).

In 2015, we found that 100 ppm of DPA was enough to eliminate CA injury at 0 kPa CO_2 and a higher dose of DPA was required for fruit stored at more elevated CO_2 levels (Fig. 2.11). 20 % CO_2, however, was too high to prevent injury by 1000 ppm of DPA. We predicted ~ 2000 ppm DPA would be required to suppress CA injury caused by 20 kPa CO_2 (Fig. 2.12 - B). The trendline in Fig. 2.12A indicates that 75 – 185 ppm of DPA was effective enough to eliminate CA injury because the CO_2 level commercially applied in most CA storage was between 1 and 3 kPa. Thus, storage operators may be able to reduce DPA residue on the fruit. DPA use is restricted or not permitted in some countries, so minimizing residue levels may be advantageous in markets that still permit its use.

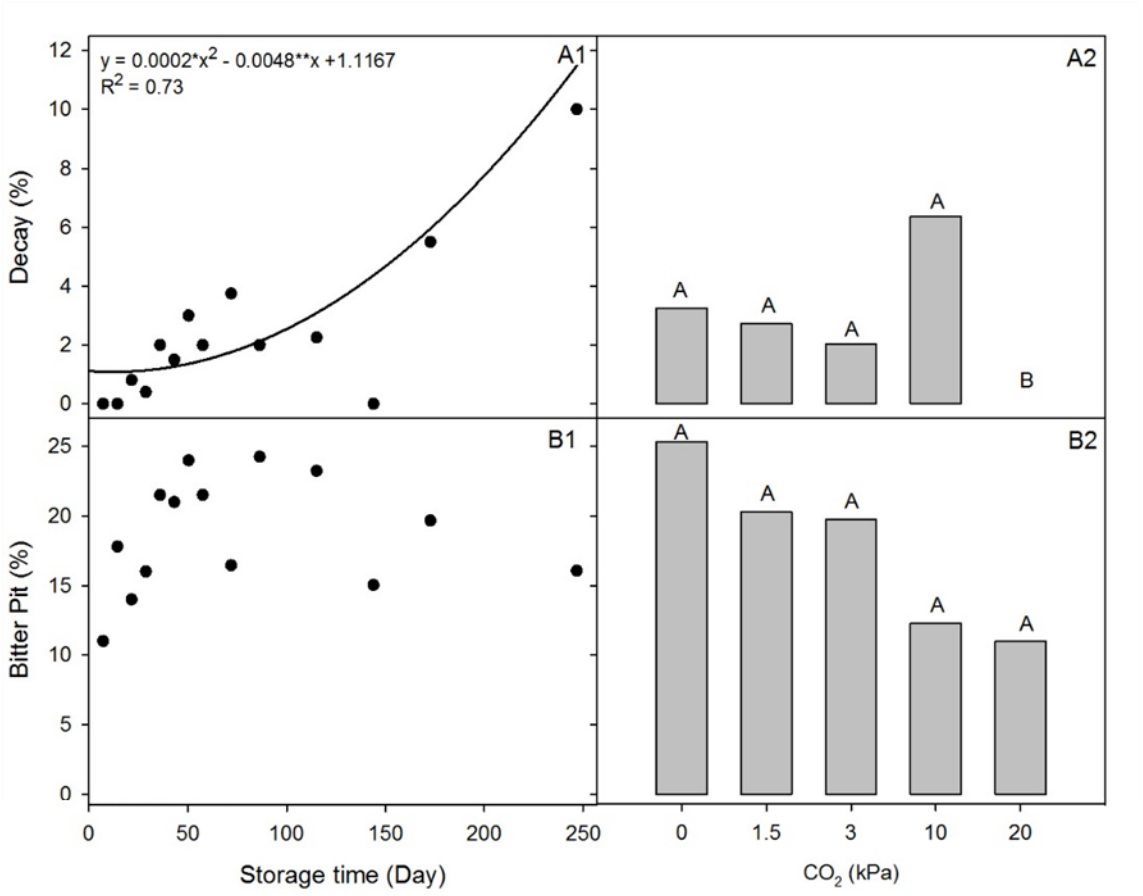
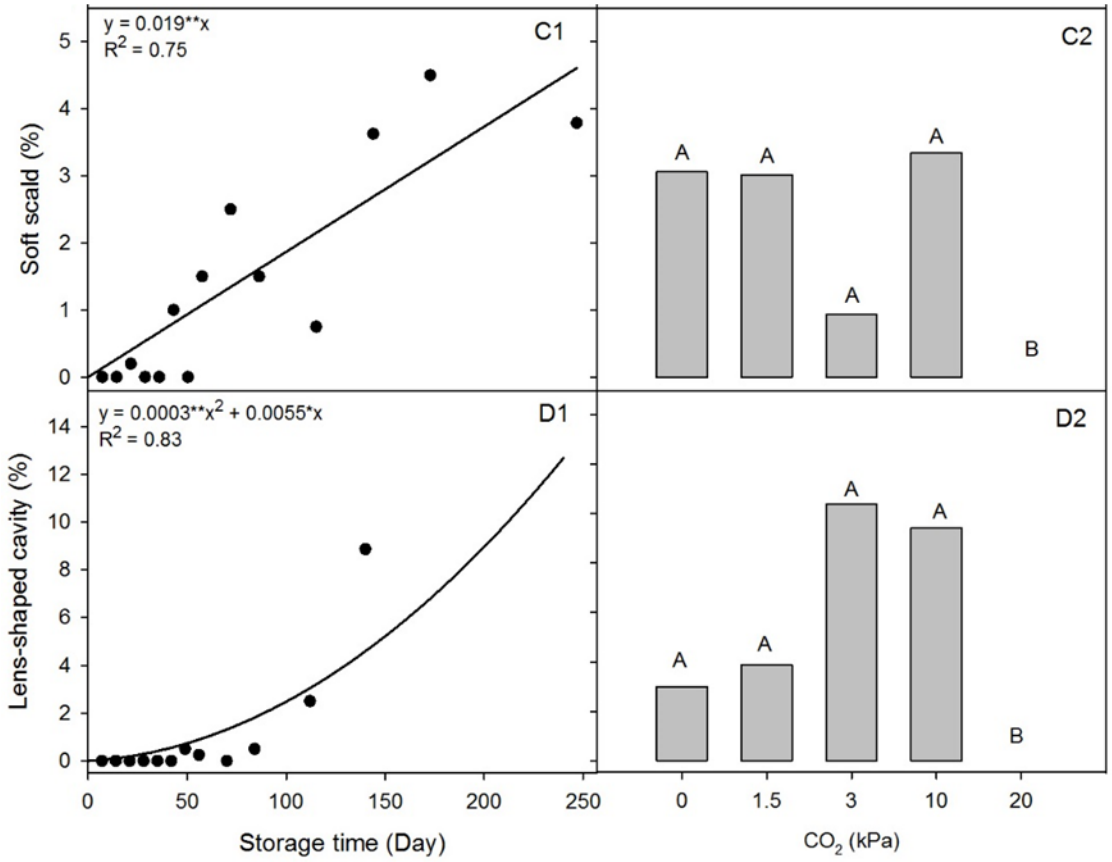


Figure 2. 10. Effect of storage time on decay (A1), bitter pit (B1), soft scald (C1), and lens-shaped cavity (D1) of 'Honeycrisp' apples stored at different CA conditions and effect of CO_2 concentrations (0 – 20kPa) on decay (A2), bitter pit (B2), soft scald (C2), and lens-shaped cavity (D2) of the fruits which received maximal injury (data pooled from day 56 to day 240). For Fig. 2.10 -A1 to D1, each symbol represent means from data of 5 orchards as replicates with approximately 10 fruits from each sampling day (except for day 240, 100 fruits). For Fig.2.10 - A2 to D2, each column represents means from data of 5 orchards as replicates with approximately 150 fruit from each orchard.

Figure 2. 10 (cont'd)



In 2015, we soaked apple in the antioxidant butylated hydroxytoluene (BHT) (Wills and Scott, 1977), (5000 $\mu L \cdot L^{-1}$, 30 s) to compare the effect of BHT and DPA on reducing/ eliminating CA injury of the fruit exposed to 0. 5, 10, and 20 kPa CO_2. However, since the fruit contained in the buckets and received CA condition from mixed CA lines, both DPA (1000 $\mu L \cdot L^{-1}$) and BHT (5000 $\mu L \cdot L^{-1}$) did not prevent CA injury (Fig. S-B2.4). In addition, CA injury symptoms of these treatments were different from those fruit stored in CA chambers (Fig. S-B2.5).

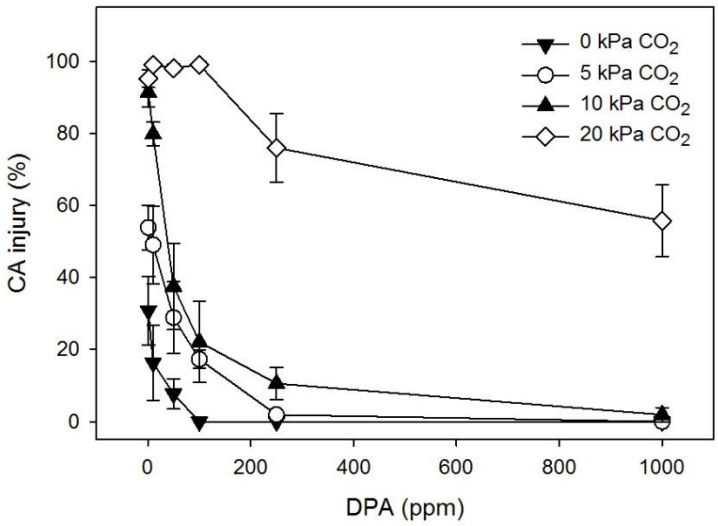


Figure 2. 11. Dose dependence of CA injury on DPA concentration and CO_2 level in 'Honeycrisp apples. Vertical bars indicate S.E of the mean.

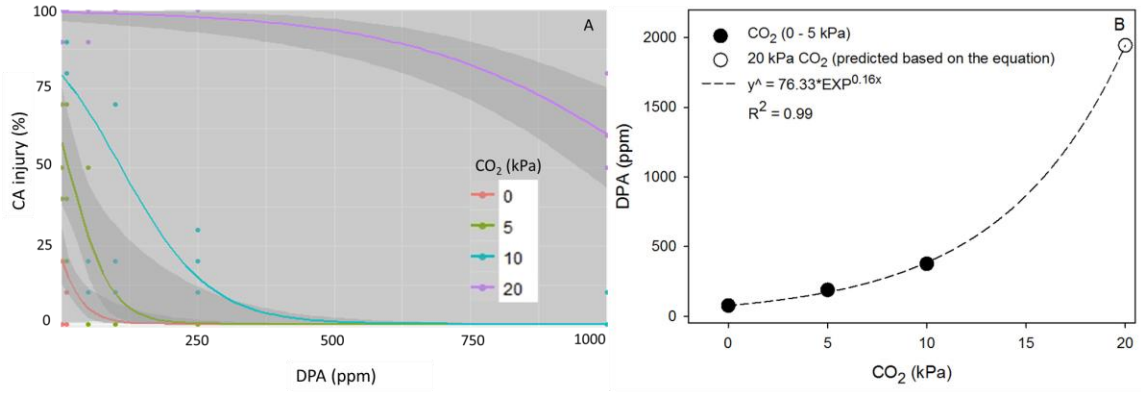


Figure 2. 12. Logistic regression models (R Studio®, ggplot, quasibinomial) was applied and the confidence-interval was used to identify interactions between DPA concentrations and CO_2 levels (A) and the DPA concentration (ppm) required to eliminate CA injury caused by a particular CO_2 concentration.

2.3.3 Experiment 3. Using 1-MCP in air storage to substitute for CA storage

Experiments on 1- MCP multi-application to ‘Honeycrisp’ apple in 2016 showed that 1-MCP reduced internal ethylene, aromatic compound production, and delayed the development of greasiness on apple skin relative to untreated fruit. Fruit treated with one, two, and three doses of 1-MCP did not differ in terms of firmness, greasiness, the incidence of CA injury (internal browning), or the concentration of internal ethylene (Fig. 2.13 and Table 2.3). Additional doses of 1-MCP delayed ripening only slightly more than a single dose. Little difference was detected between 1-MCP treatments in the production of volatile esters (Fig. 2.14). The harvest maturity of apples likely influenced the success of 1-MCP treatment since 1-MCP is not highly effective at suppressing ripening of over-mature fruit. We suggested that a single dose of 1-MCP at harvest yielded the maximum benefit in terms of quality retention and that there was still a need to control the internal injury we call 'CA injury' beyond the use of non-CA (i.e., air) storage conditions for this variety.

From the experiment in 2016, we considered whether 1.5-month intervals of 1-MCP application might be too long. During that time, ethylene receptors might be produced, and ethylene production may recover to a level that outweighs additional 1-MCP molecules. Therefore, in 2017, we applied 1-MCP at a shorter interval, increasing the frequency of application (15 days) and we extended the maximum number of repeat doses to 4 doses on fruits from two orchards in Sparta (Fig. 2.4). After two months in air storage, half of the fruits from the four 1- MCP treatments were transferred to CA chambers (3 kPa CO_2 + 3 kPa O_2). The results were consistent with the experiments in 2016 in which the fruits that received more doses of 1-MCP produced less ethylene. However, there was no difference in the ethylene levels of fruit which had been treated either once or twice with 1-MCP (Fig. 2.15).

CA conditions (3 kPa CO_2 + 3 kPa O_2) suppressed ethylene production at the rate that negatively correlated with the number of doses of 1-MCP. In addition, CA conditions contributed in reducing decay symptom for the fruit stored for 6 months (Table 2.4). While the fruits from orchard H, which were more mature (Table S-B2.1), did not show CA injury symptom, the fruits from orchard F were very sensitive to both 1-MCP treatment and CA conditions. 1-MCP with more doses appeared to enhance the sensitivity of the cultivar to CA injury (Table 2.4). Therefore, 1-MCP in combination with CA storage should not be applied to less mature fruit.

Table 2. 3. Senescent breakdown and CA injury incidence of 'Honeycrisp' apple treated with 0, 1, 2, and 3 doses of 1-MCP and stored under refrigerated air (21 kPa O_2 and 0 kPa CO_2) at 3°C for 1.5, 3, 4.5, 6, and 9 months in 2016 (n=20 per orchard/storage duration/1-MCP dosage combination)

Storage time (months)	Senescent breakdown (%)				CA injury (%)			
	No 1-MCP	1-MCP 1 dose	1-MCP 2 doses	1-MCP 3 doses	No 1-MCP	1-MCP 1 dose	1-MCP 2 doses	1-MCP 3 doses
1.5	0.0	0			0.0	0.0		
3	0.0	0	0		0.0	0.0	0.0	
4.5	7.5	2.5	0	0	2.5	4.0	2.5	2.5
6	0.0	0	2.5	2.5	0.0	7.5	5.0	0.0
9	0.0	0	0	0	0.0	0.0	0.0	0.0

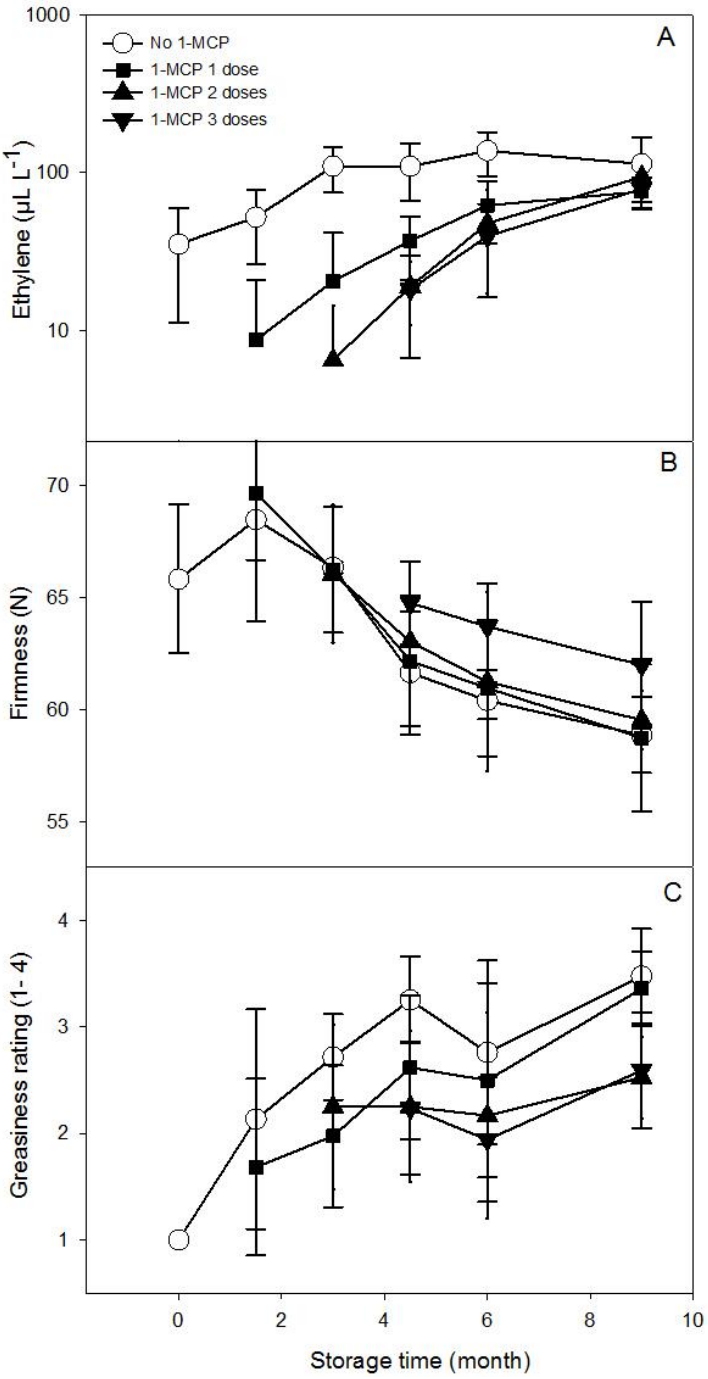


Figure 2. 13. Effect of 1-MCP multiple applications on internal ethylene concentration (A), fruit firmness (B) and skin greasiness (C) of 'Honeycrisp' apple during RA storage (21 kPa O_2 and 0 kPa CO_2) at 3 °C. Each symbol represents fruit from four orchards in 2016, n=10 fruit per orchard; bars are ± 1 SD.

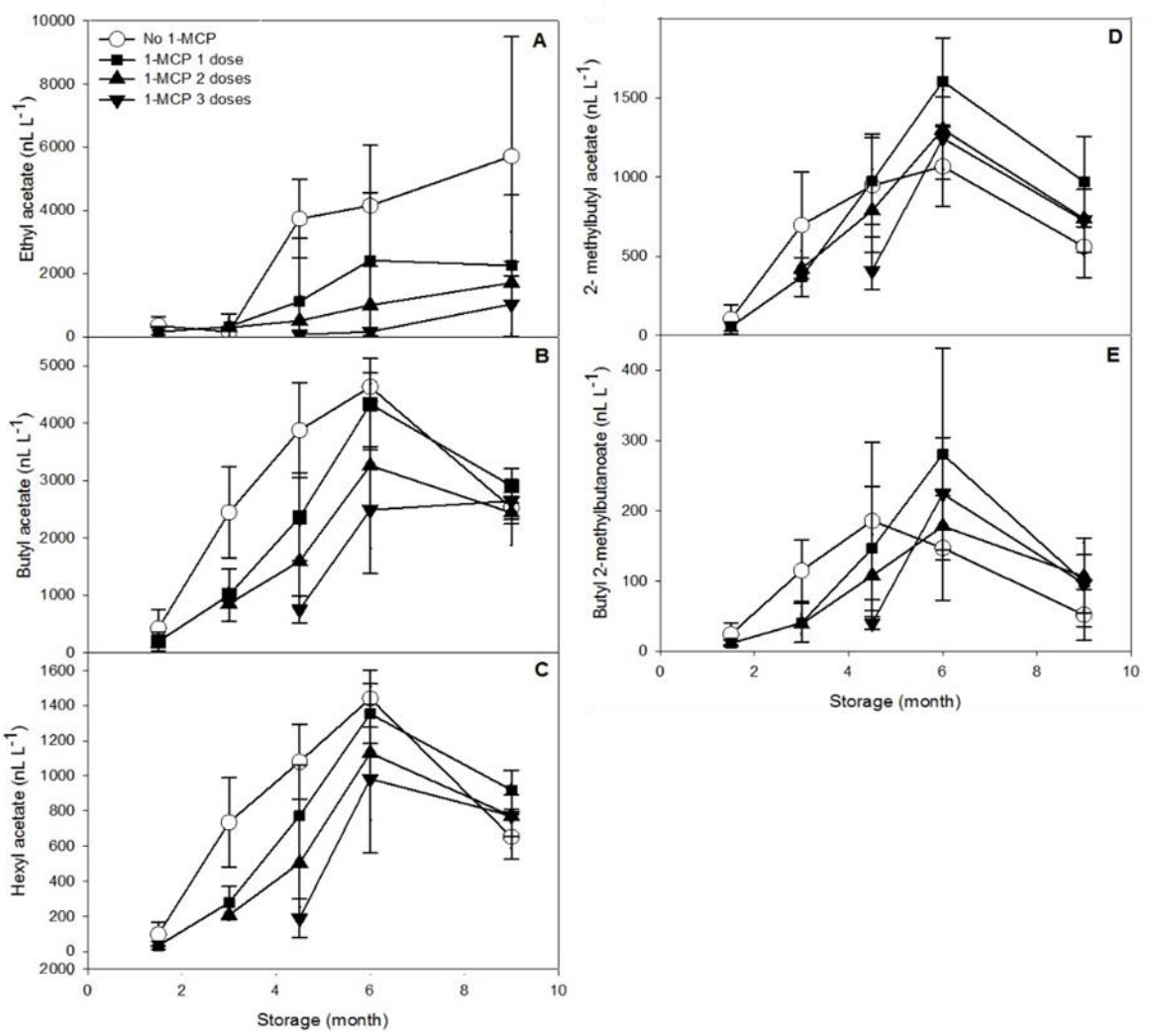


Figure 2. 14. Effect of 1-MCP on production of ethyl acetate (A), butyl acetate (B), hexyl acetate (C), 2-methylbutyl acetate (D), and butyl 2-methylbutanoate (E) of 'Honeycrisp' apple harvested from the four orchards during air storage (21 kPa O_2 and 0 kPa CO_2) at 3°C. Each symbol represents fruit from four orchards, n=5 fruit per orchard; bars are ± 1 SD.

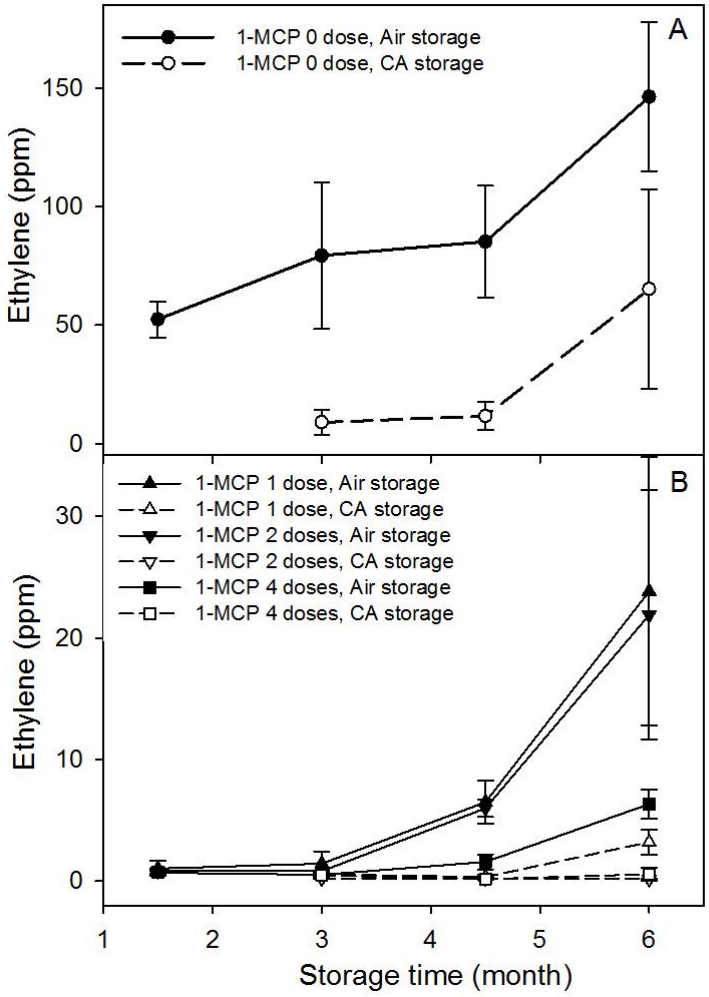


Figure 2. 15. Ethylene level of 'Honeycrisp' apple receiving 1-MCP application (1 $\mu L \cdot L^{-1}$) with 0 doses (A) or 1, 2, or 4 doses (B) with a 15-day interval between repeat doses and stored in air (21 kPa O_2+ 0 kPa CO_2) and CA (3 kPa O_2+ 3 kPa CO_2).

Table 2. 4. Storage disorders of ‘Honeycrisp’ apple received 1-MCP application (with 0, 1, 2, 4 doses at 15 day – interval) and stored in air (21 kPa O_2+ 0 kPa CO_2) and CA condition (3 kPa O_2+ 3 kPa CO_2)

Orchard	1-MCP dose	Decay (%)	Soft scald (%)	Senescent Breakdown (%)	Lens - shaped void (%)	CA Injury (%)	CA index (0-1)
				Air storage			
F	0	23.68	22.41	8.62	25.86	32.76	0.20
F	1	22.94	32.14	1.19	7.14	32.14	0.20
F	2	23.89	43.02	3.49	8.14	26.74	0.22
F	4	24.53	15.00	6.25	12.50	26.25	0.16
H	0	2.78	0	0	0	0	0
H	1	7.77	5.26	0	2.11	4.21	0.03
H	2	3.37	16.28	0	0	6.98	0.03
H	4	0	4.00	0	0	1	0
				CA storage			
F	0	0	6.67	16.67	10.00	13.3	0.05
F	1	9.09	3.33	0	8.33	16.7	0.13
F	2	6.78	12.73	3.64	7.27	20.0	0.09
F	4	0	26.67	6.67	23.33	36.7	0.28
H	0	0	0	0	0	0	0
H	1	2.50	2.56	0	0	5.13	0.02
H	2	2.50	0	0	0	2.56	0.02
H	4	0	0	0	0	0	0

2.3.4 Experiment 4. Effect of preconditioning and the combination of preconditioning and DPA on CA injury of 'Honeycrisp' apple fruit

Temperature conditioning is a required activity for successful storage of ‘Honeycrisp’ apples (and some other chilling sensitive cultivars) in refrigerated air (RA) or controlled atmosphere (CA) storage. Failure to properly condition the fruit can lead to a loss of most of the

crop. In 2014, we performed a matrix experiment of days and temperatures of preconditioning. The results showed that the minimal time for conditioning is about 2-3 days at 20 °C, 3-5 days at 15 °C, or 5-7 days at 10 °C (Beaudry et al., 2014). In 2015 and 2016, we noticed that more matured fruit was more tolerant to CA conditions.

In 2017, we implemented a new preconditioning experiment in which the fruits from two commercial orchards in Sparta were kept in the field for 0, 1, 3, 5, or 7 days before being transported to our lab and stored under CA conditions (five CO_2 levels: 0, 3, 5, 10, and 20 kPa in combination with 3kPa O_2 at 3 °C). Additionally, the fruits on the harvest day were transported to the lab and preconditioned for five days at 20 °C before storage in the same CA conditions as the fruit preconditioned in the field (Fig. 2.7). Based on our previous work, the needed degree-days for control of CO_2 injury was between 100 and 140 degree-days. It took 5 - 7 days for preconditioning in the field to receive such required degree-days. The results showed that approximately 5-7 days in the field or five days (at 20 °C at the lab) were needed to suppress storage injuries (Table S-B2.3-4) because the fruits were more matured (Table S-B2.2). Thus, field conditioning may perform well to protect 'Honeycrisp' fruit from CA and chilling injuries. It should be noted that some greasiness was found in fruit conditioned for seven days in the field. Harvesting fruit at a less mature stage could reduce greasiness (Table S-B2.3-4). In addition, preconditioning at 20 °C at least 2 – 5 days in the lab showed more effective than 10°C for 5 days in reducing CA injury (Table S-B2.3- 6). Preconditioning was a more effective approach than using 1- MCP for the fruit from orchard 1 which was less matured and very sensitive to CA injury.

2.3.5 Experiment 5. Combination of DPA and preconditioning to reduce CA injury and chilling injury of the fruits

In 2016, DPA again confirmed its ability to eliminate CA injury even though the fruit did not receive preconditioning. However, preconditioning approach alone (10°C for five days in cold storage) failed to reduce the symptoms of CA injury (Table S-B 2.5).

In 2017, we used 20 °C, instead of 10 °C like the preconditioning experiment in 2016, as preconditioning temperature. Following 4.5 months of storage, soft-scald, a chilling injury symptom at chilling temperature 0 °C, was noted in non-preconditioned fruits which were treated or not treated with DPA and stored at 2 CO_2 levels (Table S-B2.6). The fruits from orchard F, less mature than those from orchard H (Table S-B2.1), suffered more severity of soft scald if not received the preconditioning treatment (Table S-B2.7). In other words, preconditioning (2 or 5 days at 20 °C) was more effective in reducing chilling injury for the fruit from this orchard. Especially, ethylene production of orchard F increased to more than 100 ppm after five days receiving precondition treatment in the field or the lab (Table S-B2.2).

DPA might play as a supplementary factor to preconditioning. A combination of preconditioning and DPA resulted in the most effective reduction of chilling injury in apples from both orchards. From this experiment, we confirmed that DPA can eliminate CA injury at two CO_2 levels (3 and 5 kPa) (Table S-B2.6). However, preconditioning should apply at least two days for more mature apples (orchard H) and five days for less mature fruit (orchard F) to reduce both chilling injury and CA injury at 3 kPa CO_2 and 3 °C.

2.3.6 Experiment 6. The response of fruit to hypoxia condition

Fruits of all treatments did not have CA injury symptoms on the day of removal from the experimental condition. The fruit under extreme hypoxia (0.1 kPa O_2 and 0.2 kPa O_2), however,

showed injury with different symptoms after three days at air condition at 20 °C (Fig. 2.16). 0.1 kPa O_2 caused an external injury on the skin, which had an appearance like soft scald early symptoms whereas 0.1 kPa O_2 caused an internal injury of which the symptom was the same as CA injury.

We hypothesized that a sudden shift from anaerobic to aerobic conditions may have provided more oxygen for browning reactions. Dilley et al (1963) observed a superficial scald-like browning incidence of 'Red Rome' apples exposed 108 h in anaerobic condition following 36 h in aerobic one.

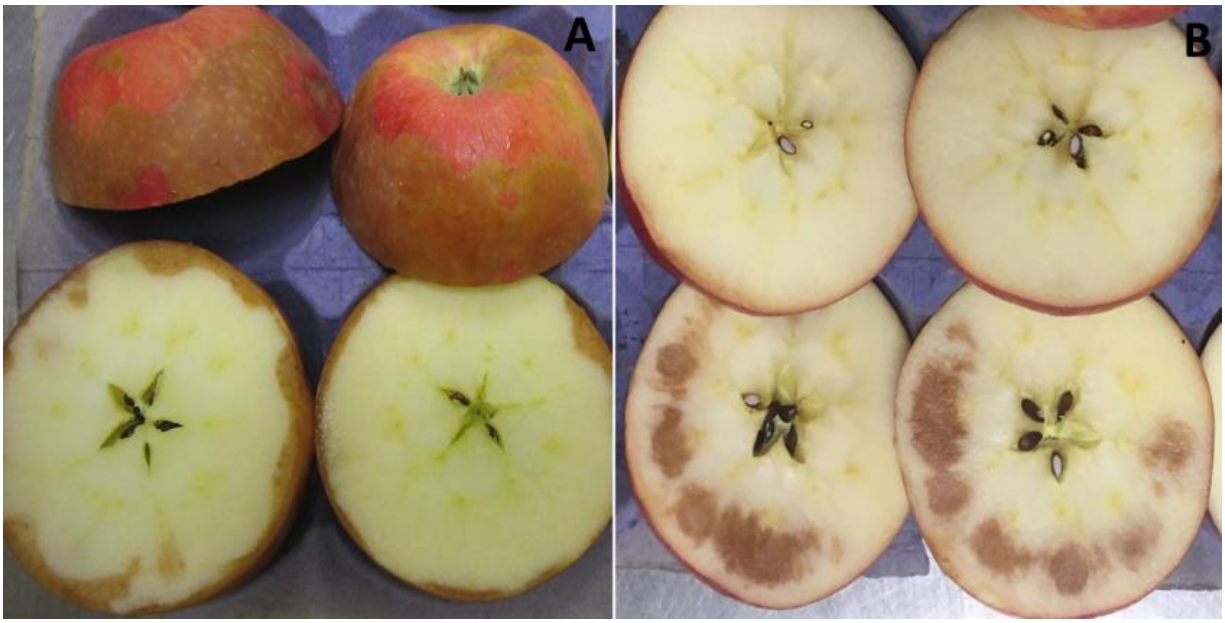


Figure 2. 16. Injury symptoms of ‘Honeycrisp’ apples treated with hypoxia for 14 days at 0.1 kPa O_2 (A) and 0.2 kPa O_2 (B) at 3 °C and then held for 3 days in normal air (21 kPa O_2) at 20 °C.

2.3.7 Relationship of the maturity stage of the fruit and CA injury

‘Honeycrisp’ apples were harvested from commercial apple orchards during the primary period of harvest so that findings would represent commercial practices in Michigan and other temperate fruit production regions. Maturity stages were determined but not controlled. Research

on ‘Honeycrisp’ apples showed that the fruit was more tolerant to CA when picked at higher maturity and this is consistent with the findings of Contreras et al. (2014). We also noted this factor when performing experiments with fruit from 2014 – 2017. Orchards B and D from Sparta and Belding respectively, which supplied the fruit for the experiments for three years (2014 – 2016), picked the fruits at different maturity stages (Table S-B1). Based on ethylene and starch index levels, fruit from orchard B were less mature than fruits from orchard D ($P < 0.05$). The fruits from these orchards had a significantly different response to CA injury. To evaluate the relationship between maturity and CA injury, the fruit damage data of the two orchards was collected from the following experiments:

1) 2014: Fruit stored at 3 kPa CO_2 for 112 days (the experiment elaborated in Fig. 2.1)

2) 2015: Fruit stored at 5 kPa CO_2 for 42 days; Fruit treated with DPA (only 1ppm, 30s) and stored at 5 kPa CO_2 for 112 days (the experiment elaborated in Fig. 2.3)

3) 2016: Control fruit stored at 5 kPa CO_2 for 120 days (the experiment elaborated in Fig. 2.5).

Even though the sampling dates of the fruit from three years were not the same, after 42 days, the fruit had suffered maximal injury. The result showed that fruit from Sparta, with lower ethylene production and lower starch index, showed more severity of fruit damage. There was a strong negative relationship between ethylene and fruit damage as well as between starch and fruit damage (Fig. 2.17).

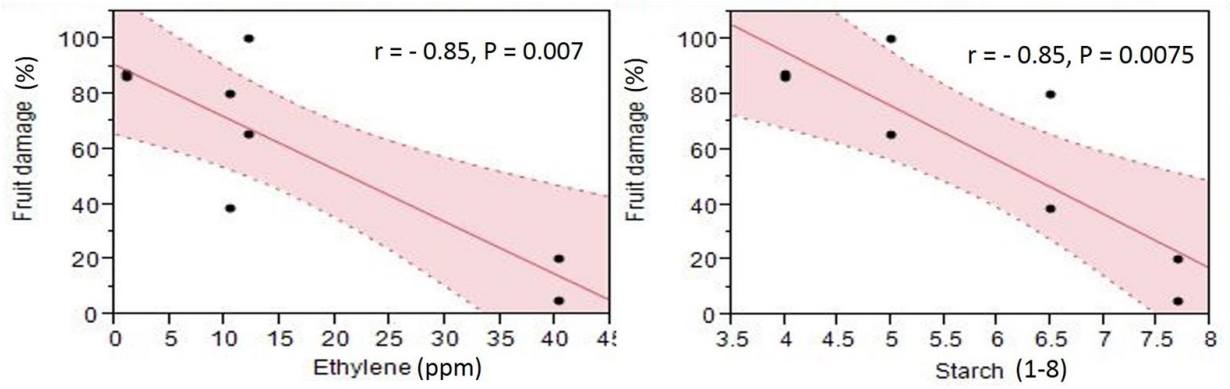


Figure 2. 17. Relationship between maturity stage (based on starch and ethylene indices) and CA injury in 'Honeycrisp apple fruit and the maximal level of injury due to 3 or 5 kPa CO_2 in CA storage.

2.3.8 Recommendations for CA technology

At higher maturity, 'Honeycrisp' apple fruit might be tolerant to standard CA condition and thus can be stored immediately in commercial and universal CA regime for apples (3 kPa CO_2 + 3 kPa O_2). 1- MCP can be applied after one day of harvest to immediately inactivate ethylene receptors. A combination of 1-MCP treatment in air storage followed by CA storage after a period of 2 months might be suitable to prolong its marketing life. Four doses of 1-MCP applications with a15 day-interval proved to be the most effective treatment.

If less mature, the fruit should be preconditioned until its starch index reaches 7-8. Preconditioning might be at the field (at least 7 days) or at packing houses (5 days at approximately 20 °C). Then, 1-MCP and CA application should be applied to prolong its market life.

Packing house managers should check maturity stage of harvested fruit before applying a suitable practice to their apples to reduce/eliminate CA injury, senescent breakdown, and soft scald.

3 °C is the optimal storage temperature for ‘Honeycrisp’ apple because even 1000 ppm DPA application cannot help eliminating soft scald, a chilling injury symptom when the fruits are stored at 0 °C. However, preconditioning can reduce (not eliminate) the symptom. Fruit must be stored with other apple varieties due to storage capacity.

The apple industry should reduce DPA concentrations from commercial dose (2000 ppm) to our recommended dose (75 – 185 ppm) if they want to apply it to the 'Honeycrisp' fruit, which is extremely sensitive to CA injury at commercial CA conditions.

2.4 Conclusion

- There was a strong correlation between CO_2 concentration and CA injury severity
- CA injury symptom reached maximum after the first two months of CA storage
- Lens-shaped voids appeared at late storage, usually after 4 months.
- External disorders (decay and soft scald) did not positively correlate with CO_2 levels but with storage time. Bitter pit was noted after the first week of storage, but did not have a relationship with CO_2 level or storage time, but was dependent on orchard factor.
- Extreme hypoxia did not cause CA injury symptoms for the fruit during storage; however, the fruits had external browning on the skin after exposure to normal air condition and holding at 20 °C for 3 days.
- DPA can be applied at low concentration (< 130 ppm) in commercial CA practices (3 kPa CO_2 + 3 kPa O_2). However, even at 0 kPa CO_2, 75 ppm DPA was a minimum concentration required to eliminate CA injury symptoms to the fruit extremely sensitive to CA storage.

- 1-MCP reduced CA injury (only 5 -7 %) since the fruit was stored in air (21 kPa O_2 + 0 kPa CO_2). Additional doses of 1-MCP further suppressed ethylene evolution but caused more injury for CA sensitive fruit. A single dose of 1-MCP application at one day after harvest might be enough to inhibit ripening for up to six months under air storage. However, for further extension storage life of the CA less sensitive fruit, 1-MCP application up to four times at 15-day intervals should be applied. In addition, a CA regime could be applied after this 1-MCP/air storage regimen.
- The maturity of apples determined the tolerance of the fruit to CA injury. The fruit that was more mature had less incidence of CA injury. Both preconditioning before CA storage and 1-MCP before air storage are approaches that could not eliminate CA injury completely but did reduce symptoms. In this case, DPA at approximately 130 $\mu L \cdot L^{-1}$ for 30 s before CA storage at 3 °C is the best option if they are stored immediately in CA storage. On the other hand, the fruits harvested at a more mature stage were more tolerant to standard CA conditions. Therefore, it is acceptable to store more mature immediately in CA storage after harvest.
- There is no single regime of postharvest storage practice for this cultivar. Depending on maturity stage and orchard factor (i.e., preharvest practices) the ideal postharvest conditions may change.

APPENDICES

APPENDIX A. Manuscript entitled "Response of air-stored 'Honeycrisp' apple fruit to repeated application of 1-MCP"

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Abstract

'Honeycrisp' apple (*Malus* × *domestica* Borkh.) fruit is one of the most profitable apples grown on a large scale in the US. However, commercial controlled atmosphere (CA) technology has not been widely applied to the fruit because of its susceptibility to low O_2- and high CO_2-induced injuries, which develop as brown lesions and lens-shaped cavities in the cortex. Our storage data for 'Honeycrisp' have consistently shown that preconditioning (holding the fruit at 10 °C for 5 to 7 days before storage) combined with diphenylamine (DPA) drench before CA storage or a single dose of 1-MCP before air storage is very helpful to maintain fruit quality and avoid CA injury. Even though 1/4-label rates of DPA were found to protect fruit from CA injury, the low tolerance for DPA in Europe precludes even this low dose. The aim of this study was to evaluate multiple 1-MCP applications combined with DPA on quality of air-stored fruit as an alternative to CA storage to avoid CA injury. Single (harvest), double (harvest and after 1.5 months) and triple (harvest and after 1.5 and 3 months) applications of 1-MCP were given to 'Honeycrisp' apples harvested from 4 orchards across Michigan in 2016 before storing the fruit in air at 3 °C. Ethylene, selected volatile esters (ethyl acetate, hexyl acetate, butyl acetate, butyl 2-methylbutanoate, and 2-methylbutyl acetate), and greasiness were used as measures of ripening behavior. These indices were measured after 1.5, 3, 4.5, 6, and 9 months of storage. 1-MCP reduced internal ethylene and aromatic compound production and delayed the development of greasiness on apple skin relative to untreated fruit. Additional doses of 1-MCP delayed ripening only slightly more than a single dose. Little difference was detected between 1-MCP treatments in the production of volatile esters. The harvest maturity of apples influenced the success of 1-MCP treatment since 1-MCP is not highly effective for over-mature fruit in suppressing ripening. We recommend a single postharvest treatment of 1-MCP immediately following harvest for air storage (3 °C) of Honeycrisp fruit and a storage duration no longer than 3 to 4 months.

Keywords: Preconditioning, CA injury, CO_2 injury, storage, diphenylamine.

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INTRODUCTION

Since its release in 1991 from the Minnesota Agricultural Experiment Station's Horticultural Research Center (Bedford, 2001), the 'Honeycrisp' apple (*Malus* × *domestica* Borkh.) has become one of the most profitable apples grown on a large scale in the United States and eastern Canada and now occupies a significant share of the apple market in the US (National Agricultural Statistics Service, 2011, 2012, 2015). To meet an increasing demand for supply of this cultivar throughout the year, two postharvest practices, i.e. controlled atmosphere (CA) storage and 1-MCP treatment, have been evaluated (Delong et al., 2006; Beaudry and Contreras, 2009; Watkins and Nock, 2012). 'Honeycrisp' apple, however, is very sensitive to CA conditions. Symptoms of CA injury include the presence of irregular brown regions in the cortex with or without lens-shaped cavities (Beaudry and Contreras, 2009; Watkins and Nock, 2012; Contreras et al., 2014). Conditioning the fruit by holding the fruit at 10 °C for 5 to 7 days before transferring them to CA storage or applying a diphenylamine (DPA) drench before CA storage (Contreras et al., 2014; Leisso et al., 2017) reduced CA injury (Contreras et al., 2014). However, even a very low dose of DPA before CA storage (250 ppm) which completely eliminates CA injury on 'Honeycrisp' apples (Diep Tran and Randolph Beaudry, unpublished data) is not acceptable to European Union countries (Calvo and Kupferman, 2012).

The application of the ethylene antagonist 1-methylcyclopropene (1-MCP), like the use of CA storage, can extend the storage life of apple fruit. 1-MCP has been commercially applied to apple industry since 2002 under the commercial name SmartFresh^{TM} (AgroFresh Inc., Spring House, PA, USA) (Beaudry and Watkins, 2003). The advantage of 1-MCP is that it can strongly and, apparently permanently bind to ethylene receptors at very low concentrations

(from 0.005 – 1 ppm), depending on the exposure durations of apples to the compound (Sisler et al., 1996; Beaudry and Watkins, 2003). With a single dose treatment of 1-MCP to 'Honeycrisp' apples before air storage, production of ethylene concentration of the fruit dramatically reduced in relative to the control (DeEll and Ehsani-Moghaddam, 2012; Watkins and Nock, 2012). Consequently, fruit development and senescence was retarded (Watkins and Nock, 2012). Ethylene receptors, however, can continue to be produced in fruit tissues especially when the fruit is at climacteric (Nakatsuka et al., 1998). To maintain the effectiveness of 1-MCP in blocking ethylene receptors, multi-application of the compound was applied to 'Northern Spy', and 'McIntosh' apples (DeEll et al., 2016), 'Redchief Delicious' apples (Mir et al., 2001) and Roma-type 'Plum Dandy' tomatoes (Mir et al., 2004). Apple firmness remained high with weekly application of 1-MCP even at room temperature (Mir et al., 2001). In tomato, continuous 1-MCP exposure for 34 days at room temperature stopped skin color development and inhibited the rate of softening on breaker and turning tomatoes (Mir et al., 2004). Multi-application of 1-MCP maintained the firmness of 'McIntosh' and 'Empire' apple after 9 months of CA storage (DeEll et al., 2016).

The objective of this study was to determine the efficacy of 1-MCP multi-application with single (harvest), double (harvest and after 1.5 months) and triple doses (harvest, 1.5 months and 3 months) on eating quality of mature 'Honeycrisp' apple during air storage. In addition to usual indices for fruit quality including greasiness, titratable acid, senescent breakdown, CA injury, etc., the production of aroma volatile compounds, which has not been investigated before on 1-MCP treated 'Honeycrisp' apples, was also included because the aroma is one of most attractive attributes to consumers contributing to apple flavor (Beaudry, 2000).

MATERIALS AND METHODS

Materials

'Honeycrisp' apple commercially grown in four orchards near Hartford (orchard 1), Belding (orchard 2), Ludington (orchard 3), and Traverse City (Orchard 4) in Michigan were collected on 14, 20, 27 September and 10 October, respectively in 2016. The fruit were harvested in the morning and immediately transported to Michigan State University.

Maturity analysis.

At the lab, 20 fruits per orchard were used for fruit maturity analysis after holding at 22 °C for one day after harvest (day 1) and seven days after harvest (day 7). Indices for evaluation of fruit maturity on day 1 include: 1) fruit weight (g) by using a calibrated balance (Mettler PE3000, Toledo Scale, Toledo, OH, USA), 2) percentage of redness and percentage of background coloration by trained assessors; 3)internal ethylene concentration (IEC) ($\mu L\ L^{-1}$) using gas chromatography, 4) firmness (N) using a drill stand-mounted penetrometer, 5) starch index (1 to 8) based on the Cornell Starch Chart, and 6) soluble solid content (°Brix) using a handheld refractometer as previously described (Sugimoto et al., 2015). The fruit weight and firmness were also evaluated for the apples kept at 22 °C for 7 days.

1-MCP treatment and storage

The fruit of each orchard was approximately evenly distributed into twelve plastic crates (about 30 fruits/crate): 3 crates for 1-MCP non-treatment as control and the others for 1-MCP application (1, 2 and 3 doses) and stored overnight in a cool room at 3 °C.

After overnight cooling, the six apple crates subjected to 1-MCP treatment were placed in a sealed plastic container (150 cm x 150 cm x 130 cm). After a capsule containing 7.051 g TruPick^{TM} (Essentiv Corp.) was dropped into a small flask containing 50 mL of water, the fan

was turned on and the container was immediately sealed with tape so that exposure of 1-MCP gas was evenly distributed at a concentration 1 ppm for 24 h at 3 °C.

Apple crates of four treatments (No 1-MCP, 1-MCP 1 dose, 1-MCP 2 doses, and 1-MCP 3 doses) were stored separately in four sealed metal chambers (143 cm x 71 cm x 91 cm) connected to a CA system set with an air regime (21 kPa O_2 and 0 kPa CO_2) at 3 °C in a cold room for 9 months. Apples from 4 orchards with the same treatment were kept in the same chamber.

The fruit subjected to 1-MCP multi-application were moved to the 1-MCP handling room after 45 days (for 2 and 3 doses) and 90 days (for 3 doses) for the same 1-MCP treatment procedure as described above and stored back to their CA chambers.

Quality analysis of apple treated and untreated with 1-MCP

The fruit quality from 4 treatments control, 1-MCP application 1 dose, 2 doses, and 3 doses were evaluated based on IEC (ppm), firmness (N), juice pH, and titratable acid, senescence breakdown (%), greasiness (rate 1-4), CA disorders (%), lens shape incidence (%), volatile compound concentration (nL L^{-1}) of following esters: ethyl acetate, hexyl acetate, butyl acetate, butyl 2-methylbutanoate, and 2-methylbutyl acetate by GC coupled with time-of-flight mass spectrometry (TOFMS). The sampling dates were after 1.5, 3, 4.5, 6, and 9 months of air storage. Measurement of the indices were implemented 1 d after removing the samples from the chambers at ambient temperature except for greasiness 3 d.

Assays

The IEC of each apple fruit was measured by using a gas chromatograph (Carle Series 400 AGC; Hach Company, Loveland, CO) equipped with a flame ionization detector (FID) and a 2-m long × 6-mm internal diameter stainless steel column packed with activated alumina

which was kept at 100 °C. Flow rates for nitrogen, hydrogen, and air were 50, 50 and 500 mL min^{-1}, respectively. A 1-mL sample of internal gas withdrawn into a disposable plastic syringe through a needle inserted into apple core cavity was injected into the machine. Ethylene concentration was calculated based on the certified standard (Matheson Gas Products Inc., Montgomeryville, PA) containing 0.979 µL L^{-1} ethylene, 4.85% CO_2, and 1.95% O_2 balanced with N_2 according to Mir et al. (2001).

For starch measurement, fruit were cut perpendicular to the fruit axis through the seed cavity and dipped into potassium iodine solution for one minute. The starch index (1-8) of the fruit was recorded based on the black stain level on an equatorial cross-section of the fruit, ranging from 1 (100 % starch) to 8 (no starch) based on Cornell Starch Chart (Blanpied and Silsby, 1992). The fruit firmness was recorded by pound-force (lb) by pressing the probe (11-mm-diameter) of the penetrometer (Effegi FT-327; McCormick Fruit Tree Inc., Yakima, Wash.) into 2 opposite sides of each fruit to a depth of a scribed line (1 cm) from the tip. The target sides were at the midway of the stem and blossom end where 1.5 - 2 cm in diameter of skin had been removed by a vegetable peeler. The unit pound-force (lb) was converted to Newton (N) by multiplying the data by 4.448 N/lb.

The apple juice extracted from the target fruit sides from firmness measurement was used for recording the soluble solid content (°Brix) using a hand-held refractometer (Atago N1, Atago Co. Ltd., Tokyo, Japan). The pH of apple juice from 5 apple fruits per treatment was recorded by using a 370 Thermo Orion pH meter (Thermo Fisher Scientific Inc., Logan, UT). Titratable acidity (TA) was determined using a Multi-T 2.2 digital automated titrator (Laboratory Synergy Inc., Goshen, NY) connected with an auto-sampler and control unit (Titroline 96; Schott-Geräte, Mainz, Germany). 10 mL of apple juice was adjusted to 110 mL

by adding 100 mL of deionized water. Aliquots of 10 mL were titrated to pH 8.2 with 0.1 N NaOH and expressed as g L^{-1} of malic acid equivalents as 0.067 following procedures of Iland et al. (2004) and Mitcham et al. (1996).

The skin greasiness of each fruit was evaluated by three trained assessors following instructions at Postharvest lab at Michigan State University on 1-4 scale (1, none; 1, slight; 2, moderate; and 4, severe).

The incidence of senescent breakdown and CA disorders including injury severity rate (0: No browning area in cross-section of apple cortex; 1: 0-<10%; 2: <11-25%; 3: 25 - <50%; 4: > 50%) following instructions at Postharvest lab at Michigan State University and lens-shaped lesions were assessed as percentage of fruit having these symptoms of the total 20-apple samples. Each fruit was chopped into 5 1-cm thick slices using an onion slicer (NSFQC Nemco Food Equipment, Hicksville, Ohio). The slice with the most severe injury was used to assess the incidence of the disorders.

Volatile ester (ethyl acetate, butyl acetate, hexyl acetate, butyl 2-methylbutanoate, and 2-methylbutyl acetate) analysis was performed as previously described (Song et al., 1997; Ferenczi et al., 2006) with minor modifications. Each apple was placed in a 1.5-L Teflon™ chamber sealed by its lid for 20 min - incubation, enough for volatile apple components to diffuse into gas phase. Five apples of each treatment were used as technical replicates. A solid phase micro extraction (SPME) fiber (65 μm thickness PDMS-DVB, Supelco Inc., Bellefonte, PA, USA) was manually inserted through a rubber septum on the Teflon lid to absorb the headspace gas on the fiber coating in 3 min and immediately desorbed for 3 min through a predrilled septum (Thermogreen LB-2, Supelco Co.) in the GC (HP-6890, Hewlett Packard Co., Wilmington, DE, USA) inlet (220 °C). During volatile desorption, liquid nitrogen was

placed under the head of the column (20 m long × 0.18 mm i.d., SP-5, Supelco Inc., Bellefonte, PA) to trap desorbed volatiles. Afterwards, the gas released and separated in the column following the program set up in the GC (an increase in temperature at 50 °C min^{-1} from 40 °C to 240 °C, then maintained at 240 °C for 1 min). Helium was used as a carrier gas (0.8 mL min^{-1}).

Volatile Collection, Separation, and Detection.

Identification of volatile compounds was confirmed by comparison of collected mass spectra with those of authenticated reference standards and spectra in the National Institute for Standards and Technology (NIST) mass spectral Search Program for the NIST/EPA/NIH Mass Spectra library Version 2. The quantification was performed relative to the known standards that had similar or approximately similar molecular weights and retention times. Volatiles were measured at 1−2 d intervals for a period of 8 d after transfer of fruit from refrigerated air (RA) or CA to 22 °C.

Five target compounds (ethyl acetate, hexyl acetate, butyl acetate, butyl 2-methylbutanoate, and 2-methylbutyl acetate) were identified by comparison with the peaks and retention times of corresponding standards and reference spectra of the compounds in the national institute for standard and technology (NIST) mass spectral library (Search Version 1.5). Quantification of the target compounds was calculated based on an absorbance area of five corresponding compounds included in the standard mixture (see below) of 21 authenticated volatile compounds sourced from Sigma Co. and Fluka Chemika.

Preparation of Volatile Aroma Standards

The aroma mixture contained equal volumes (2 µL) each of 21 compounds ethyl acetate, hexyl acetate, butyl acetate, butyl 2-methylbutanoate, 2-methylbutyl acetate, 1-

butanol, 2- methyl 1 butanol, 2 methyl propanol, 1-hexanol, 1-propanol, trans-2-hexenyl acetate, trans-2-hexenal, 3-methyl-1-butanol, acetaldehyde, ethanol, ethyl 2-methylbutanoate, ethyl butanoate, ethyl hexanoate, hexanoic acid, propyl hexanoate, propyl acetate. A glass syringe (Alltech, 1 uL SGE Zero Dead Vol. Syringe, 5-cm needle) was used to remove 0.5 µL of the standard aroma mixture and to inject this sample onto a small glass microfiber filter (Whatman^{TM} 24-mm dia.), which was then immediately dropped into a 4.4-L glass volumetric flask with a ground-glass top fitted with a Mininert valve (Supelco Inc., Bellefonte, PA). The flask was sealed and the mixture was allowed a complete evaporation of the mixture to headspace gas according to Song et al. (1997). A new standard was freshly made on analyzing dates.

Experimental design

The experimental design was completely randomized with doses of 1-MCP treatment as fixed effects. 4 orchards were used as replicates. For technical replicates, 10 fruit per treatment at each sampling date was used for analysis of IEC, firmness, greasiness, TA, and pH; 20 fruits were used per replicate for evaluation of storage disorders; 5 fruit per replicate were used for volatile aroma compound analysis.

RESULTS AND DISCUSSION

Apple maturity

Apples of the four orchards were at mature stage. Fruit cortex's starch had been hydrolyzed to reach the index 7 to 8. The fruit firmness and soluble solid contents indices were not significantly different among the orchard. At this stage, internal ethylene ranged from 6.76 µL L^{-1} in orchard 4 apples to 64.9 µL L^{-1} in orchard 1 apples even though they had the same starch index. Also, the fruit weight, redness, background varied among the orchards

(Supplementary Table 2A. 1).

Supplementary Table 2A. 1. Maturity indices of 'Honeycrisp' apples harvested near Hartford (orchard 1), Belding (orchard 2), Ludington (orchard 3), and Traverse City (Orchard 4) in Michigan on 14, 20, 27 September and 10 October 2016, analyzed after 1 d at room temperature.

	Weight (g)	Ethylene ($\mu L\ L^{-1}$)	Redness (%)	Background (1-5)	Firmness (N)	Starch (1-8)	SSC (°Brix)
Orchard 1	215.75 ± 36.22	64.90 ± 27.42	55.00 ± 21.21	2.60 ± 1.26	67.61 ± 4.25	8.00 ± 0.00	10.75 ± 2.28
Orchard 2	200.98 ± 38.11	40.32 ± 25.21	69.50 ± 21.14	1.30 ± 0.67	63.70 ± 4.13	7.70 ± 0.48	12.40 ± 1.18
Orchard 3	204.49 ± 39.77	6.76 ± 7.17	76.00 ± 21.19	1.40 ± 0.52	64.30 ± 4.14	8.00 ± 0.00	12.80 ± 0.84
Orchard 4	224.49 ± 28.89	29.49 ± 29.41	32.50 ± 21.25	4.00 ± 0.00	70.78 ± 9.61	7.70 ± 0.48	11.04 ± 0.74

Effect of 1-MCP multi-application on fruit ethylene evolution, greasiness, and firmness

Treatment with 1-MCP reduces the internal ethylene concentration relative to untreated fruit (Sisler et al., 1996). In our study, the average IEC of untreated fruit after 1.5 months storage in air increased 164% relative to day 1 while the concentration was reduced by 75% in the fruit treated with 1 dose of 1-MCP (Fig. S2-A.1-A). Additional doses of 1-MCP further suppressed ethylene content, especially for the 6-month time-point. Since constant perception of ethylene is essential to keep a continuous autocatalytic ethylene production (Nakatsuka et al., 1998), blocking of this receptor by 1-MCP likely suppressed ethylene production. Repeated application of 1-MCP might help maintain blockage of ethylene receptors. However, since mature fruit were used as plant material, a delay of 1.5 months until the first 1-MCP reapplication might be too late in the ripening process to ensure full efficacy for the 2-dose and 3-dose treatments compared to the single dose. Eventually, ethylene production of 1-MCP

treated fruit increased with storage time and almost reached to the level of untreated fruit after 9 months of air storage (Fig. S2-A.1-A).

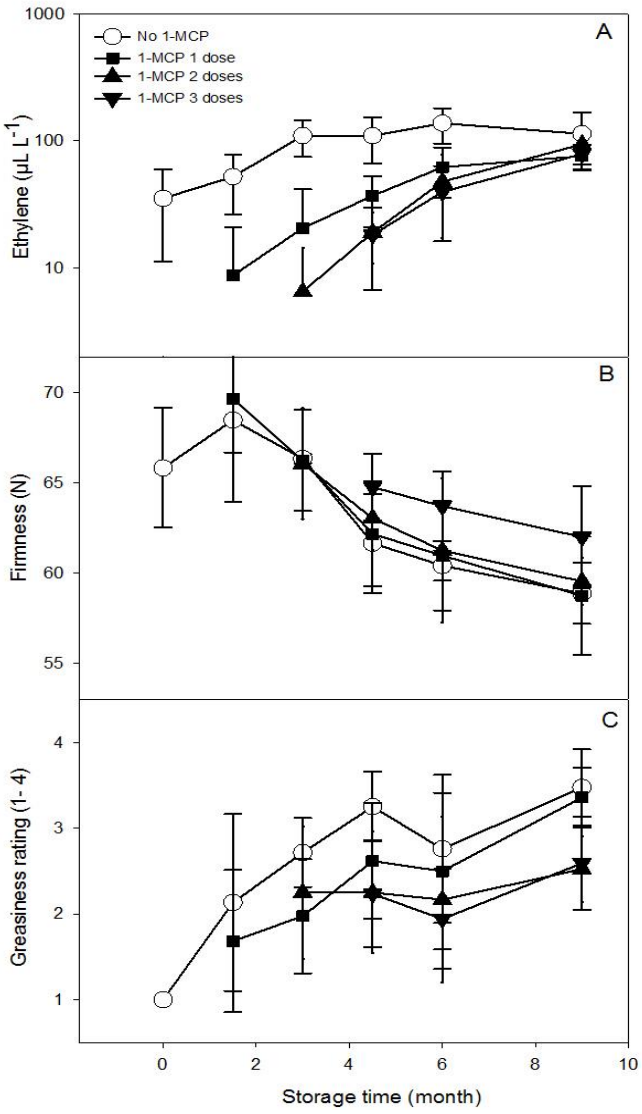
When choosing an apple, a customer is not only interested in apple flavor and skin color, but its crisp texture. The apple breeders in Minnesota University had focused on creating apple lines with superior texture like the 'Honeycrisp' apple variety (Bedford, 2001) where fruit crispness is maintained throughout extended storage and shelf life (Tong et al., 1999; Wargo and Watkins, 2004; Mann et al., 2005; Watkins et al., 2005; Harb et al., 2012). For the mature fruit used in this experiment, 1-MCP did not delay softening relative to control fruit when the fruit received one or two 1-MCP treatments (Fig. S2-A.1). However, fruit stored for 9 months after 3 1-MCP treatments were firmer than controls. The result was consistent to study of Watkins and Nock (2012) on the same variety and might be due to lower expression of genes responsible to cell wall hydrolysis to fruit softening (i.e., via arabinofuranosidase, expansin, polygalacturonase, and pectate lyase enzymes) in comparison to 'McIntosh' apples (Harb et al., 2012). The result contradicts previous research on 'Granny Smith', 'Gala', 'Red Chief Delicious', 'Ginger Gold', Empire', and 'Fuji' apples in which 1-MCP caused a significant reduction in softening (Watkins et al., 2000; Mir et al., 2001; Fan et al., 1999a, 1999b). 1-MCP gas around these fruits may not have been sufficient to completely compete with the internal ethylene. A more frequent or continuous 1-MCP treatment might be very useful for mature fruit to retard ripening.

As the apple fruit senesces, the skin becomes oily or greasy because of changes in wax and cuticular constituents. Fluid state esters composed of 18-carbon unsaturated fatty acids (linoleic or oleic acids), the 15-carbon alcohol (trans, trans, farnesol – a colorless liquid), and 3- to 5-carbon alcohols become abundant in the surface of ripe 'Jonagold' and 'Cripps Pink'

apples, which resulted in a greasy feeling when touched (Yang et al., 2017). The greasiness rating of 'Honeycrisp' apples of all treatments steadily increased with storage time (Fig. S.2-A1-C) indicating that the fruit became overripe and were likely unacceptable to consumers. Repeated doses of 1-MCP retarded greasiness development. This is consistent with previous finding in which 1-MCP treatment delayed greasiness development on other apple varieties after cold storage (Fan et al., 1999a). However, Curry (2008) illustrated that there were only minor difference in epicuticular wax, in terms of its morphology and biochemistry, between 1-MCP treated and untreated 'Autumn Gold' and 'Royal Gala' apples, even though the treated fruits showed lower greasiness rate than the untreated ones. It was suggested that several key wax components involved in greasiness were indirectly hindered by 1-MCP (Curry, 2008).

Effect of 1-MCP multi-application on fruit juice's pH, total titratable acid (TA), injury, senescence breakdown

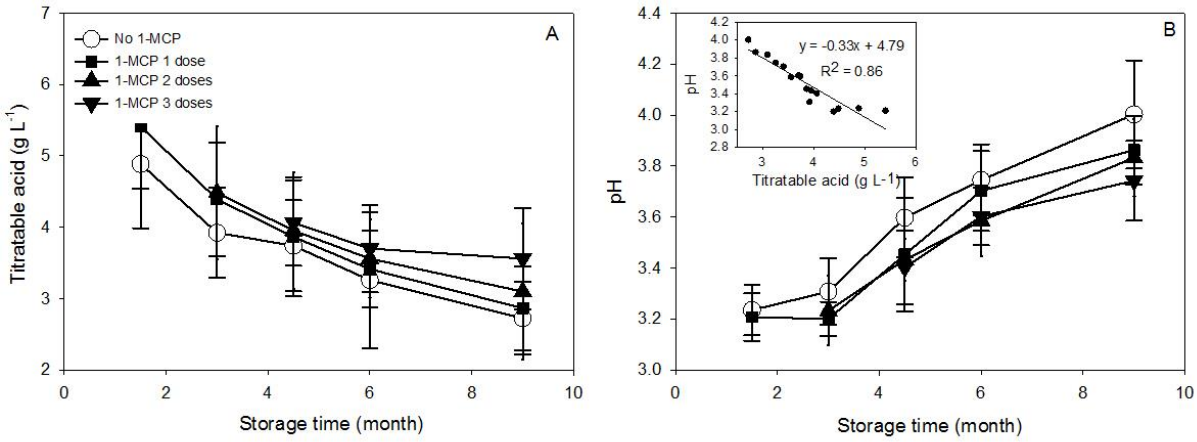
Acidity and the pH of apple juice are important contributors to the taste quality of apples (Yahia, 1994). In our study, the TA of apple juice steadily declined throughout storage, showing that acids had been used as substrates for respiration. There were no difference in the TA or pH between the fruit untreated and treated with 1-MCP for one, two or three doses (Fig. S-A. 2A and 2B). There was a highly significant linear relationship between TA and pH of the apple juice (Fig. S2A. 2-B inset). These results were consistent with previous study on 'Honeycrisp' with single dose of 1-MCP (Watkins and Nock, 2012) as well as on other varieties (Watkins et al., 2000; Mir et al., 2001; DeEll et al., 2016) with single or repeated 1-MCP applications in which the 1-MCP did not maintain tartness for the apples.



Supplementary Figure 2A. 1. Effect of 1-MCP multiple applications on internal ethylene concentration (A), fruit firmness (B) and skin greasiness (C) of 'Honeycrisp' apple during RA storage (21 kPa O_2 and 0 kPa CO_2) at 3 °C. The fruit were exposed to no 1-MCP (open circle), 1 dose 1-MCP (solid square), 2 doses of 1-MCP (solid triangle) and 3 doses of 1-MCP (solid inverted triangle). Each symbol represents fruit from four orchards, n=10 fruit per orchard; bars are ± 1 SD.

CA injury and senescent breakdown have been investigated by Watkins and Nock (2012) on 1-MCP treated 'Honeycrisp' fruit. They found that 1-MCP could enhance CA (CO_2)

injury. In our work, 1-MCP in combination with air storage did not completely suppress the low level of 'CA injury' (~ 7.5%) or senescent breakdown (~ 7.5%) despite the lack of CO_2 in the storage atmosphere (Table S-A. 2). Therefore, there is still a need to control the internal injury we term 'CA injury' beyond the use of non-CA (i.e., air) storage conditions for this variety.



Supplementary Figure 2A. 2. Effect of 1-MCP on acidity (A) and juice pH (B) of 'Honeycrisp' apple harvested from the four orchards during air storage (21 kPa O_2 and 0 kPa CO_2) at 3 °C. The fruit were exposed to no 1-MCP (open circle), 1 dose 1-MCP (solid square), 2 doses of 1-MCP (solid triangle) and 3 doses of 1-MCP (solid inverted triangle). Each symbol represents fruit from four orchards, n=5 fruit per orchard; bars are ± 1 SD. The relationship between TA and pH is indicated in B (inset).

Supplementary Table 2A. 2. Senescent breakdown and CA injury incidence of 'Honeycrisp' apple treated with 0, 1, 2, and 3 doses of 1-MCP and stored under RA (21 kPa O_2 and 0 kPa CO_2) at 3°C for 1.5, 3, 4.5, 6, and 9 months (n=20 per orchard/storage duration/1-MCP dosage combination)

Storage time (months)	Senescent breakdown (%)				CA injury (%)			
	No 1-MCP	1-MCP 1 dose	1-MCP 2 doses	1-MCP 3 doses	No 1-MCP	1-MCP 1 dose	1-MCP 2 doses	1-MCP 3 doses
1.5	0.0	0			0.0	0.0		
3	0.0	0	0		0.0	0.0	0.0	
4.5	7.5	2.5	0	0	2.5	4.0	2.5	2.5
6	0.0	0	2.5	2.5	0.0	7.5	5.0	0.0
9	0.0	0	0	0	0.0	0.0	0.0	0.0

Effect of 1-MCP multi-application on fruit volatiles production

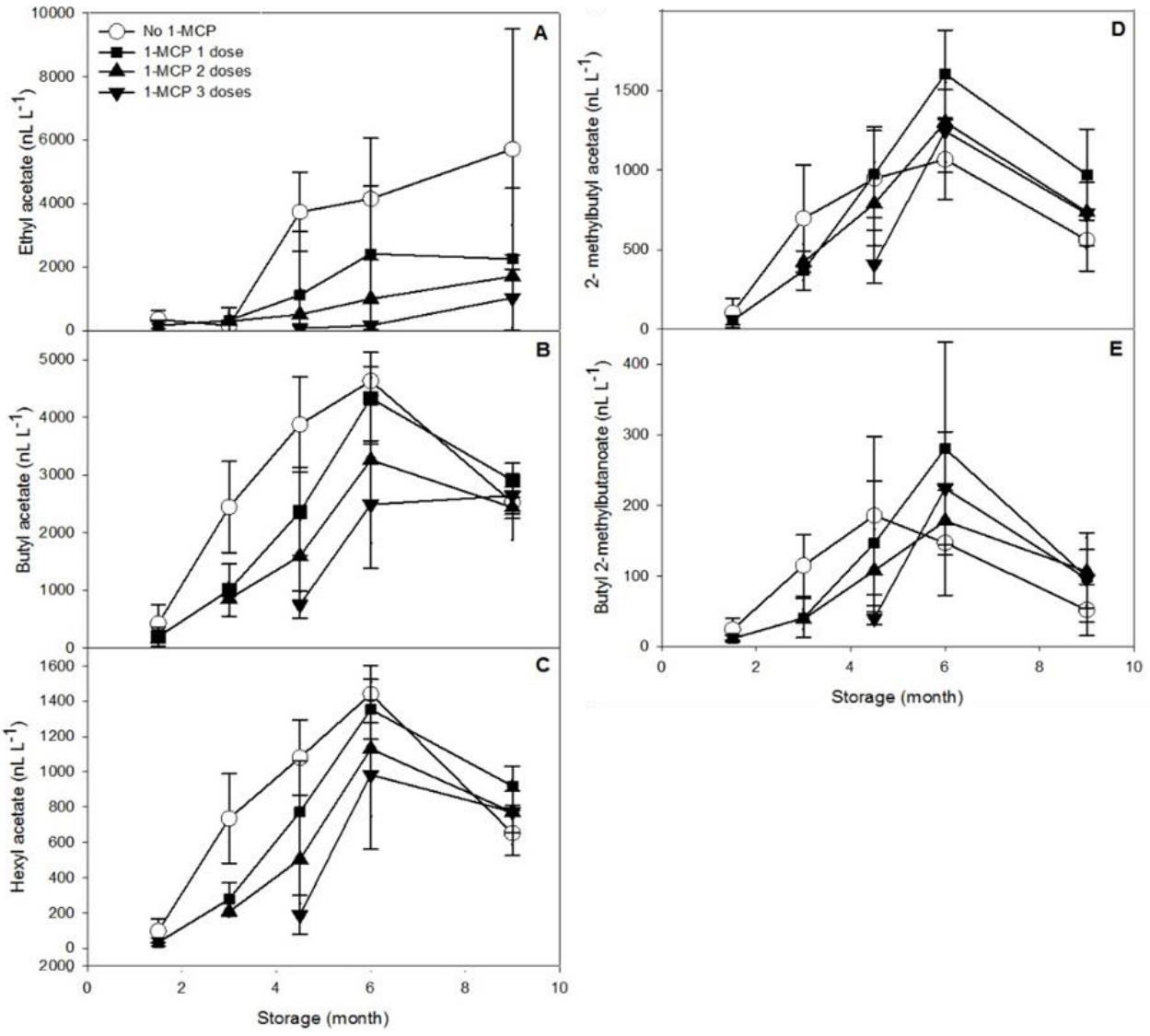
In addition to taste, aroma is a critical component for apple quality perceived by consumers (Beaudry, 2000). More than 300 apple aroma compounds have been investigated (Dimick and Hoskin, 1983). Each variety has unique aroma complex (Sugimoto et al., 2015; Espino-Díaz et al., 2016). The aroma profiles of apples change during development in which they are dominated by aldehydes at immature stages of development and alcohols and esters during maturation and ripening (Fellman et al., 1993, 2000). Esters occupy 78 – 92% of the total volatile profile by weight during ripening (Dixon and Hewett, 2000).

In this experiment, to evaluate single and repeated doses of 1-MCP on volatile production from matured 'Honeycrisp' advancing to ripening and senescence, five aroma ester compounds were investigated: ethyl acetate, butyl acetate, hexyl acetate, 2-methylbutyl acetate, and butyl 2-methyl butanoate. For many ripening apples varieties, four typical and universal aroma ester compounds are butyl acetate, hexyl acetate, 2-methyl butyl acetate, and ethyl 2-methylbutyrate (Plotto et al., 1999; Fellman et al., 2000). Because the latter is typically

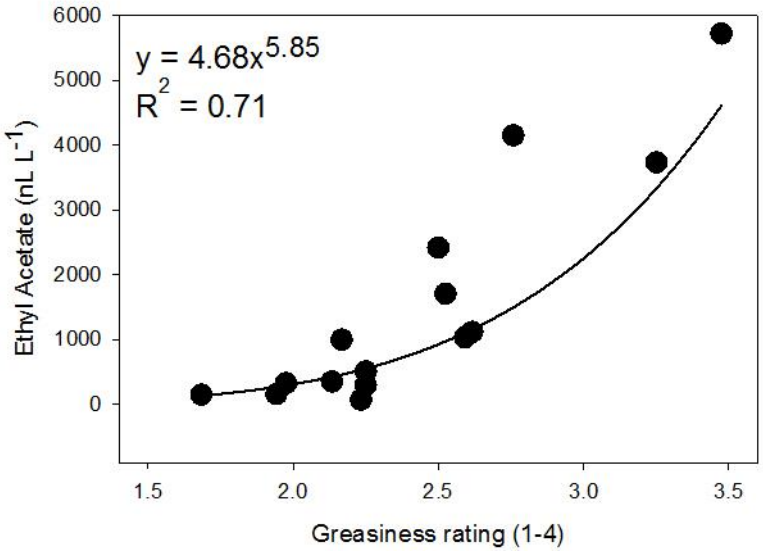
found at low levels (Fellman et al., 2000), we did not evaluate its production. Instead, another branched-chain ester with the same alkanoate group (butyl 2-methyl butanoate), which was plentiful in ripening in 'Jonagold' apple fruit (Sugimoto et al., 2011), was studied. Ethyl acetate, together with 3 other acetate esters with hexyl, butyl, and 2-methyl butyl alcohols commonly found in apples (Fellman and Mattheis, 1995; Mattheis et al., 1991) was also targeted in this study.

Production of three acetate esters with butyl, hexyl, and 2-methyl butyl alcohol groups had similar patterns with only slight variations (Fig. S2A. 3 B-D) among treated and untreated treatments. Their production steadily increased to their peaks at 6 storage months and then dropped, except for butyl acetate for the triply dosed fruit, which continued to be produced thereafter (Fig. S2A. 3B) and for butyl 2-methyl butanoate of control apple with its peak at 4.5 storage months (Fig. 3E). Ethyl acetate, on the contrary, linearly increased with storage time (Fig. S2A. 3A). When overripe, shorter chain alcohols might be dominating precursors for esterification, and ethyl esters increased advancing to senescence (Panasiuk et al., 1980; Willaert et al., 1983). The production pattern of these aroma profiles was comparable to ripening pears (Rapparini and Predieri, 2003) and 'Red Chief Delicious' apple (Ferenczi, 2004).

When using transgenic apples lacking ACC-synthase and ACC-oxidase enzymes for investigating the effect of 1-MCP on ethylene production and action, Defilippi et al. (2004) proved that a continuousness of ethylene production and perception was required for aroma biosynthesis (Fan et al., 1998). Application of 1-MCP reduced aroma production in many apple varieties (Bai et al., 2005; Kondo et al., 2005; Ferenczi et al., 2006).



Supplementary Figure 2A. 3. Effect of 1-MCP on production of ethyl acetate (3A), butyl acetate (3B), hexyl acetate (3C), 2-methylbutyl acetate (3D), and butyl 2-methylbutanoate (3E) of 'Honeycrisp' apple harvested from the four orchards during air storage (21 kPa O_2 and 0 kPa CO_2) at 3°C. The fruit were exposed to no 1-MCP (open circle), 1 dose 1-MCP (solid square), 2 doses of 1-MCP (solid triangle) and 3 doses of 1-MCP (solid inverted triangle). Each symbol represents fruit from four orchards, n=5 fruit per orchard; bars are ± 1 SD.



Supplementary Figure 2A. 4. The relationship between ethyl acetate and skin greasiness of 'Honeycrisp' apples treated with 1-MCP. Each symbol represents fruit from four orchards, n=5 fruit per orchard

In this study, 1-MCP not only suppressed ethylene perception, but reduced its production (Fig. S2-A.1). However, the reduced amount of ethylene may have been enough to activate genes involved in aroma production. As a result, the production of the volatiles of interest were not suppressed as completely as they might have been if the initial treatments took place prior to the onset of ethylene production (Ferenczi et al., 2006). After 9 months of storage, excepting for ethyl acetate, which continued to increase and had significant differences among treatments, production of other volatile compounds declined and fruit from each treatment produced these compounds at approximately the same rate (Fig. S2-A.3).

CONCLUSIONS

1-MCP reduced internal ethylene and aromatic compound production and delayed greasiness on apple skin relative to untreated 'Honeycrisp' fruit. Additional doses of 1-MCP for further delaying ripening were only slightly more effective than a single dose. With the

exception of ethyl acetate, little difference was detected between treatments in the production of volatile esters. The harvest maturity may influence the success of repeated 1-MCP treatments and should be further investigated. At this time, however, we recommend a single treatment of 1-MCP immediately following harvest for air-stored 'Honeycrisp' apple fruit and a moderate storage duration of 3 to 4 months. A shorter interval, increased frequency, or continuous application of this ethylene antagonist on mature 'Honeycrisp' needs to be investigated in the future.

Acknowledgments

This research was partially financed by the Vietnam Education Foundation, the Michigan Apple Committee and AgbioResearch at Michigan State University.

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APPENDIX B. Supplementary tables and figures

Supplementary Table 2B. 1. Maturity indices of 'Honeycrisp' apples harvest from commercial orchards in Michigan in 2014 – 2017[a].

Orchard	Area	Harvest day	Ethylene (ppm)	Redness (%)	Background (1-5)	Starch (1-8)	TSS (°Brix)	Firmness d0 (lb)	Firmness d7 (lb)
2014									
A	South Lyon	9/11	7.9 ± 3.9	54.5 ± 5.0	1.4 ± 0.2	6.1 ± 0.4	14.3 ± 0.5	14.4 ± 0.4	15.1 ± 0.4
B	Sparta	9/16	2.3 ± 1.0	66.5 ± 4.8	2.8 ± 0.3	2.6 ± 0.7	12.0 ± 0.1	14.5 ± 0.5	14.6 ± 0.4
C	Harford	9/18	11.4 ± 3.7	57.5 ± 7.3	3.7 ± 0.2	5.6 ± 0.6	13.5 ± 0.9	12.9 ± 0.4	15.0 ± 0.3
D	Belding	9/23	10.3 ± 4.0	69.3 ± 5.5	3.0 ± 0.0	7.0 ± 0.2	13.0 ± 0.5	13.0 ± 0.4	13.8 ± 0.2
E	Ludington	9/25	13.9 ± 4.9	72.3 ± 6.5	1.7 ± 0.2	5.6 ± 0.3	13.4 ± 0.4	16.1 ± 0.4	14.8 ± 0.3
2015									
F	Sparta	9/17	12.3 ± 2.5	92.5 ± 1.1	1.5 ± 0.2	6.4 ± 0.5	13.7 ± 0.3	16.3 ± 0.4	16.4 ± 0.6
B	Sparta	9/17	12.3 ± 5.0	71.0 ± 6.3	2.1 ± 0.2	5.0 ± 0.0	12.5 ± 0.4	15.4 ± 0.4	15.4 ± 0.3
D	Belding	9/24	10.5 ± 2.4	90.5 ± 2.8	1.2 ± 0.1	6.5 ± 0.2	13.5 ± 0.3	15.2 ± 0.4	12.7 ± 0.5
E	Ludington	9/24	5.2 ± 3.8	48.5 ± 4.3	1.7 ± 0.2	7.1 ± 0.1	12.2 ± 0.4	15.0 ± 0.3	17.4 ± 0.8
2016									
C	Harford	9/14	64.9 ± 8.7	55.0 ± 6.7	2.6 ± 0.4	8.0 ± 0.0	10.8 ± 0.7	14.5 ± 0.9	15.4 ± 0.4

Supplementary Table 2B.1 (cont'd)

B	Sparta	9/15	1.1 ± 0.3	73.0 ± 5.9	3.7 ± 0.3	4.0 ± 0.6	12.6 ± 0.2	17.3 ± 0.6	17.8 ± 0.6
D	Belding	9/20	40.3 ± 8.0	69.5 ± 6.7	1.3 ± 0.2	7.7 ±	12.4 ± 0.5	14.3 ± 0.4	14.5 ± 0.4
E	Ludington	9/27	6.8 ±2.3	76.0 ± 6.7	1.4 ± 0.2	8.0 ± 0.0	12.8 ± 0.4	14.5 ± 0.4	14.5 ± 0.3
G	Transverse	10/10	29.5 ± 9.3	32.5 ± 6.7	4.0 ± 0.0	7.7 ± 0.2	11.0 ± 0.3	15.9 ± 0.8	14.3 ± 0.5
2017									
F	Sparta	9/8	5.3 ± 0.6	81.0 ± 7.0	1.40 ± 0.2	5.9 ± 0.3	14.6 ± 0.4	17.4 ± 0.5	16.8 ± 0.2
H	Sparta	9/15	27.3 ± 6.2	77.0 ± 5.9	2.2 ± 0.3	7.5 ± 0	14.5 ± 0.3	15.0 ± 0.4	16.8 ± 0.4

[a] Values are means SE for analyses of 10 fruit except TSS using 5 fruit.

Supplementary Table 2B. 2. Effect of CO_2 on controlled atmosphere injury index (0-1)* of 'Honeycrisp' apples fruit from five orchards in 2014, two orchards in 2015, and four orchards in 2016. (N = 10 - 20 fruit, except day 240 in 2014 using 120 fruit. Replicates = 2 for CO_2 concentration). Tukey's test was used for multiple comparison analysis of averages.

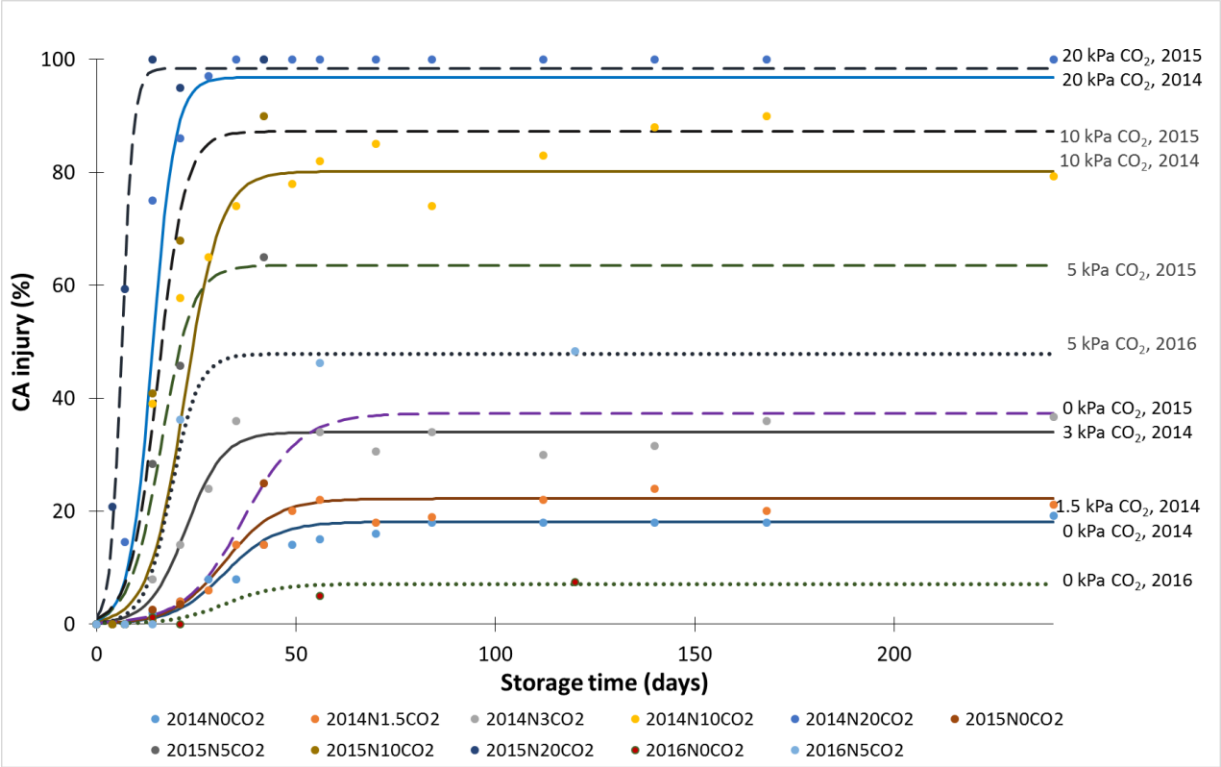
Storage Day	2014					2015				2016	
	0 kPa CO_2	1.5 kPa CO_2	3 kPa CO_2	10 kPa CO_2	20 kPa CO_2	0 kPa CO_2	5 kPa CO_2	10 kPa CO_2	20 kPa CO_2	0 kPa CO_2	5 kPa CO_2
4						0.00	0.00	0.00	0.14		
7	0.00	0.00	0.00	0.00	0.05 ± 0.03	0.00	0.00	0.00	0.45 ± 0.083	0.00	0.00
14	0.01 ± 0.01	0.00 ± 0	0.04 ± 0.04	0.18 ± 0.05	0.61 ± 0.14	0.01 ± 0.009	0.18 ± 0.068	0.33 ± 0.146	0.89 ± 0.057	0.00	0.12 ± 0.09
21	0.02 ± 0.02	0.02 ± 0.02	0.06 ± 0.02	0.37 ± 0.07	0.84 ± 0.10	0.01 ± 0.006	0.36 ± 0.164	0.49 ±0.196	0.92 ±0.057	0.00	0.22 ± 0.19
28	0.05 ± 0.04	0.02 ± 0.01	0.14 ± 0.06	0.53 ± 0.13	0.90 ± 0.06						
35	0.09 ± 0.05	0.09 ± 0.05	0.22 ± 0.08	0.52 ± 0.12	1.00 ± 0						
42	0.03 ± 0.02	0.11 ± 0.05	0.22 ± 0.10	0.51 ± 0.08		0.18 ±0.084	0.79 ±0.075	0.88 ±0.053	0.98 ±0.013		
49	0.04 ± 0.01	0.14 ± 0.04	0.27 ± 0.11	0.68 ± 0.09							
56	0.04 ± 0.02	0.13 ± 0.03	0.23 ± 0.08	0.68 ± 0.06						0.00	0.31 ± 0.17
70	0.06 ± 0.03	0.10 ± 0.03	0.21 ± 0.09	0.66 ± 0.06							
84	0.10 ± 0.06	0.10 ± 0.05	0.19 ± 0.09	0.59 ± 0.06							
112	0.11 ± 0.08	0.13 ± 0.03	0.17 ± 0.08	0.69 ± 0.07							

Supplementary Table 2B.2 (cont'd)

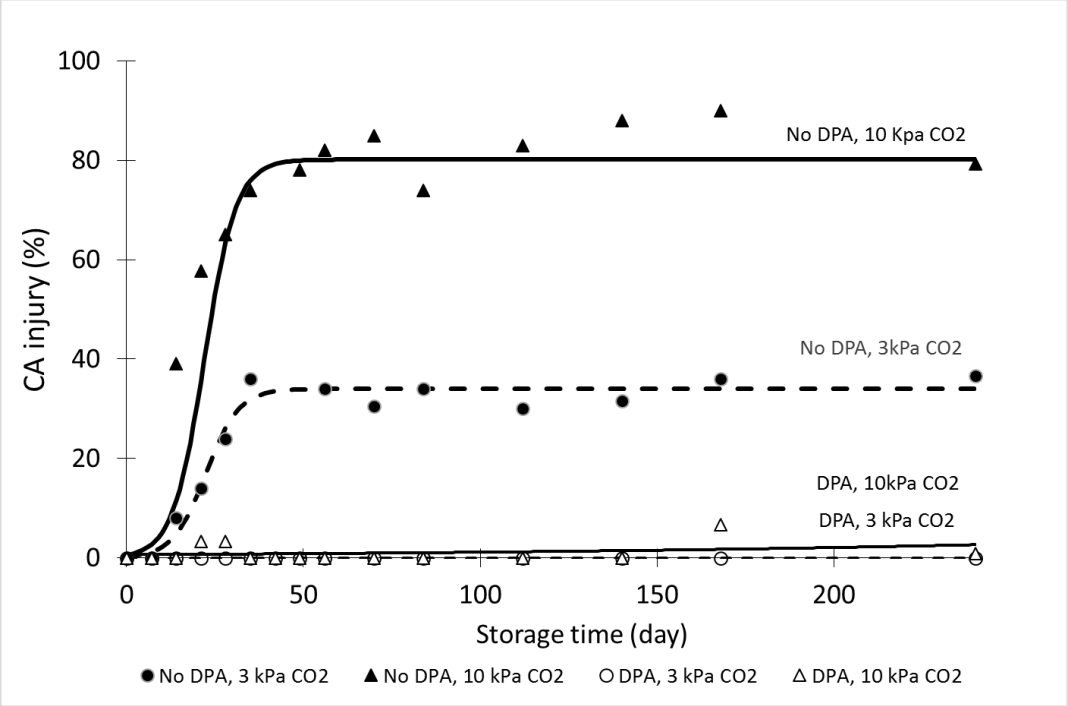
										0.006 ±0.006	0.30 ± 0.16
140	0.11 ± 0.07	0.13 ± 0.04	0.16 ± 0.09	0.66 ± 0.09							
168	0.10 ± 0.06	0.10 ± 0.04	0.24 ± 0.01	0.71 ± 0.08							
240	0.14 ± 0.08	0.12 ± 0.05	0.23 ± 0.06	0.63 ± 0.04							

	2014		2015		2016	
Source	*DF*	*Pr > F*	*DF*	*Pr > F*	*DF*	*Pr > F*
Model	*74*	*<.0001*	*23*	*<.0001*	*11*	*0.0290*
CO_2	*4*	*<.0001*	*3*	*<.0001*	*1*	*0.0068*
day	*14*	*<.0001*	*5*	*<.0001*	*5*	*0.3250*
*CO_2*day*	*56*	*<.0001*	*15*	*0.0033*	*5*	*0.3524*

* Numbers in the column followed by differing letters were different. CA injury index = ((no- injury-fruit number/total fruit)*0 + injury - rate -1- fruit number/total fruit)*1 + (injury -rate – 2 - fruit number/total fruit)*2 + (injury - rate -3- fruit number/total fruit)*3 + (injury rate – 4 - fruit number/total fruit)*4)/4

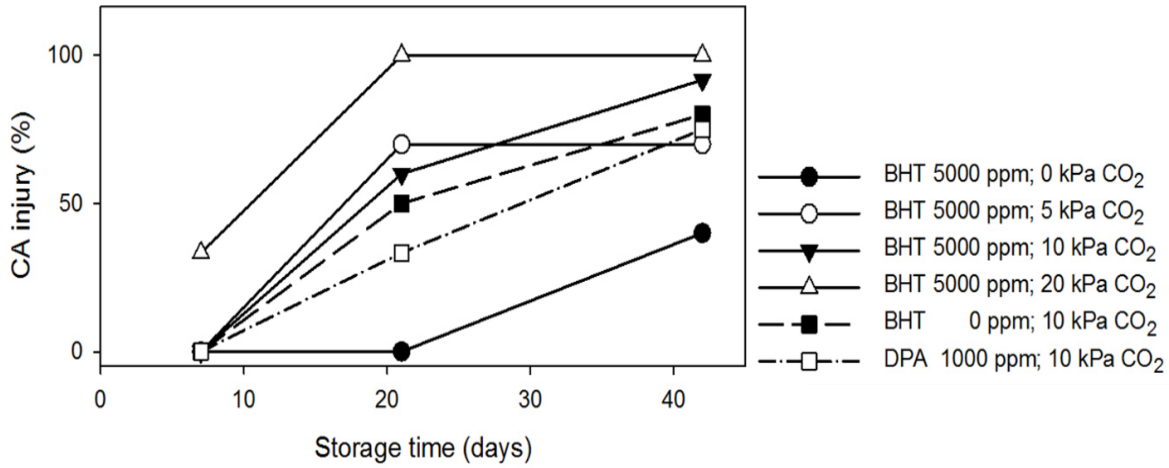


Supplementary Figure 2B. 1. Effect of CO_2 on CA injury of the fruit in 2014, 2015 and 2016 (n = 10 -20 fruit, except day 204 in 2014 using 120 fruit). Curve fit lines of each CO_2 concentration was decided by SAS function to have equation CA injury = A/(1+b*exp(-k*day), at which A, b, and k values as follows: Year 2014: 0 kPa CO_2 (A 18.22, b 137.59, k -0.15), 1.5 Kpa CO_2 (A 22.21, b 139.59, k -0.15), 3 kPa CO_2 (A 33.99, b 124.98, k -0.22), 10 kPa CO_2 (A 80.14, b 137.59, k -0.22), 20 kPa CO_2 (A 96.81, b 137.59, k -0.35); Year 2015: 0 kPa CO_2 (A 37.33, b 124.98, k -0.14), 5 kPa CO_2 (A 63.52, b 63.59, k -0.26), 10 kPa CO_2 (A 87.23, b 86.33, k -0.29), 20 kPa CO_2 (A 98.43, b 79.00, k 0.69); Year 2006: 0 kPa CO_2 (A 7.13, b 137.59, k -0.15), 5 kPa CO_2 (A 47.84, b 233.71, k -0.29)

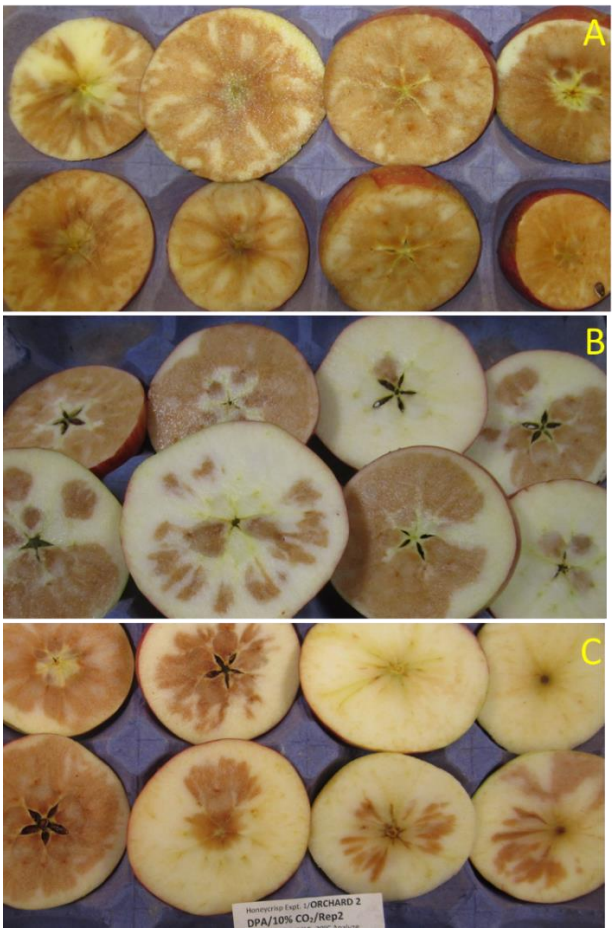


Supplementary Figure 2B. 2. Effect of DPA (1000ppm, 30s) on elimination of CA injury in 'Honeycrisp' apples harvested from orchards A, B, and C in 2014

Supplementary Figure 2B. 3. The fruit harvested from orchard A in 2014 exposed maximal CA injury when stored at 10 kPa CO_2 (A) and 20 kPa CO_2 (B) in combination with 3 kPa O_2 at 3°C at day 56 and 28, respectively.



Supplementary Figure 2B. 4. Effect of DPA (1000 ppm, 30 sec) or BHT (5000 ppm, 30 sec) on CA injury of fruit from orchard F contained in buckets and exposed to 0, 5, 10, 20 kPa CO_2 using mixed CA lines for 42 days in 2015



Supplementary Figure 2B. 5. CA injury of fruit from orchard F in 2015, control (A), treated with BHT 5000 ppm (B), or with DPA 1000 ppm (C) before contained in buckets and exposed to 10 kPa CO_2 using mixed CA lines for 42 days

Supplementary Table 2B. 3. Maturity indices of the fruit harvest from commercial orchards F and H after preconditioning treatment at the field and the lab.

Preconditioning		Analysis day 0						Analysis day 7		
Day	Place	Ethylene (ppm)	Redness (%)	Background (1-5)	Starch (1-8)	TSS (°Brix)	Firmness (lb)	TSS (°Brix)	Firmness (lb)	TA (%)
Orchard F										
0	Field	5.3 ± 0.6	81.0 ± 7.0	1.4 ± 0.2	5.9 ± 0.3	14.6 ± 0.2	17.4 ± 0.4	14.2 ± 4.5	16.8 ± 0.5	6.9 ± 2.2
1	Field	7.1 ± 2.4	56.5 ± 5.1	1.9 ± 0.1	6.5 ± 0.3	14.8 ± 0.2	21.3 ± 0.4	14.4 ± 4.5	17.1 ± 0.4	6.8 ± 2.2
3	Field	9.9 ± 3.1	78.3 ± 6.0	1.5 ± 0.2	6.1 ± 0.2	14.6 ± 0.2	20.9 ± 0.5	14.5 ± 4.6	16.8 ± 0.7	6.4 ± 2.0
5	Field	101.8 ± 20.9	74.5 ± 7.0	1.7 ± 0.2	6.6 ± 0.2	14.9 ± 0.3	18.5 ± 0.5	14.5 ± 4.6	16.9 ± 0.5	6.5 ± 2.1
7	Field	57.7 ± 10.0	78.8 ± 3.2	2.2 ± 0.2	7.3 ± 0.1	15.0 ± 0.1	16.1 ± 0.5	14.6 ± 4.6	17.9 ± 0.4	6.6 ± 2.1
5	Lab	168.8 ± 38.2	77.1 ± 4.6	1.00 ± 0	7.7 ± 0.1	15.0 ± 0.2	18.1 ± 0.4			
Orchard H										
0	Field	27.3 ± 6.2	77.0 ± 5.9	2.2 ± 0.3	7.5 ± 0	14.5 ± 0.3	15.0 ± 0.3	14.0 ± 4.4	16.8 ± 0.4	5.0 ± 1.6
1	Field	69.8 ± 9.0	68.5 ± 6.1	1.9 ± 0.3	7.7 ± 0.1	14.1 ± 0.2	16.2 ± 0.3	14.0 ± 4.4	17.4 ± 0.5	5.4 ± 1.7
3	Field	82.8 ± 10.9	72.0 ± 8.2	1.0 ± 0	8.0 ± 0	13.9 ± 0.2	19.0 ± 0.5	12.5 ± 4.0	16.6 ± 0.4	4.8 ± 1.5
5	Field	78.8 ± 17.8	77.8 ± 5.2	1.2 ± 0.1	8.0 ± 0	14.5 ± 0.2	19.1 ± 0.5	12.8 ± 4.0	20.3 ± 3.1	4.6 ± 1.5
7	Field	98.3 ± 23.1	73.5 ± 6.7	1.7 ± 0.2	8.0 ± 0	13.5 ± 0.1	16.9 ± 0.5	12.6 ± 3.9	16.5 ± 0.4	4.4 ± 1.4
5	Lab	89.6 ± 25.3	69.5 ± 7.2	1.6 ± 0.2	8.0 ± 0	13.6 ± 0.1	18.3 ± 0.5	12.8 ± 4.0	16.5 ± 0.4	4.6 ± 1.4

Supplementary Table 2B. 4. Storage disorder of ‘Honeycrisp’ apple from orchard F after preconditioning treatment at the field and the lab and stored under CA conditions at 3 °C

Preconditioning		CO_2	Greasiness	Decay	Lens-shaped	Soft scald	CA injury	Injury index
Day	Place	(kPa)	(%)	(%)	(%)	(%)	(%)	(0-1)
0	Field	0	1.43	1.28	2.50	41.92	49.20	0.38
1	Field	0	1.30	1.92	3.33	32.56	48.97	0.41
3	Field	0	1.60	2.50	1.25	46.53	35.09	0.27
5	Field	0	2.02	2.96	2.96	8.56	15.32	0.09
7	Field	0	2.23	5.03	2.33	3.31	0	0
5	Lab	0	1.80	9.45	0.00	15.34	3.57	0.03
0	Field	3	1.63	1.14	1.39	54.29	86.36	0.74
1	Field	3	1.97	5.00	0	41.07	75.02	0.67
3	Field	3	1.80	3.64	0	21.94	76.58	0.63
5	Field	3	2.42	1.11	0	1.89	31.96	0.22
7	Field	3	2.28	4.20	0	6.72	4.48	0.03
5	Lab	3	2.43	12.67	0	2.94	7.69	0.04
0	Field	5	1.65	3.22	2.44	49.29	82.59	0.72
1	Field	5	1.42	4.63	0.96	50.70	81.70	0.74
3	Field	5	1.87	1.82	6.47	28.28	70.05	0.58
5	Field	5	1.82	5.81	11.01	19.96	47.62	0.35
7	Field	5	1.98	1.97	3.23	6.00	8.06	0.05
5	Lab	5	2.37	14.82	0	2.27	6.00	0.05
0	Field	10	1.97	1.16	0	75.10	98.28	0.92
1	Field	10	2.05	7.14	0	78.57	96.94	0.96
3	Field	10	2.27	6.90	0	61.21	93.10	0.81
5	Field	10	2.42	9.14	0	57.19	79.67	0.75
7	Field	10	2.63	2.62	4.31	28.72	46.26	0.33
5	Lab	10	2.77	0.00	2.38	22.38	63.33	0.28
0	Field	20					100	1
1	Field	20					100	1
3	Field	20					100	1
5	Field	20					100	1
7	Field	20					100	1
5	Lab	20					100	1

Supplementary Table 2B. 5. Storage disorder of ‘Honeycrisp’ apple from orchard H after preconditioning treatment at the field and the lab and stored under CA conditions at 3 °C.

Preconditioning		CO_2	Greasiness	Decay	Lens-shaped	Soft scald	CA injury	Injury index
Day	Place	(kPa)	(%)	(%)	(%)	(%)	(%)	(0-1)
0	Field	0	1.17	1.85	1.04	12.33	19.15	0.13
1	Field	0	1.35	0.85	2.57	0.85	7.69	0.05
3	Field	0	1.65	1.44	0.00	0.77	2.98	0.02
5	Field	0	1.68	0	0	0	0.79	0
7	Field	0	1.95	0	0.83	0.83	0	0
5	Lab	0	1.68	0	0	0	0	0
0	Field	3	1.13	0.76	1.4	9.05	18.64	0.15
1	Field	3	1.38	1.61	0.8	3.34	15.07	0.12
3	Field	3	1.83	0	0	0	12.29	0.08
5	Field	3	2.00	0	1.9	0	2.50	0.01
7	Field	3	1.97	1.67	0.8	0.83	0	0
5	Lab	3	2.47	0	1.4	0	0.72	0.01
0	Field	5	1.40	0	4.23	10.71	40.00	0.35
1	Field	5	1.33	0	2.49	1.64	15.83	0.12
3	Field	5	1.53	0.93	1.72	0.93	10.71	0.06
5	Field	5	2.12	0	0	1.67	3.33	0.03
7	Field	5	2.17	0.83	0	1.81	0	0
5	Lab	5	2.32	1.79	0	0.89	5.12	0.02
0	Field	10	1.47	0.76	2	39.47	66.59	0.50
1	Field	10	1.52	0.00	0	15.34	50.06	0.41
3	Field	10	1.88	2.53	2.6	16.84	48.30	0.34
5	Field	10	1.02	2.31	6.2	9.23	23.08	0.13
7	Field	10	1.83	0.77	0	0.77	2.46	0.01
5	Lab	10	2.37	0	2.73	12.61	33.30	0.21
0	Field	20					100	1
1	Field	20					100	1
3	Field	20					100	1
5	Field	20					100	1
7	Field	20					100	1
5	Lab	20					100	1

Supplementary Table 2B. 6. Internal disorders of the fruit harvested from four commercial orchards in Michigan in 2016. The fruit was treated with DPA, preconditioning for 5 days at 10 °C, and stored under CA conditions with low CO_2 level (0 and 3 kPa) at 3 °C for 120 days.

Treatment factors			Internal disorders (%)			
DPA (ppm)	Preconditioning (day)	CO_2 (kPa)	Lens- shaped void (%)	Senescent breakdown (%)	CA injury (%)	Injury index (0-1)
0	0	0	0	1.63 ± 0.5	2.37 ± 1.0	0.01 ± 0.003
0	0	5	14.74 ± 4.4	1.25 ± 0.5	42.4 ± 10.7	0.30 ± 0.1
0	5	0	0.32 ± 0.2	1.56 ± 0.6	5.9 ± 1.9	0.02 ± 0.01
0	5	5	14.33 ± 5.8	0	43.5 ± 11.9	0.28 ± 0.1
1000	0	0	0	1.70 ± 0.8	0	0
1000	0	5	0	0.31 ± 0.2	0	0
1000	5	0	0	0.31 ± 0.2	0	0
1000	5	5	0	0.94 ± 0.3	0	0

Supplementary Table 2B. 7. Storage disorders of 'Honeycrisp' fruit from two commercial orchards (F and H) in Michigan. The fruit was treated with DPA, kept in the lab from 2-5 days and then stored in CA conditions at 3 °C and 0 °C for 4.5 months

Orchard	Preconditioning day	CO_2 (kPa)	Decay (%)	Senescent breakdown (%)	Lens -shaped cavity (%)	CA injury (%)	Injury index (0-1)
DPA untreated fruit							
F	0	5	2.6 ± 2.6	0	5.3 ± 5	97.4 ± 2.7	0.7 ± 0.01
H	0	5	0	0	2.5 ± 0	30 ± 10	0.2 ± 0.05
F	0	3	45.0 ± 5	3.6 ± 3.6	0	24.3± 4.3	0.2 ± 0.07
H	0	3	21.7 ± 4.3	0	6.5 ± 2.2	26.1 ± 4.4	0.2 ± 0.03
F	2	5	0	0	0	18.8 ± 0	0.2 ± 0
H	2	5	0	0	5 ± 0	15 ± 10	0.08 ± 0.05
F	2	3	7.1 ± 7.1	21.0 ± 7.6	3.3 ± 3.3	10 ± 10	0.04 ± 0.07
H	2	3	0	0	2.4 ± 2.4	0	0
F	5	5	9.4 ± 3.1	0	9.4 ± 3.2	18.8 ± 6.3	0.1 ± 0.05
H	5	5	0	0	10 ± 2.5	10 ± 5	0.05 ± 0.02
F	5	3	11.6 ± 6.0	0	0	0	0
H	5	3	1.3 ± 1.3	0	6.6 ± 1.3	2.6 ± 2.6	0.02 ± 0.02
DPA treated fruit							
F	0	5	10 ± 3.3	13.3 ± 6.7	0	0	0
H	0	5	0	0	0	0	0
F	0	3	3.6 ± 1.5	0	0	0	0
H	0	3	0	0	0	0	0
F	2	5	5 ± 5	0	0	0	0
H	2	5	0	0	0	0	0
F	2	3	7.7 ± 3.6	0	0	0	0
H	2	3	1.7 ± 1.7	0	0	0	0
F	5	5	3.13 ± 3.1	0	0	0	0
H	5	5	0	0	0	0	0

Supplementary Table 2B. 7 (cont'd)

F	5	3	8.3 ± 7.7	0	0	0	0
H	5	3	1.5 ±1.5	0	0	0	0

Supplementary Table 2B. 8. Soft scald incidence (%) of the fruit harvest from two commercial orchards (F and H) in Michigan. The fruit were treated with DPA, kept in the lab from 2-5 days and then stored in CA conditions at 3 °C and 0 °C for 4.5 months.

Orchard	Preconditioning (days)	CO_2 (kPa)	3 °C		0°C	
			DPA	No DPA	DPA	No DPA
F	0	5	0	0	70.8	54.3
H	0	5	0	10	39.3	2.9
F	0	3	3.1	3.3	60	63.3
H	0	3	0	15.2	59.3	11.1
F	2	5	0	0	4.2	0
H	2	5	0	2.5	1.4	4.2
F	2	3	7.4	0	4	10
H	2	3	1.7	0	2.5	2.9
F	5	5	0	0	9.7	0
H	5	5	0	0	0	0
F	5	3	8.3	5.9	4.5	9.4
H	5	3	0	0	0	4.4

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CHAPTER 3.

EFFECTS OF CO_2 AND O_2 ON FERMENTATIVE VOLATILE PRODUCTION OF 'HONEYCRISP' APPLE

3.1 Introduction

While controlled atmosphere (CA) storage has been used for most commercial varieties of apple (Fidler, 1965; Golding and Jobling, 2012), storage of the 'Honeycrisp' apple in modified atmospheres has been impeded by extreme sensitivity to elevated CO_2 and low O_2 (Beaudry and Contreras, 2009; Watkins and Nock, 2012b). Further, 'Honeycrisp' fruit are sensitive to storage temperatures below 3 °C, which cause soggy breakdown and soft scald (Watkins and Rosenberger, 2000; Watkins et al., 2004).

Common symptoms of CA injury include internal browning and lens-shaped voids in the flesh (Burmeister and Dilley, 1995; Elgar et al., 1998). Injury of 'Honeycrisp' under CA storage is exacerbated by elevated CO_2 and reduced O_2 levels (Contreras et al., 2014). The symptom was also found in other cultivars including 'Empire' (Razafimbelo et al., 2006; Watkins, 2010), 'Braeburn' (Elgar et al., 1998; Mattheis and Rudell, 2008), and Fuji (Argenta et al., 2002).

The mechanism of CA injury in 'Honeycrisp' and other apples has not been elucidated. Elevated CO_2 and/or low O_2 in CA conditions can initiate fermentation in apple (Beaudry, 1993), but it is not clear if the products of fermentation are toxic to fruit flesh. Ethanol and acetaldehyde increased in avocado, pears, lettuce and strawberry stored under very low O_2 (0.25 kPa) and extremely high CO_2 (20 – 80 kPa) (Fernández-Trujillo et al., 2001; Ke et al., 1995; Watkins et al., 1999). Under a lower CO_2 concentration (6 kPa) in combination with 0.5 kPa O_2, there was also an accumulation of ethanol, acetaldehyde, and methyl esters in 'Fuji' (Lumpkin et al., 2015) and 'Jonagold' apples (Saquet and Streif, 2008). However, low levels of CO_2 may not directly contribute to an increase in fermentative volatiles (Ke et al., 1995).

It is not certain if fermentative metabolism is a cause or a result of internal disorders (Lee et al., 2012; Pintó et al., 2001; Volz et al., 1998). These volatiles increased linearly with injury

rate for CA-stored apple (Lee et al., 2012). Cell death leading to browning is supposed as a cause of fermentative volatile accumulation (Fernández-Trujillo et al., 2001). Stressful conditions (i.e. ozone, sulfur dioxide, freezing temperature, and drought) caused an increase in acetaldehyde and ethanol levels in red pine and paper birch trees. The levels, however, declined when their leaves became completely necrotic (Kimmerer and Kozlowski, 1982). Under the same CA conditions, diphenylamine (DPA) treated 'Braeburn' apple showed almost no CA injury and emitted lower levels of fermentative volatiles in comparison to DPA untreated fruit and the volatiles were lower in comparison to control fruits that had been damaged (Lee et al., 2012). Although a DPA drench before CA storage suppresses CA injury for 'Honeycrisp' apples (Leisso et al., 2017; Contreras et al., 2014), the fermentative volatile production in this cultivar has not been studied.

The aim of this study was to 1) ascertain whether CO_2-related CA injury of 'Honeycrisp' causes an increased production of fermentative volatiles and 2) determine whether fermentation volatiles induce CA injury. Therefore, we evaluated fermentative volatile production of 'Honeycrisp' apples exposed to different concentrations of CO_2 ranging from 0 - 20 kPa in combination with 3 kPa O_2. DPA, which has been shown to inhibit the formation of CA injury symptoms, was also applied to fruit before CA storage to test if CO_2 caused an increase in fermentation products even when the symptoms of the CO_2-related stress were suppressed by DPA. In 2017, hypoxic (< 0.3 kPa O_2) atmospheres without CO_2 were used to determine whether the fermentation volatiles produced were similar in concentration to those induced by the CA combinations used.

3.2 Materials and methods

3.2.1 Plant Material

In 2014, fruits from five commercial orchards in South Lyon, Hartford, Cassnovia, Belding, and Ludington, Michigan, on September 11, 16, 18, 23, and 25, respectively, were used to test the effect of CO_2 on fermentative volatile accumulation in the fruit (Fig. 3.1). At each orchard, the fruits were harvested in the morning into two 18-bushel bins and the fruit were then manually transferred into to 60 x 40 x 18 cm plastic crates (model 5000206, Twinpack B.V., Netherlands) and immediately transported to the Postharvest Physiology Laboratory at Michigan State University (Fig. 3.2).

In 2017, 'Honeycrisp' apples from another commercial orchard in Sparta, Michigan was used to test the effect of hypoxia storage condition on fermentative volatile production had been stored in refrigerated air for three months.

3.2.2 Experiment 1. Impact of CO_2 and DPA on fermentation volatiles.

Five CO_2 concentrations (0, 1.5, 3, 10, and 20 kPa) in combination with 3 kPa O_2 were generated in CA chambers to test whether the atmosphere altered the accumulation of selected fermentative volatiles (acetaldehyde, ethanol, and ethyl acetate) in 'Honeycrisp' apples (Fig. 3.1). In addition, the fruits treated with DPA (1000 ppm, 30 s drench) and stored under 3 and 10 kPa CO_2 were also used for this fermentative volatile analysis (Fig. 3.2). The atmospheres were monitored and regulated by an atmosphere control system (ICA 61 Laboratory System; International Controlled Atmosphere Ltd., Paddock Wood, U.K.). The temperature of the CA chambers (3 °C) was regulated by the cold room in which the chambers were held. Fruit were stored up to six months or until all apples within a treatment had been damaged. Fruits from each treatment/orchard combination were removed from the storage chamber on day 7, 14, 21, 28, 35,

42, 56, 112, 140, and 168 after placement into storage atmospheres. On each date, emanations of fermentative volatiles (acetaldehyde, ethanol, and ethyl acetate) were measured for cortex tissue samples. Tissue samples were taken from a 2.5-cm thick center transverse slice of each of five randomly selected apples; one tissue cylinder was taken per slice using a cork borer (0.5 cm dia.). The tissue cylinders were cut to 2 cm in length and then placed into 22-mL clear glass vials (Supelco, Bellefonte, PA). The vials were capped with airtight Nylon valves with septa (Mininert, Thermo Scientific, NY) and incubated at 22 °C for 5 min. The quantification of fermentation-related volatiles using gas chromatography/mass spectrometry (GC/MS) was as described below in 3.2.4.

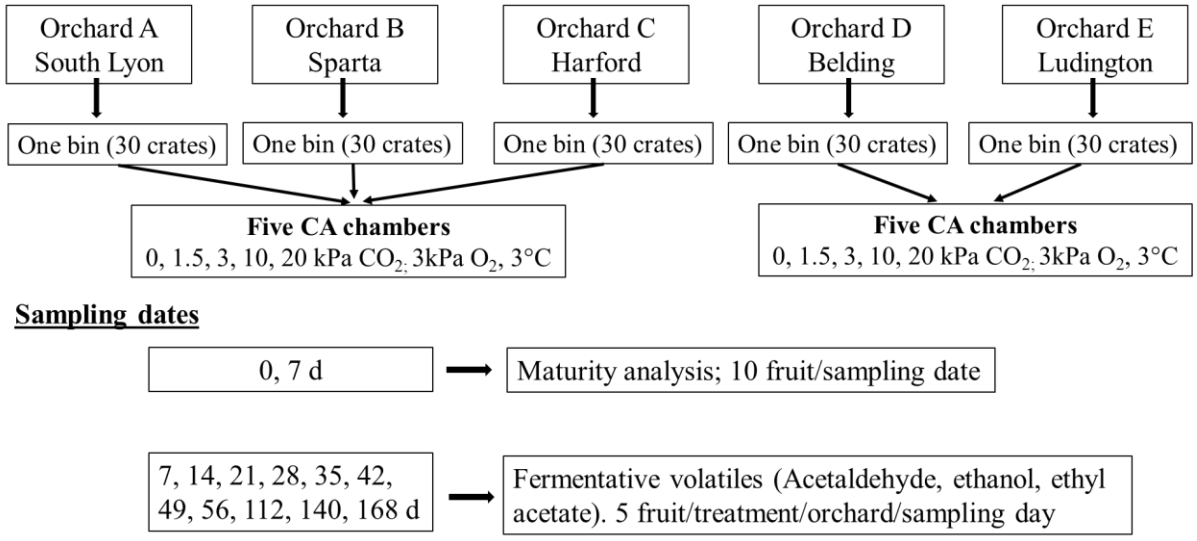


Figure 3. 1. Unbalanced completely random split-plot design using five partial pressures of CO_2 (0, 1.5, 3, 10, and 20 kPa) in combination with 3 kPa O2 at 3 °C for 'Honeycrisp' apple from five commercial orchards in Michigan in 2014. Samples were used for analysis of fermentative volatiles.

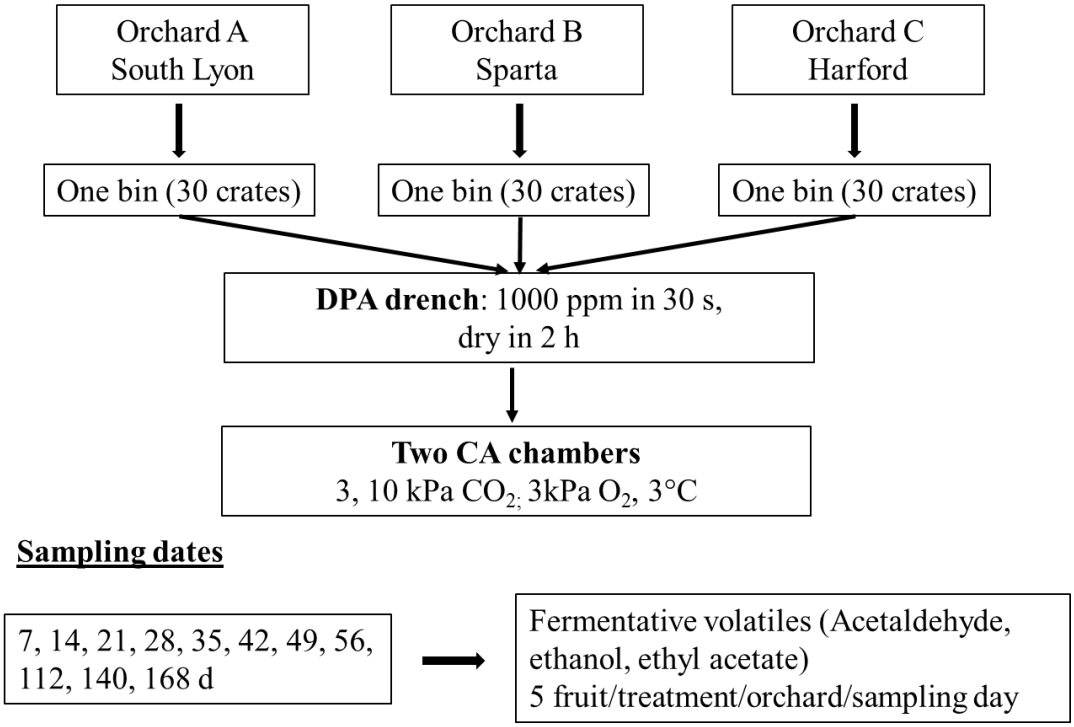


Figure 3. 2. Experiment design of the 'Honeycrisp' apple fruit harvested from three commercial orchards in Michigan in 2014, treated with DPA (1000 ppm, 30s), and then stored under 3 and 10 kPa CO_2 in combination 3kPa O_2 at 3 °C. Samples were used for analysis of fermentative volatiles.

3.2.3 Experiment 2. Effect of O_2 concentration on fermentative volatile production in 'Honeycrisp' apple

To assess the impact of low O_2 on the formation of fermentation volatile production, thirty 'Honeycrisp' fruits that had been stored in air at 3 °C for three months were put into three 20-L plastic buckets (10 fruit each) fitted with airtight gasket-sealed lids (Gamma Plastic Company) and flushed with nitrogen gas at a flow rate 20 mL min^{-1} to achieve three oxygen levels: 0.1, 0.2, and 0.4 kPa. Control fruit were placed in a CA chamber in which oxygen concentration was maintained at 21 kPa. In each environment, the CO_2 partial pressure was 0 kPa and storage temperature was 3 °C. After 14 d, the emission of fermentation-related volatiles

from five whole apples from each treatment. Each apple (approx. 250 g) was placed in a 1.5-L Teflon^{TM} chamber sealed and incubated at 20 °C for 20 min.

3.2.4 Analysis of fermentation volatiles

Emission of three fermentative volatiles (acetaldehyde, ethanol, and ethyl acetate) were measured as previously described (Ferenczi et al., 2006; Song et al., 1997) with minor modifications. For 2014 samples, headspace volatiles were collected using a solid phase micro extraction (SPME) fiber (65 μm thickness PDMS-DVB, Supelco Inc., Bellefonte, PA) for 3 min. Sorbed volatiles were immediately desorbed for 3 min in a gas chromatograph (HP-6890, Hewlett Packard Co., Wilmington, DE, USA) using an inlet temperature of 220 °C. During volatile desorption, a small pool (~10 mL) of liquid nitrogen was placed under the head of the column (20 m long × 0.18 mm i.d., SP-5, Supelco Inc., Bellefonte, PA) to trap desorbed volatiles. Afterwards, trapped gases were released by removal of the liquid nitrogen and separated in the column following programmed heating (50 °C min^{-1} from 40 °C to 240 °C, then maintained at 240 °C for 1 min). Helium was used as a carrier gas and held at a constant flow rate (0.8 mL min^{-1}).

The levels of the volatiles were quantified using gas chromatography coupled to mass spectrometry (GC–MS). The standard mixture used for quantification included equal volumes of 20 compounds (Sigma and Fluka Chemika): *ethyl acetate*, 1-butanol, 2-methyl-1-butanol, 1-propanol-2-methyl, 1-butanol-2-methyl-butyrate, 1-hexanol, 1-propanol, trans-2-hexenyl acetate, trans-2-hexenal, cis-3-hexen-1-ol, 3-methyl-1-butanol, *acetaldehyde*, n-butyl acetate, *ethanol*, ethyl 2-methylbutanoate, ethyl butyrate, ethyl hexanoate, propyl hexanoate, hexyl acetate, and propyl acetate. Only those in italics were used for quantification. A glass syringe (1 uL SGE Zero Dead Vol. Syringe, Alltech) was used to inject 0.5 μL of the standard mixture onto a

24-mm dia. glass microfiber filter, (Whatman™ 24-mm dia., GE Healthcare, Life Science, CAT no. 1822-024), which was then immediately dropped into a 4.4-L glass volumetric flask with a ground-glass top fitted with a Mininert valve (Thermo Scientific, NY) (Song et al., 1997). The flask was sealed, and the mixture was allowed to evaporate. A new standard was freshly made on each day of analysis.

Volatile compounds were identified by comparing collected mass spectra and retention times with those of authenticated reference standards and spectra in the National Institute for Standards and Technology (NIST) mass spectral Search Program (NIST Mass Spectra Library Version 2). Quantification was by comparison of the GC/MS response to that of authenticated reference standards included in the standard mixture. Mass (m/z) ranges of acetaldehyde, ethanol, and ethyl acetate were 43, 74, and 61 respectively. And retention time was ~74, ~76, and ~88 sec for acetaldehyde, ethanol and ethyl acetate, respectively.

Experimental design and statistical analyses

The experiments had a random split-plot design in which CO_2 was a whole plot treatment factor with two CA chambers. For DPA treatment, the experimental design was a completely randomized factorial with two CO_2 levels (3 and 10 kPa) and two DPA levels (0 and 1 g L^{-1}). As before, storage day was a split-plot treatment factor for each treatment.

For experiment 2 in which O_2 treatments were applied, the experimental design was completely randomized with 4 levels of O_2 as a fixed effect. Five fruits per replicate were used for volatile aroma compound analysis.

For volatile analyses, all data for acetaldehyde, ethanol, and ethyl acetate were subjected to test normality and assumptions for ANOVA using SAS 'Proc mixed' procedure (Version 9.4; SAS Institute Inc., Cary, NC). Mean separations were examined using

Duncan's multiple range test and only differences significant at $P \leq 0.05$ were discussed. In order to identify correlated or related pairs of variables and injury index (Supplementary Table 1), a scatterplot matrix displayed all pairwise plots of the data (JMP® Version 9.0). Pearson correlation coefficients were obtained with 'Proc Corr' module in SAS.

3.3 Results

3.3.1 Experiment 1. Effect of CO_2 concentration alone and in combination with DPA on fermentative volatile production

The rate of development and the extent of CA injury of 'Honeycrisp' apples were positively correlated with CO_2 concentration and storage period (Fig. S-B2.1 and Table S-B3.1; see also CHAPTER 2). High CO_2 concentrations (10 and 20 kPa) caused a dramatic escalation in the production of acetaldehyde, ethanol, and ethyl acetate in comparison to lower CO_2 concentrations (0, 1.5, and 3 kPa) (Fig. 3.3). Stored under the low CO_2 levels (0, 1.5 and 3 kPa), the fruit accumulated fermentative volatiles at lower levels, which did not change with storage time (Fig. 3.3). Since all fruit at 20 kPa CO_2 were damaged after four weeks (Fig. S-B2.1 and Table S-B3.1), no fruit were analyzed after that. For 10 kPa CO_2 treatment, the compounds increased and reached their peaks when the fruit received maximal injury at day 42 (Table S-B3.1). After that, ethanol and ethyl acetate had a slight decline, but acetaldehyde level dropped rapidly.

Acetaldehyde levels ranged from approximately 2 to 10 µL L^{-1} over the storage duration except for day 42 which accumulated 20 µL L^{-1}. This difference was not statistically significant among the CO_2 treatments and did not differ with storage time.

Ethanol accumulated in the fruit stored under 20 kPa CO_2. On day 28, ethanol level of fruit stored at this treatment was approximately 15 times higher than that for the 10 kPa CO_2

treatment. Ethanol level did not change with storage time and was not different among the lower CO_2 concentrations (0, 1.5, and 3 kPa) (Fig. 3.3).

Ethyl acetate levels, together with ethanol and acetaldehyde levels, increased exponentially with storage day under 20 kPa CO_2 (Fig S3.1). Under 10 kPa CO_2, the injury level increased at a lower rate, reaching the peak at day 49, and then slightly declined when fruit reached maximal CA injury (Fig 3.3, Fig. S-B2.1 and Table S-B3.1; see also CHAPTER 2).

The ratio of ethanol: acetaldehyde was > 1 at 10 and 20 kPa CO_2; however, it began below 1 for 0, 1.5 and 3 kPa CO_2 treatment, but became larger than 1 when fruit suffered maximal CA injury, and subsequently dropped (Fig 3.3., Fig. S-B2.1 and Table S-B3.1; see also CHAPTER 2). There was a strong positive correlation between these two compounds (r = 0.83) (Fig. 3.4). On the contrary, acetaldehyde showed higher with ethyl acetate (Fig. 3.4). Ethanol might be an indicator showing CA injury severity of 'Honeycrisp' apples because there was a strong positive correlation between injury index and ethanol (r = 0.78) (Fig. 3.4).

DPA treatment prevented CA injury despite storage of apples under 3 or 10 kPa CO_2 (Fig. S-B2.2). Control fruits, however, were severely injured by these CO_2 levels, with half-maximal injury (14%) occurring at day 21 and maximal injury (34%) occurring at day 42 if stored under 3 kPa CO_2 and 34% injury occurring at day 14 and 78% injury occurring at day 42 if stored under 10 kPa CO_2 (Fig. S-B2.2).

DPA treatment had no effect on fermentative volatile emissions for fruit stored under 10 kPa CO_2 before day 35 (Fig. 3.5); at this time, non-DPA treated fruit had already exceeded half maximal injury (Fig. S-B2.1 and Table S-B3.1; see also CHAPTER 2). This suggests that fermentative volatiles did not induce CA injury symptoms. However, after day 35, DPA suppressed the CO_2-induced increase in ethanol and ethyl acetate (Fig. 3.5). The greater injury in

non-DPA treated fruit might cause more production of ethanol and ethyl acetate. Acetaldehyde in DPA-treated fruit reached the peak on day 42, concurrent with the development of maximal injury, and then dropped to levels that did not differ from other treatments. The 3 kPa CO_2 treatment caused CA injury for the control fruit, but the injury was not associated with a significantly higher accumulation of fermentative volatiles than controls (Figure 3.5).

3.3.2 Experiment 2. Effect of O_2 on accumulation of fermentative volatiles in 'Honeycrisp apples

When the fruit were stored under very low O_2 concentration (hypoxia conditions), anaerobic fermentation took place. Ethanol accumulated rapidly in the apple tissue, causing the ratio of ethanol to acetaldehyde to get as high as 50:1. In a preliminary experiment in 2015, the fruits on the day of harvest were stored under extremely low oxygen by letting nitrogen gas run through their chambers. The fruits stored under hypoxia produced much more fermentative volatiles than the fruits stored at 3 kPa O_2 and 5 kPa CO_2 despite no CA injury observed in hypoxia-treated fruit (data not shown).

In 2017, when fruits stored in refrigerated air (RA) for 140 storage days were placed under hypoxia for 3 weeks, low O_2 stress caused fruit to produce much higher ethanol and acetaldehyde levels than those held in air. The fermentative volatile levels were negatively correlated with oxygen concentrations (Table 3.1).

3.3.3 Discussion

Ethanol and acetaldehyde are produced at trace amounts in air-stored apples during ripening process at 0 or 20 °C (Thomas, 1925). Ethyl acetate, however, was not detected in 'Delicious' apples in normal air condition by (Mattheis et al., 1991). The O_2 concentration used in the experiments in the current study was 3 kPa which was higher than the Pasteur point and

ensured that low oxygen stress and/or fermentation did not occur; the low oxygen threshold limit is approximately 0.9% for 'Honeycrisp' apple. (DeLong et al., 2004c; Prange et al., 2013a). Typically, between 2 and 21 kPa O_2, the respiration quotient of apples does not change no matter the storage temperature (Fidler and North, 1967; Gran and Beaudry, 1993). However, accumulation of fermentative volatiles in the fruit can be affected by CO_2 and may vary depending on CO_2 concentration. Since we did not save the fruit under 20 kPa CO_2, which were completely damaged after 28 days of storage, we did not see changes in accumulation of the compounds after that. However, when 10 kPa CO_2 stored fruit reached maximal injury (day 49), the volume of stressed and/or dying tissue would have declined and might not have generated enough substrate for further fermentation product accumulation and ethanol production declined as a consequence (Kimmerer and Kozlowski, 1982). Acetaldehyde, the first product of fermentative respiration, is sometimes considered a toxic metabolite (Smagula et al., 1968) (Dasgupta and Klein, 2014). If acetaldehyde exceeds a certain threshold level in apple fruit it can cause cellular disorganization and browning (Smagula et al., 1968).

Even when oxygen concentration is maintained above the Pasteur limit, CO_2 may have changed the respiration pattern from aerobic to CO_2-zymastic type (Thomas, 1931) by activation of pyruvate decarboxylase for the conversion of pyruvate into acetaldehyde, which would then be converted to ethanol. After that, ethyl acetate is created via an energy requiring pathway, using ethanol and acetyl CoA as substrates (Knee and Hatfield, 1981). At extreme low oxygen (0.1 kPa O_2), the ratio of ethanol to acetaldehyde was only 2:1 on 'Honeycrisp' apples in this experiment (Table 3.1) in comparison to the ratio 50:1 that Thomas (1929) found on 'Newton Wonder' apples.

The suppression of fermentative volatile accumulation by DPA application for the fruit stored at under low levels of CO_2 in DPA-treated 'Honeycrisp' apples was consistent with results of 'Braeburn' apple by Lee et al. (2012). Nonetheless, there has been no evidence to explain why DPA helps maintain tissue integrity for 'Honeycrisp' apples and prevents fermentative respiration. The DPA treated fruit tissue might have been stressed by the CO_2, but not to the extent to cause tissue death and browning. Acetaldehyde was thought to be toxic for plant tissues (Smagula et al., 1968). In this experiment, however, ethanol had a strong positive correlation with the injury than acetaldehyde.

3.4 Conclusion

The production of fermentation volatiles may not be indicative of CA-related injury *per se* because their production did not precede the development of injury. That DPA application prevented the accumulation of fermentation volatiles in an atmosphere of 10 kPa CO_2 suggests that the accumulation of fermentation volatiles is not in direct response to the applied atmospheres, but rather is a downstream response to the injury itself. Further, there was about the same low level of these volatile compounds in both DPA treated and untreated fruit before day 56 (i.e. when control fruit stored at 3 or 10 kPa CO_2 received maximal injury). We, therefore, suggest that the production of fermentation volatiles is a marker for damage, rather than a marker for stress that will eventually result in tissue damage. The nature of the stress induced by CO_2 still needs to be elucidated, likely there are metabolites that may serve as indicators to predict CA injury and provide clues as to how DPA acts to suppress CA-related injury.

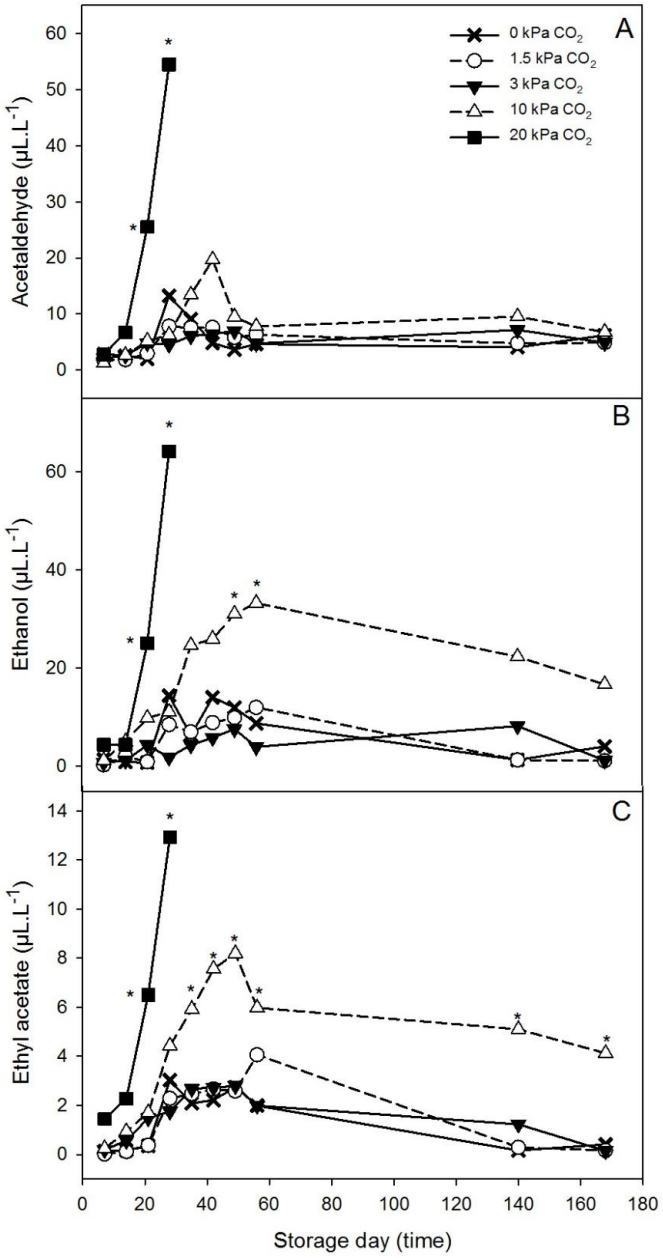


Figure 3. 3. Effect of CO_2 concentrations on the emissions of acetaldehyde (A), ethanol (B) and ethyl acetate (C) of whole 'Honeycrisp' apple during CA storage (0 kPa O_2 with 0- 20 kPa CO_2) at 3 °C. Each symbol represents fruit from five commercial orchards in Michigan in 2014, n=5 fruit per orchard. * indicates significant difference ($P < 0.05$) among the treatments at a particular time.

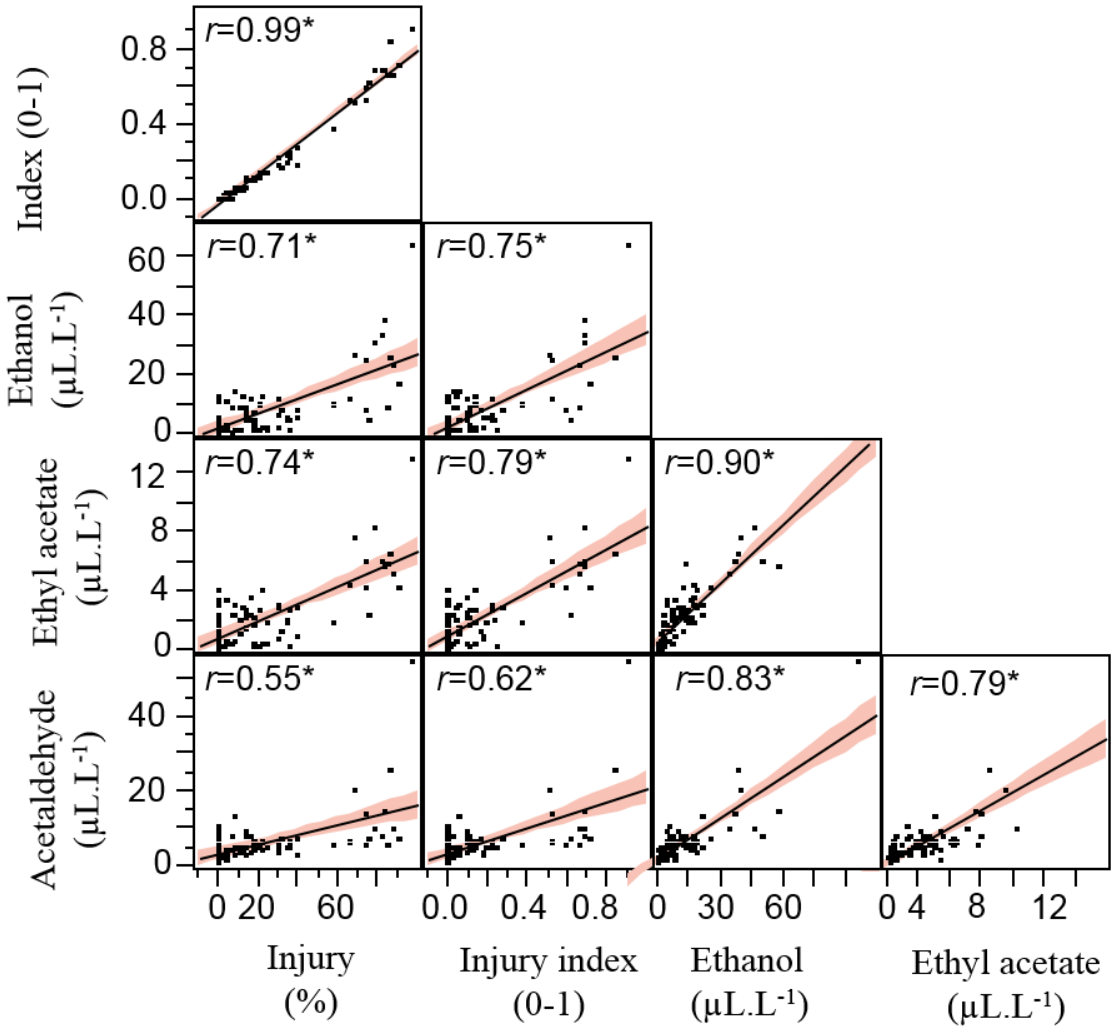


Figure 3. 4. A scatterplot matrix with all pairwise plots of the data of CA injury index and the fermentative variables for CA stored 'Honeycrisp' apple fruit harvested from five commercial orchards in Michigan in 2014. CA storage conditions were 3 kPa O_2 with 0-20 kPa CO_2 at 3 °C. P values all pairwise correlations <0.00001.

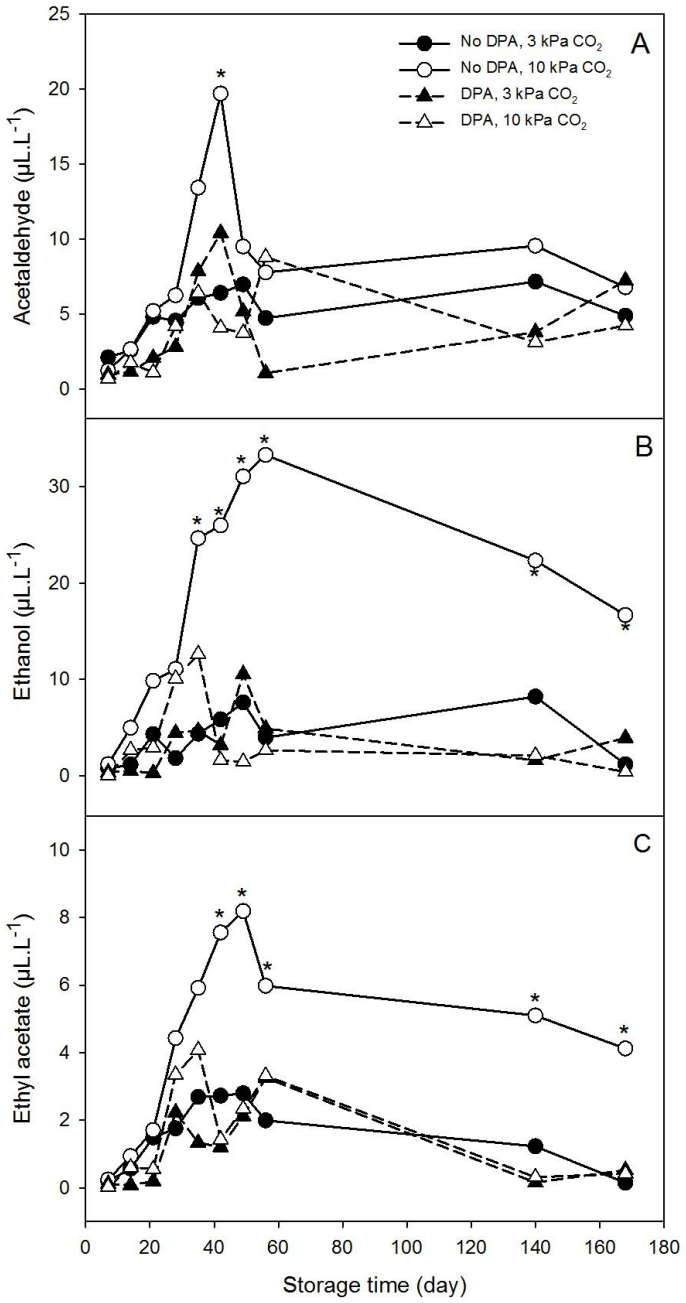


Figure 3. 5. Effect of DPA (1 g·L^{-1} a.i.) applications on emissions of acetaldehyde (A), ethanol (B) and ethyl acetate (C) of whole 'Honeycrisp' apple fruit during CA storage (0 kPa O_2 with 3 kPa CO_2 and 10 kPa CO_2) at 3 °C. Each symbol represents fruit from five commercial orchards in Michigan in 2014, n=5 fruit per orchard. * indicates significant difference ($P \leq 0.05$) among the treatments at a particular time.

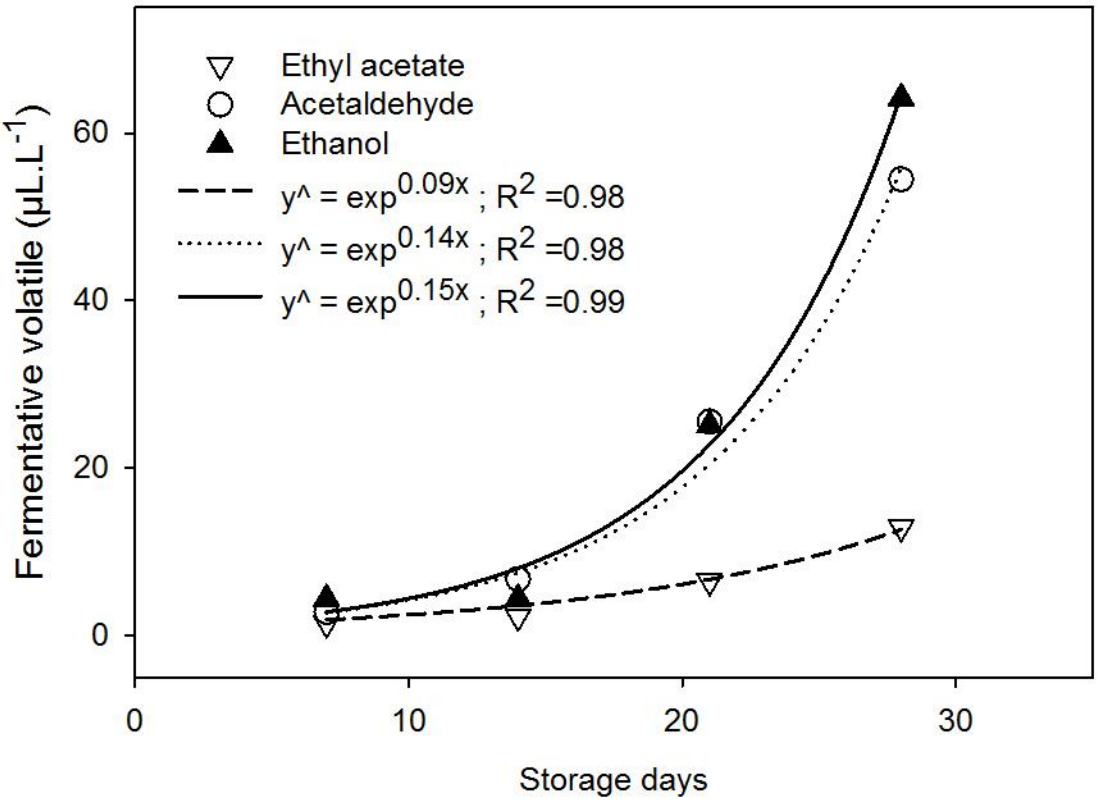
Table 3. 1. Fermentative volatile levels of 'Honeycrisp' apples stored in different low O_2 concentrations (0.1, 0.2, and 0.4 kPa) for 14 days. Control fruit were stored in refrigerated air. N = 5 fruit as replicate. Means were separated by LSD (P = 0.05). Means followed by the same letter within a column are not significantly different.

Treatment		Fermentative volatiles (µL L^{-1})			Fermentative volatiles (Ratio)		
O_2 (kPa)	CO_2 (kPa)	Acetaldehyde	Ethanol	Ethyl acetate	Acetaldehyde	Ethanol	Ethyl acetate
0.1	0	28.05a	59.88a	2.75a	72	684	164
0.2	0	4.15b	27.35b	3.07a	11	312	183
0.4	0	2.64b	7.56c	0.69b	7	86	41
21	0	0.29c	1.72c	0.068c	1	1	1

APPENDIX

Supplementary Table 3. 1. CA injury (percentage of sampled fruit) and CA index (0-1) in Honeycrisp apple stored at different CO_2 concentrations (n = 5 orchards)

Day	Injury (%)					Index (0-1)				
	CO_2 (kPa)					CO_2 (kPa)				
	0	1.5	3	10	20	0	1.5	3	10	20
7	0a	0a	0a	0a	14.6a	0a	0a	0a	0a	0.05a
14	2c	1c	8c	39b	75a	0.01b	0b	0.04b	0.18b	0.61a
21	4c	4c	14c	57.8b	86a	0.02c	0.02c	0.06c	0.37b	0.84a
28	8c	6c	24c	65b	97a	0.05c	0.02c	0.14c	0.53b	0.90c
35	14b	14b	36b	74a		0.09b	0.09b	0.22b	0.52a	
42	8b	14b	30b	68a		0.03b	0.11b	0.22b	0.51a	
49	12b	20b	40b	78a		0.04b	0.14b	0.27b	0.68a	
56	10b	22b	34b	82a		0.04b	0.13b	0.23b	0.68a	
112	18b	22b	30b	83a		0.11b	0.13b	0.17b	0.69a	
140	18b	24b	31.6b	88a		0.11b	0.13b	0.16b	0.66a	
168	18b	20b	36b	90a		0.10b	0.10b	0.24b	0.71a	
Source	DF	Pr > F				DF	Pr > F			
Model	56	<.0001				56	<.0001			
CO_2	4	<.0001				4	<.0001			
day	12	<.0001				12	<.0001			
*CO_2*day*	40	<.0001				40	0.0033			



Supplementary Figure 3. 1. Fermentative volatile production of acetaldehyde, ethanol, and ethyl acetate in the fruit stored at 20 kPa CO_2 with storage time

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CHAPTER 4.

THE CO_2 CONCENTRATION IN CONTROLLED ATMOSPHERE (CA) STORAGE IMPACTS KEY METABOLITES OF 'HONEYCRISP' APPLES

4.1 Introduction

'Honeycrisp' apple shows an extreme sensitivity to elevated CO_2 and low O_2 in CA storage (Beaudry and Contreras, 2009; Watkins and Nock, 2012b). We hypothesize that CA storage causes cellular carbohydrate metabolism in apple cortex to behave abnormally in both the glycolytic pathway and tricarboxylic acid (TCA) cycle, leading to a shift in metabolites that might be toxic to fruit cells. The condition might cause a shortage in NADH and/or NADPH, which are necessary for maintaining pools of key antioxidants GSH and Asc as reductants for scavenging free oxygen radicals abundant in CA stressful conditions. Additionally, ATP synthesis might be hindered if there is an insufficient source of NADH and NADPH, which transfer their electrons via multiple electron carriers in the electron transport chain (ETC). Therefore, the tissue may not have a high enough value of adenylate energy charge (AEC, i.e., [ATP] + 0.5[ADP])/([ATP+] [ADP] + [AMP]) for cellular survival. Consequently, cell death follows, resulting in browning area which is a symptom of CA injury.

4.1.1. Roles of ATP, ADP, and ADP as energy state compounds in cells

ATP shortage might be one of the reasons that cause cell death in 'Honeycrisp' and CA conditions might be a cause of this shortage. A restriction in aerobic respiration would be expected to lead to a decrease in ATP biosynthesis in the cells. In response, the expectation is that there would be an induction of anaerobic (fermentative) respiration and the production of fermentation-related volatiles such as ethanol, acetaldehyde, and ethyl acetate.

ATP is the major compounds preserving and transferring energy in cells. AEC is one way of describing an energy status of a cell and "represents the relative saturation of the adenylate pool in phosphoric anhydride bonds" (ATKINSON and Walton, 1967). Its value ranges from 0 to 1 but mostly around 0.8 to 0.85 in healthy plant cells (Raymond et al., 1985). The energy level of

cells helps regulate biochemical and physiological activities such as glycolysis, the Krebs' cycle, the electron transport system and oxidative phosphorylation. To maintain energy levels, ATP is synthesized through two important processes: photosynthesis and cellular respiration (Nelson and Cox, 2013; Taiz and Zieger, 2010).

At harvest, the fruit requires ATP for its normal developmental metabolism. The fruit experiences an increase in respiration during ripening, producing an increase of ATP during the ripening process (Bennett et al., 1987; Saquet and Streif, 2008). Carbohydrate metabolism will break down sugars, starch (energy-rich compounds) to smaller compounds (often called "carbon skeletons") and ATP. Under conditions of normal oxidative respiration, the plant cells produce ATP to maintain all metabolic pathways within the cell. There are 36 ATP molecules produced per glucose molecule during aerobic respiration, but only 2 ATP equivalents are synthesized per glucose during fermentative respiration (Nelson and Cox, 2013; Taiz and Zieger, 2010). With ^{31}P nuclear magnetic resonance spectroscopy (NMR), Bennett et al. (1987) found that ATP levels rose in accordance with the rate of CO_2 production of avocado during ripening. Then, both declined at day 300 after harvest (Bennett et al., 1987). ATP level peaked during the second month in 'Conference' pear and during the fourth month in 'Jonagold' apples under refrigerated air storage, but the level of ATP was lower in the fruit under CA storage (Saquet et al., 2000). ATP levels also increased during development and ripening of litchi fruit. However, the level dramatically declined during storage (Wang et al., 2013). Exogenous ATP (1 mM) application reduced browning of litchi skin. It was believed to be a result of delayed senescence of the cells and the maintenance of higher concentrations of ascorbic acid (Song et al., 2006a; Wang et al., 2013). Application of exogenous ATP also delayed senescence of cut carnation flowers because the ATP level in flower tissues maintained at higher levels than control (Song et al., 2008).

Programmed cell death can be distinguished by typical characteristics in the morphology of the cell and by intracellular biochemical mechanisms depending on ATP (Elmore, 2007). Therefore, programmed cell death on tulips was triggered by inadequate supplies of ATP and reduced efficiency of cellular energy regeneration (Azad et al., 2008)

CA technology applied to the storage of apple fruit helps to extend storage life, primarily through the inhibitory effect of low oxygen on ethylene perception (Burg and Burg, 1967). However, the reduction of aerobic respiration and increase of anaerobic respiration under stressful CA conditions hinders ATP synthesis (Kader, 1989; Ke et al., 1995). Under severe hypoxic conditions, the fruit will switch to fermentation, resulting in severe scarcity of ATP in the cells. (Ho et al., 2013b) developed a permeation–diffusion-reaction model to investigate gas exchange and predict ATP production on apple cultivars ‘Kanzi’, ‘Jonagold’, and ‘Braeburn’ under CA conditions. The results suggested that CA conditions could cause local ATP deficiencies. ATP/ADP ratio of 'Bartlett' pears under CA condition with 0.25% O_2 was only 0.97, which was five times lower than that under air storage only after 2 days (Nanos and Kader, 1993). Compared to normoxia condition, ATP levels and ATP/ADP ratios were lower in low O_2 and much lower when in combination with elevated CO_2 levels. Under 6% CO_2 + 0.5% O_2, ‘Conference' pears and ‘Jonagold' apples had the lowest level of ATP and ADP in comparison with other treatments that had higher O_2 levels and/or lower CO_2 (Saquet et al., 2000). The fruit kept under 6% CO_2 + 0.5% O_2 showed the most severe disorder incidence (Saquet et al., 2000). When ATP levels fall below a critical level, fruit cell death is triggered (Azad et al., 2008). ATP scarcity, however, is not the unique cause of disorders in pears stored under anoxia (0% O_2 with or without CO_2) since under anoxia, the fruit showed no browning disorders despite very low ATP level in the tissue (Veltman and Peppelenbos, 2003; Veltman et al., 2003).

4.1.2 Roles of NAD^+, NADH, $NADP^+$, and NADPH in maintaining redox state in cells

The compounds NADH, NAD^+, NADPH, and $NADP^+$ are the main electron transport metabolites for oxidative and reductive reactions in plant cells. In aerobic respiration, NADH transfers electrons to O_2 to produce ATP via mitochondrial oxidative phosphorylation. Meanwhile, some of the electrons reduce oxygen to free radicals that, at high levels, are harmful to proteins, lipids, and DNA. While there are many studies on the roles of NAD(P)H on the redox balance in plant cells under osmotic, drought, and pathological stresses, its role in fruits under CA storage has not been extensively investigated. For example, under oxygen and carbon dioxide stresses of CA conditions (0.25% O_2 and 0.25% O_2 + 80% CO_2), NADH increased in avocado in addition to accumulation of acetaldehyde and ethanol, leading to an increased NADH/NAD ratio (Ke et al., 1994, 1995). However, no changes in NADH levels of 'Golden Delicious' during long-term storage under 3% CO_2 + 1% O_2 were observed. NAD^+ and $NADP^+$ decreased while NADH and NADPH increased in 'Conference' pears during storage. However, there was little difference in these compounds at different CA conditions: 0.5% CO_2 + 0.5% O_2; 1.5% CO_2 + 1.5% O_2; 6.0% CO_2 + 0.5% O_2; 6.0% CO_2 + 0% O_2; and air. NADH and NADPH levels in all storage conditions also increased in 'Jonagold' apples during. $NADP^+$ increased to reach its peaks after 2 months, then sharply declined. There were no changes in NAD^+, NADH, $NADP^+$, and NADPH levels of 'Jonagold' apples or 'Conference' pears of all treatments excepting that NAD^+ level of the RA stored apple fruit after four months was much higher than other treatments (Saquet et al., 2000). Therefore, the study did not prove the roles of the compounds in handling stresses caused by CA conditions. Blanch et al. (2013) suggested the role of NADP-malic enzyme (NADP-ME) in producing NADPH under high CO_2 stress for regeneration of glutathione (GSH) in strawberries treated with 20% CO_2.

4.1.3 Roles of antioxidants in scavenging ROS state in cells

There are two kinds of antioxidants: enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), etc. and nonenzymatic antioxidants such as ascorbate (Asc), glutathione (GSH), and carotenoids (Sharma et al., 2012; Noctor and Foyer, 1998). In plants, Asc and GSH are abundant low molecular weight compounds.

Asc reduces H_2O_2 to H_2O via a series of reactions in the Asc-GSH cycle (Noctor and Foyer, 1998). Asc and GSH are not consumed in this cycle. Instead, they join in the cyclic transfer of reducing equivalents, involving four antioxidant enzymes: glutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and ascorbate peroxidase (APX), which permits the reduction of H_2O_2 to H_2O using electrons derived from NAD(P)H (Noctor and Foyer, 1998).

Asc has been considered one of the most powerful plant antioxidants (Noctor and Foyer, 1998). If Asc levels are below a certain threshold to scavenge ROS, oxidative stress happens and is proposed to cause browning in fruits (Veltman and Peppelenbos, 2003). Fruits held in modified atmosphere packaging (MAP) with low O_2 levels (1.0–2.5%) develop internal browning that appears to be associated with a reduction in ascorbic acid (Asc) and an accumulation of malondialdehyde (MDA)(Wang et al., 2013). MDA is considered a very toxic aldehyde since it involves in peroxidation of polyunsaturated fatty acid in the cell membrane (Del Rio et al., 2005), and has been associated with an increase in membrane leakage (Wang et al., 2013). CO_2 in CA conditions also decreases Asc levels by about 46% in pears, coincident with the induction of browning disorders (Veltman and Peppelenbos, 2003).

GSH is another important antioxidant in plants. In addition to regeneration of Asc via the Asc-GSH cycle, GSH can directly eliminate $O_2^{\bullet-}$, $^{\bullet}OH$, and H_2O_2 (Noctor and Foyer, 1998). Additionally, by producing adducts (compounds formed by an additional reaction) and donating hydrogen atoms in the presence of ROS, GSH can protect cellular proteins, lipids, and DNA from damage. Oxidation of GSH is accompanied by net glutathione degradation. Under oxidative stresses, glutathione was proven to be produced in plants (Noctor and Foyer, 1998). Plant cells have a defensive mechanism in which GSH is an important protector (Ghosh et al., 2012). "Although NAD(P)H acts as redox energy currency, GSH acts as a dynamic redox energy buffer" (Das and White, 2002). The GSH level increases when receiving electrons from NADPH via the ascorbate-glutathione cycle. At the same time, the cycle also regenerates Asc, another important antioxidant in scavenging ROS (Noctor and Foyer, 1998).

4.1.4 DPA (Diphenylamine) and its role as an antioxidant

It was suggested that due to its antioxidant function, DPA could control oxidation of α-farnesene, resulting in eliminating superficial scald of 'Granny Smith', 'Crofton', and 'Cortland' apple peel (Huelin and Coggiola, 1970)(Mir and Beaudry, 1999). A DPA drench (1000 ppm) before any preconditioning treatments almost completely eliminated CA injury in 'Honeycrisp' apples under 3% O_2 and 3% CO_2 (Contreras et al., 2014). DPA and its derivatives also prevented internal browning of 'Braeburn' apples under CA conditions (Lee et al., 2012; Mattheis and Rudell, 2008). It was suggested that in the presence of $^{\bullet}OH$, DPA was oxidized to form hydroxydiphenylamine derivatives, resulting in accumulation of 2-, 3- and 4-hydroxydiphenylamine in 'Delicious' apples and 'Granny Smith' peel, and 4-hydroxydiphenylamine in 'Braeburn' cortex tissues (Noctor and Foyer, 1998).

When cells die, proteolysis is enhanced, resulting in an accumulation of free amino acids (Muntz, 2007). Many amino acids were vigorously produced in ‘Braeburn’ cortex tissue after 12 weeks of CA storage when the incidence of internal disorder symptom was high (Lee et al., 2012). However, the fruit with DPA treatment before CA storage had lower levels of these amino acids. It is suggested that DPA suppressed amino acid production. Consistent with this, amino acid levels were low in the fruits treated with DPA and free of browning symptoms. Therefore, DPA’s role in obstructing amino acid accumulation from proteolysis seems clear.

4.1.5 Carbohydrate metabolites and damaged fruit cells

High concentrations of CO_2 in CA influence carbohydrate metabolic pathways. For example, 10% CO_2 caused a decrease in ATP: phosphofructokinase and CA conditions in which high CO_2 levels in the atmosphere also interfere with normal metabolism of the TCA cycle in ‘Braeburn’ apple (Kerbel et al., 1988). During CA storage, concurrent with internal browning in ‘Braeburn’ apple, there were increases in alanine, galactose, mannitol, sorbitol, and xylose and a decrease in malic acid, and sucrose (Hatoum et al., 2016).

Hatoum et al. (2016) found no significant difference in pyruvate level between asymptomatic control fruit and fruit experiencing browning due to applied CO_2 in the storage environment. Therefore, alanine accumulation might be the result of, not the cause for ‘‘Braeburn’ browning disorder (BBD) (Hatoum et al., 2016).

Under a normal physiological status of the cell, succinic acid is maintained at trace amounts because of its rapid turnover in carbohydrate metabolism in the TCA cycle (Hulme, 1956). In fact, high concentrations of exogenous succinate applications to apple peels caused browning on the tissue (Hulme, 1956). When stored under very high CO_2 levels (20%), succinic acid dehydrogenase activity was obstructed, resulting in an accumulation of succinic acid, which

can be toxic for plant tissues (Hulme, 1956). Succinate elevation in ‘Braeburn’ cortex (Hatoum et al., 2016) might result from inhibition of the enzyme succinate dehydrogenase (Gonzalez-Meler et al., 1996). Elevated CO_2 concentrations of CA storage also caused a short-term increase in succinic acid in ‘Cortland’ and ‘Law Rome’ apples (Fernández-Trujillo et al., 2001). However, there was no significant difference in succinic acid levels in ‘Braeburn’ apples stored under high CO_2 while protected from tissue damage by DPA drench before CA storage (Lee et al., 2012)). The result implied that succinate is not directly related to CO_2 injury or presumed that DPA might prevent toxic effects of elevated succinate levels (Lee et al., 2012).

In conclusion, there have been many studies on internal disorders of pome fruits under CA storage. Most recent results are from metabolomics (Hatoum et al., 2016) and transcriptomics (Mellidou et al., 2014) of events that cause internal browning for ‘Braeburn’ apples under CA storage. However, it was unclear which metabolites would be reliable biomarkers for early detection of internal browning (Hatoum et al., 2016). The model for browning development in apples during CA storage that Mellidou et al. (2014) proposed was based on metabolomic analysis of inner and outer cortexes at harvest and after 4 months of CA storage, which is well after CO_2-related damage occurs in 'Braeburn' apple tissue. Browning at 4 months might be the result of many critical metabolic changes that start to happen early CA storage. In addition, browning in ‘Honeycrisp’ usually happens in the area between the inner and the outer cortexes of which were sampled for analysis by ‘Braeburn’ apples observed by (Hatoum et al., 2016) and (Mellidou et al., 2014).

The aim of this study was to test the following five hypotheses:

1. Insufficient energy availability due to CA storage conditions causes CA injury in ‘Honeycrisp’ apple cortex.

2. Unbalanced redox state due to CA storage conditions causes CA injury in Honeycrisp' apple cortex.
3. Insufficient antioxidants due to CA storage conditions causes CA injury in Honeycrisp' apple cortex.
4. Abnormal levels of carbohydrate metabolites due to CA storage conditions causes CA injury in Honeycrisp' apple cortex.
5. When DPA drench or preconditioning practices applied to the fruit, levels of some of key mentioned metabolites shift to prevent CA injury in Honeycrisp' apple cortex. DPA, an antioxidant, likely protects the fruit from oxidative stress under CA conditions. Preconditioning might be a duration for the fruit to prepare enough NADH and NADPH to ready itself for an oxidative stress and/or enhance ATP synthesis for cellular survival.

Therefore, we evaluated changes in levels of key metabolites mentioned above in apple cortex when 'Honeycrisp' apple were exposed to CO_2 ranging from 0 – 20 kPa in combination with 3 kPa O_2 in CA storage at 3 °C. Changes of the metabolites were also quantified in apple cortex when the fruit was stored in hypoxic (< 0.3 kPa O_2) atmospheres without CO_2. In addition, we also observe alterations of the metabolites in apple cortex of the fruit drenched in DPA (1000 ppm, 30 s) or a preconditioning treatment before storage at 5 or 10 kPa CO_2 in CA storage.

4.2 Materials and methods

4.2.1 Plant materials

'Honeycrisp' apples at commercial maturity stage were harvested from commercial orchards (A-F) in 2014, 2016, and 2017 in Michigan (Table S-B2.1). At each orchard, two 18-bushel bins of fruit were harvested in the morning and fruit were transferred to 60 x 40 x 18 cm

plastic crates (model 5000206, Twinpack B.V., Netherlands) and immediately transported to the Postharvest Physiology Laboratory at Michigan State University. The fruit used in the DPA experiments were from three orchards (A, B, and C) in 2014, from three orchards (B, C, and D) in 2016, and from two orchards (F and H) in 2017. The fruit used in the preconditioning experiments were from two orchards (F and H) in 2017. The apples used in hypoxia atmosphere experiments were from one orchard (F) and had been stored in refrigerated air for three months.

4.2.2 Experiment 1. Impact of CO2, DPA, and preconditioning to key metabolites in apple cortex.

In 2014, fruit were handled as depicted in the flow chart of Figure 4.1. In brief, fruit from five orchards were given four CO_2 concentrations (0, 3, 10, and 20 kPa) in combination with 3 kPa O_2 using CA systems as previously described in method section in Chapter 2. Fruit were held at 3 °C to create a CA environment which was similar to that found commercially, but with O_2 levels high enough to avoid hypoxic stress. In addition, fruit from six orchards in 2014, 2016, and 2017 were treated with DPA (1000 ppm, 30 s) and stored at 10 kPa CO_2 (Fig. 4.1). DPA was applied by submerging the fruit in the treatment solution for 30 s and allowing them to dry for 2 hours in the laboratory before placing them into CA storage.

Fruit were stored up to six months or until all apples within a treatment had been damaged. During storage, the fruits from each treatment and each orchard were removed from the chamber on day 7, 14, 21, 28, 35, 42, 49, 56, 84, 112, 140, and 168 after placement into storage atmospheres. On each day, the fruit cortex tissues were frozen and stored at -80 °C. Samples used for metabolite analysis were from fruits prior to receiving CO_2 stress (day 0), after imposition of the stress, but prior to symptom development (day 3), after imposition of the stress and after the first signs of symptom development in any treatment (day 7), the day of half

maximal injury, and the day of maximal injury. Half-maximal injury (see CHAPTER 1) was on day 21 for 0 and 3 kPa CO_2 and on day 14 for 10 and 20 kPa CO_2. Maximal injury day was day 56 for 0, 3, and 10 kPa and day 28 for 20 kPa CO_2. Even though DPA-treated fruit did not have CA injury when held at 10 kPa CO_2, we also sampled fruit 14 and 56 d after storage for comparison with fruit receiving 10 kPa CO_2 without DPA (Fig. 4.1). For each target date (Fig. 4.1), stored tissue samples were later used for quantification of key metabolites (see below).

In 2016, 5 kPa CO_2 was used in combination with DPA (1000 ppm) on fruit from three orchards to test the effect of DPA on metabolite pools (Fig. 4.1). Control fruit were not treated with DPA. Fruit were placed into two CA chambers (as replicates) at which atmosphere of 5 kPa CO_2 + 3 kPa O_2 was established at 3 °C. We selected tissue samples from fruit with evident damage and removed tissue samples from brown, damaged areas (B) and healthy areas (H) of cortex tissue samples taken from 1-cm thick transverse slices removed from the middle of the fruit. Samples were from fruit with injury ratings of 1 (10 – 25% browning area on the cut surface) or 2 (25 - 50% browning area on the cut surface) (Fig. S-B4.1). Samples were from fruits from three orchards after 21 days of storage at 5 kPa CO_2 in two CA chambers (as replicates).

In 2017, the effects of DPA and preconditioning were compared for fruits exposed to 5 kPa CO_2 to induce injury on fruit from two orchards (Fig. 4.1). There were two treatments given to the fruit immediately following harvest: DPA and preconditioning. DPA (1000 ppm) was applied as previously described. After being dried at the lab for two hours, the fruit was put into two CA chambers (as replicates) having an atmosphere of 5 kPa CO_2 + 3 kPa O_2 and a temperature of 3 °C. Preconditioning was accomplished by holding the fruit at 20 °C for five days before being stored in two CA chambers (as replicates) having an atmosphere of 5 kPa CO_2

+ 3 kPa O_2 at 3 °C. Control (non-preconditioned) fruit were put in the same CA chambers with preconditioning fruit (Fig. 4.1).

In 2016 and 2017, fruit cortex tissues were sampled and stored weekly at -80 °C until day 56. After evaluation of CA injury caused by 5 kPa CO_2, days 21 and 56 were selected as half maximal injury day and maximal injury day, respectively.

4.2.3 Experiment 2. Impact of O2 on key metabolites in apple cortex.

In 2017, we performed an additional experiment to test the effect of low oxygen on CA injury using 'Honeycrisp' apples after 3 months of refrigerated air storage. The fruits were put into 20-L plastic buckets fitted with airtight gasket-sealed lids (Gamma Plastic Company) and flushed with nitrogen gas at a flow rate 20 mL min^{-1} to achieve three oxygen levels: 0.1, 0.2, and 0.4 kPa. Control fruit were from a CA chamber in which oxygen concentration was maintained at 21 kPa. In each environment, the CO_2 partial pressure was 0 kPa and storage temperature was 3 °C. After 14 d, the apple cortex tissues were sampled for quantification of key metabolites.

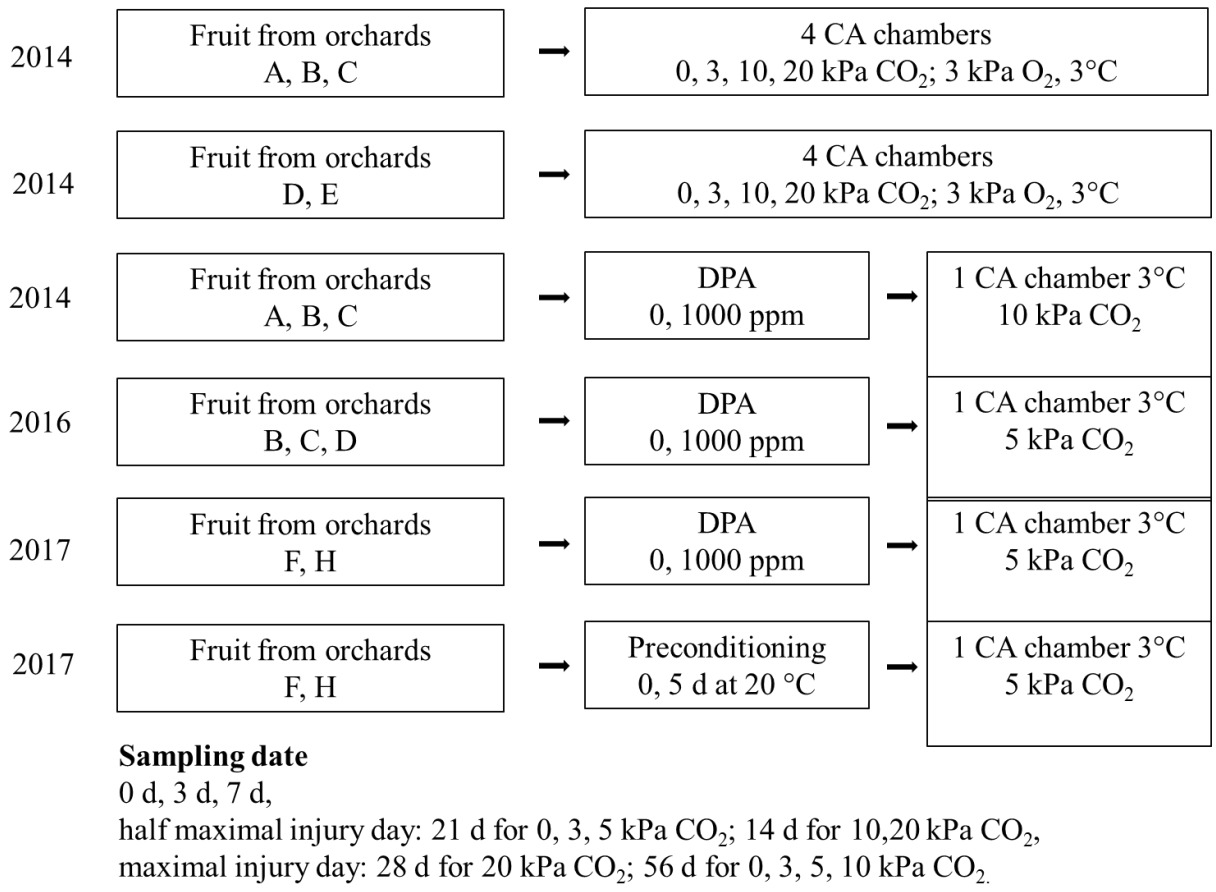


Figure 4. 1. Experimental design for the fruit harvested from commercial orchards in Michigan in 2014, 2016 and 2017. The fruit were stored immediately in CA chambers on the day of harvest. A portion of the fruit were drenched with DPA (1000 ppm, 30 s), and then stored under 5 or 10 kPa CO_2 in combination 3 kPa O_2 at 3 °C. A portion of the fruit were preconditioned (five days at 20 °C). Samples were taken on the indicated days for 15 important metabolites in the fruit cortex.

4.2.4 Quantification of fifteen important metabolites in apple tissues by using ultra-high-performance liquid chromatography-tandem mass spectrometry

4.2.4.1 Chemicals

Metabolites evaluated are listed in Table 4.1. Adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), glutathione (GSH), glutathione disulfide (GSSG), oxidized nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide adenine dinucleotide (NADH), oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$), reduced nicotinamide adenine dinucleotide phosphate (NADPH), coenzyme A (CoA), acetyl coenzyme-A (Acetyl CoA), succinic acid (SA), phosphoenolpyruvate (PEP), uridine diphosphate glucose (UDP-G), acetonitrile (HPLC grade), methanol (HPLC grade), and formic acid (FA) were purchased from Sigma-Aldrich (St. Louis, MO). Propyl paraben (Sigma-Aldrich, St. Louis, MO), used as internal standards, and ascorbic acid (Sigma-Aldrich, St. Louis, MO) were generously provided by Dr. Daniel Jones and MSU Mass Spectrometry and Metabolomics Core.

Water (>18 MΩ) was purified using the Milli-Q System (Millipore Corp, Bedford, MA). To eliminate free oxygen in the water, nitrogen gas from a compressed gas cylinder (UN 1066-Airgas, PA) was flushed (approx. 50 mL/min) through the purified water in a 500 mL Erlenmeyer flask for over 2 h.

4.2.4.2 UHPLC-MS/MS conditions

A Waters Quattro Premier XE mass spectrometer coupled to a Waters ACQUITY UPLC system with a binary solvent manager was used for the analyses. The mobile phases consisted of mobile phase A1 (97:3 water: methanol + 10 mM tributylamine + 15 mM acetic acid) and mobile phase B2 (100% methanol). A Waters BEH C18 column (2.1 mm × 50 mm, 1.7 µm particle size, S/N: 024134119157) coupled with an Acquity UPLC™ column in-line filter kit

(0.2 µm filter) was used and the column temperature was held at 50 °C when running samples. Analytes were separated using a gradient program (Table 4.1) with a flow rate of 0.3 mL min^{-1} for 10 min at 50 °C (Table 1). The injection volume was 10 µL. Dwell time for each multiple reaction monitoring (MRM) transition was set to 0.1 s, and inter-scan delay was 5 ms.

Analytes were detected using electrospray ionization in a negative-ion mode using multiple reaction monitoring (MRM) and processed using Masslynx 4.1 software (Waters Corporation, Milford, MA, USA). Source and desolvation temperatures were 120 °C and 350 °C, respectively. Cone gas and desolvation gas flows were 4 and 800 L h^{-1} respectively. MRM parameters including cone voltage and collision cell potentials were individually optimized for each compound using commercial software (QuanOptimize, Waters) (Table 4. 2).

Table 4. 1. The mobile phase gradient for solvent A1 and B2

Time (min)	% A1	% B2
0	99.0	1.00
1	99.0	1.00
2.5	80.0	20.0
4.00	80.0	20.0
7.00	35.0	65.0
7.50	5.00	95.0
9.00	5.00	95.0
9.01	99.0	1.00
10.00	99.0	1.00

4.2.4.3 Preparation of stock solutions and calibration solutions

It was impossible to make good standard curves for each standard included in a mixture of all 15 compounds because the reactions among them might take place and influence actual concentrations. After several trials of grouping several standards into one mixture aiming to get high value of R^2 (> 0.9) of response linear regression of five concentrations of each compound,

we decided to divide 15 compounds into six groups:1) Group A: Asc, GSH, NADH, NADPH; 2) Group B: GSSG, NAD, NADP, SA; 3) Group C: PEP, UDP-G; 4) Group D: AMP, Acetyl CoA; 5) Group E: ADP, CoA; 6) Group F: ATP.

Stock solutions (30 mM) of each standard compound were prepared in 0.1% oxygen-free water (ATP, ADP, AMP, UDP-G, SA, PEP, Asc) and oxygen-free formic acid (NAD, NADH, NADP, NADPH, GSH, GSSG, Acetyl CoA, CoA). Then the stock solution of each compound was diluted 10-fold (to 3 mM) and transferred into twenty 1.5 mL-vials, each of which was used for one round of further dilutions. The stock solutions were stored at -80 °C for three months. On the day of analysis, each stock solution was diluted and combined into group stock solution (150 µM). After that, each group was diluted into five concentrations 50, 25, 12.5, 6.75, and 0 µM using mobile phase A1 which had been purged with nitrogen, autoclaved and stored at 4 °C. ATP and Asc were two-fold concentrated due to their limit of detection by the Quattro Premier XE. Each standard contained 1 µM propyl paraben (IS1) and 1 µM butyl paraben (IS2). Stock and calibration solutions were made in the cold and under nitrogen gas environments.

4.2.4.4 Method performance

Response linearity of each standard was assessed using standard spiked calibration solutions with five concentrations ranging from 0 to 50 µM (except Asc and ATP, which were 0 to 100 µM). The calibration curve was built by plotting the ratio of peak areas of unlabeled compounds to that of the internal standard against concentrations of the unlabeled analytes, fitted by a weighted (1/x) least squares linear regression using the TargetLynx component of MassLynx v. 4.1software (Waters). The lower limit of detection was defined as the concentration at which the peak height was three times that of the RMS noise (S/N=3), and the lower limit of

quantification was the concentration with a peak height corresponding to 10 times that of the RMS noise (S/N=10).

Note: Retention time of SA was at 3.34 min. in standard mixtures and at 2.15 min. in apple extract. This had been proven using SA standard spiked into apple extracts. With the same extract solvent and analysis instrument, we also detected three keto acids in TCA cycle: pyruvic acid (PA), ketoglutaric acid, and oxaloacetic acid after incubation of 100 µL of 1 M hydroxylamine·HCl in 200 µL apple supernatant 25 °C for 16 h for oxime formation. However, since we focused on other key metabolites that were easily degraded at room temperature, we did not analyze these compounds. Fumaric acid, citric acid, and malic acid were also detected in standard solutions but resulted in large, poorly resolved peaks due to their high abundances in apple tissues.

4.2.4.5 Tissue Sample Preparation, Extraction, and Quantification

4.2.4.5.1 Sampling and lyophilization of apple flesh tissues

Five randomly selected apples from each orchard of each treatment were used. On each sampling day, each fruit was immediately chopped into five 1-cm thick slices using an onion slicer (NSFQC Nemco Food Equipment, Hicksville, Ohio), in which a center transverse slice was cut into four tissue cylinders at four opposite corners using a cork borer (1 cm dia.). The apple cylinders were frozen in liquid nitrogen in a Styrofoam box and randomly transferred to 1) 15-mL polycarbonate vial containing two chrome steel bearing balls (0.25-inch dia.) followed by lyophilization and 2) Ziploc double zipper freezer bags (17.7 x 18.8 cm) for -80 °C storage. Every handling step performed as quickly as possible before the apple tissues were kept completely frozen. The vials, each of which consisted of two balls and eight frozen cylinders, together with one vial containing liquid nitrogen were put into Genesis Pilot Lyophilizer.

After three days of lyophilization or when the machine's pressure was constantly low at 10 mTorr, the vials were removed from the machine, immediately placed into liquid nitrogen, and tightly closed with unlined caps once the liquid nitrogen had evaporated. The lyophilized apple tissues were homogenized at frequency 20/s for 10 s using the mixer mill MM 400 (Retsch®). After that, the lyophilized apple powders were transferred to 2-mL microcentrifuge tubes, the weights of which had been recorded. The filled tubes were then weighed to record apple powder weight (0.05 – 0.15 g).

4.2.4.5.2 Extracting metabolites from the lyophilized apple powder

The extraction solvent for apple metabolites consisted of isopropanol, acetonitrile and oxygen-free water at the volume ratio 3:3:2 in 0.1% formic acid and 1 µM propyl paraben was used as an internal standard. The solution was transferred to 10-mL glass bottles, occupying approximately 80% of the bottle volume to avoid glass breaking, and stored in a refrigerator at -20 °C. On the day of extraction, 1 mL of cold extraction solvent was transferred to the tube containing 0.05 – 0.15 g lyophilized and homogenized apple. We prepared 12 samples each time. All procedures of solvent preparation and extraction were implemented under liquid nitrogen to reduce risk of re-absorbance of oxygen in the extracts. After incubation for 4 h at 0 °C, the tubes were centrifuged at 21,000 x g at 0 °C for 30 min using IEC Micromax RF refrigerated microcentrifuge. After centrifugation, the tubes were gently placed on ice in a Styrofoam box that was put inside a larger container box containing liquid nitrogen. Within the box, 200 µL of supernatant from each tube was pipetted to each of four 2-mL microfuge tubes (200 µL x 4 tubes). While one tube was stored at -80 °C, the supernatants of the other three were concentrated for 1 h at low temperature under vacuum using a SpeedVac concentrator (Savant instrument, Inc. Farmingdale, New York, Model No DNA1200P-120). To minimize oxidation,

100 mL of liquid nitrogen was used to flush the SpeedVac centrifuge compartment (containing open tubes) before sample drying. During operation, The SpeedVac was connected with nitrogen gas flow from a nitrogen separation system (Prism® Alpha, PERMEA, Amonanto Company, Missouri). The SpeedVac was situated in the fume hood. After one hour, about 1/4 volume of supernatant (i.e., about 50 µL) remained. The liquid that contained apple metabolites was frozen again when the tubes were transferred back to the liquid nitrogen box after speed vacuuming. Solutions were lyophilized (Genesis Pilot Lyophilizer, SP Scientific). To avoid foaming, 2-mL (instead of 1.5-mL microfuge tubes were used and the shelf heat for the lyophilizer was not turned on until after 12 h when the pressure reading was at about 35 mTorr. In addition, 2-mL microfuge tubes were used (instead of 1.5 mL tubs) to ensure no loss from foaming and that the extracted metabolites stayed inside the tubes. After 1 d of lyophilization, the tubes were quickly transferred into a liquid nitrogen-containing box to minimize exposure to oxygen and water. The reasons for using two drying steps (i.e., SpeedVac and lyophilization) are 1) apple juice contained a lot of sugar which took a long time to dry using only the SpeedVac concentrator; 2) the final product of the SpeedVac was a gel, which was not easily dissolved, and 3) NADH, NADPH, and ascorbic acid levels were very low if only the SpeedVac was used. Because of its sensitivity to degradation, we suspected that NADH may degrade or oxidize during lyophilizing or extracting procedures. For our final protocol, we tested recovery rates by spiking NADH standards in 1) fresh frozen apple tissues and 2) lyophilized apple powder. The result showed that their recovery rates were > 80% (data not shown).

Table 4. 2. Optimized UHPLC-MS/MS parameters for each analyte.

Abbreviations: *m/z corresponds to mass to charge ratios of precursor ion ([M-H]-) and product ion; CV, cone voltage; CE, collision voltage; RT, retention time.* R^2*, correlation coefficient; LOD, limit of detection; LOQ, limit of quantitation.*

Product Code	Compounds	MRM transitions *m/z*	CV (V)	CE (V)	RT (min)	R^2	LOD (nM)	Calibration curve Slope	LOQ (nM)
G4251	Glutathione (GSH)	306 —> 143	28	20	2.44	0.99	57	849	110
A2252	Adenosine monophosphate (AMP)	346 —> 97	34	22	3.31	0.99	5	1,548	167
A5285	Adenosine diphosphate (ADP)	426 —> 134	28	25	5.56	0.99	2	6,815	8
A2252	Adenosine triphosphate (ATP)	506 —> 159	34	28	6.37	0.98	0.6	13,401	6
U4625	Uridine diphosphate glucose (UDP-G)	565 —> 323	40	25	5.01	0.99	--	18,061	5
G4376	Glutathione disulfide (GSSG)	611 —> 306	28	28	4.48	0.99	2	6,299	5
N6522	Oxidized nicotinamide adenine dinucleotide (NAD^+)	662 —> 540	20	16	2.62	0.99	0.7	5,346	25
N8129	Reduced nicotinamide adenine dinucleotide (NADH)	664 —> 408	50	35	5.59	0.98	9	584	35
N8035	Oxidized nicotinamide adenine dinucleotide phosphate ($NADP^{+)}$	742 —> 620	22	16	5.4	0.99	1.6	15,253	3.4
7505	Reduced Nicotinamide adenine dinucleotide phosphate (NADPH)	744 —> 426	50	35	6.34	0.99	1.7	2,097	9.8
C3144	Coenzyme A (CoA)	766 —> 408	52	36	6.57	0.99	0.4	14,829	1.3
A2056	Acetyl coenzyme A (Acetyl CoA)	808 —> 461	52	36	6.64	0.99	0.8	11,532	1.8
S-7501	Succinic acid (SA)	117 —> 73	20	10	3.34*	0.98	5.0	5,564	70
860077	Phosphoenolpyruvate (PEP)	167 —> 79	16	20	5.56	0.99	7.9	3,013	24
A5960	L-Ascorbic acid (Asc)	175 —> 87	28	28	1.82	0.99	33	373	164
	Propyl paraben (IS1)	179 —> 92	28	22	7.06	0.99	--	45633	--
	Butyl paraben (IS2)	193 —> 92	28	22	7.67	0.99	--	79768	--

4.2.4.5.3 Apple extract analysis

The analysis of apple metabolites was implemented at the Mass Spectrometry and Metabolomics Core in Michigan State University. All the tubes containing lyophilized apple metabolites were kept in a Styrofoam box containing liquid nitrogen. Using the same pipette which had been used in extraction, 200 µL of solvent phase A1 in 1 µM butyl paraben (IS2) was injected into the sample microfuge tube. The metabolite was re-dissolved and a portion of the solution transferred to a glass insert, which was then put into a 1 mL-autosampler vial for the first analysis with Quattro Premier XE mass spectrometer coupled to a Waters ACQUITY UPLC system.

Since the LC/MS/MS analysis time of each sample was ten minutes, the next sample was prepared after eight minutes. All procedures were implemented in a cold and nitrogen gas saturated environment using a large Styrofoam box containing liquid nitrogen. After running about 24 samples, the cone of the mass spectrometer ion source was washed three times with water, methanol, and isopropanol to maintain adequate transmission of ions from the mass spectrometer's ion source: apple extracts contain abundant sugars, which accumulate on the cone. The column filter was changed after each batch of samples, and the column was washed with 100% methanol at a rate 0.2 mL min^{-1} for 12 h. The column pressure was recorded at washing time with 100% methanol and at starting time with 99% of solvent A1.

4.2.4.5.3 Experimental design and statistical analyses

The experiments had a completely random split-plot design in which CO_2 was a whole plot treatment factor with two CA chambers as replicates. For the DPA experiment, DPA concentration was fixed factor. For preconditioning experiment, precondition and orchards were treated as fixed factors. Storage day was a split-plot treatment factor. Since we used

different fruit trays at each sampling time, this observational unit was treated as a random factor. All data for the variables of the experiments were subjected to test normality and assumptions for ANOVA using SAS Proc mixed procedure (Version 9.4; SAS Institute Inc., Cary, NC). Mean separations are examined using Duncan's multiple range test and only differences significant at $P \leq 0.05$ are discussed.

To answer the questions mentioned in the introduction, we classified results of key metabolites into four following groups: 1) Energy state compounds: ATP, ADP, AMP, and AEC; 2) Redox energy state compounds: NAD^+, $NADP^+$, NADH, NADPH, NADH/NAD^+, NADPH/$NADP^+$; 3) Antioxidants: Asc, GSH, GSSG, and GSH/SGGS; and 4) Carbohydrate metabolites: UPD-G, PEP, SA, CoA and Acetyl CoA

The effects of CO_2 concentrations under CA condition, low O_2 concentration in hypoxia atmosphere, and DPA and precondition applications before CA storage at different CO_2 concentrations will be evaluated and discussed.

4.3 Results

4.3.1 Effect of CO_2 concentration, DPA, or preconditioning treatment on CA injury

CA injury of the fruit was CO_2 concentration-dependent. DPA treatment eliminated CA injury while precondition suppressed completely the symptom for the fruit from orchard F, which was less mature at harvest, and therefore more susceptible to CA injury (Table 4.3).

Table 4. 3. CA injury (%) in the fruit stored at 0, 3, 5,10, and 20 kPa CO_2, in the fruit treated with DPA and stored at 5 and 10 kPa CO_2, and in the fruit receiving preconditioning treatment before stored at 5 kPa CO_2 [a]

	No DPA[b] CO_2 (kPa)					DPA CO_2 (kPa)		Preconditioning ([c]) No	Preconditioning ([c]) Yes
Day	0	3	5	10	20	5	10	5	5
0			0			0		0	
3	0a	0a	0a	0a	16a	0	0	0	0
7	0b	0b	0b	0b	78a	0	0	0	0
HMI	4c	14c	38.8b	39b	99a	0	0	46.3	0
MI	10c	34c	56.3b	82a	100a	0	0	64.5	0

[a] Half maximal injury (HMI) day of 0, 3, and 5 kPa CO_2 was day 21, of 10 and 20 kPa CO_2 was day 14. HM (Maximal injury) day for all CO_2 treatment was 56 days, except 20 kPa CO_2 as 28 days, after storage.

[b] Means within row within sampling date followed by different letters indicate significant differences by Turkey's HSD test, $P \leq 0.01$ (n = 5 for 0, 3, 10, 20 kPa CO_2; n=3 for 5 kPa CO_2)

[c] Data only from orchard F since no CA injury observed in fruit from orchard H

4.3.2 Energy Compounds

4.3.2.1 Effect of CO_2 and O_2 concentration on energy status of 'Honeycrisp' apple

ATP levels for tissue samples from all CO_2 concentrations, except 0 kPa, decreased with storage time (Fig. 4.2). 0 kPa CO_2 treatment caused an increase in ATP level at day 7, followed by a slight decrease, but the ATP level was considerably higher than the levels for other treatments at each sampling date. At day 7, ATP levels of 5, 10, and 20 kPa CO_2 were about the same (average at 15 nmol g^{-1} on a dry weight basis). After that, the level of the fruit stored at 10 and 20 kPa CO_2 dropped sharply to 2 and 0.1 nmol g^{-1}, respectively, when the fruit received

maximal CA injury. ATP content in the fruit under 3 and 5 kPa CO_2 decreased by 64 and 44%, respectively, relative to initial levels after 56 days of storage.

The ADP contents of the fruit in the 0 -10 kPa CO_2 treatments were relatively stable with storage time and declined by only 15 – 25% at the time of half maximal injury (Fig. 4.2). However, the ADP level of fruit from the 20 kPa CO_2 treatment declined markedly by day 7 and lost 90% of its initial value in dead tissue collected at the time of maximal injury (Fig. 4.2).

The AMP level for 0 kPa CO_2 did not change with storage time and kept the level at approximately 3 nmol g^{-1} while the levels of AMP in the CO2 treatments was very dynamic. 10 and 20 kPa CO_2 caused a sharp increase of AMP levels, which peaked on day 7, followed by a sharp decrease for 20 kPa (84% loss) while remaining unchanged for the 10 kPa CO_2 treatment. In contrast, after seven days of storage, AMP levels for 3 or 5 kPa CO_2 treatments increased by 15 – 20% at maximal injury day (Fig. 4.2).

Energy state compounds ATP, ADP, and AMP in the fruit tissues were used to calculate the AEC to test the effect of CO_2 concentration on energy status of the fruit stored in CA conditions. AEC was calculated as the ratio of sum of ATP and half of ADP levels divided by sum of ATP, ADP, and AMP levels. The fruit tissues under 0 kPa CO_2 maintained AEC at high values (0.7-0.9) during storage time. However, for all CO_2 treatments, after seven days of storage, AEC started to decline (Fig. 4.2). When the fruit tissue turned brown as CA injury symptom, AEC veaues were always below 0.7. We first noted the symptom on the fruit stored at 20 kPa CO_2 at day 7 at which AEC ratio dropped to 0.6. AEC ratios decreased with storage time and were somewhat linear with CO_2 concentration. When the fruit 10 kPa CO_2 or 20 kPa CO_2 had 80 % or 100% damage respectively, ACEs for the treatments were only 0.3 (data not shown). AEC ratios remained high (above 0.7) in control (0 kPa CO_2) and in DPA-treated tissues

(Fig. 4.2). The fruit stored at 0 kPa CO_2 also had a low level of CA injury symptom 4% and 10% on day 21 and 56, respectively. However, AEC ratios in sampled tissues of these sampling dates were above 0.7 probably due to healthy tissues were randomly selected.

To compare energy status of brown tissues, healthy tissues of CO_2-injured fruit at injury rate 1 (10 to 25% browning area on the fruit cut surface) and at injury rate 2 (25 to 50% browning area on the fruit cut surface) were compared. Even though AMP and ADP levels were not very different in the brown tissues, the ATP level was very low in the damaged tissue (Fig. 4.3). Consequently, AEC ratios in browned tissues were only 0.41 and 0.51 at injury rating 1 and 2, respectively.

The CO_2 concentration was positively correlated with CA injury (see CHAPTER 1). To test effect of low oxygen on sensitiveness of the fruit to CA injury, we applied hypoxic conditions of 0.1, 0.2, and 0.4 kPa O_2 at 3 °C. Control fruit were stored in refrigerated air (21 kPa O_2 at 3 °C). When the fruit exposed to 0.4 kPa O_2, ATP levels were higher and AMP levels lower than control and other treatments (Fig. 4.4A). ADP contents were not significantly different among the treatments. As a result, AEC for the 0.1 kPa O_2 treatment was very low at 0.2. AEC increased as O_2 increased and, the fruit exposed to 0.4 kPa O_2 had the same AEC (about 0.8) as control fruit (Fig. 4.4B). There was a high, positive correlation ($r = 0.9$) between ATP levels and adenylate energy charge (AEC) for low O_2-treated fruit (data not shown). The correlation between ADP and AEC and between AMP and AEC were very weak, having correlation coefficients of $r = 0.4$ and $r = -0.3$, respectively (data not shown).

At the time when fruit had received half maximal CA injury, the CO_2 concentration of CA storage was negatively correlated with AEC ($r = -0.89$) (Fig. 4.5). However, the O_2 partial pressure of 14 d storage in hypoxia was positively correlated with AEC ($r = 0.90$) (Fig. 4.5).

4.3.2.2 Effect of DPA on cellular energy state of 'Honeycrisp' apple

When the fruit was treated with DPA prior to storage, ATP levels declined approximately 17% or 32% after 56 d when stored at 5 or 10 kPa CO_2, respectively (Fig. 4.2). However, fruit from the same CO_2 atmospheres, but without DPA treatment lost 60% and 90% of their initial ATP, respectively.

The ADP level of DPA treated fruit was largely stable with storage time, remaining somewhat elevated relative to untreated fruit held in 5 and 10 kPa CO_2. ADP levels of DPA treated fruit at 10 kPa CO_2 rose to maximum day 7 and then sharply dropped to 1.5 nmol g^{-1} (i.e. 53% reduction) after 56 days of storage.

While AMP levels of DPA-treated fruit at 5 kPa CO_2 remained at about 4 nmol g^{-1} during storage, the level in untreated fruit started to increase abruptly at day 7 for these treatments and reached 8.8 nmol g^{-1} at day 56 (maximal injury day) (Fig. 4.2). AMP levels of 10 kPa CO_2 increased approximately by 75% after seven days of storage in both DPA and non-DPA treatments, followed by an 80% reduction in response to DPA-treatment.

For DPA-treated fruit held in 5 or 10 kPa CO_2, the AEC remained high, around 0.7 – 0.8, during CA storage (Fig. 4.2). This is in sharp contrast to the no-DPA fruit, for which the AEC dropped below 0.6 when reaching half maximal CA injury. AEC ratio declined to 0.6 when 5 kPa CO_2 caused 46% CA injury and to only 0.3 when 10 kPa CO_2 induced 85% CA injury (Table 4.4).

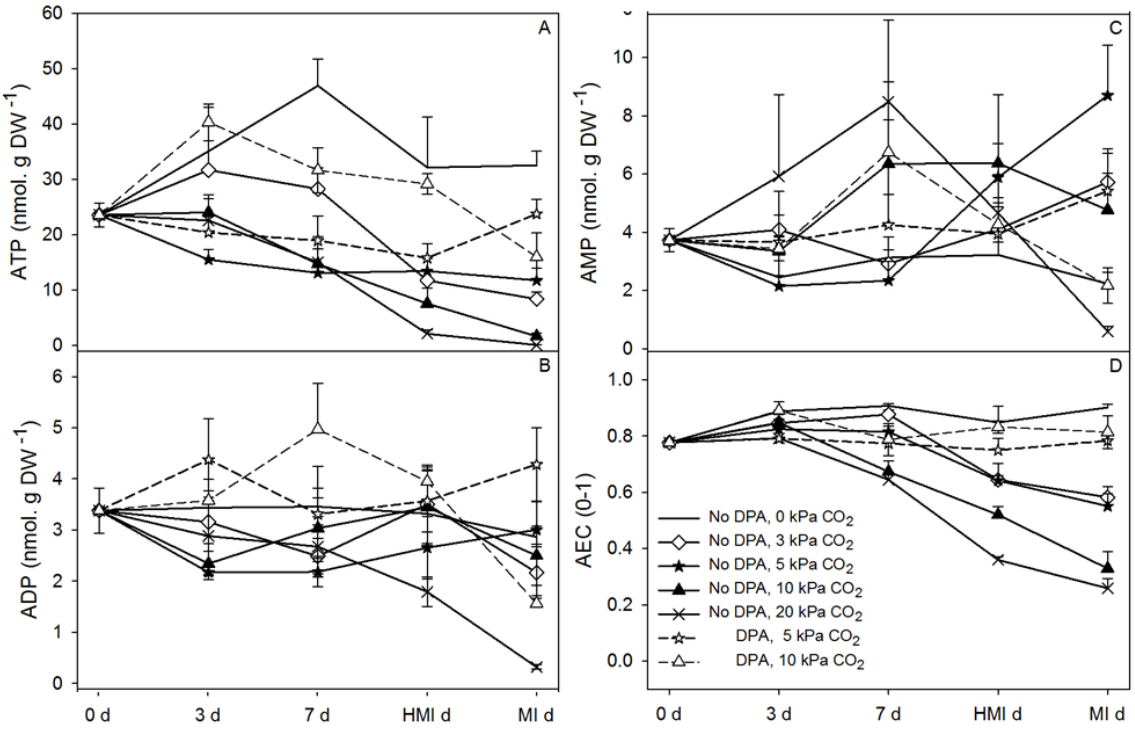


Figure 4. 2. Effect of CO_2 concentrations on levels of ATP (A), ADP (B), AMP (C), and AEC (D) of 'Honeycrisp' apple during CA storage (3 kPa O_2 with 0, 3, 5, 10 and 20 kPa CO_2) at 3 °C. Some of the fruit held in 5 and 10 kPa CO_2 were treated with DPA (1000 ppm, 30 s). Each symbol represents fruit from five orchards in 2014, three orchards in 2016 and two orchards in 2017 for two replicates (for CO_2 factor), n = 5 fruit per orchard. Sampling dates were 0 d (harvest day), 3 d, 7 d, HMI d (day of half maximal injury and MI d (day of maximal injury). Statistical analysis of the mean values was elaborated in Supplementary Table 4. 1. Vertical bars represent the SE of the mean.

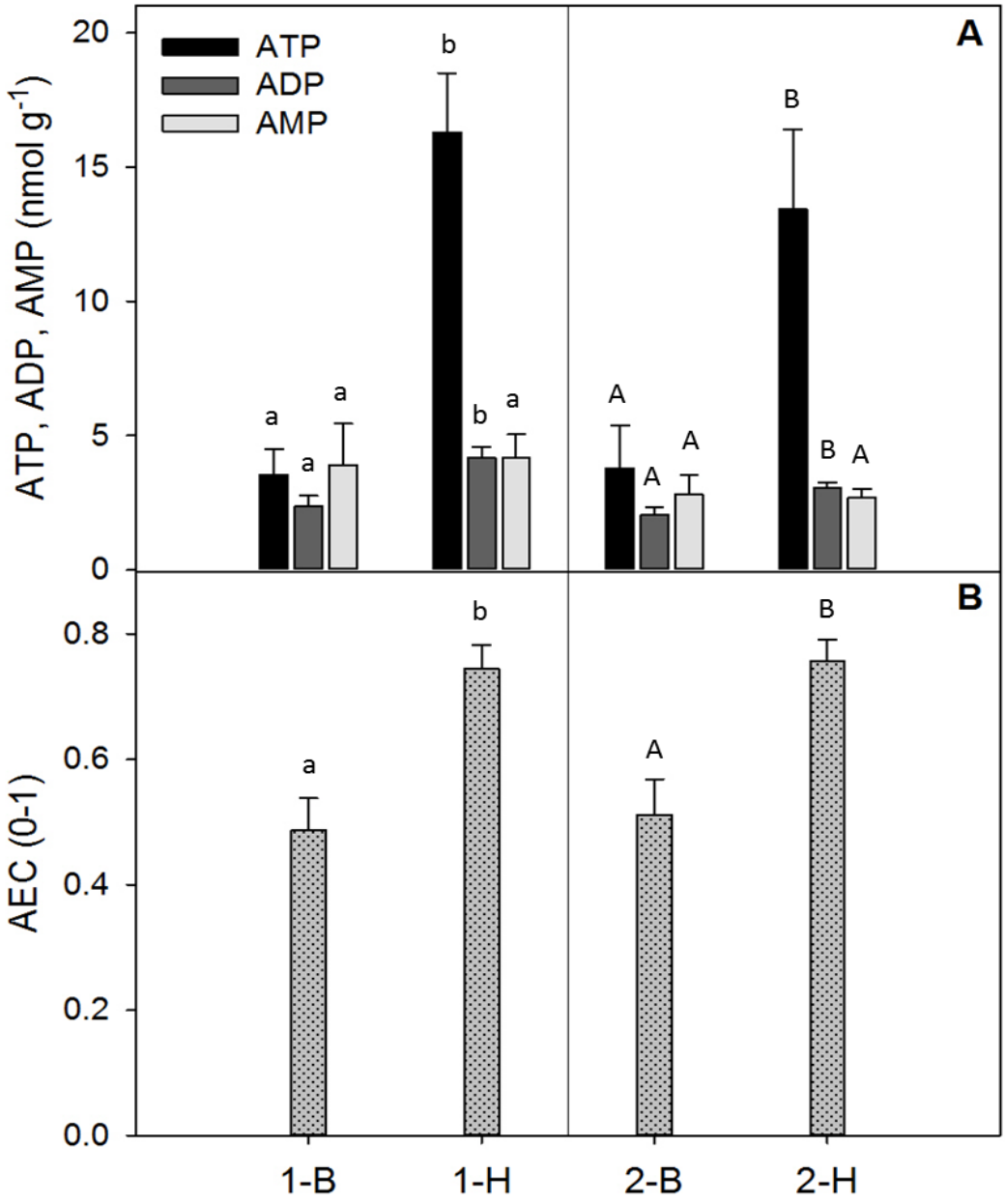


Figure 4. 3. Levels of energy state compounds and AEC values in 'Honeycrisp' apple tissues suffered CA injury at rating 1 and 2 when stored at 5 kPa CO_2 + 3 kPa O_2 at 3 °C for 21 d. The samples were browning area (B) and healthy area (H) of the injured apple slice. Error bars were SE of the mean from three orchards stored at 5 kPa CO_2 of two CA chambers (2 replicates). N = 5 fruits. Means followed by the same letter within a treatment are not significantly different ($P < 0.05$).

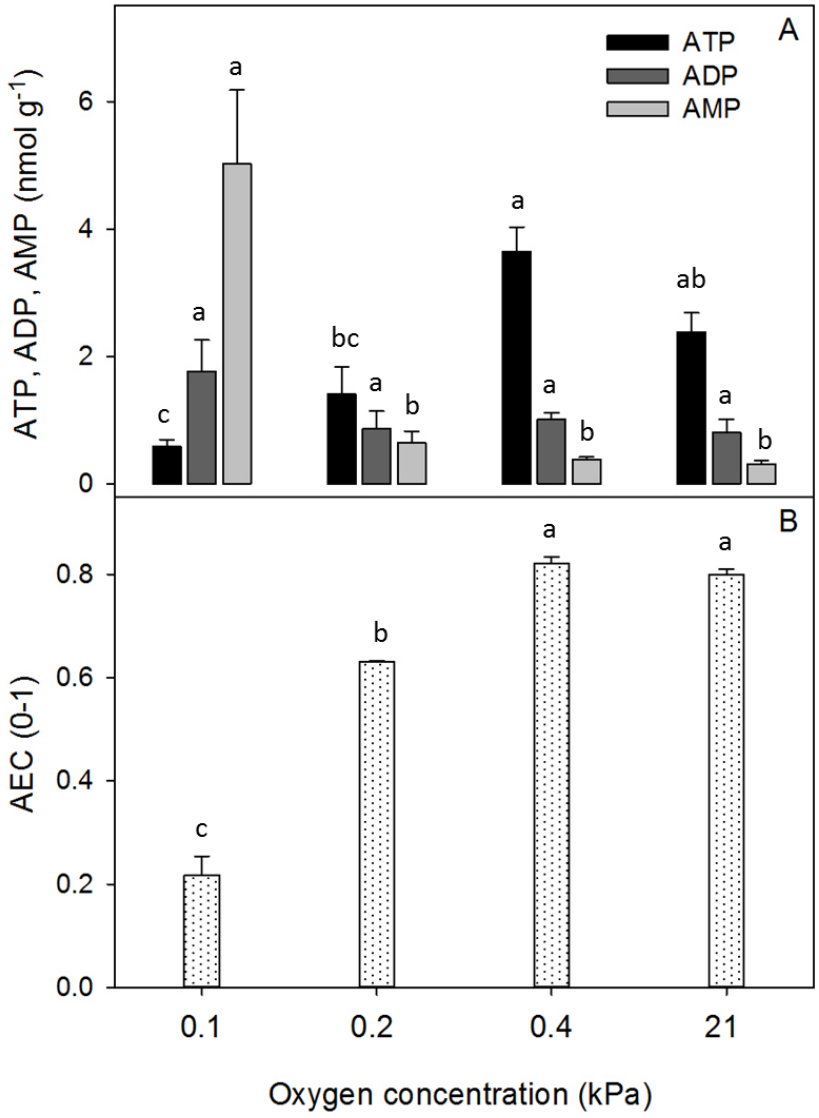


Figure 4. 4. Effect of O_2 concentrations on levels of ATP, ADP, AMP, and AEC of 'Honeycrisp' apple under hypoxia conditions (0 kPa CO_2 with 0.1, 0.2, or 0.4 kPa O_2) at 3 °C. Control was RA stored fruit (21 kPa O_2 + 0 kPa CO_2) at 3 °C. The sampling date was after two weeks of storage. Error bars represent SE of average four replicates using five fruits for each treatment. Means followed by the same letter within a treatment are not significantly different ($P < 0.05$).

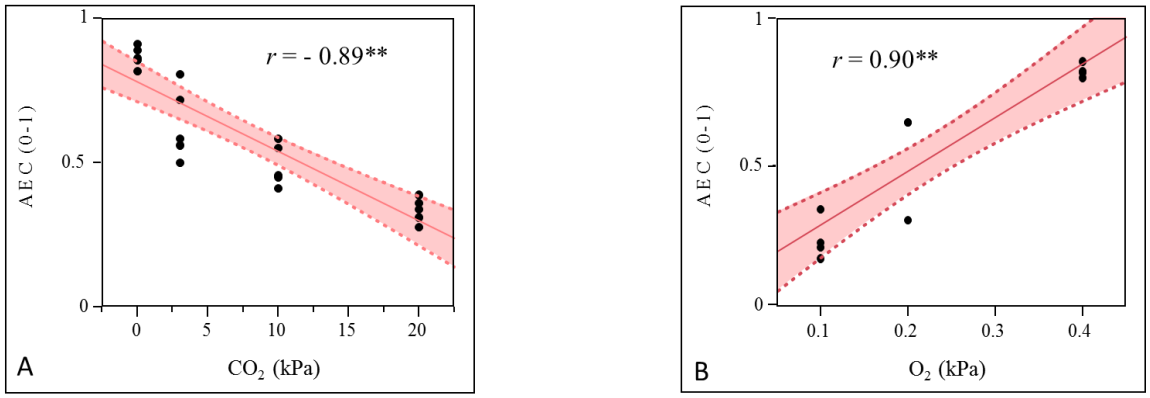


Figure 4. 5. Correlation and regression confidence intervals between values of adenylate energy charge (AEC) and CO_2 concentration for fruit reaching maximal injury in CA conditions (A), and between AEC value and O_2 concentration for fruit stored 14 days in hypoxia conditions (B).

4.3.2.3 Effect of preconditioning on energy compounds

Preconditioned 'Honeycrisp' apple after harvest for five days at 20 °C increased fruit tolerance to CA conditions (Table 4.1). However, there was a distinct difference in the sensitivity of the fruit form the two orchards to CO_2, with orchard F being the more sensitive. Since fruit of orchard F were less mature than those from orchard H at harvest (Table 4.2) and sensitivity to CA condition differed as well (Table 4.1), we compared the changes in these compounds levels based on two matrix factors: orchard and preconditioning.

Table 4. 4. Maturity indices of 'Honeycrisp' apples harvest from commercial orchard across Michigan in 2014 – 2017[a].

Orchard	Area	Harvest day	Ethylene (ppm)	Redness (%)	Background (1-5)	Starch (1-8)	TSS (°Brix)	Firmness d0 (lb)
F	Sparta	9/8	5.31 ± 0.6	81.00 ± 7.0	1.40 ± 0.2	5.90 ± 0.3	14.60 ± 0.4	17.40 ± 0.5
H	Sparta	9/15	27.30 ± 6.2	77.00 ± 5.9	2.15 ± 0.3	7.50 ± 0	14.45 ± 0.3	15.01 ± 0.4

[a] Values are means SE for analyses of 10 fruit except TSS using 5 fruits.

ATP levels of the preconditioned fruit from orchard F and H at harvest (day 0) were 3 times and 6 times, respectively, higher than those that were not preconditioned (Fig. 4.6). ATP levels in preconditioned fruit afterward decreased more extensively for orchard F than for orchard H. ATP levels of non-preconditioned fruit did not dramatically change with storage time. Eventually, ATP levels of all four treatments were very low on day 56.

ADP levels of the four treatments had about the same changing patterns as ATP levels. They were higher in preconditioned fruit but declined steadily until fruit from all treatments had about the same amount after 56 days of storage (Fig. 4.6).

AMP levels of the fruit were raised by preconditioning and declined with storage time. AMP levels of non-preconditioned fruit, however, started to increase at day 7 and eventually became higher than those in preconditioned fruit when maximal CA injury occurred (Fig. 4.6).

AEC ratios remained near 0.8 for preconditioned fruit for both orchards and for non-preconditioned fruit from orchard H (Fig. 4.6). However, the AEC of non-preconditioned fruit from orchard F declined markedly by the time of half-maximal damage.

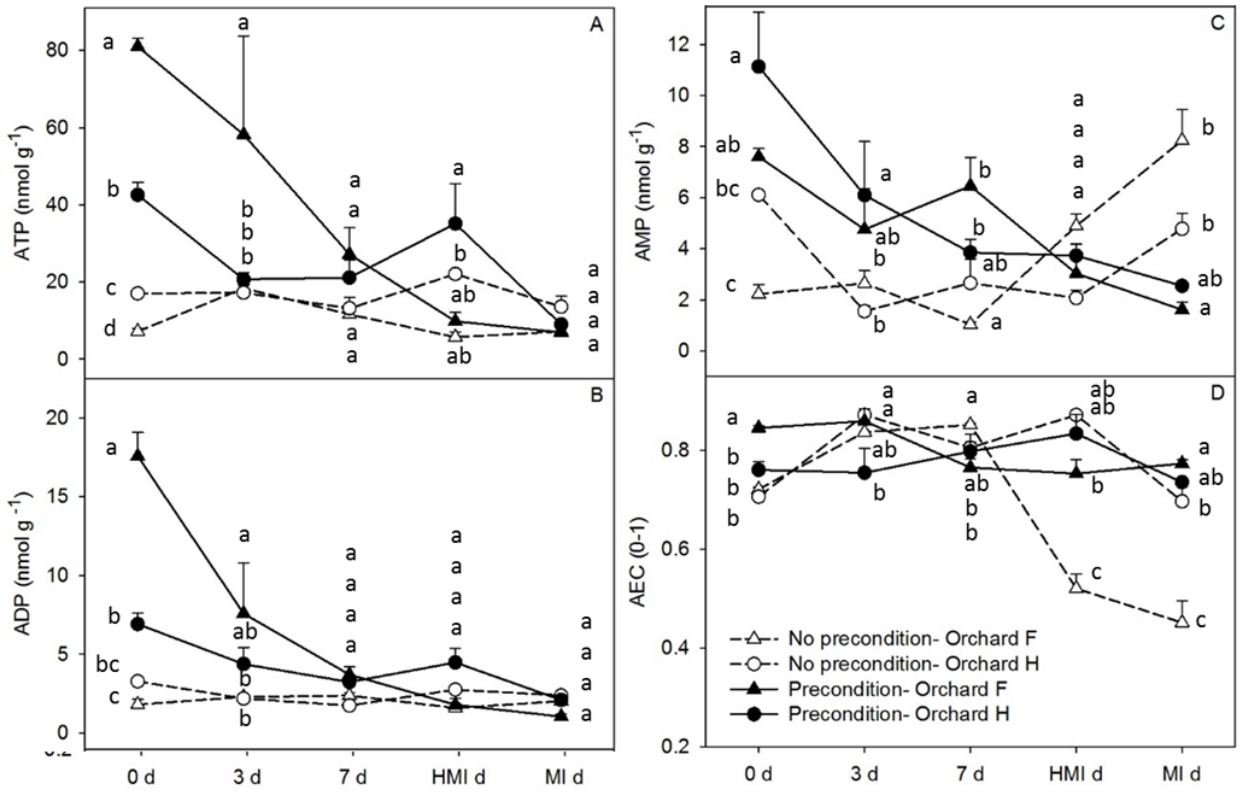


Figure 4. 6. Effect of preconditioning (20 °C for 5 d) on levels of ATP (A), ADP (B), AMP (C), and AEC values (D) of 'Honeycrisp' apple during CA storage (0 kPa O_2 and 5 kPa CO_2 at 3 °C) from two orchards (F and H) harvested in Michigan in 2017. Each symbol represents 10 fruits of two precondition replicates. At each sampling date, means followed by the same letter within a treatment are not significantly different ($P < 0.05$).

4.3.3 Energy redox compounds (NAD^+, NADH, $NADP^+$, NADPH)

4.3.3.1 Effect of CO_2 and O_2 concentration on redox energy status of 'Honeycrisp' apple

NAD^+, NADH, $NADP^+$, and NAPDH levels, as well as their ratios, fluctuated with storage time for all CO_2 treatments (Fig. 4.7). NADH level of 5 kPa CO_2 was stable during storage time, however, NAD^+ levels of 3 and 10 kPa changed in different ways. They decreased at day 7 and rose back to near initial levels by HMI day. The content declined sharply for 10 kPa CO_2 but remained steady for 3 kPa CO_2 until the fruit receiving maximal CA injury (MI day). At

HI day, only the NAD+ level of 0 kPa CO_2 remained similar to day 0 while the levels in fruit receiving higher CO_2 concentrations were reduced in comparison to day 0. NADH levels of all treatment decreased with storage time (Fig. 4.7). When fruit stored at 20 kPa CO_2 had become completely damaged (day 28), the NADH level had declined 80% relative to day 0.

The ratios of $NADH/NAD^+$ of all treatments changed dynamically with storage time (Fig. 4.7). Since NADH levels of all treatments at any sampling dates were always smaller than 1 nmol g^{-1} and much smaller than the contents of its oxidized forms, the ratios of NADH/NAD+ were always smaller than 1. On the date of maximum injury, the treatments exhibiting the greatest amount of damage (10 and 20 kPa CO_2), had NADH/NAD+ ratios increase markedly relative to the half-maximal injury date. The levels of NADP+ and NADPH for all CO_2 treatments decreased with storage time (Fig. 4.7). NADPH levels of 0 or 3 kPa CO_2 reached peaks on day 7 and day 21 (HMI) respectively, followed by a slight decrease but remained higher than other CO_2 levels. 20 kPa CO_2 caused a marked reduction in levels of the compounds: 95% and 98% for NADP and NADPH, respectively, at maximal injury. The 5 and 10 kPa CO_2 treatments caused approximately 50% loss of NADH and NADPH levels when the fruit reached maximal injury. For the 20 kPa CO_2 treatment, NADPH levels always remained high at all sampling dates until the date of maximum injury on which the ratio of $NADPH/NADP^+$ was only 0.7 (Fig. 4.7) and the fruit exhibited the greatest amount of damage. The NADH/NAD+ ratios did not differ between brown and healthy tissue of the fruit injured by 5 kPa CO_2 at injury rating 1 and injury rating 2 (Fig. 4.8). The $NADPH/NADP^+$ ratio was the highest in healthy tissue of the fruit at injury rating 1. $NADH/NAD^+$ and $NADPH/NADP^+$ ratios of control fruit (21 kPa O_2) were higher than those held under hypoxic conditions (0.1, 0.2 and 0.4 kPa O_2) (Fig. 4.9). However, the ratios did not differ among low oxygen concentrations.

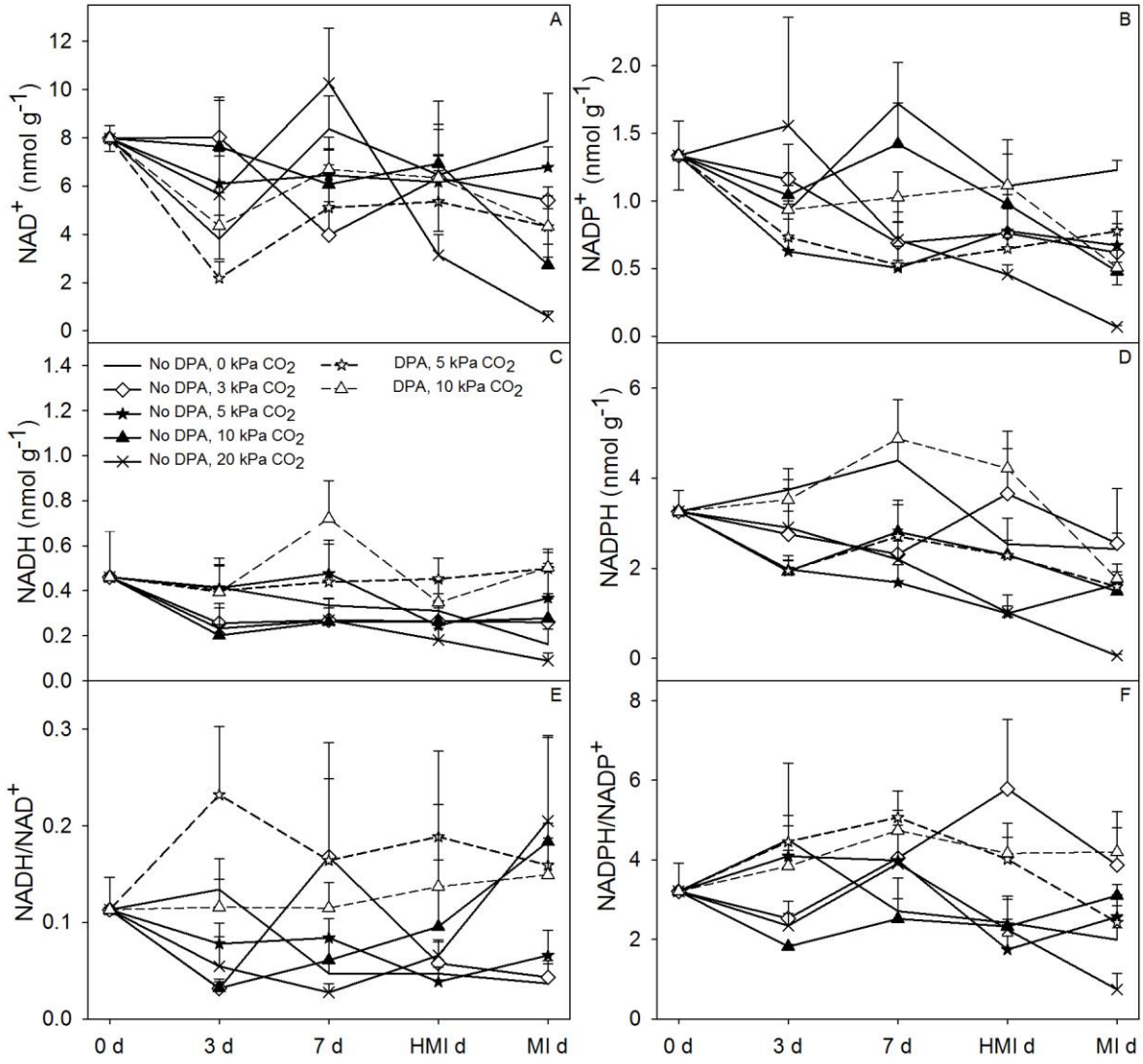


Figure 4. 7. Effect of CO_2 concentrations on levels of NAD^+ (A), NADP (B), NADH (C), NADPH (D), and ratios of NADH/NAD+ (E) and of NADPH/NADP+ (F) of 'Honeycrisp' apple during CA storage (0 kPa O_2 with 0, 5, 10 and 20 kPa CO_2) at 3 °C. A portion of the fruit was treated with DPA (1000 ppm, 30 s) and stored at 5 kPa CO_2 and 10 kPa CO_2 at 3 °C. Each symbol represents fruit from five orchards in 2014, three orchards in 2016 and two orchards in 2017 for two replicates (for CO_2), n = 5 fruit per orchard at each sampling date of each treatment. Statistical analysis of the means is elaborated in Supplementary Table 4. 1.

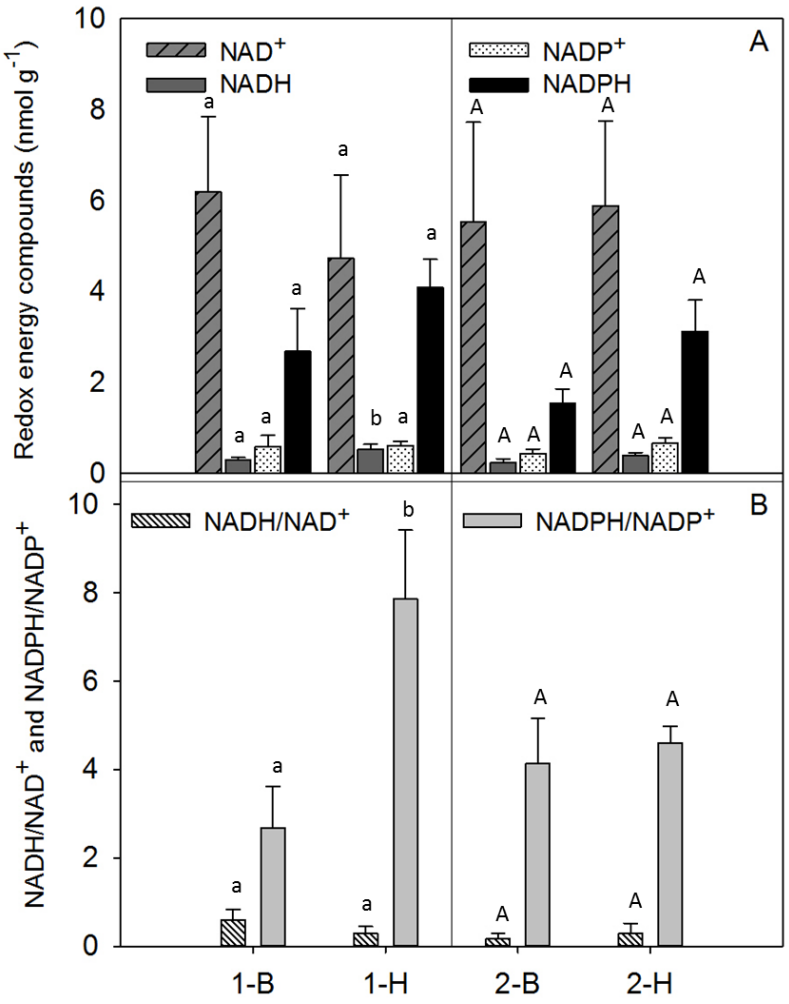


Figure 4. 8. Levels of NAD^+, NADH, $NADP^+$, NADPH and ratios of reduced over oxidized compounds in 'Honeycrisp' apple tissues suffered CA injury at rating 1 and 2 when stored at 5 kPa CO_2 + 3 kPa O_2 at 3 °C for 21 d. The samples were browning area (B) and healthy area (H) of the injured apple slice. Error bars were SE of fruit from three orchards stored at 5 kPa CO_2 of two CA chambers (2 replicates). N = 5 fruits. Means followed by the same letter within a treatment are not significantly different ($P < 0.05$).

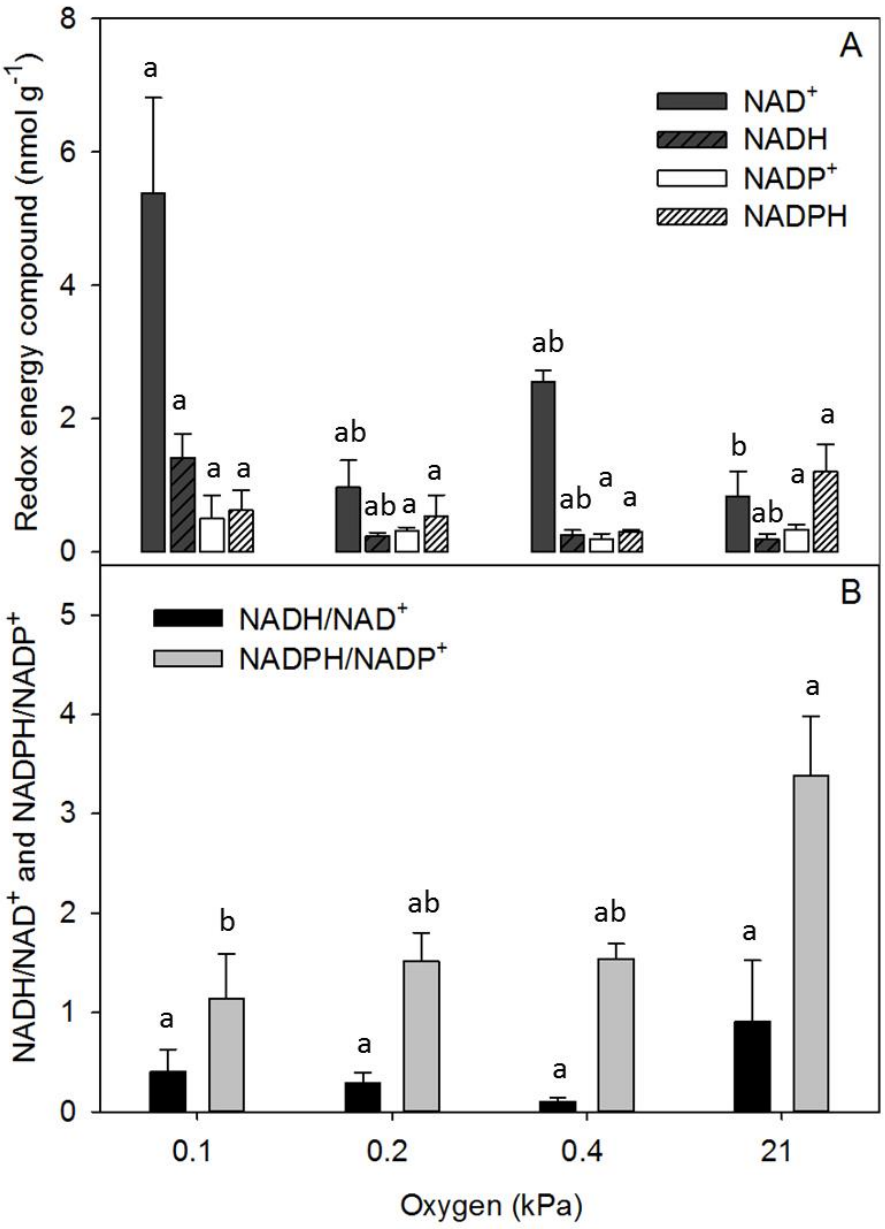


Figure 4. 9. Effect of O_2 concentrations on levels of NAD^+, $NADP^+$, NADH, NADPH, and ratios of $NADH/NAD^+$ and of $NADPH/NADP^+$ of 'Honeycrisp' apple under hypoxia conditions (0 kPa CO_2 with 0.1, 0.2, or 0.4 kPa O_2) at 3 °C. Control was RA stored fruit (21 kPa O_2 + 0 kPa CO_2) at 3 °C. The sampling date was after two weeks of storage. Error bars represent SE of average four replicates using five fruits for each treatment. Means followed by the same letter within a treatment are not significantly different ($P < 0.05$).

4.3.3.2 Effect of DPA on redox energy status of 'Honeycrisp' apple

There are four treatments of two factors CO_2 (5 and 10 kPa) and DPA (0 and 1000 ppm) in this experiment in 2014, 2016, and 2017. NAD^+, $NADP^+$, NADH, and NADPH of the fruit were also quantified to test if DPA treatment could maintain a higher ratio of reduced over oxidized state of these compounds in the fruit under CA conditions. After three days of storage, there was a 73% (for 5 kPa CO_2) and 45% (for 10 kPa CO_2) reduction of NAD^+ levels of DPA treated fruit (Fig. 4.7). Then, they recovered and remained at the same level (4.3 nmol g^{-1}) for day 56. The NAD^+ level of fruit held in 5 kPa CO_2, but not treated with DPA was steady during storage time while the level for 10 kPa CO_2 declined by 66%.

NADH levels of the four treatments had differing patterns during storage time (Fig. 4.7). However, when the fruit reached half-maximal and maximal injury, NADH level remained higher in DPA-treated fruit. As a result, NADH/NAD^+ ratios of DPA-treated fruit were higher in the DPA-treated fruit at half maximal injury than all non-DPA treated fruit at this point.

The $NADP^+$ levels of DPA-treated and untreated fruit at 5 kPa CO_2 had similar patterns (Fig. 4.7). They steadily declined to day 7 and increased slightly thereafter. $NADP^+$ levels of the fruit given 10 kPa CO_2 increased and reached maximum at day 7 for non-DPA treated fruit and at day 21 for DPA-treated fruit. After that, they declined to lower levels lower than those at 5 kPa CO_2. NADPH level of all four treatments, except DPA untreated 5kPa CO_2 which declined steadily, increased by day 7 and subsequently decreased to approximately 46 % loss after 56 days of storage at 5 or 10 kPa CO_2. The NADPH level of DPA-treated fruit at 10 kPa CO_2 was much higher than other treatments during the first two weeks before it dropped to the same level as other treatments. The NADPH/$NADP^+$ ratio for DPA-treated fruit, therefore, was also higher than untreated ones at either CO_2 concentration.

4.3.3.3 Effect of preconditioning on redox energy status of 'Honeycrisp' apple

The NAD^+ levels of non-preconditioning of fruit from both orchards was low on the day of harvest (Fig. 4.10). They, however, increased quickly with storage time and after 56 days of storage, the levels were 4.5 times and 7.2 times higher than at the beginning. NAD^+ levels of preconditioned fruit from orchard F reached the peak on day 7 and declined afterward. The same pattern for NAD^+ was found for the preconditioned fruit from orchard H. Preconditioning and orchard factor did not influence NADH levels, which declined with storage time. The NADH/NAD^+ ratios of preconditioned fruit were higher than those of other treatments in the first week.

The $NADP^+$ level in preconditioned and non-preconditioned fruits from orchards H and F declined with storage time (Fig. 4.10). However, preconditioning and orchard factors did not influence these levels. On the other hand, preconditioning helped maintain NADPH at a higher level than control fruit initially. The concentrations of NADPH did not differ among the treatments after 56 days of storage. The NADPH/$NADP^+$ ratio of the non-preconditioned less mature fruit (orchard F), which were more susceptible to CO_2 injury, was always lower than fruit from the other three treatments during storage time.

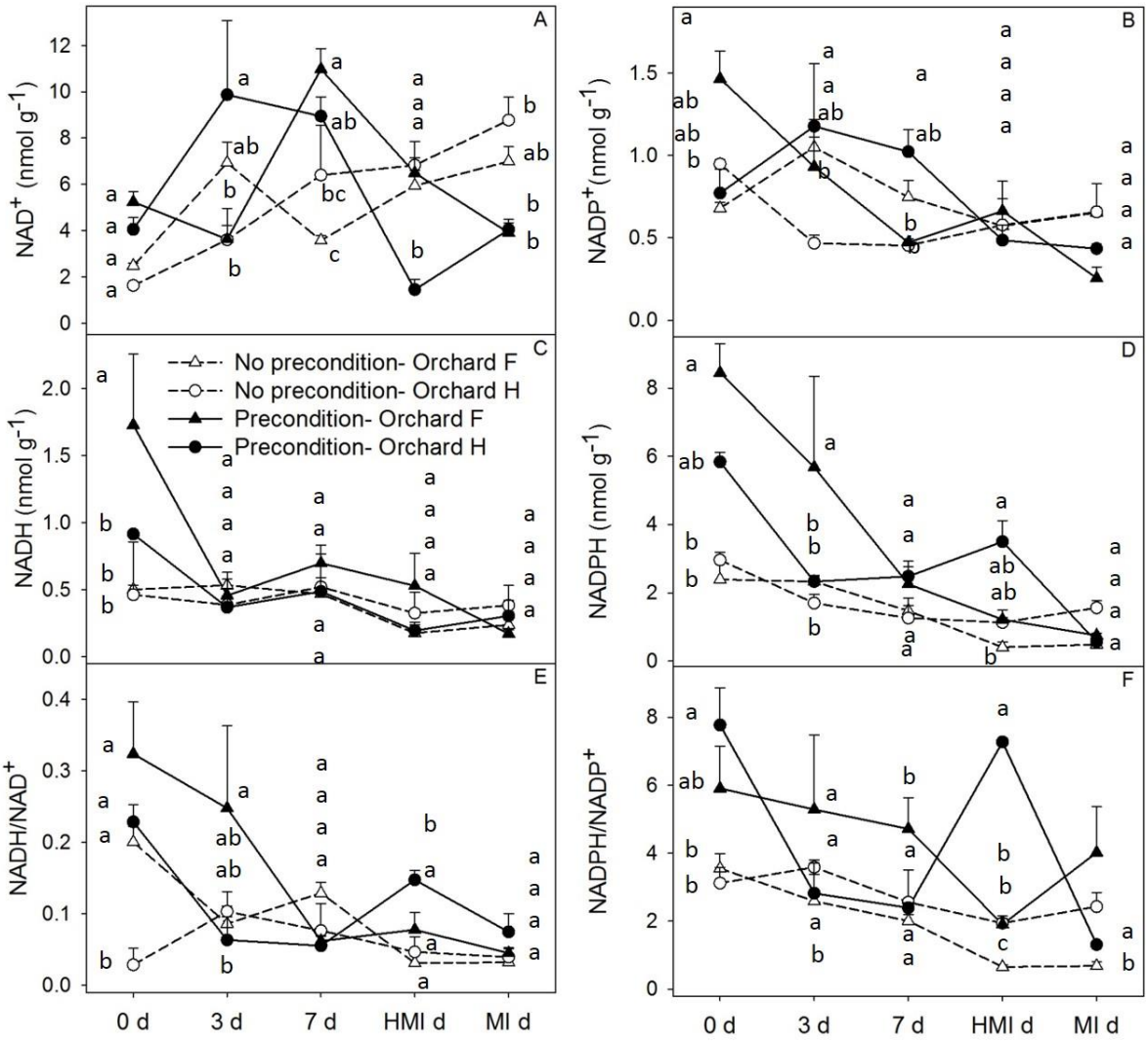


Figure 4. 10. Effect of preconditioning (the fruit at harvest was kept at 20 °C for five days in the lab before CA storage) on levels of NAD^+ (A), $NADP^+$ (B), NADH (C), NADPH (D), $NADH/NAD^+$ (E), and $NADPH/NADP^+$ (F) in 'Honeycrisp' apple during CA storage (0 kPa O_2 and 5 kPa CO_2) at 3 °C from two orchards (H and F). Each symbol represents two replicates of 5. On each sampling date, means followed by the same letter within a treatment are not significantly different ($P < 0.05$).

4.3.4 Antioxidants (Asc, GSH, and GSSG)

4.3.4.1 Effect of CO_2 and O_2 concentration on the antioxidative status of 'Honeycrisp' apple

The CO_2 partial pressure influenced Asc, GSH, and GSSH levels in different ways (Fig. 4.11). Asc levels of all CO_2 treatments decreased with storage time. At each sampling date, there was no significant difference in Asc level among the CO_2 partial pressures, but they did differ from the 0 kPa CO_2 treatment, which maintained higher Asc levels. When the fruit attained maximal injury, Asc levels of 3, 5, 10, and 20 kPa CO_2 decreased by 82%, 86%, 93%, and 99.95%, respectively but they are not significantly different.

The GSH level in fruit from the 20 kPa CO_2 treatment reached 285% of its initial value on day 7, followed by a sharp decrease, reaching a 99.4% reduction relative to initial values when the fruit tissues had been maximally injured (Fig. 4.11). Day 7 coincided with the first signs of injury of these fruits. GSH level of the fruit receiving 10 kPa CO_2 rose to 206% of initial values on day 14 (half maximal injury day of this treatment) and then declined sharply. GSH levels for other CO_2 partial pressures were stable with storage time, except 10 kPa CO_2, which declined by 50% at maximal injury day (day 56).

GSSG levels of fruit from all CO_2 treatments were maintained around 0.1 – 0.2 nmol g^{-1}, except for 20 kPa for which GSSG level started to sharply increase after day 3, maximizing when fruit had been fully damaged after 28 d storage (Fig. 4.11). GSSG levels of all treatments at all sampling dates were always considerably lower than the reduced state of glutathione, GSH. Therefore, the ratios of GSH/GSSG were extremely high and tended to track GSH levels. The ratios of 20 kPa CO_2 and 10 kPa CO_2 reached their peaks on day 7 and day 21, respectively, while those of other CO_2 concentrations remained relatively constant and low over the storage period.

Asc levels in healthy tissues (H) were about two times higher than that in brown (B) tissue (Fig. 4.12). The Asc level in H tissues having an injury rating of 2 (25 - 50% brown on its cut surface) was about 31% less than for fruits with injury rating 1 (10-25% brown on its cut surface). For fruits with injury rating of 2, Asc levels of B tissues were reduced by about 70% compared to H tissues. GSSG levels remained about the same in H and B tissues at both injury ratings (Fig. 4.12).

Under hypoxic conditions, the Asc and GSH levels for 0.1 kPa O_2 were considerably higher than those of 0.2 kPa, 0.4 kPa, and 21 kPa O_2 (Fig. 4.13). As the result, the ratio GSH/GSSG for 0.1 kPa O_2 treatment was higher than the other O_2 treatments.

4.3.4.2 Effect of DPA on the antioxidative status of 'Honeycrisp' apple

When the fruit was drenched with DPA (1000 uL/L, 30 s), Asc levels were maintained higher than in DPA untreated fruit at either 5 or 10 kPa CO_2 (Fig. 4.11). After 56 d storage, the Asc levels decreased with storage time and lost about 90% of their initial concentration for DPA-untreated at both CO_2 levels, but only approximately 10% or 50% for DPA-treated fruits stored at 5 or 10 kPa CO_2, respectively.

DPA treatment kept GSH and GSSG levels as well as the GSH/GSSG ratios at both CO_2 concentrations (5 and 10 kPa) stable during storage time. The GSH levels of DPA-untreated fruit were considerably higher than those of DPA-treated fruits at both CO_2 concentrations. The GSSG levels in DPA – untreated fruit at 5 kPa CO_2 were higher than those of other treatments. GSH levels and the GSH/GSSG ratios in DPA-untreated fruit at 10 kPa CO_2 at day 14 the highest when the fruit exhibited reached half-maximal injury before they dropped to lower values than those in other treatments at maximal injury days.

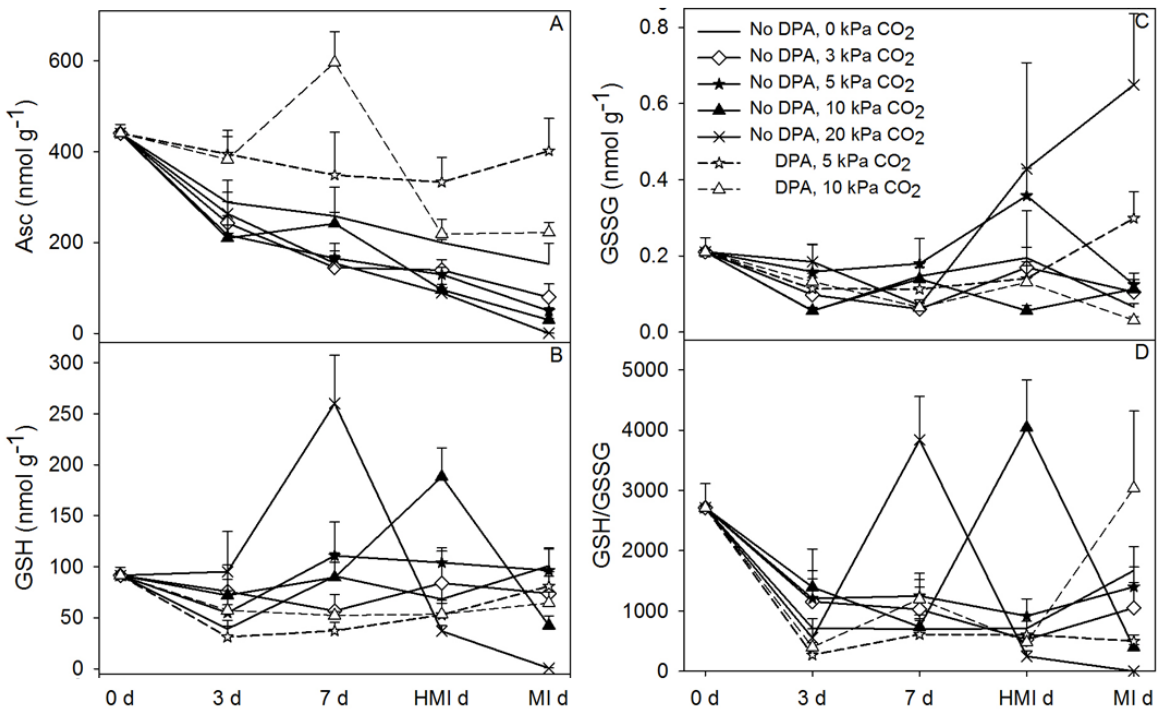


Figure 4. 11. Asc (A), GSH (B), GSSG (C), and the ratio of GSH/GSSG (D) of 'Honeycrisp' apple during CA storage in 3 kPa O_2 with 0, 3, 5, 10 and 20 kPa CO_2 at 3 °C. Some fruit were treated with DPA (1000 ppm, 30 s) and stored at 5 kPa CO_2 and 10 kPa CO_2 at 3 °C. Each symbol represents fruit from five orchards in 2014, three orchards in 2016 and two orchards in 2017 for two replicates (for CO_2 factor), n = 5 fruit per orchard at sampling dates of each treatment. Statistical analysis of the mean values was elaborated in Supplementary Table 4. 1

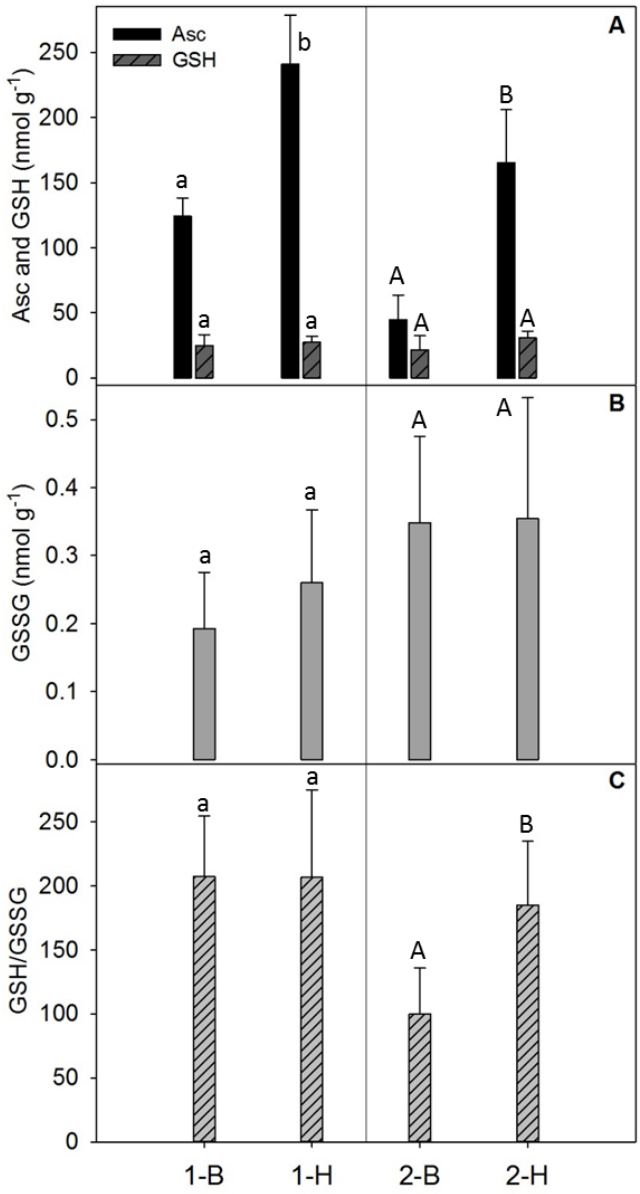


Figure 4. 12. Levels of Asc, GSH, GSSG and ratio of GSH/GSSG in 'Honeycrisp' apple tissues suffered CA injury at rating 1 and 2 when stored at 5 kPa CO2 + 3 kPa O_2 at 3 °C for 21 d. The samples were browning area (B) and healthy area (H) of the injured apple slice. Error bars were SE of fruit from three orchards stored at 5 kPa CO2 of two CA chambers (replicates). N = 5 fruits. Means followed by the same letter within a treatment are not significantly different ($P < 0.05$).

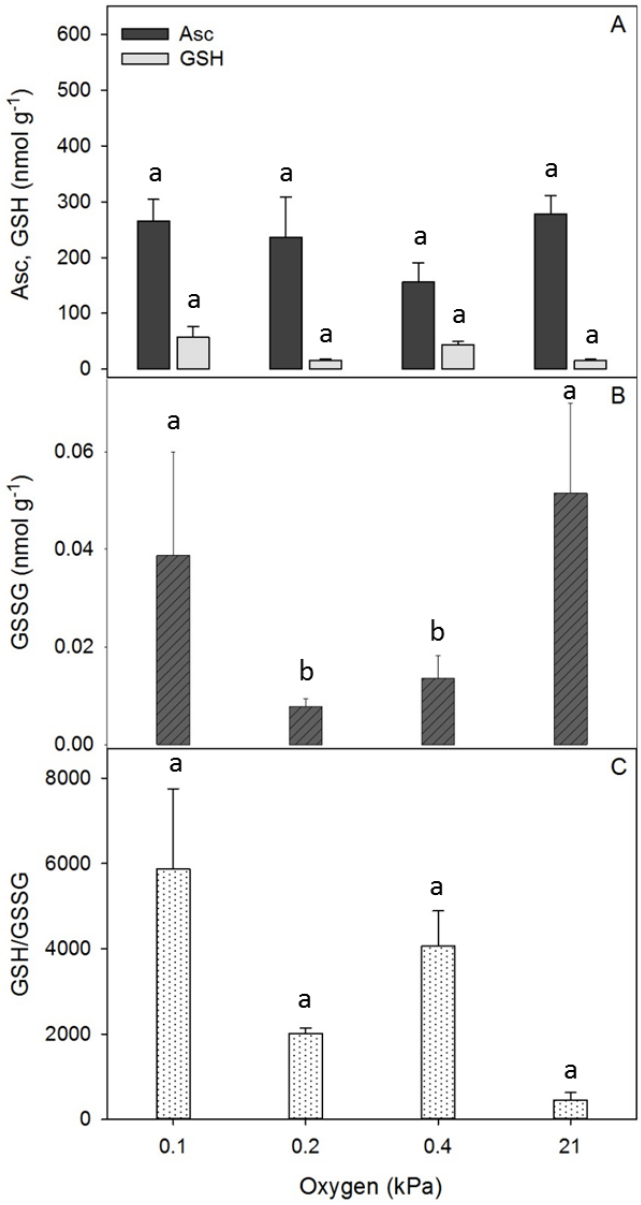


Figure 4. 13. Effect of O_2 concentrations on levels of Asc, GSH, GSSG, and the ratio of GSH/GSSG of 'Honeycrisp' apple fruit under hypoxic conditions (0 kPa O_2 with 0.1, 0.2, or 0.4 kPa O_2) at 3 °C. Control was RA stored fruit (21 kPa O_2 + 0 kPa CO_2) at 3 °C. The sampling date was after two weeks. Error bars represent SE of average four replicates using five fruits for each treatment. Means followed by the same letter within a treatment are not significantly different ($P < 0.05$).

4.3.4.3 Effect of preconditioning on antioxidant status of 'Honeycrisp' apple

The Asc level of preconditioned and non-preconditioned fruit decreased with storage time (Fig. 4.14). The Asc level of the more mature fruit from orchard H remained at a somewhat elevated level and only lost 43% loss after 56 days of storage, Asc levels of the other treatments retained only approximately 55 - 75 nmol g^{-1}.

The fruit receiving preconditioning condition (5 days at 20 °C) has about the same GSH level in comparison to harvest day. The fruit from orchard H which was more mature had higher GSH levels than that from orchard F. The preconditioning treatment also did not affect of GSSH level. Except for the GSH/GSSG ratio of non-preconditioning fruit from orchard H which was very high, the ratios of three other treatment were about the same at day 0. After that, the GSH and GSSG levels and the GSH/GSSG ratio changed unpredictably during storage time (Fig. 4.14). The GSH/GSSG ratios in the less mature (and more susceptible) and preconditioned fruit from orchard F increased relative to initial values and was higher than those in other treatments at half maximal injury day and maximal injury day when the fruit did not show CA injury (Figure 4.14 and Table 4.3)

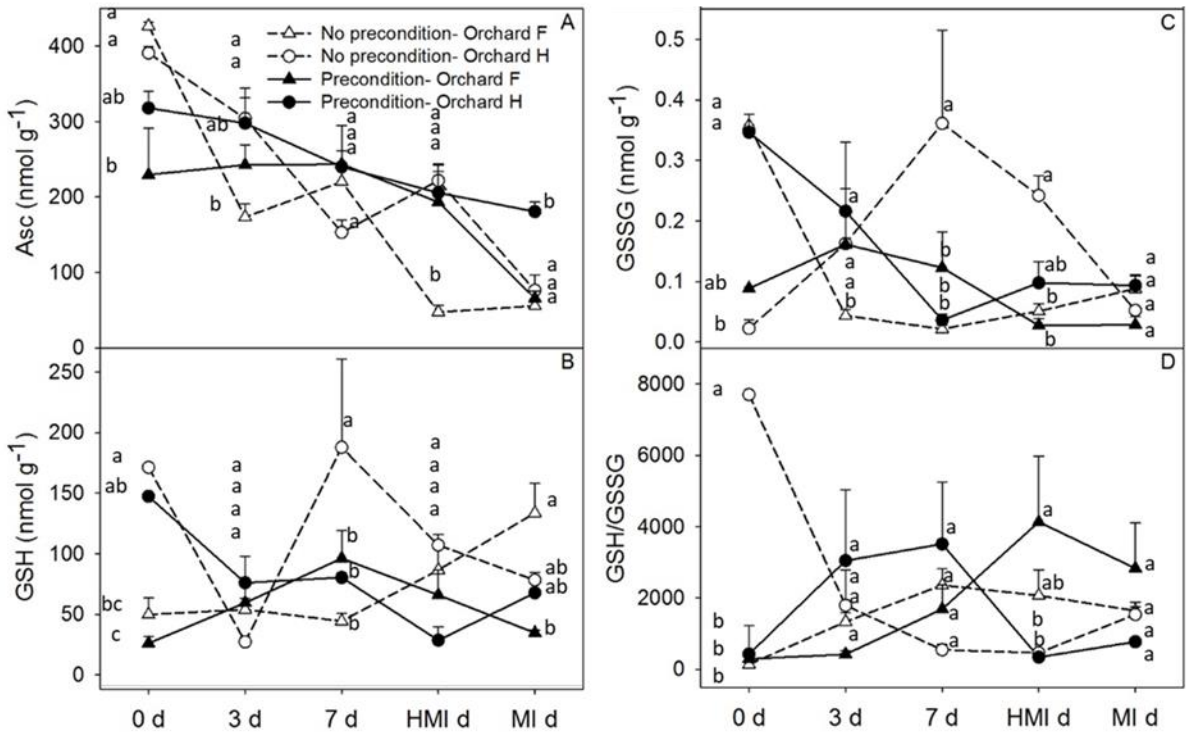


Figure 4. 14. Effect of preconditioning (20 °C for five d before CA storage) on levels of Asc, GSH, GSSG, and the GSH/GSSG ratio in 'Honeycrisp' apple during CA storage (3 kPa O_2 and 5 kPa CO_2 at 3 °C) from two orchards (F and H) in Michigan in 2017. N=5 fruit per orchard for each sampling/treatment combination. At each sampling date, means followed by the same letter within a treatment are not significantly different ($P < 0.05$)

4.3.5 Carbohydrate metabolites (UDP-G, SA, PEP, CoA, and Acetyl CoA)

4.3.5.1 Effect of CO_2 and O_2 concentration on carbohydrate metabolites

The UDP-G level of 20 kPa CO_2 treatment declined very quickly with storage time and lost 96% of its initial value when the damage level reached its maximum (100%) (Fig. 4.15). Atmospheres of 3 and 10 kPa CO_2 resulted in a slight decrease of UDP-G levels after seven days of storage, followed by a sharp rise when the fruit reached half-maximal injury (HMI) and subsequently dropped at maximal injury (MI) day. at HMI day.

The CoA level of control (0 CO_2 kPa treated) fruit increased and reached its peak on day 7, followed by a slight reduction (Fig. 4.15). However, the CoA level was lower for all CO_2 treatments compared to the control throughout storage. The CoA content of fruit receiving 3, 5, 10 and 20 kPa CO_2 treatment declined and eventually achieved its lowest levels after the fruit reached their maximal injury. At maximal injury, CoA levels were positively and linearly correlated with the applied CO_2 concentration.

Acetyl CoA levels of the control fruit increased initially and were higher than levels in the fruit exposed to CO_2 for the first seven days of storage, followed by a reduction to a similar level (0.2 nmol g^{-1}) as day 0 (Fig. 4.15). The acetyl CoA level of fruit from the 20 kPa CO_2, however, declined to only 0.01 nmol g^{-1} when fruit reached maximum injury.

The elevated CO_2 treatments resulted in an increase in SA levels (Fig. 4.15). SA levels of fruit from the 20 kPa CO_2 treatment rose quickly with storage time, remaining higher than other treatments. After 28 d storage, the level had increased approximately 25 times in comparison to the day of harvest. While 0 kPa CO_2 maintained SA at a low level, 3 to 10 kPa CO_2 caused an increase of SA levels at day 7 and then dropped as the injury of the fruit increased. When the fruit had half maximal CA injury, SA levels of fruit treated with 5 and 10 kPa CO_2 were, respectively, 67 and 83 nmol g^{-1} and were elevated relative to fruit in the 3 kPa CO_2 treatment.

The brown tissue of fruit exposed to 5 kPa CO_2 did not differ from healthy tissue in terms of UDP-G, CoA, Acetyl CoA, and PEP contents for fruit at half maximal injury (rating 1) or maximal injury level (rating 2) (Fig. 14.16). However, the SA level for brown tissue from the fruit having a rating 2 injury was significantly elevated relative to healthy tissue.

Low oxygen had little impact on the carbohydrate analytes CoA, Acetyl CoA and UDP-G (Fig 14.17). However, the content of SA was elevated, and PEP diminished in the 0.1 kPa O_2 treatment relative to other O_2 partial pressures (Fig.14.17).

4.3.5.2 Effect of DPA on carbohydrate metabolites

The succinic acid (SA) levels of DPA-treated fruit stored at 5 and 10 kPa and DPA-untreated fruit stored at 0 kPa CO_2 remained low throughout storage (Fig. 4.15). SA level at 20 kPa CO_2 had increased by day 7 to levels that were much higher than other CO_2 treatments. In contrast, SA level of DPA-untreated fruit stored at 3, 5 and 10 kPa CO_2 was elevated relative to controls (0 kPa CO_2) at the initial stages of disorder development, but later declined to the same level as DPA-treated and control fruit when fruit reached maximal injury (i.e., injury progression ceased).

DPA treatment also helped maintain PEP and Acetyl CoA of the fruit stored at 5 or 10 kPa CO_2 at a higher level in comparison to control (0 kPa CO_2) fruit during the first week of storage (Fig. 4.15). However, these treatments were not significantly different once the fruit reached half-maximal injury. The contents of CoA and UDP-G did not differ between the treatments and during storage time with the exception of the 20 kPa CO_2 treatment on maximal injury day, which showed lowest levels of these two compounds (Fig. 4.15).

4.3.5.3 Effect of preconditioning on carbohydrate metabolites of 'Honeycrisp' apples

UDP-G level was influenced by preconditioning treatment (Fig. 4.18). After five days at 20 °C, UDP-G levels rose 6.8-fold and 1.6-fold in fruit from orchards F and H, respectively, in comparison to the day of harvest. However, the level of UDP-G decreased sharply after 3 d storage. Afterwards, UDP-G levels were about the same across all treatments.

PEP, Acetyl CoA, and CoA levels of preconditioned fruit were also higher than non-preconditioned fruit and they dropped when stored in CA conditions during the first week (Fig. 4.18). The content of these metabolites remained unchanged afterwards.

SA levels of the more mature fruit of orchard H were initially significant different from those of the less mature fruit of orchard F. When stored at 5 kPa CO_2, the levels of all treatments were at a maximum on day 7 or afterwards. After day 7 the levels of SA dropped in the non-preconditioned fruit from orchard F only. SA levels of the other three treatments maintained at about 50 nmol g^{-1}, about the same as in fruit stored at 0 kPa CO_2 (Figure 4.15), which had a very low percentage of CA injury (Table 4.3)

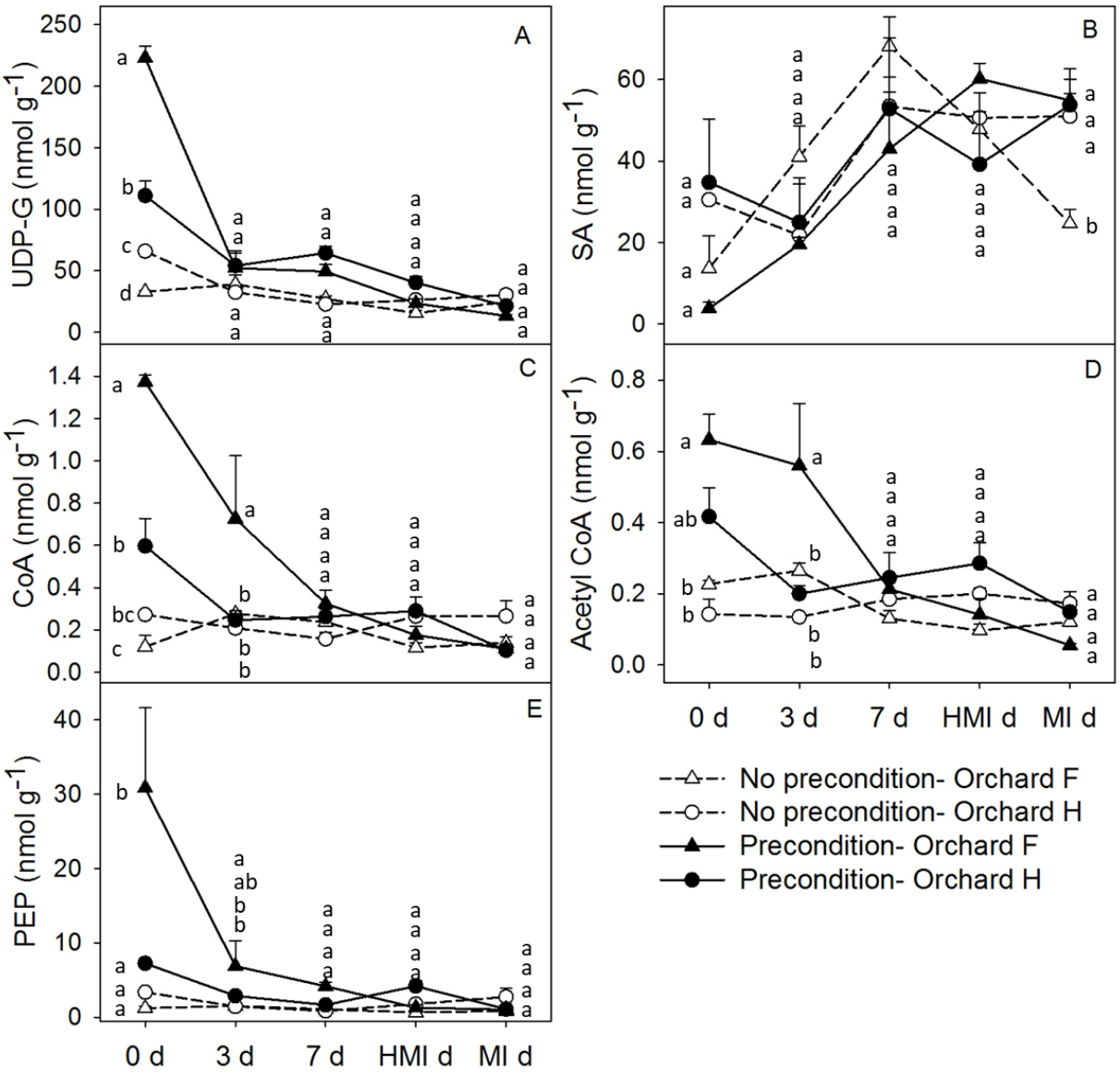


Figure 4. 15. Effect of CO_2 concentration on levels of UDP-G (A), SA (B), CoA (C), Acetyl CoA (D), and PEP (E) of 'Honeycrisp' apple during CA storage (0 kPa O_2 with 0-20 kPa CO_2) at 3 °C. The fruit were treated with DPA (1000 ppm, 30 s) and stored at 5 kPa CO_2 and 10 kPa CO_2 at 3 °C. Each symbol represents fruit from five orchards in 2014, three orchards in 2016 and two orchards in 2017 for two replicates (for CO_2 factor), n = 5 fruit per orchard at sampling dates of each treatment. The vertical bars represent the SE of the mean.

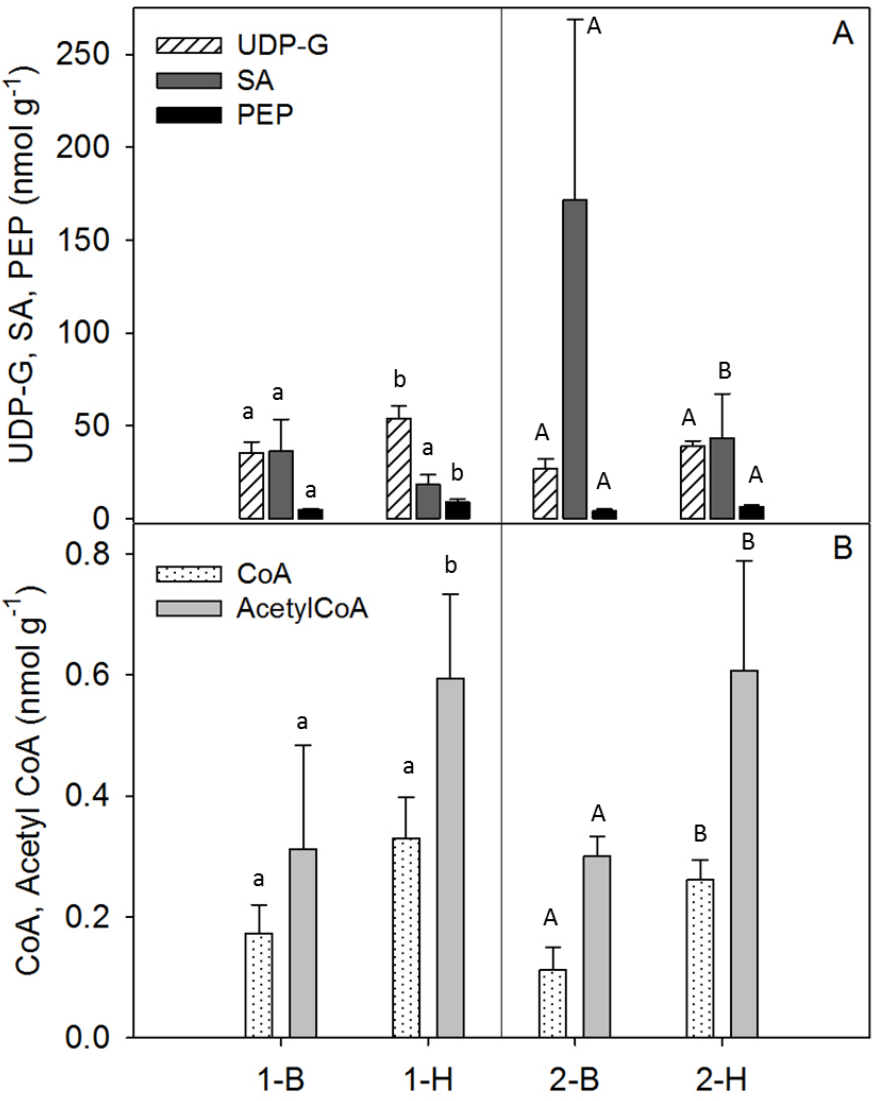


Figure 4. 16. Levels of UDP-G, SA, CoA, Acetyl CoA, PEP in 'Honeycrisp' apple tissues suffered CA injury at rating 1 and 2 when stored at 5 kPa CO_2 + 3 kPa O_2 at 3 °C for 21 d. The samples were browning area (B) and healthy area (H) of the injured apple slice. Error bars were SE of fruit from three orchards stored at 5 kPa CO_2 of two CA chambers (replicates). N = 5 fruits. Means followed by the same letter within a treatment are not significantly different ($P < 0.05$).

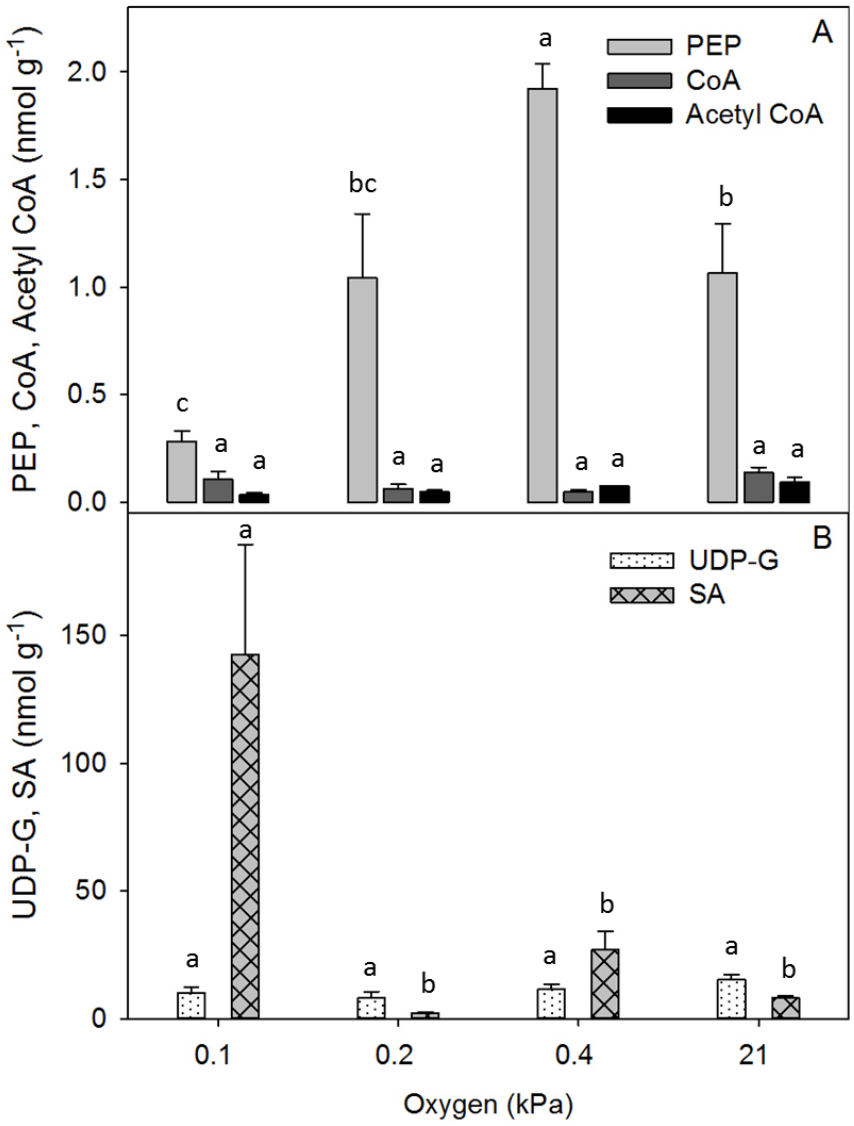


Figure 4. 17. Effect of O_2 concentration on UDP-G, SA, CoA, Acetyl CoA, and PEP levels of 'Honeycrisp' apple fruit under hypoxic conditions (0 kPa CO_2 with 0.1, 0.2, or 0.4 kPa O_2) at 3 °C. Control fruit were held continuously in 21 kPa O_2 + 0 kPa CO_2 at 3 °C. The sampling date was after two weeks of exposure to hypoxia. The error bars represent the SE of average four replicates composed of five fruits for each treatment. Means within a particular O_2 partial pressure treatment followed by the same letter are not significantly different ($P < 0.05$).

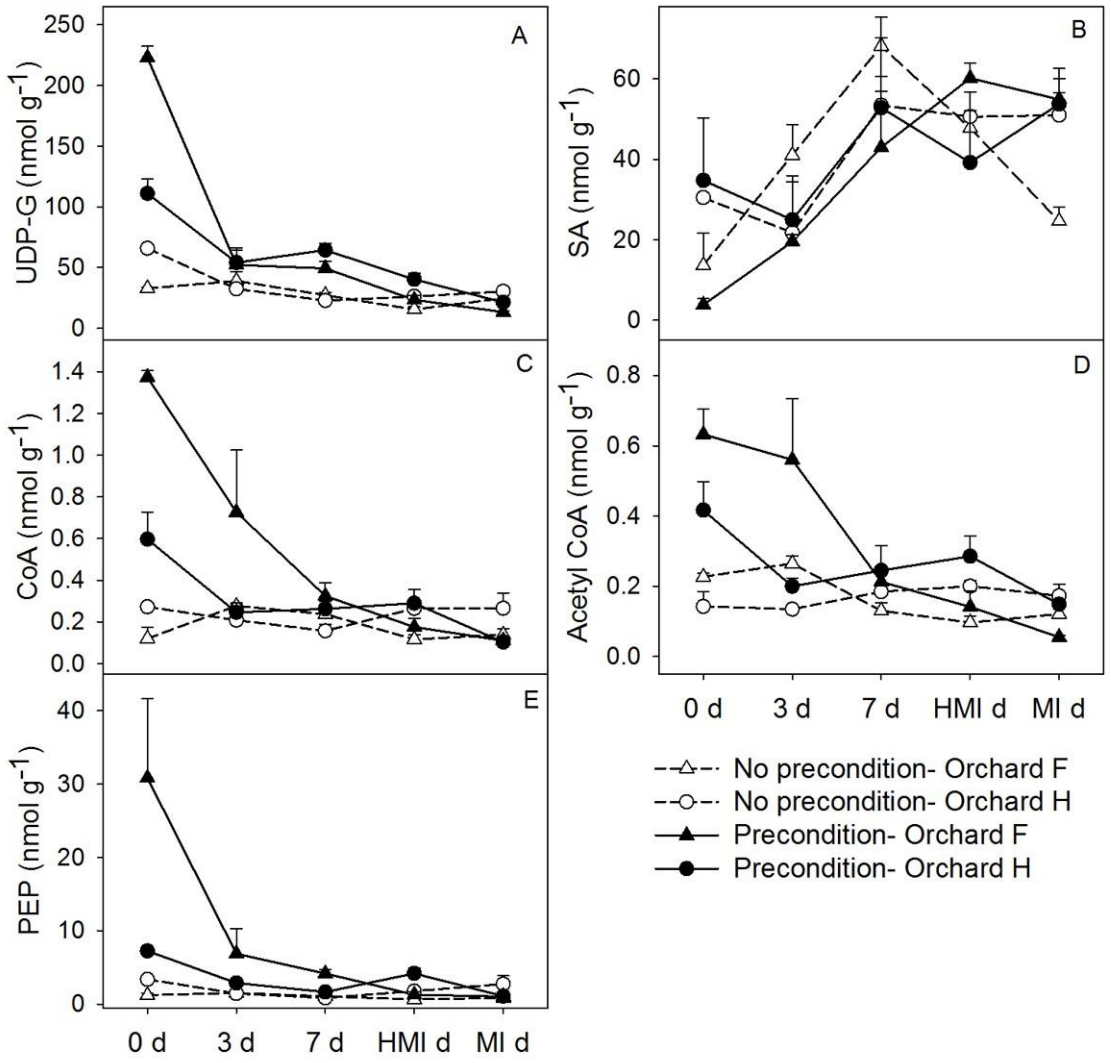


Figure 4. 18. Effect of preconditioning (the fruit at harvest was kept at 20°C for five days in the lab before CA storage) and fruit maturity (fruit from Orchard F were less mature than fruit from orchard H) on levels of UDP-G (A), SA (B), CoA (C), Acetyl CoA (D), and PEP (E) in 'Honeycrisp' apple during CA storage (0 kPa O_2 and 5 kPa CO_2 at 3 °C). Each symbol represents fruit from orchard F (less mature) and H (more mature) harvested in Michigan in 2017 from two preconditioning replicates. N=5 fruit per orchard at each sampling date of each treatment. At each sampling date, means followed by the same letter within a treatment are not significantly different ($P < 0.05$).

4.3.6 Principal component and Hierarchical clustering analysis.

A principal component and hierarchical clustering analysis was performed on mean values for each group of replicates of the variables elaborated in Supplementary Table B4.2. Based on hierarchical clustering analysis, we classified the variables of the treatments into two groups X and Y (Fig. 4.19). Group X includes treatments consisting of DPA application and 5 and 10 kPa CO_2 after 3, 7, and 14 or 21 days of CA storage, of treatment using 0 kPa CO_2 after 3 and 7 days of CA storage, and treatment using 3 kPa CO_2 after 21 days of CA storage. Group Y contains treatments of non-DPA application and 3, 5, 10, 20 kPa CO_2 after 3, 7, and 21 days of CA storage and non-DPA application and 0 kPa CO_2 after 21 days of CA storage (Fig. 4.19).

An unsupervised PCA was performed based on the concentrations of 15 metabolites to identify the variables that significantly contributed to the observed differences among treatments. The results indicated that the first three principal components PC1, PC2, and PC3 explained 39.7%, 24.1%, and 12.3% of the total variance of the dataset, respectively (Fig 4.20. A-C). As shown in Fig 4.20-A, approximately 65% of the total variance can be explained by principal components PC1 and PC2. The axis of PC1 separates variables of treatments of groups X and Y. Group X presented positive values in the PC1 axis. Group Y related to negative values in the PC1 axis. The two groups were also separated in combination of PC1 and PC3 (Fig 4. 4.20-B).

Table S-B4.2 summarizes the eigenvalues obtained from the correlation matrix of the PCA model. The variables that are largely associated with the PC1 include energy compounds (ATP, ADP, and AEC), antioxidants (Asc), reduced groups and ratios of reduced/oxidized compounds (NADH, NADPH, NADH/NAD^+, NADPH/$NADP^+$), reduced redox (NADH + NADPH), the ratio of reduced compounds to oxidized compounds (GSH + NADH + NADPH)/oxidized compounds (GSSG + NAD + NADP), the ratio of reduced redox compounds

to its oxidized ones (NADH + NADPH)/oxidized agent (NAD^+ + $NADP^+$), and carbohydrate compounds (UDP-G, CoA, Acetyl CoA, and PEP), which exhibited a strong positive correlation with the PC1. On the other hand, CA injury, GSH, AMP, and SA were negatively correlated with the PC1.

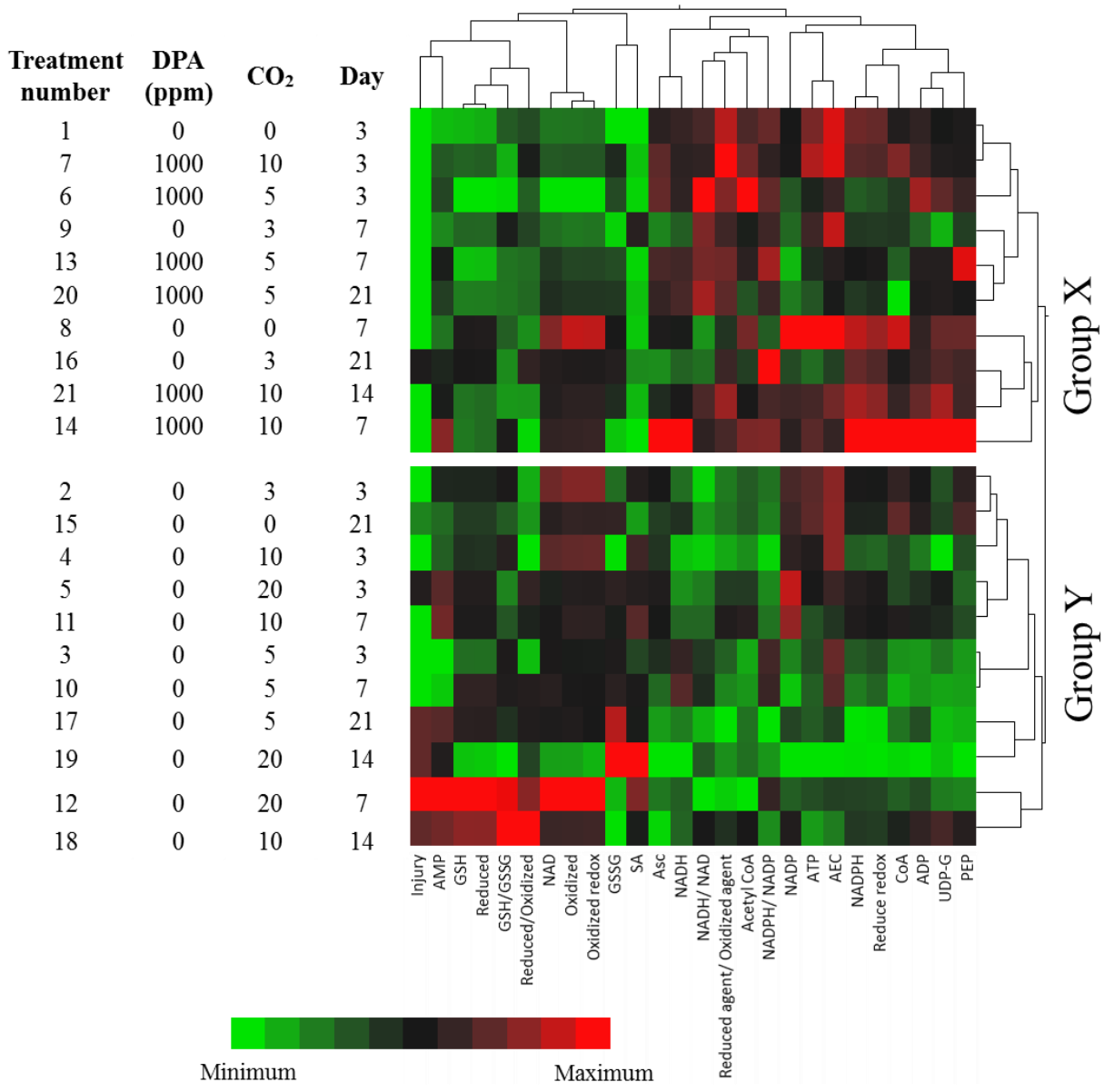


Figure 4. 19. The dendrogram with a color map describes the contribution of the variables for treatments classified into group X and Y.

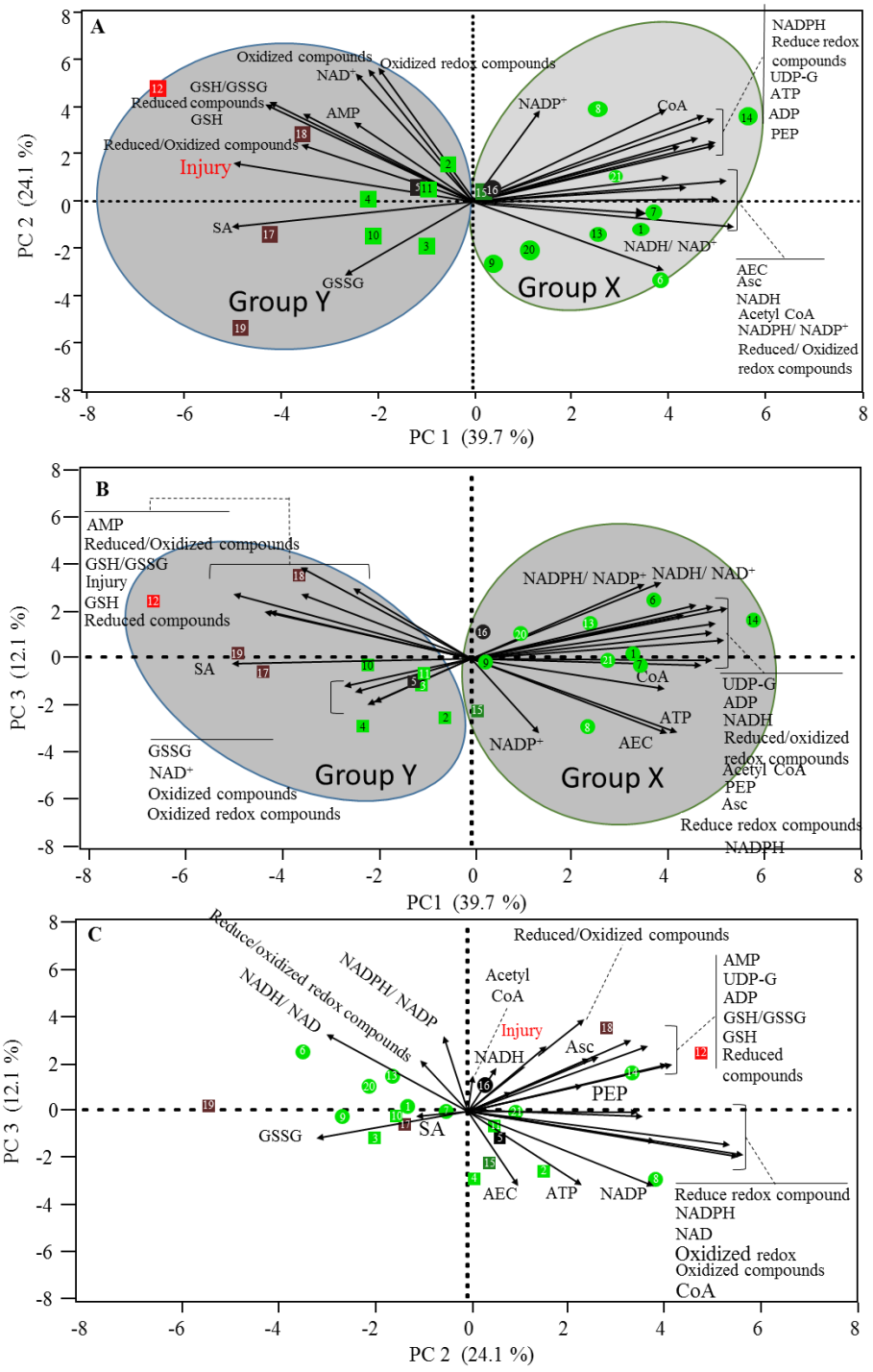


Figure 4. 20. Principal component analysis (PCA) in ‘Honeycrisp’ fruit receiving CA injury when stored at different CO_2 concentrations (0-20 kPa CO_2) at 3°C at day 7 and half maximal

day). Biplots based on loading values of variables and sample scores of PC1 vs. PC2 are presented.

GSH, in addition to other reduced compounds, also exhibited positive correlation with the PC2. NAD, oxidized compounds (GSSG + NAD^+ + $NADP^+$), oxidized redox compounds (NAD^+ + $NADP^+$) were strongly positive with the PC2. In addition, ATP and AEC were negatively correlated with the PC3 and positively correlated with the PC1, respectively. In contrast, AMP exhibited a strong positive correlation with the PC3 and negative correlation with the PC1. Variables including injury, GSH/GSSG, NADH/NAD^+, NADPH/$NADP^+$, and ratio of reduced/oxidized compounds [i.e. (GSH + NADH + NADPH)/ (GSSG + NAD^+ + $NADP^+$) showed positive correlation with the PC3 (Table S-B4.2).

4.4 Discussion

4.4.1 Energy compounds (ATP, ADP, and AMP)

We hypothesized that CA conditions might cause an alteration in the energy status in the apple cortex cells. Saquet et al. (2000) proposed a mode of action in which respiration decreased and hindered ATP synthesis when the fruit were stored at elevated CO_2 and low O_2. We did not measure respiration rate for the fruit stored at our experimental CA conditions. In support of this theory, we found that ATP levels and the AEC responded negatively to elevated CO_2 concentrations. In addition, ATP and ADP levels and AEC values decreased with increasing CA injury and with storage time under CO_2 stress, consistent with finding of Bennett et al. (1987). After harvest, fruit use their resources (i.e., sugars and organic acids) for ATP synthesis and maintain AEC values around 0.8 to 0.85 (Atkinson, 1977). Our data showed that AEC values were above 0.7 if the fruit had no CA injury symptoms or was protected by DPA or preconditioning. AEC value was only about 0.3 for fruit stored at 20 kPa CO_2 for 28 days.

The strong negative relation between CO_2 level and AEC and between injury and AEC does not necessarily prove that the low AEC values initiated CA injury. Similarly, our finding that brown tissues had AEC values lower than in healthy tissue in the same CA-injured slices simply demonstrates a correlation, rather than cause and effect. It may well be that injury is simultaneous with a decline in AEC or that ATP was below some threshold required for maintaining homeostasis of cells in fruit cortex.

Under hypoxic conditions, AEC value of the fruit at 0.1 kPa O_2 was only 0.25 but the fruit did not show CA injury symptom. This suggests that low AEC or low ATP alone may not be sufficient to induce oxidation of phenolic compounds when the fruit cells might undergo de-compartmentation. Nevertheless, we are not sure if the fruit cells under this condition were already doomed, but they were not brown at the time of the assay. We did, however, encounter browning of the fruit skin (but not the cortex) in these fruit three days after removal from hypoxia and transfer to normal oxygen (i.e., 21 kPa O_2) at room temperature. AEC value of the fruit stored at 0.4 kPa O_2 + 0 kPa remained above 0.7. Acetaldehyde, ethanol, and ethyl acetate levels produced in the fruit of this later treatment were very low: 2.6, 7.6 and 0.7 $\mu L\ L^{-1}$, respectively (Table 3.2 in Chapter 3). The O_2 partial pressure of 0.4 kPa might be near the safe low oxygen limit (LOL) for low O_2 controlled atmosphere (Prange et al., 2013b).

These experiments demonstrated that DPA application helped maintain near normal AEC values (i.e. above 0.7) during storage even when the fruit were stored at 10 kPa CO_2. This suggests that DPA treatment does more than simply prevent CA injury symptoms by preventing plant stress at some level. To our knowledge, there are no publications quantifying ATP levels and AEC values on DPA-treated apple fruit. Similar to DPA, the prevention of CO_2-related depression in AEC by preconditioning suggests that this treatment also protects the fruit from the

stressful effects of CO_2. This could be related to advancing the maturity of the conditioned fruit; more matured fruit are less sensitive to CO_2 injury (Contreras et al., 2014). Even so, it is not clear that the protective mechanisms of DPA and preconditioning are related.

4.4.2. Energy redox compounds (NAD^+, NADH, $NADP^+$, and NADPH)

We hypothesized that stressful levels of CO_2 might compromise the "redox energy currency" (i.e., NADH and NADPH metabolite pools) of apple fruit cortex cells. NADH is likely always in need by apple tissue for ATP synthesis and for sustaining redox reactions. A reduction in NADH relative to NAD^+ would be consistent with the findings for ATP and AEC. We did not find this, however; the control fruit had similar NADH/NAD^+ ratios to the CO_2-treated fruit. Similarly, NADPH/$NADP^+$ levels were not obviously affected in CO_2-treated fruit. NADPH might be synthesized via malic acid oxidation via NADP-malic enzyme; malic acid is very abundant in apple cytosol. This possibility was suggested by Blanch et al. (2013) for strawberry stored at 20 kPa CO_2. They found that NADP-malic enzyme and glutathione reductase were activated and GSH level increased when the fruit were stored at 20 kPa CO_2.

No pattern of alteration of NAD^+, NADH, $NADP^+$, and NADPH levels as well as their ratio values emerged from our analysis, suggesting no role for CO_2 on the alteration of redox state of these compounds. The fruit stored in RA had NADPH/NADP higher than those stored in hypoxia. DPA drench or preconditioning before CA storage might help to maintain the ratio at higher level than control treatment. It is probably because energy status of the cell and ROS state of the cell of the treatments might not require these "redox energy currency".

4.4.3 Antioxidants

Given that DPA, an antioxidant, very effectively suppresses CO_2 injury, it seemed reasonable to expect that antioxidants such as DPA that scavenge free radicals, might help

alleviate the stressful conditions caused by CO_2. Ascorbic acid (Asc) is one of the most powerful antioxidants in plant tissues (Noctor and Foyer, 1998). The reduction in Asc brought about by high CO_2 and its negative correlation with CO_2 injury suggests a possible role in protecting apple cortex cells from damage. Our data showed that Asc levels in healthy apple tissues in any treatment regimen (i.e., CO_2 concentration, DPA or preconditioning) or in healthy tissue in injured apples were always above 150 nmol g^{-1}. It may be there is an Asc threshold, below which apple tissues might lack the capacity to scavenge free radicals at a rate needed to maintain cellular integrity; browning in fruits may be a consequence (Veltman and Peppelenbos, 2003). Asc levels in hypoxic conditions remained high, suggesting that the conditions might not provide oxygen for oxidation of ascorbic acid to dehydroascorbic acid (DHA) (Bolin and Book, 1947) or the conditions might not support an accumulation of free oxygen radicals. It is interesting that AEC value of 0.1 kPa O_2 treatment was only 0.25 and yet there was no damage during the treatment period. Finally, it is worth noting that that aging apple fruit with no injury due to CO_2 also experienced a modest decline in Asc, a fact that argues against the direct involvement of Asc in the expression of CO_2 injury.

GSH existed at a much higher level than its oxidized form in apple tissue under any treatment regimen. The ratios reported here are consistent with finding of Noctor et al. (2002) in barley leaves. Average GSH levels of 31 apples ranged from 20 – 128 nmol g^{-1} on a fresh weight basis and GSSG levels from 0 – 84.5 nmol g^{-1} using metaphosphoric acid, EDTA, and PVPP in the extraction solvent (Davey and Keulemans, 2004). GSH is a potent antioxidant that donates of hydrogen atoms to dehydroascorbic acid for Asc regeneration (Noctor and Foyer, 1998). It has been stated that "Although NAD(P)H acts as redox energy currency, GSH acts as a dynamic redox energy buffer" (Das and White, 2002) and thus contributes to the stabilization of NADH

and NADPH levels. NADH and NADPH might be always necessary for regeneration of GSH under stress. The sharp increase and decline in GSH at 10 kPa and 20 kPa CO_2 during the early stages of symptom development argues that the GSH spike may be a signal of impending or developing damage. The noted increase in GSH might result from the ascorbate-glutathione pathway (Noctor and Foyer, 1998) or new synthesis in cytosol from the three amino acids glutamate, cysteine, and glycine (Lu, 2013).

The decline in GSH levels after its early rise in 5- and 10-kPa treated fruit might be due to the shortage of ATP under these conditions since 2 ATP molecules are required for the synthesis of one GSH molecule (Lu, 2013). GSH level and its ratios to GSSG in browned tissue and healthy tissue within the same injured slice were about the same. Like Asc, GSH level and GSH/GSSG ratio of hypoxia treatment were higher than in normoxia. It means that they were not oxidized due to limited oxygen source under hypoxia.

The fruit treated with DPA or receiving preconditioning might have a reduced requirement for GSH if DPA can substitute in the role of GSH to some extent. One might expect DPA to help maintain energy charge, antioxidative capacity, and ROS states to protect the fruit cells from disruption of cellular membranes and decomparmentation. The fruit from orchard F were very sensitive to CA injury also had an early accumulation of GSH level only after 7 days of storage.

4.4.4 Glycolytic metabolites (UDP-G, SA, PEP, CoA, and Acetyl CoA)

One of our hypotheses of CA injury in apple stored under CA conditions is that the metabolism of fruit carbohydrates might have abnormal response in adenylate and antioxidant pools, which act to compromise membrane function. UDP-G, PEP, CoA, Acetyl CoA, and SA were selected as likely candidates for assessing metabolite disruption. After harvest, the fruit

does not receive nutrition (e.g., energy-rich sucrose and sorbitol) from its mother plant. Instead, it has to reconfigure its metabolism to extract energy-rich metabolites from its stored reserves for synthesis of ATP and carbon skeleton precursors useful for its survival. Pathways in glycolysis and in TCA cycle might be interrupted under stressful conditions. PEP, acetyl CoA, and CoA levels were higher in healthy tissue than in browned tissue, suggesting carbohydrate metabolism might occur more normally in the healthy tissue.

Our data shows that succinic acid (SA) level increased for the first seven days of storage in the fruit stored at 3 - 20 kPa CO_2 and then declined. SA accumulation in mitochondria was suggested to result from a dysfunction of succinic dehydrogenase in vivo; SA dehydrogenase is sensitive to CO_2 (Gonzalez-Meler et al., 1996; Hulme, 1956; Shipway and Bramlage, 1973; Williams and Patterson, 1962). The decrease in SA level might be explained by pH change in mitochondria (Shipway and Bramlage, 1973), which might help succinic dehydrogenase activity increase again and oxidize SA. It is proposed that that high CO_2 caused a decrease in intracellular pH in lettuce and avocado tissues (Hess et al., 1993; Lange and Kader, 1997; Siriphanich and Kader, 1986). However, the pH changes in cytosol were minor (Lange and Kader, 1997) and those in vacuole were not detected due to technical limitations of Nuclear Magnetic Resonance (NRM) spectroscopy (Lange and Kader, 1997). However, SA dehydrogenase is not known to be any more sensitive to pH changes than most other enzymes. The SA level did not return to original levels under to the 20 kPa CO_2 treatment although it caused a dramatic and complete damage to the fruit within four weeks. This suggests that the apple cortex tissues could not adapt to this high level of CO_2.

For hypoxia-treated fruit, the SA level in the fruit with 0.1 kPa O_2 was extremely high. At this partial pressure of O_2, cytochrome *c* oxidase might not have enough oxygen for a normal

electron transport chain for ATP synthesis. Therefore, TCA cycle might be hindered, resulting in SA accumulation. SA also accumulated in browning tissue when browning covered about 50% of the cut surface (i.e., at injury rate 2). DPA and preconditioning treatments helped maintain SA levels at or below ~ 50 nmol g^{-1}, which might be a threshold for apple tissue toxicity.

During the preconditioning experiment, starch is hydrolyzed to sucrose, causing the starch index to become high. Sucrose is a source for UDP- glucose synthesis (Janse van Rensburg and Van den Ende, 2018). The elevated UDP-G levels of preconditioned fruit suggested that preconditioning promoted glycolysis. UDP-G is proposed as a precursor for ascorbic acid when it forms UDP-D-glucuronic acid using enzyme UDP-glucose dehydrogenase (Valpuesta and Botella, 2004). However, preconditioned fruit, in our study, while having elevated levels of most glycolytic metabolites, had lightly lower Asc levels.

4.4.5 PCA analysis

The dendrogram (Fig. 4.19) describes the contribution of the variables for treatments classified into group X and Y. Interestingly, these two groups are divided by high injury rate (X) and less injury rate (Y). The red symbols in this figure are for the fruit greater injury rates and are comprised of fruit treated with either 10 or 20 kPa CO_2. In group X, GSH is high and AMP level is high as well.

Through PCA and hierarchical clustering analysis, two distinguishable groups X and Y were formed depending on the DPA treatments and CO_2 concentration. The DPA-treated fruits and the untreated-fruits maintained at 0 kPa of CO_2 were capable of preserving their key metabolites, including AEC, Asc, ATP, ADP, CoA, Acetyl Co, PEP, UDPA and reduced compounds, above the threshold levels required for maintaining the cellular integrity. In contrast, DPA-untreated fruits in high atmospheres with CO_2 partial pressures and which also caused CA

injury and increased GSH levels, the ratio of GSH/GSSG, and SA levels. GSSG also was found to have negative loadings in PC2 and PC3. Therefore, we assumed that GSH was from emergent synthesis and/or from regeneration of its oxidized form GSSG to deal with the stress caused by high CO_2 levels. CA injury was also found to be positively correlated to AMP, GSH, and SA concentrations in the DPA-untreated fruits stored at 0 - 20 kPa of CO_2 (Supplementary Table B4.3).

4.5 Conclusion

We proposed a mechanism of actions for cell death in apple flesh tissues under CA conditions (Fig. 4.21-A) and for the protective influence of DPA or preconditioning in preventing or eliminating the injury (Fig. 4.21-B). More importantly, what we need is to know how pH changes in mitochondria to the level at which SA cannot be converted to fumaric acid in the TCA cycle since SDH enzyme inactivates (Hulme, 1956). Intracellular pH might influence other mitochondrial enzymes, however, because oxidation of organic acids in TCA cycles were hindered (Shipway and Bramlage, 1973). Therefore, it is still unclear if high CO_2 decreases mitochondrial pH and how much CO_2 is needed to challenge the pH buffering capacity of cells.

When CO_2 concentration is higher than its threshold in cells, it can support a reaction with PEP in the cytosol. PEP forms OAA which had been proved as a direct precursor for malonic acid synthesis in legumes (Bentley, 1952). Malonic acid was detected in apple juice (Gokhale and Rohrer, 2016). Malonic acid had been proved as a causal agent for cell death via inhibition of activity of the mitochondrial complex II which incorporates SA dehydrogenase (Fernandez-Gomez et al., 2005). If true, malonic acid could cause SA to build up, electron builds up, and ROS are produced, stressing the cell as a consequence (Belt et al., 2017). Asc and GSH are involved in scavenging free oxygen radicals. NADPH and NADH need to spend their redox

currency for the antioxidants (Noctor and Foyer, 1998). ATP might be needed for GSH *de novo* synthesis (Lu, 2013). As a result, A deficit in ATP might cause the TCA cycle slow and induce anaerobic respiration, with its lower production of NADH and ATP and increased production of fermentative volatiles. When there is an unbalance between "oxidants" and antioxidants, ROS steals electrons from cellular membranes, the membranes then become damaged and the cells die, and polymerization of phenolic compound takes place. However, if the fruit was drenched with DPA, an antioxidant, DPA will scavenge free oxygen radical immediately. Therefore, the fruit does not need to try to make more ATP, NADH, NADP, GSH, and Asc. (Fig. 4.21-B). In addition, DPA is a base (pKa = 0.8). Despite as an extremely weak base, it might react with HCO_3^-, which would accumulate when the fruit exposed to very high CO_2. If this reaction takes place, SA might not be built up, indicating normal electron transport activity within the mitochondria. We proved that SA is maintained at low levels in DPA-treated fruit (Fig. 4.16). If the fruit kept at 20 ºC for 5 days before CA storage (i.e. receiving preconditioning treatment), the preconditioning duration and temperature would promote starch hydrolysis to sucrose (Supplementary Table 2B. 3 in CHAPTER 2). UDP-G level increased in preconditioning fruit (Fig 4.18) and might be a precursor for UDP-D-glucuronic acid which might be used for *de novo* Asc synthesis (Valpuesta and Botella, 2004), which, in turn, would help to prevent damage caused by accumulating ROS .

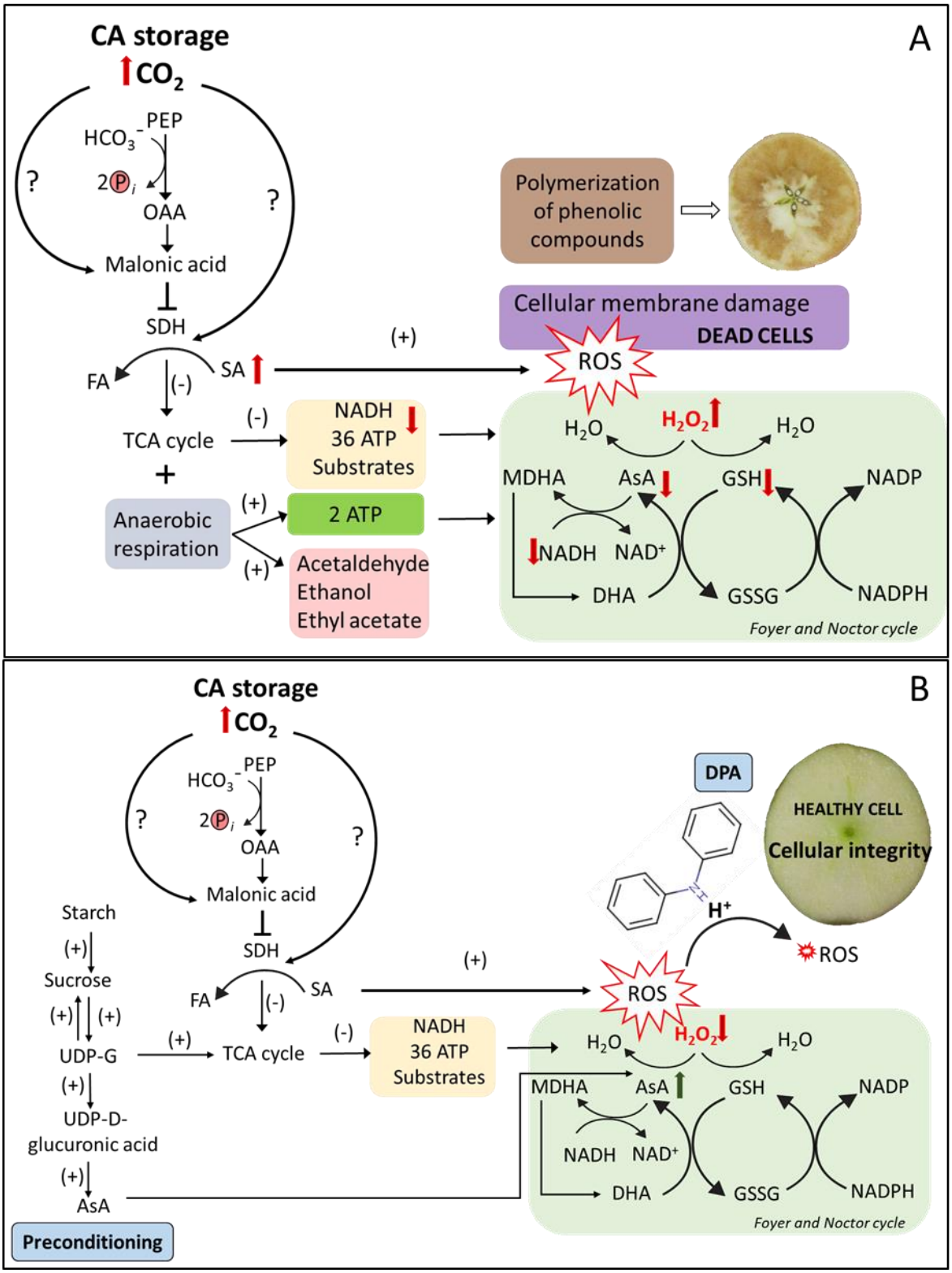
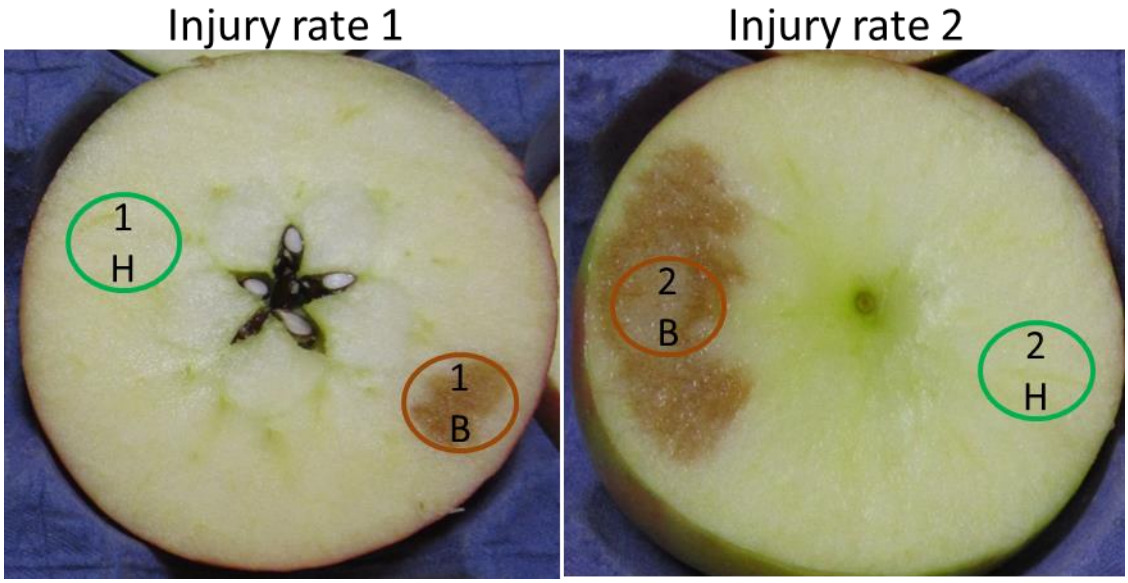


Figure 4. 21. Proposed mechanisms causing cell death in flesh tissues under CA conditions (A) and effect of DPA or preconditioning in preventing or eliminating the injury (B)

APPENDIX



Supplementary Figure 4. 1. The samples for metabolite analysis were browning area (B) and healthy area (H) of the injured apple slice

Supplementary Table 4. 1. Levels of metabolic analytes of apple cortex from seven treatments 1) No DPA-0 kPa CO_2, 2) No DPA-3 kPa CO_2, 3) No DPA-5 kPa CO_2, 4) No DPA-10 kPa CO_2, 5) No DPA-20 kPa CO_2, 6) DPA-5 kPa CO_2, 7) DPA-10 kPa CO_2. Each symbol represents fruit from five orchards in 2014, three orchards in 2016 and two orchards in 2017 for two replicates (for CO_2 factor), n = 5 fruit per orchard. Sampling dates were 0 d (harvest day), 3 d, 7 d, HMI d (day of half maximal injury and MI d (day of maximal injury). At each sampling date, means followed by the same letter within a treatment are not significantly different ($P < 0.05$). [a] The values have been log- transformed before ANOVA tests.

Day	No DPA 0 kPa CO_2	No DPA 3 kPa CO_2	No DPA 5 kPa CO_2	No DPA 10 kPa CO_2	No DPA 20 kPa CO_2	DPA 5 kPa CO_2	DPA 10 kPa CO_2
ATP[a]							
0	23.4						
3	35.1a	31.7a	15.5a	24.1a	22.6a	20.4a	40.3a
7	46.9a	28.3ab	13.1b	14.7b	15.0b	19.0ab	31.7ab
HMI	32.1a	11.8bc	13.6bc	7.6c	2.1d	15.8ab	29.2a
MI	32.5a	8.4b	10.7b	1.7c	0.1d	23.8ab	16.0ab

Source	*Pr > F*	*Source*	*Pr > F*
CO_2	*<.0001*	*Treatment*	*<.0001*
DPA	*<.0001*	*Day*	*<.0001*
Day	*<.0001*	*Treatment*Day*	*<.0001*

ADP[a]							
0	3.4						
3	3.4ab	3.2ab	2.2b	2.3ab	2.9ab	4.4a	3.6ab
7	3.5ab	2.5ab	2.2b	3.0ab	2.7ab	3.3ab	5.0a
HMI	3.3a	3.5a	2.7a	3.4a	1.8a	3.2a	3.9a
MI	2.9a	2.2a	2.8a	2.5a	0.3b	3.3a	1.6a

Source	*Pr > F*	*Source*	*Pr > F*
CO_2	*<.0001*	*Treatment*	*<.0001*
DPA	*0.0002*	*Day*	*<.0001*
Day	*0.0065*	*Treatment*Day*	*<.0001*

Supplementary Table 4. 1 (cont'd)

Day	No DPA 0 kPa CO_2	No DPA 3 kPa CO_2	No DPA 5 kPa CO_2	No DPA 10 kPa CO_2	No DPA 20 kPa CO_2	DPA 5 kPa CO_2	DPA 10 kPa CO_2
AMP[a]							
0	3.7						
3	2.5a	4.1a	2.1a	3.4a	5.9a	3.7a	3.4a
7	3.1a	2.9a	2.3a	6.4a	8.5a	4.3a	6.7a
HMI	3.2a	4.1a	5.9a	6.4a	4.7a	3.8a	4.3a
MI	2.2b	5.7ab	8.8a	4.8ab	0.6c	4.3b	2.2bc
Source		*Pr > F*		*Source*		*Pr > F*	
CO_2		*0.1083*		*Treatment*		*0.1209*	
DPA		*0.2674*		*Day*		*0.1242*	
Day		*0.2058*		*Treatment*Day*		*<.0001*	
AEC							
0	0.78						
3	0.89a	0.85a	0.82a	0.85a	0.79a	0.79a	0.89a
7	0.91a	0.88a	0.82ab	0.67bc	0.64c	0.77abc	0.79abc
HMI	0.85a	0.64bc	0.64c	0.52cd	0.36d	0.75ab	0.83ab
MI	0.90a	0.58b	0.53b	0.33c	0.26c	0.78a	0.81a
Source		*Pr > F*		*Source*		*Pr > F*	
CO_2		*<.0001*		*Treatment*		*<.0001*	
DPA		*<.0001*		*Day*		*<.0001*	
Day		*<.0001*		*Treatment*Day*		*<.0001*	
NAD[a]							
0	8.1						
3	3.8ab	8.0a	6.1a	7.6a	5.7ab	2.2b	4.3ab
7	8.4a	4.0a	6.4a	6.1a	10.3a	5.1a	6.7a
HMI	6.5a	6.3a	5.8a	6.9a	3.1a	5.4a	6.3a
MI	7.9a	5.4a	7.2a	2.7a	0.6b	4.3a	4.3a
Source		*Pr > F*		*Source*		*Pr > F*	
CO_2		*0.0036*		*Treatment*		*<.0001*	
DPA		*0.0044*		*Day*		*0.0020*	
Day		*0.076*		*Treatment*Day*		*<.0001*	

Supplementary Table 4. 1 (cont'd)

Day	No DPA 0 kPa CO_2	No DPA 3 kPa CO_2	No DPA 5 kPa CO_2	No DPA 10 kPa CO_2	No DPA 20 kPa CO_2	DPA 5 kPa CO_2	DPA 10 kPa CO_2
NADH[a]							
0	0.5						
3	0.4a	0.3a	0.4a	0.2a	0.2a	0.4a	0.4a
7	0.3a	0.3a	0.5a	0.3a	0.3a	0.4a	0.7a
HMI	0.3a	0.3a	0.3a	0.3a	0.2a	0.5a	0.3a
MI	0.2ab	0.3ab	0.4a	0.3ab	0.1b	0.4a	0.5a
Source		*Pr > F*		*Source*		*Pr > F*	
CO_2		*0.0098*		*Treatment*		*0.0002*	
DPA		*0.0073*		*Day*		*0.1657*	
Day		*0.1555*		*Treatment*Day*		*0.2520*	
NADP							
0	0.5						
3	0.9ab	1.2ab	0.6b	1.0ab	1.6a	0.7ab	0.9ab
7	1.7a	0.7abc	0.5c	1.4ab	0.7abc	0.5bc	1.0abc
HMI	1.1a	0.8a	0.8a	1.0a	0.5a	0.7a	1.1a
MI	1.2a	0.6ab	0.6ab	0.5ab	0.1b	1.0ab	0.5ab
Source		*Pr > F*		*Source*		*Pr > F*	
CO_2		*0.0013*		*Treatment*		*0.0017*	
DPA		*0.7327*		*Day*		*0.0260*	
Day		*0.3629*		*Treatment*Day*		*0.0068*	
NADPH							
0	3.3						
3	3.7a	2.8a	2.0a	1.9a	2.9a	1.9a	3.5a
7	4.4ab	2.3abc	1.7c	2.8abc	2.2abc	2.7abc	4.9a
HMI	2.5ab	3.7a	1.0b	2.3ab	1.0b	2.3ab	4.2a
MI	2.4a	2.6a	1.6ab	1.5ab	0.06b	1.6ab	1.8ab
Source		*Pr > F*		*Source*		*Pr > F*	
CO_2		*<.0001*		*Treatment*		*<.0001*	
DPA		*0.0007*		*Day*		*<.0001*	
Day		*0.0018*		*Treatment*Day*		*0.0192*	

Supplementary Table 4. 1 (cont'd)

Day	No DPA 0 kPa CO_2	No DPA 3 kPa CO_2	No DPA 5 kPa CO_2	No DPA 10 kPa CO_2	No DPA 20 kPa CO_2	DPA 5 kPa CO_2	DPA 10 kPa CO_2
NADH/NAD[a]							
0	0.11						
3	0.13a	0.03a	0.08a	0.03a	0.05a	0.23a	0.12a
7	0.05a	0.17a	0.08a	0.06a	0.03a	0.16a	0.11a
HMI	0.05ab	0.06ab	0.04b	0.10ab	0.07ab	0.19a	0.14ab
MI	0.04b	0.04ab	0.07ab	0.10a	0.21a	0.16a	0.15a

Source	*Pr > F*	*Source*	*Pr > F*
CO_2	*0.9792*	*Treatment*	*<.0001*
DPA	*<.0001*	*Day*	*<.0001*
Day	*0.6012*	*Treatment*Day*	*0.0192*

NADPH/NADP							
0	3.2						
3	4.5a	2.5a	4.1a	1.8a	2.3a	4.5a	3.8a
7	2.7a	4.0a	4.0a	2.5a	3.9a	5.1a	4.7a
HMI	2.4ab	5.8a	1.7b	2.3ab	2.2ab	4.0ab	4.2ab
MI	2.0a	3.9a	2.8a	3.1a	0.7a	2.4a	4.2a

Source	*Pr > F*	*Source*	*Pr > F*
CO_2	*0.0271*	*Day*	*0.1649*
DPA	*0.0117*	*Treatment*Day*	*0.1366*
Day	*0.1214*	*Treatment*	*0.0151*

Asc[a]							
0	440.6						
3	288.3a	243.1a	216.7a	209.8a	263.2a	395.0a	383.0a
7	258.3ab	144.9b	165.3b	241.6ab	154.3b	348.6ab	596.4a
HMI	199.9ab	139.2ab	129.4bc	96.1bc	89.0c	332.9a	219.0ab
MI	152.8ab	79.7bc	62.5bc	29.5c	0.2d	401.9a	222.0a

Source	*Pr > F*	*Source*	*Pr > F*
CO_2	*<.0001*	*Treatment*	*<.0001*
DPA	*<.0001*	*Day*	*<.0001*
Day	*<.0001*	*Treatment*Day*	*<.0001*

Supplementary Table 4. 1 (cont'd)

Day	No DPA 0 kPa CO_2	No DPA 3 kPa CO_2	No DPA 5 kPa CO_2	No DPA 10 kPa CO_2	No DPA 20 kPa CO_2	DPA 5 kPa CO_2	DPA 10 kPa CO_2
GSH							
0	91.4						
3	39.1a	75.8a	54.9a	71.9a	95.1a	31.1a	57.3a
7	90.7b	56.7b	110.8b	89.5b	260.3a	37.2b	52.3b
HMI	68.2b	84.0b	104.1b	188.2b	36.8a	52.8b	53.5b
MI	100.8a	73.8ab	101.5a	42.4ab	0.6b	81.0ab	64.5ab

Source	*Pr > F*	*Source*	*Pr > F*
CO_2	*0.4195*	*Treatment*	*0.0001*
DPA	*<.0001*	*Day*	*0.0035*
Day	*0.0093*	*Treatment*Day*	*<.0001*

Day	No DPA 0 kPa CO_2	No DPA 3 kPa CO_2	No DPA 5 kPa CO_2	No DPA 10 kPa CO_2	No DPA 20 kPa CO_2	DPA 5 kPa CO_2	DPA 10 kPa CO_2
GSSG							
0	0.21						
3	0.06a	0.1a	0.16a	0.06a	0.18a	0.11a	0.21a
7	0.15a	0.06a	0.18a	0.14a	0.07a	0.11a	0.13a
HMI	0.20a	0.17a	0.36a	0.06a	0.43a	0.14a	0.06a
MI	0.07a	0.11a	0.12a	0.11a	0.65a	0.30a	0.03a

Source	*Pr > F*	*Source*	*Pr > F*
CO_2	*0.4195*	*Treatment*	*0.0001*
DPA	*<.0001*	*Day*	*0.0035*
Day	*0.0093*	*Treatment*Day*	*<.0001*

Day	No DPA 0 kPa CO_2	No DPA 3 kPa CO_2	No DPA 5 kPa CO_2	No DPA 10 kPa CO_2	No DPA 20 kPa CO_2	DPA 5 kPa CO_2	DPA 10 kPa CO_2
GSH/GSSG[a]							
0	2715.3						
3	715.3a	1156.5a	1205.9a	1394.9a	552.0a	270.4a	399.5a
7	698.7ab	1026.4ab	1247.9ab	736.6ab	3836.4a	610.7b	1196.0ab
HMI	710.5b	526.2b	917.4b	4044.2b	247.5a	606.8b	478.9b
MI	1672.0a	1051.4ab	1241.7ab	397.1ab	0.9c	499.7b	3036.2a

Source	*Pr > F*	*Source*	*Pr > F*
CO_2	*<.0001*	*Treatment*	*<.0001*
DPA	*0.0576*	*Day*	*<.0001*
Day	*0.0092*	*Treatment*Day*	*<.0001*

Supplementary Table 4. 1 (cont'd)

Day	No DPA 0 kPa CO_2	No DPA 3 kPa CO_2	No DPA 5 kPa CO_2	No DPA 10 kPa CO_2	No DPA 20 kPa CO_2	DPA 5 kPa CO_2	DPA 10 kPa CO_2
UDP-G[a]							
0	47.0						
3	40.6ab	34.6ab	31.5ab	24.7b	40.1ab	52.8a	42.9b
7	53.9a	27.5a	28.4a	38.5a	31.2b	43.3a	70.6a
HMI	36.1ab	50.8ab	26.9b	49.2ab	26.5b	43.0ab	61.5a
MI	28.4ab	20.2b	36.0ab	33.9ab	1.8c	52.4a	21.3ab

Source	*Pr > F*	*Source*	*Pr > F*
CO_2	*<.0001*	*Treatment*	*<.0001*
DPA	*<.0001*	*Day*	*<.0001*
Day	*<.0001*	*Treatment*Day*	*<.0001*

CoA							
0	0.3						
3	0.4a	0.4a	0.2a	0.3a	0.3a	0.3a	0.5a
7	0.6a	0.3ab	0.2b	0.4ab	0.3b	0.3b	0.7a
HMI	0.5a	0.4ab	0.2ab	0.3ab	0.2ab	0.2b	0.4ab
MI	0.4a	0.3ab	0.3a	0.2ab	0.01b	0.3a	0.2ab

Source	*Pr > F*	*Source*	*Pr > F*
CO_2	*<.0001*	*Treatment*	*<.0001*
DPA	*<.0001*	*Day*	*<.0001*
Day	*<.0001*	*Treatment*Day*	*<.0001*

Acetyl CoA[a]							
0	0.2						
3	0.4ab	0.3b	0.2b	0.2b	0.3b	0.6a	0.5ab
7	0.5a	0.3a	0.2a	0.3a	0.2a	0.4a	0.5a
HMI	0.3a	0.3a	0.2a	0.3a	0.2a	0.3a	0.3a
MI	0.3ab	0.2ab	0.2ab	0.3ab	0.014b	0.4a	0.2ab

Source	*Pr > F*	*Source*	*Pr > F*
CO_2	*<.0001*	*Treatment*	*<.0001*
DPA	*0.0001*	*Day*	*<.0001*
Day	*0.0005*	*Treatment*Day*	*<.0001*

Supplementary Table 4. 1 (cont'd)

Day	No DPA 0 kPa CO_2	No DPA 3 kPa CO_2	No DPA 5 kPa CO_2	No DPA 10 kPa CO_2	No DPA 20 kPa CO_2	DPA 5 kPa CO_2	DPA 10 kPa CO_2
SA[a]							
0	16.2						
3	3.1c	113.5a	60.4ab	164.1a	115.6a	7.6c	12.7bc
7	11.1b	121.3a	97.0a	202.8a	256.8a	6.9b	3.5b
HMI	24.9c	31.6bc	84.2ab	82.5ab	399.7a	14.2c	16.0bc
MI	52.4b	27.7b	52.2b	6.4c	401.5a	20.6bc	14.5bc

Source	*Pr > F*	*Source*	*Pr > F*
CO_2	*<.0001*	*Treatment*	*<.0001*
DPA	*<.0001*	*Day*	*0.4803*
Day	*0.3538*	*Treatment*Day*	*<.0001*

PEP							
0	2.9						
3	4.5a	4.9a	2.7a	3.5a	3.2a	5.1a	4.6a
7	5.9ab	3.7ab	2.6b	3.6ab	2.9b	7.5a	7.8a
HMI	5.4a	5.3a	2.4a	4.7a	2.0a	4.8a	5.3a
MI	5.1a	3.5a	3.5a	3.1a	0.1b	5.4a	2.8a

Source	*Pr > F*	*Source*	*Pr > F*
CO_2	*<.0001*	*Treatment*	*<.0001*
DPA	*<.0001*	*Day*	*<.0001*
Day	*0.0078*	*Treatment*Day*	*<.0001*

Supplementary Table 4. 2. Eigenvectors of three principal components (PC1, PC2, and PC3) of the variables from seven treatments 1) No DPA-0 kPa CO_2, 2) No DPA-3 kPa CO_2, 3) No DPA-5 kPa CO_2, 4) No DPA-10 kPa CO_2, 5) No DPA-20 kPa CO_2, 6) DPA-5 kPa CO_2, 7) DPA-10 kPa CO_2

Variables	PC1	PC2	PC3
Injury	-0.236	0.100	0.240
Asc	0.250	0.055	0.070
GSH	-0.205	0.254	0.172
GSSG	-0.127	-0.192	-0.101
GSH/GSSG	-0.168	0.228	0.239
NAD	-0.116	0.333	-0.123
NADH	0.211	0.036	0.161
NADP	0.066	0.237	-0.273
NADPH	0.228	0.225	-0.023
NADH/ NAD	0.188	-0.180	0.284
NADPH/ NADP	0.171	-0.031	0.276
Reduced (GSH + NADH + NADPH)	-0.201	0.259	0.173
Oxidized (GSSG + NAD^+ + $NADP^+$)	-0.103	0.344	-0.167
Reduce redox (NADH + NADPH)	0.238	0.216	-0.004
Oxidized redox (NAD^+ + $NADP^+$)	-0.096	0.349	-0.160
Reduced/Oxidized compounds	-0.169	0.148	0.338
Reduced / Oxidized redox compounds	0.253	-0.061	0.187
ATP	0.204	0.144	-0.272
ADP	0.239	0.154	0.194
AMP	-0.117	0.208	0.262
AEC	0.194	0.064	-0.274
UDP-G	0.222	0.166	0.199
CoA	0.191	0.239	-0.111
Acetyl CoA	0.242	0.006	0.132
SA	-0.237	-0.066	-0.017
PEP	0.239	0.145	0.097

Supplementary Table 4. 3. Pairwise correlations of variables in the fruit from seven treatments: 1) No DPA-0 kPa CO_2, 2) No DPA-3 kPa CO_2, 3) No DPA-5 kPa CO_2, 4) No DPA-10 kPa CO_2, 5) No DPA-20 kPa CO_2, 6) DPA-5 kPa CO_2, 7) DPA-10 kPa CO_2 from day 3 to day when receiving half maximal injury (HMI day)

Variable	by Variable	Correlation	Count	Lower 95%	Upper 95%	Signif Prob
Acetyl CoA	NADPH	0.7515	15	0.389	0.9125	0.0012
Acetyl CoA	CoA	0.7148	15	0.3196	0.8982	0.0027
Acetyl CoA	UDP-G	0.6871	15	0.2697	0.8871	0.0047
Acetyl CoA	ADP	0.6333	15	0.1791	0.8649	0.0113
Acetyl CoA	NADP	0.615	15	0.15	0.8572	0.0147
Acetyl CoA	ATP	0.585	15	0.1038	0.8442	0.022
Acetyl CoA	Asc	0.4484	15	-0.083	0.7812	0.0937
Acetyl CoA	AEC	0.2728	15	-0.2784	0.6888	0.3253
Acetyl CoA	NADH	0.1469	15	-0.3951	0.613	0.6014
Acetyl CoA	AMP	-0.2021	15	-0.6473	0.346	0.4701
Acetyl CoA	NAD	-0.2075	15	-0.6506	0.341	0.4581
Acetyl CoA	GSSG	-0.2325	15	-0.6655	0.3176	0.4044
Acetyl CoA	GSH	-0.2635	15	-0.6835	0.2875	0.3426
ADP	NADPH	0.7896	15	0.4657	0.9269	0.0005
ADP	NADP	0.5993	15	0.1255	0.8505	0.0182
ADP	ATP	0.4784	15	-0.0449	0.7956	0.0713
ADP	Asc	0.3521	15	-0.1954	0.7323	0.1981
ADP	NAD	0.2541	15	-0.2968	0.6781	0.3609
ADP	GSH	0.1468	15	-0.3952	0.613	0.6017
ADP	NADH	0.052	15	-0.4729	0.5496	0.8541
ADP	GSSG	-0.4242	15	-0.7693	0.1125	0.1151
AEC	ATP	0.8291	15	0.5508	0.9415	0.0001
AEC	Asc	0.7428	15	0.372	0.9091	0.0015
AEC	NADH	0.5002	15	-0.0162	0.806	0.0576
AEC	NADPH	0.4855	15	-0.0357	0.799	0.0666
AEC	NADP	0.3835	15	-0.1603	0.7487	0.1583
AEC	ADP	0.2422	15	-0.3083	0.6712	0.3844
AEC	NAD	0.1668	15	-0.3777	0.6256	0.5523
AEC	GSH	-0.2925	15	-0.6999	0.2585	0.29
AEC	GSSG	-0.5494	15	-0.8285	-0.0516	0.0339
AEC	AMP	-0.5902	15	-0.8465	-0.1117	0.0205
AMP	GSH	0.7494	15	0.3849	0.9117	0.0013
AMP	NAD	0.3898	15	-0.153	0.7519	0.1509
AMP	NADP	0.1367	15	-0.4038	0.6065	0.627

Supplementary Table 4. 3. (cont'd)

AMP	ADP	0.104	15	-0.4312	0.5851	0.7123
AMP	GSSG	0.0713	15	-0.4577	0.563	0.8006
AMP	NADPH	-0.1861	15	-0.6376	0.3605	0.5065
AMP	Asc	-0.2981	15	-0.703	0.2527	0.2805
AMP	ATP	-0.4351	15	-0.7747	0.0993	0.1051
AMP	NADH	-0.5629	15	-0.8345	-0.0711	0.0289
ATP	Asc	0.7276	15	0.3433	0.9032	0.0021
ATP	NADPH	0.673	15	0.2453	0.8814	0.006
ATP	NADP	0.6105	15	0.1429	0.8553	0.0156
ATP	NADH	0.2447	15	-0.3059	0.6727	0.3794
ATP	NAD	0.1761	15	-0.3695	0.6314	0.53
ATP	GSH	-0.2779	15	-0.6917	0.2732	0.3159
ATP	GSSG	-0.4237	15	-0.769	0.1131	0.1155
CoA	NADPH	0.797	15	0.4811	0.9297	0.0004
CoA	ADP	0.7876	15	0.4616	0.9262	0.0005
CoA	ATP	0.7861	15	0.4584	0.9256	0.0005
CoA	NADP	0.7418	15	0.3701	0.9087	0.0015
CoA	UDP-G	0.6879	15	0.2712	0.8874	0.0046
CoA	Asc	0.4937	15	-0.0248	0.8029	0.0614
CoA	AEC	0.4651	15	-0.0619	0.7893	0.0806
CoA	NAD	0.3079	15	-0.2427	0.7084	0.2643
CoA	NADH	0.0619	15	-0.4651	0.5565	0.8266
CoA	GSH	-0.0839	15	-0.5716	0.4476	0.7662
CoA	AMP	-0.1613	15	-0.6222	0.3825	0.5656
CoA	GSSG	-0.3227	15	-0.7165	0.2271	0.2407
GSH	Asc	-0.3166	15	-0.7132	0.2335	0.2502
GSSG	GSH	-0.2854	15	-0.6959	0.2658	0.3026
GSSG	Asc	-0.3949	15	-0.7545	0.1471	0.1452
Injury	AMP	0.8019	15	0.4916	0.9315	0.0003
Injury	GSH	0.7521	15	0.3901	0.9127	0.0012
Injury	SA	0.5102	15	-0.0028	0.8106	0.052
Injury	NAD	0.3043	15	-0.2464	0.7064	0.2702
Injury	GSSG	0.236	15	-0.3143	0.6675	0.3972
Injury	UDP-G	-0.1039	15	-0.585	0.4313	0.7126
Injury	ADP	-0.1357	15	-0.6059	0.4047	0.6296
Injury	NADP	-0.3144	15	-0.712	0.2358	0.2537
Injury	CoA	-0.3632	15	-0.7381	0.1831	0.1833
Injury	PEP	-0.3651	15	-0.7391	0.181	0.1809
Injury	NADPH	-0.3885	15	-0.7513	0.1545	0.1524
Injury	NADH	-0.3888	15	-0.7514	0.1542	0.1521
Injury	Acetyl CoA	-0.3981	15	-0.7561	0.1435	0.1417

Supplementary Table 4. 3. (cont'd)

Injury	ATP	-0.5255	15	-0.8177	-0.0181	0.0442
Injury	Asc	-0.587	15	-0.8451	-0.1068	0.0214
Injury	AEC	-0.6874	15	-0.8873	-0.2704	0.0046
NAD	GSH	0.705	15	0.3017	0.8943	0.0033
NAD	Asc	0.1101	15	-0.4262	0.5891	0.6961
NAD	GSSG	-0.3819	15	-0.7479	0.1621	0.1601
NADH	Asc	0.3286	15	-0.2208	0.7197	0.2317
NADH	NAD	-0.0244	15	-0.53	0.4941	0.9313
NADH	GSH	-0.0872	15	-0.5738	0.445	0.7574
NADH	GSSG	-0.2371	15	-0.6682	0.3132	0.3949
NADP	Asc	0.6723	15	0.2441	0.8811	0.006
NADP	NAD	0.2984	15	-0.2524	0.7032	0.2799
NADP	GSH	-0.0222	15	-0.5284	0.4957	0.9374
NADP	NADH	-0.1719	15	-0.6288	0.3732	0.5401
NADP	GSSG	-0.2612	15	-0.6822	0.2899	0.3471
NADPH	NADP	0.6469	15	0.2013	0.8706	0.0092
NADPH	Asc	0.6272	15	0.1693	0.8624	0.0123
NADPH	NADH	0.2254	15	-0.3243	0.6613	0.4193
NADPH	NAD	0.1718	15	-0.3733	0.6287	0.5403
NADPH	GSH	-0.0854	15	-0.5726	0.4464	0.7622
NADPH	GSSG	-0.5065	15	-0.8089	0.0078	0.054
PEP	CoA	0.903	15	0.7271	0.9677	<.0001
PEP	ADP	0.8752	15	0.6579	0.958	<.0001
PEP	NADPH	0.8118	15	0.5127	0.9352	0.0002
PEP	UDP-G	0.7653	15	0.4163	0.9178	0.0009
PEP	ATP	0.6481	15	0.2033	0.8711	0.009
PEP	Acetyl CoA	0.6236	15	0.1635	0.8608	0.013
PEP	NADP	0.5578	15	0.0638	0.8323	0.0307
PEP	AEC	0.3982	15	-0.1433	0.7562	0.1415
PEP	Asc	0.3409	15	-0.2076	0.7263	0.2136
PEP	NAD	0.2472	15	-0.3035	0.6741	0.3744
PEP	NADH	0.0725	15	-0.4567	0.5638	0.7973

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CONCLUSION REMARKS

Since introduced in 1991, ‘Honeycrisp’ apple now occupies a significant share of the apple market in the U.S. The cultivar has become a favorite fruit of consumers because of its crisp texture and unique flavor. Like most commercial varieties of apples in the U.S., the ‘Honeycrisp’ apple is also a candidate for controlled atmosphere (CA) storage to maintain fresh quality throughout the year. CA storage conditions are typically kept with O_2 levels below 3 kPa and CO_2 levels between 1 and 5 kPa. However, very low O_2 and/or very high CO_2 concentrations of CA conditions can induce the development of physiological disorders in many cultivars, especially to ‘Honeycrisp’ apples. Typical CA injury symptoms of the fruit are jagged-edged brown lesions in the fruit cortex and lens-shaped voids. The brown lesions develop rapidly within the first 1.5 months and the voids develop more slowly, continuously increasing in frequency with storage time. Therefore, our objective was to study mechanisms by which CA storage conditions cause physiological injury to ‘Honeycrisp’ apple fruit.

5.1 Research contribution to the field

We found a strong positive correlation of CO_2 concentration to CA condition and CA injury. As the CO_2 level increased, the maximum degree of damage increased, with 20% CO_2 leading to 100% injury. CO_2 injury increased with time, reaching maximum injury in approximately 30 to 40 days. The maturity of apples determined the tolerance of the fruit to CA. More mature fruit had lower incidences of CA injury. The three approaches to control or avoid CA injury we used were DPA, 1- MCP multi-application, and preconditioning. Our results were used to determine safe recommendations for the apple industry for ‘Honeycrisp’ apple storage. Both preconditioning before CA storage and 1-MCP before and during air storage are approaches that could not completely eliminate CA injury but did reduce injury. In this case, DPA at approximately 130 ppm for 30 s before CA storage at 3 °C is the best option if the fruit

are stored immediately in CA storage. On the other hand, the fruits harvested at a more mature stage are more tolerant to standard CA conditions. Therefore, it is acceptable to store fruit immediately in CA storage after harvest.

Extensive injury developed before fermentation volatile emanations exceeded those of control fruit. This suggests that fermentative volatiles were induced by high CO_2 concentration, but they did not initiate the injury. This assessment was further supported by our demonstration that fermentative volatiles caused by very low O_2 levels far exceeded the production of fermentation volatiles induced by CO_2, but without causing a browning injury.

We also studied the impact of CO_2, DPA, and preconditioning on 15 important metabolites. We developed a protocol for the quantification of fifteen compounds using only one extract by UHPLC-MS/MS, which has not been accomplished previously. Except for succinic acid, which had been measured on 'Braeburn' apple subject to browning, the remaining compounds had never been analyzed in a single extract of apples in response to CO_2-induced injury. Sampling dates focused on several time points before browning symptoms appear, including the date on which the fruit reached maximal injury.

Adenylate energy charge (AEC) and ascorbic acid surfaced as important indicators for healthy fruits/tissue. CO_2 concentration was negatively correlated with AEC and ATP when the fruit injury reached its half maximal rate. The glutathione (GSH) pool increased when the fruit was subjected to oxidative stress and the ratio of this compound to its oxidized form fluctuated with storage time. The ratio of NADPH to its oxidized form did not change with storage time, but it declined after the fruit reached maximal injury. CO_2 concentration did not make a significant effect on carbohydrate metabolite except at 20 kPa CO_2, which caused substantial declines in UDP-glucose, acetyl CoA, CoA, and phosphoenolpyruvate and a marked

accumulation of succinic acid. The results also showed the importance of DPA and preconditioning practices in maintaining key metabolites for healthy apple tissues.

5.2 Research limitations

Based on limitation of facilities, we cannot measure change in pH levels within mitochondria or the cytosol of apple to prove if high CO_2 would create a low pH in the organelles. Based on our data, we only hypothesized an influence of low pH on enzyme activity, especially on succinate dehydrogenase, an enzyme necessary for converting succinic acid to fumaric acid in the TCA cycle.

We could apply a metabolomics approach using GC-MS to detect and quantify more key metabolites to have a general picture since sugar compound, organic acid, amino acids can be simultaneously detected in one single extract.

Lipid components in cellular membranes and their oxidized products should be analyzed to determine when membranes are degraded and also the rate at which they are degraded. This is extremely difficult, as it only takes a tiny fraction of damaged lipids to yield leakage across membranes.

A comparison of metabolites and buffering capacity to pH between CA-sensitive Honeycrisp apple and certain apple variety that is very tolerant to CA condition should be implemented.