METABOLIC ALTERATION IN 1-MCP TREATED ‘EMPIRE’ APPLES DURING STORAGE

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METABOLIC ALTERATION IN 1-MCP TREATED 'EMPIRE' APPLES DURING STORAGE

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'Empire' apples are susceptible to firm flesh browning when stored at temperatures close to 0 ^{o}C, or after 1-methylcyclopropene (1-MCP) treatment when stored at warmer temperatures such as 3 or 4 ^{o}C. This study was designed to examine the effects of 1-MCP on metabolic responses of fruit stored at 2 kPa O_2/2 kPa CO_2 at either 0.5 or 3.3 ^{o}C for up to 40 weeks. The incidence of flesh browning was higher in 1-MCP treated fruit than in untreated fruit at either storage temperature. 1-MCP reduced nitroblue tetrazolium reducing activity but inconsistently affected H_2O_2 concentrations. 1-MCP increased malondialdehyde concentrations at 0.5 ^{o}C but not at 3.3 ^{o}C. 1-MCP treated fruit had lower ascorbic acid concentrations at the end of storage. 1-MCP resulted in lower glutathione concentrations but higher oxidized glutathione concentrations at the end of storage at 3.3 ^{o}C. While 1-MCP reduced copper/zinc-superoxide dismutase activity at 3.3 ^{o}C, it increased peroxidase activity at 3.3 ^{o}C. Overall, however, 1-MCP did not affect the activities of the other antioxidant enzymes consistently, and no direct association between antioxidant metabolism and flesh browning development was revealed. Partial least squares discriminant analysis revealed different metabolic divergence and separation by 1-MCP and storage temperature. Regardless of 1-MCP and storage temperature, malic, succinic and 2-oxoglutaric acid concentrations decreased, while carbohydrates were not affected. 1-MCP increased sorbitol, tryptophan, phenylalanine, glutamate, 5-oxoproline, aspartate,

homoserine, threonine, isoleucine, valine, leucine, serine, chlorogenic acid, phloridzin and catechin concentrations at 3.3 ^{o}C. 1-MCP reduced volatile compounds, and levels were highest in untreated fruit at 3.3 ^{o}C. The partial least squares discriminant analysis loading plots indicated that sorbitol and γ-aminobutyric acid could be associated with the development of flesh browning in 1-MCP treated fruit stored at 3.3 ^{o}C. Overall, the development of flesh browning is likely to be complex, resulting from the interaction of several metabolic pathways.

BIOGRAPHICAL SKETCH

Jinwook Lee was born to Youngran Jung and Yongsoo Lee on November 24, 1972 in Gyeongju, the Republic of Korea. With his one elder brother, Sangwook, three sisters, Hyunsook, Kyongsook, and Sungsook, and one younger brother, Jungwook, he grew up in Gyeongju, Korea. In March of 1991, he entered Yeungnam University in Gyongsan, Korea. He had participated in Korea Army service for 18 months as an undergraduate student. In February of 1998, he received a Bachelor of Science in Agronomy from Yeungnam University. In March of 1998, he began his master's studies with focusing on turfgrass physiology and ecology, and received his Master of Science in Agronomy from Seoul National University in Suwon in February 2000. He worked at the National Institute of Crop Science, RDA as an internship. After then, he moved to work at the National Plant Quarantine Service, Ministry of Agriculture and Forestry, the Republic of Korea from December 2001 to July 2007. On June 1, 2002, he married Eun Jeong Koh. They had a son, Irwin Minhyung Lee, born on April 22, 2004. In August 2002, he enrolled the Graduate program at the Department of Horticulture, Cornell University at Ithaca, New York U.S.A. to study on the environmental and genotypic factors on the ginsenosides in American ginseng and received his Master of Science in January 2007. After then, he continued to work for Ph.D. program on the metabolic alteration in 1-MCP treated 'Empire' apple during CA storage under the direction of Dr. Christopher B. Watkins. He will continue to work on apple storage disorders and metabolomics with Dr. David Rudell at Tree Fruit Research Laboratory, USDA-ARS in Wenatchee, Washington.

Dedicated to my father, Yongsoo Lee as my spiritual supporter

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LIST OF ABBREVIATIONS

1-MCP: 1-methylcyclopropene

2-OG: 2-oxoglutarate

3PGA (G3P): 3-phosphoglyceraldehyde or glyceraldehydes 3-phosphate

4HNE: 4-hydroxynonenal

AAT: alcohol acyltransferase / alcohol acetyltransferase

ACC: 1-aminocyclopropane-1-carboxylic acid

ACO: 1-aminocyclopropane-1-carboxylic acid oxidase

ACS: 1-aminocyclopropane-1-carboxylic acid synthase

ADC: arginine decarboxylase

ADH: alcohol dehydrogenase

ADP: adenosine diphosphate

AIH: agmatine iminohydrolase

AMDIS: automated mass spectral deconvolution and identification system

AOA: aminooxyacetic acid

APX: ascorbate peroxidase

AsA: ascorbic acid

ATP: adenosine triphosphate

ATP-PFK: ATP:phosphofructokinase

AVG: aminoethoxyvinyl glycine

BHT: butylated hydroxyltoluene

BSTFA: *N*,*O*-bis(trimethylsilyl)trifluoroacetaminide

CA: controlled atmosphere

CAT: catalase

CDC: citrulline decarboxylase

CoA: coenzyme A

CPA: *N*-carbamoylputrescine amidohydrolase

Cu/Zn-SOD: cupper/zinc-superoxide dismutase

dcSAM: decarboxylated *S*-adenosylmethionine

DAO: diamine oxidase

DHA: dehydroascorbate

DHAR: dehydroascorbate reductase

DHS: dynamic headspace sampler

DNA: deoxyribonucleic acid

DPA: diphenylamine

DTNB: 5,5'-dithiobis-2-nitrobenzoic acid

EDTA: ethylenediaminetetraacetic acid

EPA: The U.S. Environmental Protection Agency

ER: endoplasmic reticulum

F1,6P: fructose 1,6-bisphosphate

F2,6P: fructose 2,6-bisphosphate

F6P: fructose 6-phosphate

$FADH_2$ (FAD^+): flavin adenine dinucleotide

Fe-SOD: iron superoxide dismutases

G1P: glucose 1-phosphate

G6P: glucose 6-phosphate

GABA: γ-aminobutyric acid

GABA-T: GABA transaminase

GAD: glutamate decarboxylase

GC-MS: gas chromatography-mass spectrometry

GDH: glutamate dehydrogenase

GPX: glutathione peroxidase

GR: glutathione reductase

GSH: reduced glutathione

GSSG: oxidized glutathione

GST: glutathione *S*-transferase

HPLC: high-performance liquid chromatography

IEC: internal ethylene concentration

LOOH: lipid hydroperoxides

LOX: lipoxygenase

LV: latent variable

MACC: 1-(malonylamino)cyclopropane-1-carboxylic acid

MAP: mitogen-activated protein

MDA: malondialdehyde

MDHA: monodehydroascorbic acid

MDHAR: monodehydroascorbate reductase

Mn-SOD: manganese superoxide dismutase

MPS: multipurpose sampler

MSD: mass selective detector

MSTs: mass spectral tags

MTA: methylthioadenosine

NADH (NAD^+): nicotinamide adenine dinucleotide

NADPH ($NADP^+$): nicotinamide adenine dinucleotide phosphate

NBT: nitroblue tetrazolium

OD: optical density

ODC: ornithine decarboxylase

PAL: phenylalanine ammonia lyase

PAO: polyamine oxidase

PDC: pyruvate decarboxylase

PEP: phosphoenolpyruvate

PLS-DA: partial least squares-discriminant analysis

PMSF: phenylmethylsulfonyl fluoride

POD: peroxidase

POX: peroxidase

PPi: inorganic pyrophosphate

PPi-PFK: PPi:phosphofructokinase

PPO: polyphenol oxidase

PUFA: polyunsaturated fatty acids

PVPP: polyvinylpolypyrrolidone

RH: relative humidity

RI: retention indices

RNA: ribonucleic acid

ROS: reactive oxygen species

SAM: *S*-adenosylmethionine

SAMDC: SAM decarboxylase

SDH: sorbitol dehydrogenase

SOD: superoxide dismutase

SPDS: spermidine synthase

SPMS: spermine synthase

SSADH: succinyl semialdehyde dehydrogenase

SSC: soluble solids concentrations

SUSY: sucrose synthase

TBA: thiobarbituric acid

TCA: tricarboxyl acid

TCA: trichloacetic acid

UDP: uracil-diphosphate

CHAPTER 1

GENERAL INTRODUCTION

1.1. The 'Empire' apple

The 'Empire' apple (*Malus sylvestris* (L.) Mill var. *domestica* (Borkh.) Mansf.) is a cross between 'McIntosh' and 'Red Delicious' (Derkacz et al., 1993) and it is the second most important apple cultivar by volume grown in New York State. The 'Empire' apple is the leading export cultivar for the New York apple industry.

'Empire' apples are attractive to growers and consumers because of the attractive red coverage, firmness and juiciness at harvest and the storage potential during air and controlled atmosphere (CA) storage. The fruit is medium in size and fruit weight, averaging 6.5 to 8.0 cm diameter, and 100 to 170 g $fruit^{-1}$, respectively (Goffinet et al., 1995; Stover et al., 2002). The firmness and soluble solids concentrations (SSC) of 'Empire' apple fruit are around 70 – 80 N, and about 11-12%, respectively, depending on orchard location and maturity (DeEll et al., 2005; DeEll et al., 2010). Its taste is a blend of sweet and tart, juicy and very crisp, and its flesh is creamy white. Sensory analysis involving blind-taste testing of several late harvest cultivars, indicated that 'Empire' apple had the greater acceptability than 'McIntosh' and 'Red Delicious' (Cheng et al., 1997).

1.2. Storage condition

'Empire' apple can be stored at 0 ^{o}C in air, but the potential storage period in this storage is quite shorter than in CA storage. In general, the cultivar is stored in CA (2-3 kPa O_2/1-3 kPa CO_2) at 1 to 2 ^{o}C, a storage temperature that is a compromise between chilling injury at lower storage temperature and senescent breakdown at warmer temperatures (Watkins et al., 2002; Watkins et al., 2005). Partial pressures of

CO_2 (pCO_2) of 1-2 kPa are recommended to minimize risk of flesh browning development (Watkins and Liu, 2010a).

The storage potential of any cultivar to specific CA regimes is influenced greatly by both pre- and post-harvest factors. Preharvest factors include fruit maturity, growing climate, the application of agro-chemicals including plant growth regulators and cultural practices (Bramlage, 1993; Kays, 1999), while postharvest factors include storage atmosphere, storage temperature, storage humidity and postharvest agro-chemical applications (Yahia, 1994). These pre- and post-harvest factors affect the potential of CA to maintain fruit quality factors, including the ability of fruit to withstand physiological and pathological disorders. The development of physiological disorders such as low O_2 injury, external and internal CO_2 injuries, internal browning and superficial scald can limit the storage of many cultivars (Blanpied et al., 1990; Watkins, 2003).

1.3. 1-MCP application

1-Methylcyclopropene (1-MCP) is an inhibitor of ethylene perception that was patented by Sisler and Blankenship (1996). 1-MCP is a simple gas molecule, which has a single double bond in its structure and a methyl group on the first carbon of the cyclopropene. Due to this double bond structure, it mimics ethylene, and thus has more ability to inhibit ethylene action and perception on the ethylene receptors on cell wall membranes and/or endoplasmic reticulum (ER) membranes (Sisler, 2006).

The effectiveness of 1-MCP in controlling ethylene-mediated responses of plant tissues has been widely shown (Blankenship and Dole, 2003; Watkins, 2006). Since1-MCP was registered for food uses in the Chile in 2001, the US in 2002 and subsequently around the world, 1-MCP technology has been used commercially on many horticultural crops (Huber, 2008; Watkins, 2008). The product is used as

Ethylbloc for flowers and ornamental plants, SmartFresh for fruits and vegetables, and Harvista and Invinsa for preharvest application to horticultural and field crops, respectively. SmartFresh technology has been adopted internationally most widely by apple industries (Watkins, 2008).

In apple fruit, 1-MCP delays loss of fruit firmness and titratable acidity, delays the initiation of system II ethylene production, and reduces respiration and ethylene production, thereby extending storage and shelf life (Fan et al., 1999a; Watkins et al., 2000; DeLong et al., 2004; Fawbush et al., 2009). The success of 1-MCP based technology for apple lies in its ability to keep the fruit fresh with a firm texture. In contrast, fruit such as avocado, banana and tomato, where a delay but not an inhibition of ripening is desirable, an apple with ripening characteristics close to those at harvest is usually optimal in the marketplace. 1-MCP can also inhibit the development of physiological disorders such as superficial scald, core flush, senescence breakdown and core browning (DeLong et al., 2004; Zanella, 2005; Jung and Watkins, 2008). However, 1-MCP can induce internal and external CO_2 injuries, flesh browning and brown core in some cultivars (Fawbush et al., 2008). The most serious of these disorders in 'Empire' apples is the apparent enhancement of firm flesh browning in 1-MCP treated fruit, especially in CA stored fruit kept at warmer storage temperatures of 2-3 ^{o}C to avoid development of chilling injury (Watkins and Nock, 2004; Watkins, 2008). This browning has resulted in serious losses to industries that grow the 'Empire' apple, and is the subject of study to find practical methods to avoid development of the disorder.

1.4. Flesh browning disorders

The symptoms of flesh browning range from light to dark brown coloration on the tissues in core, coreline, vascular and inner and outer cortex for core browning

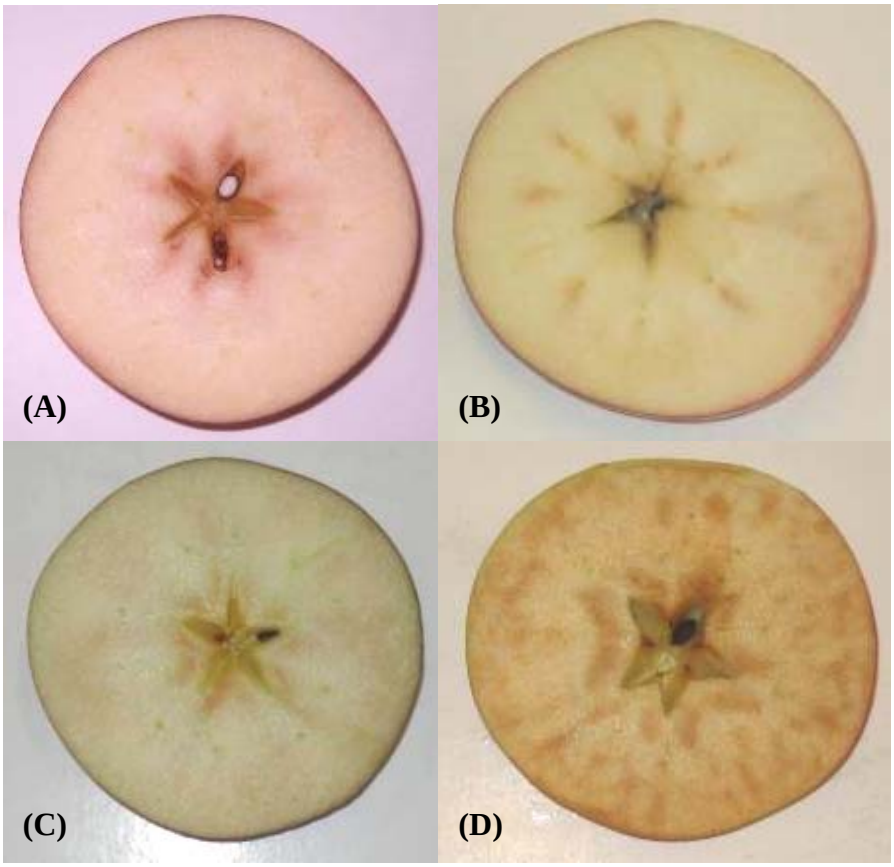

Figure 1.1. Core browning (A), vascular breakdown (B), and moderate (C) and severe (D) flesh browning disorders in ‘Empire’ apple fruit after CA storage.

(Figure 1.1 A), vascular breakdown (Figure 1.1 B) and flesh browning (Figure 1.1 C and D), respectively. These damaged tissues are firm and moist. Core browning occurs next to carpel or randomly throughout core flesh tissue. Vascular breakdown develops in the main vascular tissue of the core line. Flesh browning is sporadically detected at the inner and outer cortex tissues of the flesh. The form of flesh browning found in the 'Empire' apple is shown in Figure 1.1 C and D. The flesh browning is diffuse on the flesh tissue with no definite outline of the injured area and also it does not have external symptoms. The flesh browning is typically detected at the later stage of storage. The disorder is similar to that described by Meheriuk et al. (1994).

The physiological, biochemical, and molecular mechanism of the incidence of internal browning disorder is still not elucidated. In general, flesh browning is associated with polyphenol oxidase (PPO, EC 1.10.3.1) activity on phenolic compounds in the presence of oxygen, causing the hydroxylation of monophenolic compounds to *o*-diphenols and consequently oxidizing to *o*-quinones, following the more complex brown polymerization finally developing brown discoloration (Vamos-Vigyazo, 1981; Sapers, 1993). Also, laccase (EC 1.10.3.2), tyrosinase (EC 1.14.18.1), and peroxidase (EC 1.11.1.1-2) activities are associated with flesh browning. Laccase oxidizes *o*-diphenols and *p*-diphenols to their corresponding quinines. Tyrosinase referred to as monophenol monooxygenase catalyzes the hydroxylation of monophenols. The peroxidase functions to oxidize hydrogen donors at the expense of peroxides (Nicolas et al., 1994). Jung and Watkins (submitted) suggested that browning is associated with inhibition of ethylene production by chilling temperatures with and without 1-MCP, and inhibition by 1-MCP at warmer temperatures, that results in reduced ability of the fruit to resist stress. They found that higher PPO activity in flesh tissues was associated with 1-MCP treatment.

1.5. Research objectives

The objective of the research in this thesis is to investigate the metabolic responses that are associated with internal browning disorder of untreated and 1-MCP treated 'Empire' apple fruit during CA storage. I have taken two approaches:

1. Antioxidant metabolism. CA storage conditions may cause the oxidative stress to apple fruit by the combination of lower partial pressures of oxygen (pO_2) and higher pCO_2 levels concurrently with lower storage temperature. The oxidative stress during CA storage may cause increased reactive oxygen species (ROS) on the cellular level, and thus, these ROS can disturb the redox balance of enzymatic- and non-enzymatic antioxidant scavenging systems and at some point, accumulate to the toxic level. Finally, the accumulated ROS can rapidly react with DNA, RNA, protein and cell membranes to cause damage to cellular function. Homeostasis in the cell is maintained by enzymatic- and non-enzymatic antioxidant systems. The enzymatic antioxidant scavenging system is composed of superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX) and glutathione reductase (GR), whereas the non-enzymatic antioxidant scavenging system consists of reduced and oxidized forms of ascorbic acid and glutathione (Mittler, 2002; Apel and Hirt, 2004).
2. Metabolomic profiling. To identify metabolic alterations in 'Empire' apples during storage, an untargeted metabolic profiling approach was employed using in full (GC-MS). Using this technique, partial least squares discriminant analyses can be used to characterize any metabolomic changes

associated with disorder development (Lisec et al., 2006; Rudell et al., 2008; Rudell et al., 2009).

Chapter 3 examines the effects of storage temperature on antioxidant scavenging systems of untreated and 1-MCP treated 'Empire' apple fruit during CA storage. This chapter covers enzymatic- and non-enzymatic antioxidant scavenging systems at 0.5 and 3.3 ^{o}C during CA storage.

Chapter 4 examines the influence of two different storage temperature systems on any metabolomic alterations of 1-MCP treated 'Empire' apple fruit during CA storage. Metabolomic changes of 1-MCP treated 'Empire' apple fruit are identified using PLS discriminant analyses with GC-MS, and key metabolites further investigated by detailed description of changes over time.

1.6. Hypothesis

This thesis is based on experimental results that indicate that 1-MCP may cause more flesh browning in 'Empire' apple fruit during CA storage with a higher storage temperature than a lower storage temperature. Based on the results, the hypothesis has been developed that 1-MCP may change antioxidant scavenging systems and metabolomic responses at 3.3 ^{o}C compared with 0.5 ^{o}C.

REFERENCES

Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. 55, 373-399.

Blanpied, G.D., Johnson, D.S., Lau, O.L., Lidster, P.D., Lougheed, E.C., Porritt, S.W., 1990. Apples. Agriculture Canada Publications, 7-22.

Bramlage, W.J., 1993. Interactions of orchard factors and mineral nutrition on quality of pome fruit. Acta Hort. 326, 15-28.

Cheng, H.-t., Criner, G.K., Kezis, A.S., 1997. The impact of consumer characteristics on preferences for selected apple varieties. J. Food Products Marketing 3, 1 - 11.

DeEll, J.R., Murr, D.P., Mueller, R., Wiley, L., Porteous, M.D., 2005. Influence of 1-methylcyclopropene (1-MCP), diphenylamine (DPA), and CO_2 concentration during storage on ‘Empire’ apple quality. Postharvest Biol. Technol. 38, 1-8.

DeEll, J.R., Ayres, J.T., Murr, D.P., 2010. Commercial application of 1-methylcyclopropene affects storability and disorders of 'Empire' apples. Acta Hort. 857, 107-114.

DeLong, J.M., Prange, R.K., Harrison, P.A., 2004. The influence of 1-methylcyclopropene on 'Cortland' and 'McIntosh' apple quality following long-term storage. HortScience 39, 1062-1065.

Derkacz, M., Elfving, D.C., Forshey, C.G., 1993. The history of the 'Empire' apple. Fruit Var. J. 47, 70-71.

Fan, X., Blankenship, S.M., Mattheis, J.P., 1999. 1-Methylcyclopropene inhibits apple ripening. J. Amer. Soc. Hort. Sci. 124, 690-695.

Fawbush, F., Nock, J.F., Watkins, C.B., 2008. External carbon dioxide injury and 1-methylcyclopropene (1-MCP) in the 'Empire' apple. Postharvest Biol. Technol. 48, 92-98.

Fawbush, F., Nock, J.F., Watkins, C.B., 2009. Antioxidant contents and activity of 1-methylcyclopropene (1-MCP)-treated 'Empire' apples in air and controlled atmosphere storage. Postharvest Biol. Technol. 52, 30-37.

Goffinet, M.C., Robinson, T.L., Lakso, A.N., 1995. A comparison of ‘Empire’ apple fruit size and anatomy in unthinned and hand-thinned trees. J. Hort. Sci. Biotech. 70, 375-387.

Jung, S.-K., Watkins, C.B., 2008. Superficial scald control after delayed treatment of apple fruit with diphenylamine (DPA) and 1-methylcyclopropene (1-MCP). Postharvest Biol. Technol. 50, 45-52.

Jung, S.-K., Watkins, C.B., 2011. Involvement of ethylene in browning development of controlled atmosphere-stored 'Empire' apple fruit. Postharvest Biol. Technol. In press

Kays, S.J., 1999. Preharvest factors affecting appearance. Postharvest Biol. Technol. 15, 233-247.

Lisec, J., Schauer, N., Kopka, J., Willmitzer, L., Fernie, A.R., 2006. Gas chromatography mass spectrometry-based metabolite profiling in plants. Nat. Protocols 1, 387-396.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405-410.

Nicolas, J.J., Richard-Forget, F.C., Goupy, P.M., Amiot, M.J., Aubert, S.Y., 1994. Enzymatic browning reactions in apple and apple products. Crit. Rev. Food Sci. Nutr. 34, 109-157.

Rudell, D.R., Mattheis, J.P., Curry, E.A., 2008. Prestorage ultraviolet-white light irradiation alters apple peel metabolome. J. Agric. Food Chem. 56, 1138-1147.

Rudell, D.R., Mattheis, J.P., Hertog, M.L.A.T.M., 2009. Metabolomic change precedes apple superficial scald symptoms. J. Agric. Food Chem. 57, 8459-8466.

Sapers, G.M., 1993. Browning of foods: control by sulfites, antioxidants, and other means. Food Technol. 47, 75-84.

Sisler, E.C., Blankenship, S.M., 1996. Methods of counteracting an ethylene response in plants. U.S.

Sisler, E.C., 2006. The discovery and development of compounds counteracting ethylene at the receptor level. Biotechnol. Adv. 24, 357-367.

Stover, E., Fargione, M., Risio, R., Yang, X., 2002. Crop load reduction and fruit size following multi-step thinning of 'Empire' apple. HortScience 37, 130-133.

Vamos-Vigyazo, L., 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. Crit. Rev. Food Sci. Nutr. 15, 49-127.

Watkins, C., Liu, F.W., 2010. Temperature and carbon dioxide interactions on quality of controlled atmosphere-stored 'Empire' apples. HortScience 45, 1708-1712.

Watkins, C.B., Nock, J.F., Whitaker, B.D., 2000. Responses of early, mid and late season apple cultivars to postharvest application of 1-methylcyclopropene (1-MCP) under air and controlled atmosphere storage conditions. Postharvest Biol. Technol. 19, 17-32.

Watkins, C.B., Kupferman, E.M., Rosenberger, D.A., 2002. Apple. Postharvest quality management guidelines. In: Gross, K.C., Wang, Y., Saltveit, M.E. (Eds.), The Commercial Storage of Fruits, Vegetables and Florist and Nursery Crops Agriculture Handbook no. 66. USDA.

Watkins, C.B., 2003. Postharvest physiological disorders of fresh crops. Encyclopedia of Applied Plant Science, 2. Academic press.

Watkins, C.B., Nock, J.F., 2004. SmartFresh™ (1-MCP) - The good and bad as we head into the 2004 season! New York Fruit Quart. 12, 3-8.

Watkins, C.B., Nock, J.F., Razafimbelo, F., 2005. Storage disorders of apples with and without 1-MCP (SmartFresh™). Cornell Fruit Handling and Storage Newsletter, 2-17.

Watkins, C.B., 2008. Overview of 1-methylcyclopropene trials and uses for edible horticultural crops. HortScience 43, 86-94.

Yahia, E.M., 1994. Apple flavor. Hort. Rev. 16, 197-234.

Zanella, A., Cazzanelli, P., Panarese, A., Coser, M., Chistè, C., Zeni, F., 2005. Fruit fluorecence response to low oxygen stress: modern storage technologies compared to 1-MCP treatment of apple. Acta Hort. 682, 1535-1542.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

The 'Empire' apple, which is a major New York apple cultivar that is grown both for domestic and overseas markets, is susceptible to chilling injury at 0 ^{o}C and senescent breakdown at 3 ^{o}C. Therefore, the storage recommendations for 'Empire' apples are 1.5-2.0 ^{o}C, as a compromise between the two disorders (Watkins et al., 2005; Watkins and Liu, 2010a). In addition, unpublished observations indicate that there is a relationship between sensitivity to chilling injury and preharvest temperatures, where cooler July and August temperatures are associated with higher risk of injury development. As a result, it is common to increase the storage temperatures slightly to avoid chilling injury after cooler summers.

A recent phenomenon has challenged these practices. A new storage technology based on 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception, is now used widely by apple industries throughout the world (Watkins, 2006b). 1-MCP reduces ethylene production and respiration and thereby extends storage life of apple fruit (Watkins, 2008). In addition, 1-MCP can suppress certain storage disorders, such as superficial scald, soggy breakdown and soft scald (Fan et al., 1999b; Watkins et al., 2000; Zanella, 2003; DeEll and Ehsani-Moghaddam, 2010). However, 1-MCP can aggravate other disorders, especially those that are CO_2-related (Argenta et al., 2007; Fawbush et al., 2008; Watkins and Liu, 2010a). 1-MCP treated fruit stored at 3 ^{o}C can have a higher incidence of firm flesh browning (Watkins, 2008). This firm flesh browning has resulted in high economic losses for affected apple industries.

2.2. Induction factors for storage disorders

2.2.1. CO_2 and O_2 levels

After harvest, apples are stored at elevated CO_2 concentrations to maintain quality and extend storage life. Ripening of fruit is slowed down by inhibition of ethylene biosynthesis and action by the gas. The optimum CO_2 concentrations for CA storage of apples vary from 0.5 kPa for 'Braeburn', 'Empire', 'Fuji', and 'Granny Smith' to 5 kPa for 'McIntosh', depending on a combination with other factors, such as O_2 concentration, storage temperature and storage duration (Kupferman, 2003). In addition to reduction of respiration and ripening delay, high CO_2 directly influences secondary metabolism, such as phenolic biosynthesis and degradation in plants (Beaudry, 1999). Phenylalanine ammonia lyase (PAL) activity is enhanced, but total phenolic content and polyphenol oxidase (PPO) activity are reduced by elevated CO_2 (Beaudry, 1999; Kader, 2003).

High CO_2 can also affect metabolism by decreasing cellular pH. A CO_2-enriched atmosphere around plant tissues can cause diffusion of CO_2 into the cell, which dissolves in the cellular solution to yield carbonic acid (H_2CO_3). Carbonic acid can then disassociate to yield bicarbonate (HCO_3^-) and carbonate ions (CO_3^{2-}). These two ionic intermediates continuously deprotonate to yield protons (H^+) in the cellular media. Consequently, the acidity of cellular milieu increases (Smock, 1979; El-Goorani and Sommer, 1981; Kader, 1995; Mathooko, 1996). Bown (1985) reported that CO_2 concentrations as high as 5 kPa increase intercellular acidity. pH alteration in cellular compartments by high CO_2 probably affects the apparent *Km* values (Harel et al., 1964), enzyme activities and specificity, cofactors to the corresponding enzymes, and oxidation rates (Mathooko, 1996). Janovitz-Klapp et al. (1989) reported that although the optimum pH range of 4-methylcatechol, chlorogenic acid and (+)-catechin activities was between 4.5 and 5, chlorogenic acid was a better substrate for

apple PPO activity than (+)-catechin at pH 4, which is close to the vacuole pH in apples. PPO specificity toward substrates, in order of decreasing activity, was 4-methylcatechol, chlorogenic acid, catechol, caffeic acid, and 3,4-dihydroxyphenylalanine (Trejo-Gonzalez and Soto-Valdez, 1991). The optimum pH range of PPO activity was between 4.2 and 7.3 based on the localization, solubilization and extraction from the bulk of apple PPO (Vamos-Vigyazo, 1981; Janovitz-Klapp et al., 1989; Trejo-Gonzalez and Soto-Valdez, 1991) but PPO activity was still relatively stable to high acidity with activity at pH 3 still being 40% of the maximum (Nicolas et al., 1994). Also, Zhou et al.(1993) showed a similar result in that the activity of PPO was relatively stable between pH 4 and 8, but below pH 4, the activity was sharply reduced to approximately 20%. In addition to PPO activity, peroxidase (POD or POX, EC 1.11.1.7) activity can be affected by high acidity because the optimum for POD activity is around pH 5.8 (Vamos-Vigyazo, 1981). However, the optimum pH range for phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) activity is 8.0-9.5 and the most reported *Km* values range between 0.3×10^{-4} and 1.5×10^{-2} M (Camm and Towers, 1973).

In addition to elevated CO_2, reduced O_2 has been applied to reduce respiration and inhibit ethylene production of apples during CA storage (Smock, 1979). The optimum O_2 concentrations for CA storage of apple fruit, which are between 1 kPa for ‘Golden Delicious’, ‘Jonagold’, and ‘Delicious’ and 3 kPa for ‘Braeburn’ depending on the numerous other storage factors, such as CO_2 concentrations, storage temperature and storage life, are less variable than for CO_2 (Kupferman, 2003).

2.2.2. Storage temperature

Low temperatures can result in an increase of the degree of unsaturation of fatty acids of their membrane lipids by the modification of polar head group composition or

by enhancement of membrane phospholipid concentrations to maintain membrane fluidity and membrane function (Graham and Patterson, 1982). In cortex tissue of 'Cox's Orange Pippin' stored at 3.5 ^{o}C in air, the concentrations of sitosterol and phospholipid increased but the concentration of phospholipid increases less in 2 kPa O_2 than in air storage (Bartley, 1986). The viscosity and the concentration of phospholipids in the membranes was inversely associated with low storage temperature; the degree of unsaturation of the fatty acids decreased in the membrane phospholipids but the ratio of sterols to phospholipids increased in 'Calville de San Sauveur' apple stored at 0 ^{o}C in air (Lurie et al., 1987). However, low storage temperature can induce storage injuries including low temperature breakdown, which can be further exacerbated by storage under a threshold concentration of pO_2 (Meheriuk et al., 1994). In 'Jonathan' apple, low temperature breakdown was less developed on blushed side than on unblushed side (Wills and Patterson, 1972). In 'Jonathan' apple, the more acetic acid injected into apple fruits, the greater the low temperature breakdown incidence (Wills et al., 1970). However, gibberellic acid reduced the incidence of low temperature breakdown (Wills and Patterson, 1971).

2.2.3. Storage humidity

After harvest, water loss is one of the major single contributors to postharvest deterioration, directly affecting quantitative and qualitative damage such as loss of visual quality (shrinkage and wilting), and degradation of certain metabolites (Grierson and Wardowski, 1978; Kader, 1986). For apples, shriveling is significant at less than 60% relative humidity (RH) (Grierson and Wardowski, 1978). Water loss occurs by water vapor pressure deficit, the difference in water vapor pressure between the inside and outside of an apple at a given temperature (Gaffney, 1978; Paull, 1999). Weight loss, which also occurs by respiration, is positively correlated with RH at a

constant temperature (Grierson and Wardowski, 1978; Tu et al., 2000). Therefore, RH can control the transpiration rate and thereby reduce water loss or weight loss of apples during storage. The generally recommended RH for apples is 90-95% (Paull, 1999). At lower RH, apples will easily lose water because of the higher water vapor pressure deficit, resulting in significant wilting, softening, shriveling, shrinkage and an adverse taste. Unlike temperature which is fairly manageable, RH relies on the efficiency of the cooling system, such as the surface area of the refrigeration evaporator coil in the storage, the temperature difference and distribution (Paull, 1999).

Lower RH can impact on loss of cell turgor; tissue under lower cell turgor fails by cell separation (Johnston et al., 2002). Lower RH during storage results in an increase in internal air space and cellular pH, but a decrease in soluble solid concentrations in 'Braeburn' and 'Jonagold' (Tu et al., 2000). Alteration of cytoplasmic, cellular, or intercellular pH and soluble osmoticum is sometimes accompanied by the remodeling of the specificity, affinity, or activity of PAL, PPO and POD (Mihalyi et al., 1978). The shifts in the pH optimum might be explained by partial denaturation of those enzymes and/or conformational changes (Mayer and Harel, 1979).

2.2.4. Fruit senescence

Senescence results in softening and sometimes, browning of cortex tissues immediately under the skin tissue (Figs 1 and 2). Breakdown occurs initially in the outer cortex and then inner cortex tissues, finally reaching the core. Damaged tissues become soft and dry with dark brown discoloration, and eventually, the skin becomes

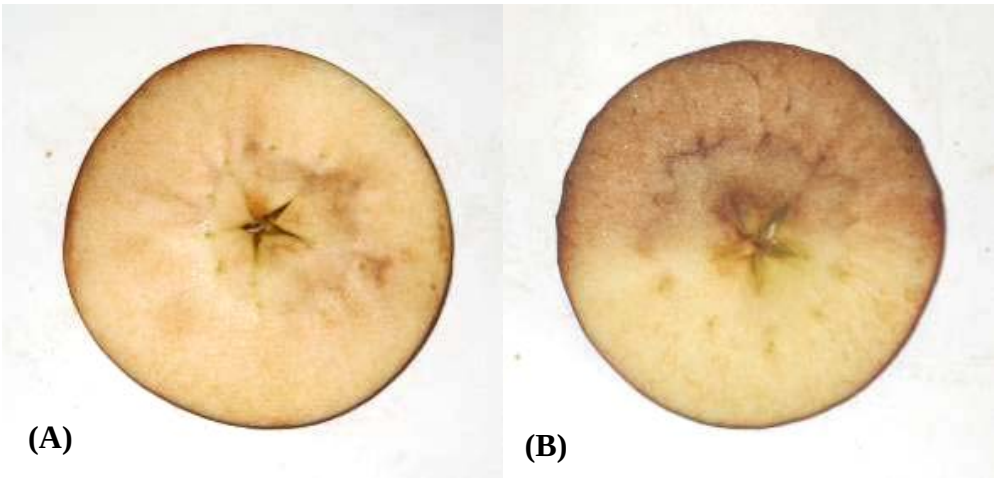

Figure 2.1. Moderate (A) and severe (B) senescence breakdown of ‘McIntosh’ apple after prolonged CA storage.

dark brown and soft and may crack. Accordingly, these symptoms are called senescence breakdown because they are related with aging and maturity during CA storage (Fidler et al., 1973; Lidster et al., 1990). Lougheed et al. (1983) reported that the incidence of senescence breakdown of 'McIntosh' apples was associated with advanced maturity. Preharvest calcium sprays reduced the incidence of senescent breakdown in 'Cox's Orange Pippin' apples (Watkins et al., 1989).

2.3. Metabolic responses

2.3.1. Carbohydrates

In apple, carbohydrates are used as the primary energy source for respiration and the other metabolic pathways after harvest. Sugars in apple fruit include erythrose, xylose, arabinose, ribose, rhamnose, fucose, fructose, glucose, sucrose, maltose, D-erythro-tetrafuranose, talose, galactose, lactose, turanose, maltotriose and raffinose. Sugar alcohols include erythritol, xylitol, arabinitol, adonitol, mannitol, sorbitol and inositol (Tisza et al., 1994; Rudell et al., 2008). Fructose, glucose and sucrose are the major sugars and sorbitol is the principal sugar alcohol (Berüter, 1985; Ackermann et al., 1992; Suni et al., 2000; Veberic et al., 2007; Wu et al., 2007). However, the proportion of these sugars is dependent on the cultivar and cultivation practice (Suni et al., 2000; Hecke et al., 2006; Róth et al., 2007; Wu et al., 2007), strain within a cultivar and year to year (Veberic et al., 2007) as well as storage condition and shelf life (Suni et al., 2000; Róth et al., 2007).

Sorbitol and sucrose can be translocated from leaves into apple fruit tissues as main carbohydrate transported materials but sorbitol has been known as primary translocated carbohydrate in apple and *Rosaceae* family (Webb and Burley, 1962). The translocated carbohydrates consist of approximately 70% sorbitol and 30% sucrose (Hansen, 1970; Klages et al., 2001). These transported carbohydrates can be converted into fructose or/and glucose, catalyzed by sorbitol dehydrogenase (SDH, EC 1.1.1.14), sorbitol oxidase,

acid and neutral invertase, and sucrose synthase (SUSY, EC 2.4.1.13). SDH using NAD^+ as a cofactor converts sorbitol into fructose in cytosol, sorbitol oxidase into glucose, and neutral invertase converts sucrose into fructose and glucose in cytosol. Of those enzymes, SDH has been revealed as a primary enzyme for the first step of sorbitol degradation and ubiquitously localized in a cell and may also be present in multiple forms (Park et al., 2002; Berüter, 2004).

Of one of major transported carbohydrates, sucrose transported from leaves, pass through the phloem, enter the cell either by diffusion through the plasmodesmata against a concentration gradient or by symplasmic transport, and then degraded by either invertase or SUSY. Sucrose can be passed through the sucrose transporter on parenchyma cell wall as unchanged form or converted into fructose and glucose catalyzed by cell wall-bound invertase (EC 3.2.1.26) in the apoplast or intracellularly. Then, both fructose and glucose can pass through hexose transporters on parenchyma cell wall. Sucrose entered via sucrose transporters can be converted into UDP-glucose and fructose catalyzed by SUSY with UDP in the cytosol. Furthermore, sucrose in vacuole can be converted into fructose and glucose by vacuolar acid invertase (Teo et al., 2006). Yamaki (1984) reported that most fructose, glucose, malic acid and sorbitol are localized and compartmentalized in the vacuole, and sucrose in the free space and in the cytosol.

In the cytosol, glucose-1-phosphate (G1P), which is converted from glucose-6-phosphate (G6P) by the action of phosphoglucomutase, or from UDP-glucose by the action of UDP-glucose pyrophosphorylase, can enter into a plastid via a transporter on a membrane. G1P can be further polymerized for starch synthesis in the plastid. For this metabolic event, starch synthases are applied to polymerize glucose monomers into α-1,4-glucans using ADP-glucose as a substrate for the production of amylose, which has only α-1,4 linkage. On the other hand, the starch branching enzymes introduce α-1,6-glycosidic branch points for the production of amylopectin, which is a branched α-1,4: α-1,6 D-

glucan polymer (Smith et al., 1997). Starch is a very complicate quaternary structure made of the two glucose polymers, amylose and amylopectin and is localized as semicrystalline particles inside plastids (Ball and Morell, 2003; Lloyd et al., 2005). The size of these starch granule is various from 0.1 to over 50 μm in diameter (Buléon et al., 1998). Typically, weight percentage of starch granule is 15-35% for amylose and 65-85% for amylopectin (Ball et al., 1998). In apple fruit, the amylose content accounts for 25% of the total starch (Potter et al., 1949). During apple fruit development and growth, semicrystalline granules are deposited more in the outer part of cortex tissue than at both middle and inner part of cortex tissues (Ohmiya and Kakiuchi, 1990). After cell enlargement, starch can be deposited from the most outer part of cortex tissue into core region, and then the localized starch granules are hydrolyzed from the core tissue to the outer cortex tissue (Smock and Neubert, 1950). Furthermore, these starch granules were not homogeneously distributed within single apple cell (Ernst et al., 1999). As apple fruit mature, total starch and amylose content decrease (Fan et al., 1995). Furthermore, the percentage of amylose in the total starch content is reduced (Bartley, 1976; Fan et al., 1995). While starch content in cortical tissues of apple fruit gradually decreased while ripening on the tree, the contents are more rapidly reduced off the tree (Knee, 1973).

Starch degradation in plastids provides carbohydrates as the primary energy source within the same cells for the cellular maintenance. During ripening, apple starch in the plastid can be hydrolyzed into glucan, maltose, glucose and fructose by α-amylase (endo-amylase, EC 3.2.1.1), β-amylase (exo-amylase, EC 3.2.1.2), maltase, starch phosphorylase (EC 2.4.1.1), pullulanase (EC 3.2.1.41), disproportionating enzyme (D-enzyme, EC 2.4.1.25) and debranching enzyme (EC 3.2.1.10). Possibly, starch in plastids can be first catalyzed by α-amylase, which converts starch into large branched glucan. In this case, α-amylase can randomly hydrolyze internal α-1,4 glycosidic linkages within linear or branched glucose polymers exposed on the surface or in channels within granules,

releasing soluble linear and branched glucans that are the substrate for further degradation. Second, the large branched glucan can be converted into long linear glucan by debranching enzyme. Third, these long linear glucans can be cleaved into G1P by starch phosphatase, converted into short glucans by α-amylase or maltose by β-amylase. Fourth, the short glucans can be cleaved into either long linear glucans or glucose by disproportionating enzyme (D-enzyme). At the same time, the maltose from the long linear glucans can be cleaved into glucose by α-glucosidase. G1P can be further remobilized into triose-phosphate. Those final carbohydrate metabolites, which are triose-phosphate, maltose, and glucose, can be exported into the cytosol by using triose phosphate transporter, maltose transporter, and glucose transporter, respectively, on the plastid membranes for the further metabolism (Smith et al., 2003; Smith et al., 2005).

2.3.2. Intermediates of glycolysis and TCA cycle

Generally, high CO_2 and low O_2 concentrations in the storage atmosphere reduce respiration rate, starch breakdown, sugar consumption as primary metabolism. Furthermore, secondary processes such as ethylene metabolism, pigment metabolism, phenolic metabolism, cell wall metabolism and volatile compound metabolism can be affected (Beaudry, 1999).

High CO_2 concentrations reduce the activity of several respiratory enzymes and metabolites of the TCA cycle (Hulme, 1956; Williams and Patterson, 1964; Frenkel and Patterson, 1973). Hulme (1956) reported that succinic acid accumulated in apple tissue as CO_2 concentration elevated, but malic acid was not affected. This result is associated with reduction of succinate dehydrogenase activity with increased CO_2 concentration in the storage atmosphere (Frenkel and Patterson, 1973; Ke et al., 1993). Williams and Patterson (1964) also reported that succinic and citric acid accumulated in flesh tissue of 'Bartlett' pear stored with elevated CO_2 but malic acid concentrations

were lower. CO_2 injury was accompanied by accumulation of the succinic acid in both core and flesh tissues. Fernández-Trujillo et al. (2001) reported that elevated CO_2 treatments induced the accumulation of succinate in peel and flesh of 'Cortland' and 'Law Rome' apples. However, accumulation also occurred in DPA treated fruit in which CO_2 injury was absent, suggesting that succinate accumulation is not the cause of CO_2 injury.

In 'Bartlett' pear fruit, the levels of fructose 6-phosphate (F6P) and fructose 2,6-bisphosphate (F2,6P) accumulated with elevated CO_2 concentrations during storage, while the level of fructose 1,6-bisphosphate (F1,6P) declined (Kerbel et al., 1988). The activities of ATP:phosphofructokinase (ATP-PFK) and PPi:phosphofructokinase (PPi-PFK) also declined. Under reduced O_2, F1,6P increased in carrots shreds but the other glycolytic intermediates were not influenced (Kato-Noguchi and Watada, 1996). Kato-Noguchi and Watada (1996) suggested that PPi-PFK may be associated with the regulation of glycolytic metabolism in fresh-cut carrots under reduced O_2 concentrations.

2.3.3. Fermentation metabolites

Fermentation is induced under conditions of elevated CO_2 and/or reduced O_2 concentration outside of limits that vary by crop, species, plant part and maturity (Beaudry, 2000; Watkins, 2000). The fermentation pathway from pyruvate to acetaldehyde to ethanol permits continued generation of ATP under stress conditions. Under anaerobic condition, NADH and pyruvate can be oxidized to NAD^+ and acetaldehyde, respectively (Kader, 1995). Pyruvate produced by glycolysis is irreversibly decarboxylated to acetaldehyde by pyruvate decarboxylase (PDC, EC 4.1.1.1) and then the acetaldehyde may be reduced to ethanol by alcohol dehydrogenase (ADH, EC 1.1.1.1), when NADH is present (Smagula and Bramlage, 1977; Ke et al., 1994a). Therefore, the

TCA cycle and electron transport chain are inhibited and ATP is only generated through fermentation rather than oxidative phosphorylation by TCA cycle (Geigenberger, 2003). The product of fermentative metabolism, ethanol can be converted to ethyl acetate with acyl CoA, catalyzed by alcohol acyltransferase (AAT, EC 2.3.1.84) (Ke et al., 1994b).

The concentrations of fermentative volatile metabolites in 'Fuji' apples increased with increased fresh browning as duration of exposure and concentration of CO_2 increased (Volz et al., 1998). Flesh browning was not related to tissue ethyl acetate concentration but positively correlated with ethanol concentrations. However, there was no strong relationship between accumulation of these volatile metabolites and high CO_2-induced flesh browning. Also, Argenta et al. (2004) reported that ultralow O_2 or high CO_2 conditions apples reduced the production of most volatiles in 'Fuji', but ethanol and ethyl acetate were increased by both CA regimes. In contrast to Volz et al. (1998), Argenta et al. (2004) found no causal relationship between development of CO_2 injury and accumulation of volatile products by high CO_2.

2.3.4. Volatile metabolism

Apples produce a versatile mixture of volatile metabolites, which include alcohols, esters, aldehydes, ketones, acetic acids and sesquiterpenes (Dimick and Hoskin, 1983; Yahia, 1994; Dixon and Hewett, 2000; Kondo et al., 2005). The quantification and qualification of these volatiles can be affected by several factors, such as cultivar, maturity and storage regime (Patterson et al., 1974; Sapers et al., 1977; Streif and Bangerth, 1988; Zhu et al., 2008). During ripening, the major volatile compounds in apples are volatile esters, which account for up to 80-98% of total volatile production (Flath et al., 1967; López et al., 1998; Kondo et al., 2005). The production of these ester volatiles is an ethylene dependent process (Song and Bangerth, 1996; Fan et al., 1998). These volatile esters are produced by the reaction of catalytic esterification with transferring an acyl

moiety from acyl-CoAs, derived from β-oxidation of fatty acids, into the corresponding alcohol by alcohol acyltransferase (AAT, EC 2.3.1.84), or with transferring an acetyl moiety from acetyl-CoAs, derived from pyruvate, into the corresponding alcohol by alcohol acetyltransferase (AAT) (Harada et al., 1985; Aharoni et al., 2000; Dixon and Hewett, 2000; Shalit et al., 2001). Defilippi et al. (2005a) reported that alcohol acyltransferase plays an important role in ester biosynthesis for apple aroma as a rate limiting step.

More volatile compounds were produced in fruit harvested at the climacteric stage than at preclimacteric stage when stored in either air or CA, but CA storage suppressed aroma production (Brackmann et al., 1993; Fellman et al., 2003). 1-MCP treatment reduced volatile compounds in 'Delicious' and 'Golden Delicious' (Kondo et al., 2005; Ferenczi et al., 2006). 1-MCP treatment inhibited not only biosynthesis of ester volatiles but also production of alcohols in 'Fuji' (Fan and Mattheis, 1999) and suppressed gene expression of specific AATs and ACSs in 'Golden Delicious' and 'Granny Smith' fruit (Zhu et al., 2008). Along with the reduction of AAT activity, 1-MCP enhanced ADH activity in 'Greensleeves' apple (Defilippi et al., 2005a). However, the activity of AAT did not change in fruit flesh during on-tree ripening of 'Fuji' apple fruit and activity even decreased in peel, while the production of aroma compounds was increased (Echeverría et al., 2004b). These results indicate that precursor availability is a more significant factor than AAT activity during the development of volatile compounds. Moreover, lipoxygenase (LOX, EC 1.13.11) activity for the production of corresponding volatile precursors was strongly associated with the production of volatile compounds in CA stored 'Fuji' apple (Altisent et al., 2009). However, in 'Greensleeves' apple from a transgenic line with a high suppression of ethylene biosynthesis, AAT activity was more strongly associated with ethylene regulation than ADH and LOX activities (Defilippi et al., 2005b). The production of ester volatiles were strongly associated with expression of

putative genes for amino acid, β-oxidation, ester formation and fatty acid oxidation (Sugimoto, 2008). Levels of an important precursor of volatile compounds, isoleucine increased during ripening in 'Greensleeves' apple (Defilippi et al., 2005b).

2.3.5. Energy status (ATP/ADP, $NAD(P)^+$/NAD(P)H)

Stored commodities require energy. However, the extreme CA storage regimes with ultralow O_2 and/or elevated CO_2 concentration can cause reduced ATP concentrations in stored products (Streif et al., 2003). Oxidative phosphorylation, which is an O_2-dependent process occurring in the inner membrane of mitochondria, should be negatively affected by low O_2 storage regimes. NADH and $FADH_2$ produced by glycolysis and TCA cycle can be oxidized to NAD^+ and FAD^+ respectively to release protons and these protons are transferred through the electron transport chain on the inner membrane of mitochondria to cytochrome oxidase finally to react with O_2 to generate H_2O. Also, protons in the matrix of mitochondria are pumped out to intermembrane space by each protein complex of mitochondrial electron-transfer chain and thereby a proton gradient is generated. This electrochemical potential driven by proton concentration can activate ATP synthase on the inner membrane of mitochondria and thereby ATP synthesis is driven by proton motive force between the matrix and intermembrane space of mitochondria. Therefore, ATP production at normoxia is coupled with O_2 consumption.

Under conditions of anoxia and hypoxia, ATP levels and synthesis are reduced in the tissue of 'Conference' pear, compared with those in air storage (Veltman et al., 2003). They also showed that at 5 kPa CO_2, ATP levels and synthesis was correlated with decreasing O_2 concentration during storage. In 'Jonagold' apple and 'Conference' pear, CA storage altered the energy levels as ATP concentrations at CA storage regime were lower than in air storage but ADP concentration showed opposite responses to the storage regime (Saquet et al., 2000). Also, NAD^+ and $NADP^+$ decreased with storage time in both

CA and air storage, while the concentrations of NADH and NADPH in both fruit tissues were enhanced under either storage regime. The incidence of flesh browning in 'Conference' pears was associated with low ATP concentrations during CA storage (Saquet et al., 2000), however, Veltman et al. (2003) concluded that ATP concentrations and estimated ATP generation were not directly correlated with the development of internal browning in 'Conference' pears. Nevertheless, the energy status change by the suppresion of oxidative phosphorylation may play a detrimental role in development of storage disorders such as core browning, flesh browning, senescence breakdown and cavities. Rawyler et al. (1999) found that under anoxia, ATP concentration and synthesis rate of the incubated potato cells declined with incubation time, while ethanol and lactate accumulated. In the long run, anoxia or hypoxia can decrease ATP levels and suppress ATP synthesis. Moreover, these conditions can contribute to the generation and accumulation of fermentative metabolites, leading to develop off-flavors and loss of cell membrane integrity. These consecutive events or reactions are associated with induction of internal browning activated by the oxidation of phenolic compounds.

2.3.6. Ethylene

Ethylene is a plant growth hormone that has many effects on plant growth and development including leaf abscission, senescence and ripening. It is a simple hydrocarbon (C_2H_4) that can easily diffuse into and out of plant tissues (Saltveit, 1999). Ethylene can be induced by wounding and abiotic stresses such as drought stress and extreme temperature. Methionine is used as a precursor to biosynthesize ethylene and then it is regenerated by Yang cycle. *S*-Adenosylmethionine (SAM) is synthesized from methionine and ATP, catalyzed by AdoMet synthetase, an intermediate in ethylene biosynthesis. ACC synthase (ACS) converts SAM to 1-aminocyclopropane-1-carboxylic acid (ACC). Finally, ethylene can be produced from ACC catalyzed by ACC oxidase

(ACO) (Adams and Yang, 1979). ACC synthase can be regulated by fruit ripening, flower senescence, auxin, wounding, chilling injury, drought stress and flooding. These biotic and abiotic stimuli can trigger production of ethylene. During ripening, ACC oxidase is activated to synthesize ethylene (Kieber, 2006). Ethylene production can be inhibited by aminoethoxyvinyl glycine (AVG) and aminooxyacetic acid (AOA), which inhibit ACC synthase activity or suppressed by anaerobiosis, cobalt, more than 35 ^{o}C temperature, and free radical scavengers, which negatively affect the activity of ACC oxidase (Saltveit, 1999).

Fruits have been classified as climacteric and nonclimacteric on the basis of respiration and ethylene production during ripening. The apple is a climacteric fruit because of the presence of a respiratory climacteric and autocatalytic ethylene production. Other climacteric fruits include the avocado, banana, mango, peach, pear, persimmon, plum and tomato. In contrast, citrus, cherry, watermelon, strawberry and pineapple are categorized as nonclimacteric fruits because those fruits do not show increased ethylene production and respiration after harvest (Burg and Burg, 1965).

2.3.7. Polyamines

Polyamines are low molecular weight aliphatic amines, which are ubiquitously presented in all living organisms. In plants, of many kinds of polyamines, diamine putrescine [butane-1,4-diamine], triamine spermidine [*N*-(3-aminopropyl) butane-1,4-diamine], and tetraamine spermine [*NN'-bis*-(3-aminopropyl)butane-1,4-diamine] are predominant and further, in general, putrescine and spermidine are much more abundant than spermine. Polyamines can be localized in the vacuole, mitochondria and chloroplasts. Polyamines play an important role as a positive charged compound because the amine groups of the polyamines are protonated at physiological pH. Thus, putrescine, spermidine and spermine possess two, three and four positive charges, respectively. Therefore, this

essential characteristic makes polyamines electrostatically react with negative charged macromolecules such as, DNA, RNA, protein, acidic phospholipids and cell wall components, for example, pectic polysaccharides. Polyamines are presented as not only free forms but also conjugates bound to phenolic compounds, low molecular weight compounds, and macromolecular ones. Polyamines have been also known to function for numerous plant growth and development events, and abiotic and biotic stress responses by preventing leakage and causing stabilization under adverse environmental conditions (Smith, 1985; Heby and Persson, 1990; Kumar et al., 1997; Kaur-Sawhney et al., 2003; Alcázar et al., 2006).

The biosynthetic pathways of polyamines have been well characterized and documented in detail. There are three main anabolic pathways revealed for the production of diamine putrescine from arginine. First, amino acid arginine is decarboxylated to agmatine, catalyzed by arginine decarboxylase (ADC, EC 4.1.1.19). At the same time, one molecule of CO_2 is released during this reaction. Agmatine can then be converted to *N*-carbamoylputrescine by agmatine iminohydrolase (AIH), and putrescine is produced from *N*-carbamoylputrescine by *N*-carbamoylputrescine amidohydrolase (CPA). Another pathway for the biosynthesis of putrescine uses ornithine, which is converted from arginine catalyzed by arginase. Ornithine is then converted to putrescine by ornithine decarboxylase (ODC, EC 4.1.1.17) which removes carboxyl group from ornithine. At this step, CO_2 one molecule is also released. The other anabolic pathway is producing citruline from either arginine or ornithine, and then citruline can be transformed into *N*-carbamoylputrescine catalyzed by citrulline decarboxylase (CDC). These putrescines produced by three anabolic pathways can react with decarboxylated *S*-adenosylmethionine (dcSAM) produced by SAM decarboxylase (SAMDC, EC 4.1.1.50) to synthesize spermidine, catalyzed by spermidine synthase (SPDS, EC 2.5.1.16) and then the spermidine and dcSAM are converted to spermine, catalyzed by spermine synthase

(SPMS, EC 2.5.1.22). At the same time, dcSAM as an aminopropyl donor provides aminopropyl groups to both putrescine and spermidine and is converted to methylthioadenosine (MTA) catalyzed by either SPDS or SPMS, respectively. dcSAM is yielded from SAM, which is the intermediate compound of methionine cycle and the precursor of ethylene as well (Kumar et al., 1997; Bagni and Tassoni, 2001; Martin-Tanguy, 2001; Kaur-Sawhney et al., 2003; Alcázar et al., 2006; Mattoo and Handa, 2008).

Putrescine is degraded into Δ^1-pyrroline, ammonia and H_2O_2, catalyzed by diamine oxidase (DAO, EC 1.4.3.6) under the presence of O_2. Spermidine can be degraded into 1-(3-aminopropyl)-pyrroline, ammonia and H_2O_2, catalyzed by DAO, or converted into 1,3-diaminopropane, Δ^1-pyrroline and H_2O_2, catalyzed by polyamine oxidase (PAO, EC 1.4.3.3) under the presence of O_2. Then, Δ^1-pyrroline degraded from either putrescine or spermidine can react with NAD^+ to biosynthesize γ-aminobutyric acid (GABA) and NADH, catalyzed by pyrroline dehydrogenase (PDH). GABA can be transaminated into succinyl semialdehyde by GABA transaminase (GABA-T, EC 2.6.1.19) and then succinyl semialdehyde can be oxidized into succinate, catalyzed by succinyl semialdehyde dehydrogenase (SSADH, EC 1.2.1.24). Finally, the resulting product succinate can be incorporated into the Krebs cycle. Spermine can also be degraded into 1,3-diaminopropane and 1-(3-aminopropyl)-pyrroline, catalyzed by PAO. 1,3-diaminopropane produced from either spermidine or spermine can be converted into β-alanine. 1-(3-aminopropyl)-pyrroline is subsequently converted into 1,5-diazabicylononane. The catalytic activity of PAO also produces H_2O_2. H_2O_2, generated by the catalytic activity of either DAO or PAO can potentially provide defense signaling against biotic and abiotic stress responses (Smith, 1985; Bouchereau et al., 1999; Bagni and Tassoni, 2001; Mattoo and Handa, 2008). Both DAO and PAO are cell wall localized enzymes (Kaur-Sawhney et al., 1981).

Spermidine and spermine inhibited ethylene production in apple fruit and protoplasts and in leaf tissues (Apelbaum et al., 1981). Also, the polyamines reduced the amount of ethylene produced by senescing petals of *Tradescatia.* Spermidine and spermine suppressed the ethylene production induced by the exogenous application of 1-aminocyclopropane-1-carboxylic acid (ACC) (Suttle, 1981). Apple fruit with watercore produced more ethylene, putrescine, spermidine, ACC and 1-(malonylamino) cyclopropane-1-carboxylic acid (MACC) than those without the disorder. While SAM was remained at a steady-state level, ACS and ACO activities in watercored apple were much higher than controlled ones (Wang and Faust, 1992). Pressure infiltration of polyamines increased fruit firmness of 'Golden Delicious' and 'McIntosh' apples, immediately and during storage (Kramer et al., 1991). In 1-MCP treated 'Orin' apple, free spermidine and spermine levels were not different than untreated ones (Pang et al., 2006).

2.3.8. Phenolic compounds

Phenolic compounds are generally described as a broad range of substances which contain one or more aromatic rings and hydroxyl group and are classified to phenolic acids, flavonoids, stibenes, coumarins and tannins. Phenolic acids are subdivided to hydroxylbenzoic acids and hydroxylcinnamic acids. Gallic, *p*-hydroxybenzoic, protocatechuic, vannilic and syringic acids are belonging to hydroxybenzoic acids derivatives and *p*-coumaric, caffeic, ferulic and sinapic acids to hydroxycinnamic acids derivatives. Flavonoids are also subcategorized to flavonols, flavones, flavanols, flavanones, anthocyanidins and isoflavonoids. Of the flavonoids, flavonols contain quercetin, kaempferol, myricetin, galangin and fisetin. Flavones include apigenin, chrysin and luteolin. Flavanols, known as catechins, contain catechin, epicatechin, epigallocatechin, epicatechin, gallate, epigallocatechin and gallate. Flvanones have eriodictyol, hesperitin and naringenin. Anthocyanidins contain cyaniding, pelargonidin,

delphinidin, peonidin and malvidin. Last, isoflavonoids include genistein, daidzein, glycitein and formononetin (Liu, 2004).

In apple fruit and products, the main phenolic compounds are the phenolic acids, flavan-3-Ols, flavonols, dihydrochalcones and anthocyanins. 5'-caffeoylquinic (chlorogenic acid), 4'-caffeoylquinic (cryptochlorogenic acid), 3'-caffeoylquinic (neochlorogenic acid), 5'-*p*-coumaroylquinic, 4'-*p*-coumaroylquinic, 3'-*p*-coumaroylquinic, *p*-coumaroylglucose, caffeoylglucose and feruloylglucose are belonging to phenolic acids. (-)-Epicatechin, (+)-catechin, B1, B2, B5 and C1 are categorized to favan-3-Ols. Flavonols contain quecetin-3-*O*-β-D-galactopyranoside (hyperin), quecetin-3-*O*-β-D-glucopyranoside (isoquercitrin), quercetin-3-*O*-β-D-xyloside (reynoutrin), quercetin-3-*O*-α-L-rhamnopyranoside (quercitrin), quercetin-3-*O*-α-L-aravinofuranoside (avicularin), quercetin-3-*O*-rutinoside (rutin). Dihydrochalcones are more subclassified to phloretin-2'-*O*-glucoside (phlorizin), and phloretin-2'-xyloglucoside. The anthocyanins include cyaniding-3-galactoside (ideain), cyaniding-3-glucoside (kuromarin), cyaniding-3-xyloside, and cyaniding-3-arabinoside (Nicolas et al., 1994).

Both the shikimic acid pathway and the malonic acid pathway are involved in the biosynthesis of phenolic compounds, although the former contributes to the biosynthesis of most plant phenolic compounds. It produces the three essential aromatic amino acids, phenylalanine, tyrosine and tryptophan from carbohydrate precursors derived from the pentose phosphate pathway and glycolysis. Of the three aromatic amino acids, phenylalanine basically functions as a substrate to produce the most abundant classes of phenolic compounds in plants catalyzed by PAL. The malonic acid pathway, which is more important as a source of phenolic compounds in fungi and bacteria, uses acetyl-CoA as a precursor to produce phenolic compounds (Engelberth, 2006).

Flesh browning is associated with polyphenol oxidase (PPO, EC 1.10.3.1) activity on phenolic compounds in presence of oxygen, causing the hydroxylation of

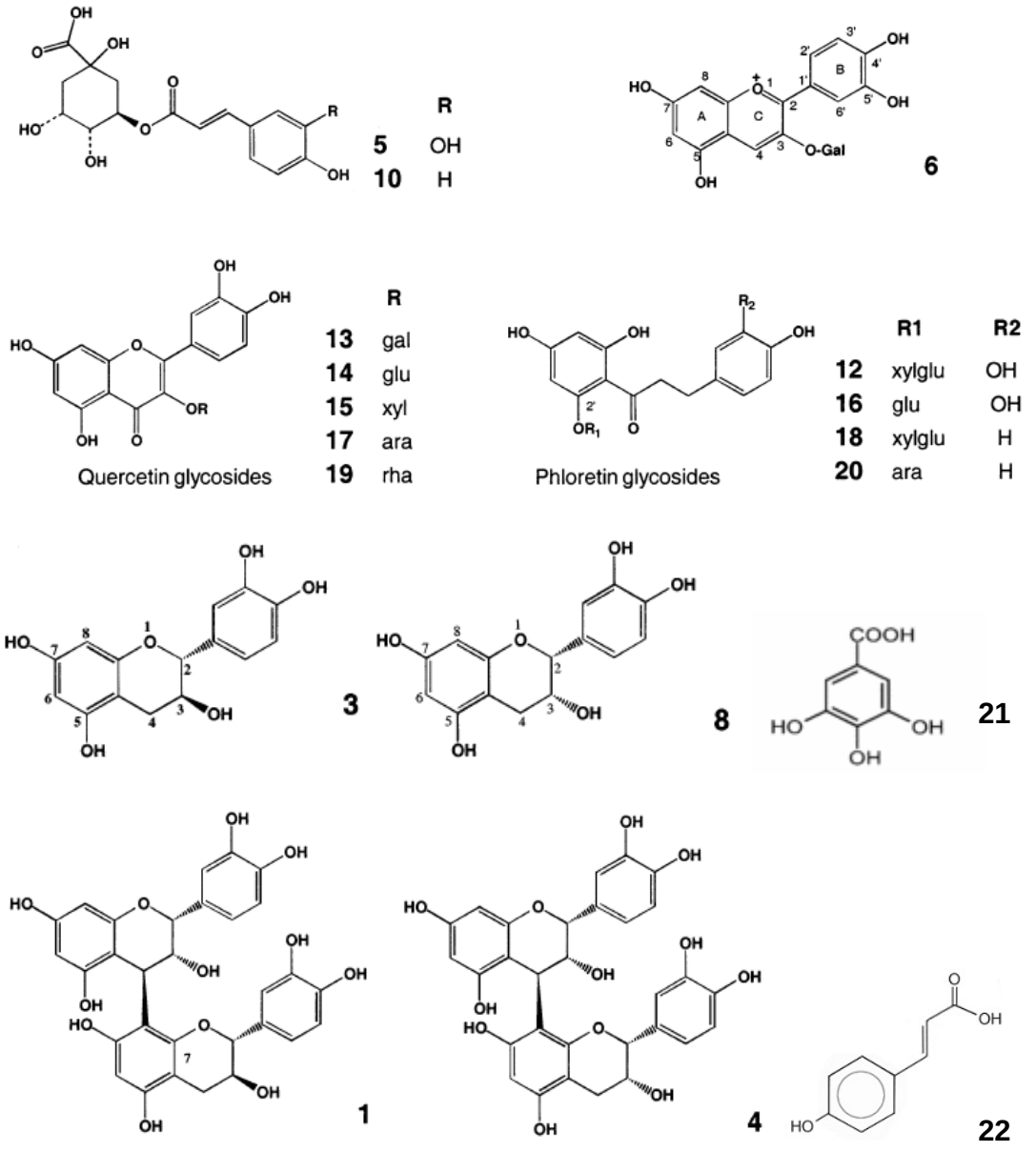

Figure 2.2. The chemical structures of the most commonly found phenolic compounds in apple fruits. **1**, procyanidin B1; **3**, catechin; **4**, procyanidin B2; **5**, chlorogenic acid; **6**, cyanidin 3-galactoside; **8**, epicatechin; 10, *p*-coumaroylquinic acid; 12, 3-hydroxyphloretin 2'-xyloglucoside; 13, quercetin 3-galactoside; 14, quercetin 3-glucoside; 15, quercetin 3-xyloside; 16, 3-hydroxyphloretin 2'-glucoside; 17, quercetin 3-arabinoside; 18, phloretin 2'-xyloglucoside; **19**, quercetin 3-rhamnoside; **20**, phloridzin; **21**, gallic acid; **22**, *p*-coumaric acid (Tsao et al., 2003; Boyer and Liu, 2004).

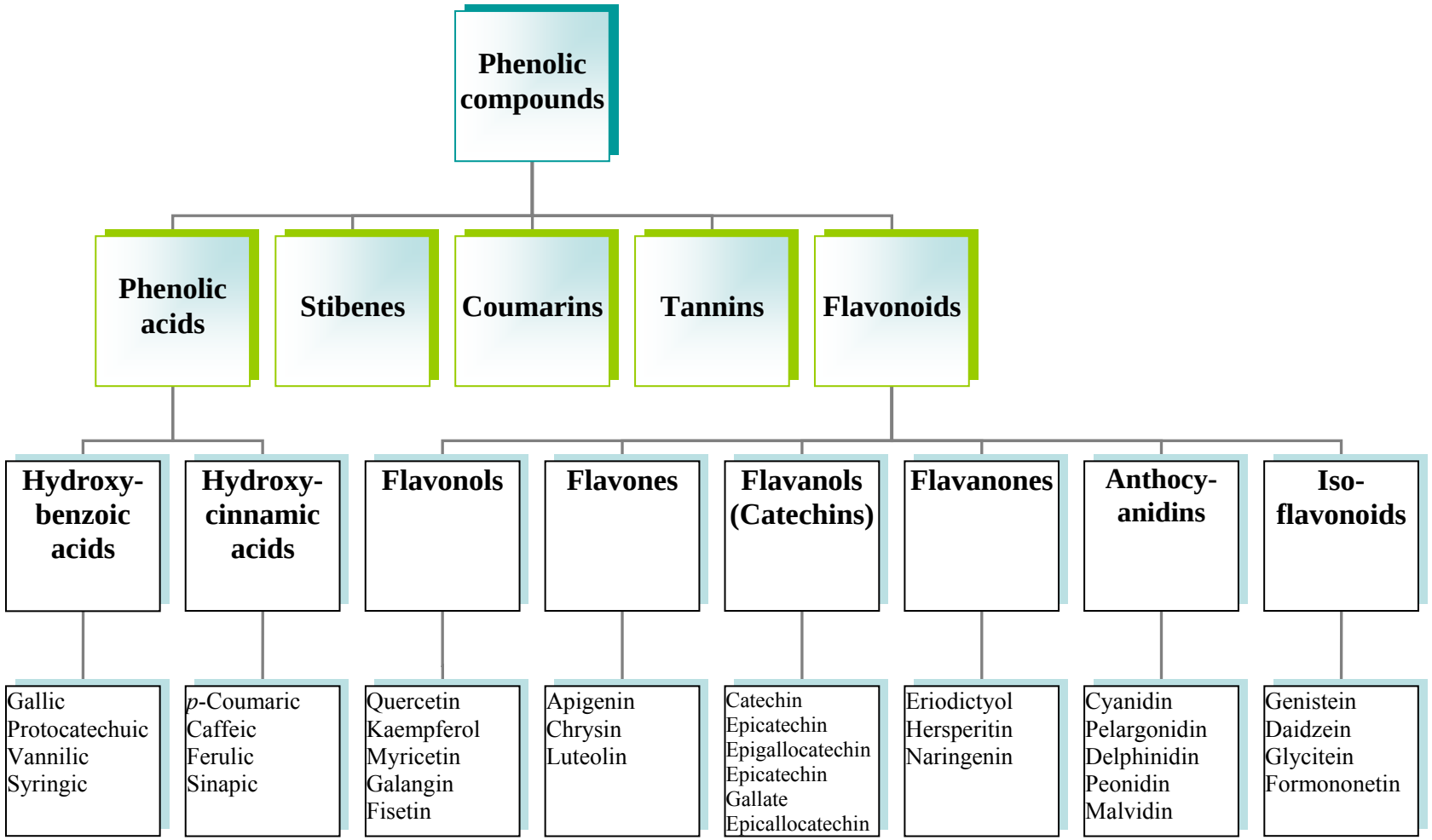

Figure 2.3. General classification of phenolic compounds in plants (Liu, 2004).

monophenolic compounds to *o*-diphenols and consequently oxidizing to *o*-quinones, via polymerization (Vamos-Vigyazo, 1981; Sapers, 1993). PPO in apples is present in chloroplasts and mitochondria (Nicolas et al., 1994). Also, laccase (EC 1.10.3.2), tyrosinase (EC1.14.18.1), and peroxidase (EC 1.11.1.1-2) activities are associated with browning. Laccase oxidizes *o*-diphenols and *p*-diphenols to their corresponding quinines. Tyrosinase referred to as monophenol monooxygenase catalyzes the hydroxylation of monophenols. POX (or POD) functions to oxidize hydrogen donors at the expense of peroxides (Nicolas et al., 1994).

PPO activity remained relatively constant in the seven apple cultivars during cold storage (Coseteng and Lee, 1987). PPO activities were directly associated with browning in 'Delicious', 'Rhode Island Greening', 'McIntosh' and 'Cortland' but the concentration of phenolic compounds was more related to the browning in 'Empire', 'Rome' and 'Golden Delicious' (Coseteng and Lee, 1987). PPO activity decreased with the higher CO_2 concentrations (Galvis-Sanchez et al., 2006). In 'Conference' pear, PPO activity was lower in brown tissue than in healthy tissues without damage (Veltman et al., 1999a). Tyrosinase activity was inversely associated with incidence of brown core. Furthermore, as CO_2 concentrations increased, the activities of total tyrosinase and active tyrosinase decreased (Veltman et al., 1999a).

2.3.9. Antioxidant scavenging system

Elevated CO_2 and reduced O_2 concentrations can lead to stress to the commodity during storage. Under these abiotic stresses, oxidative processes induce accumulation of reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\cdot-}$), H_2O_2, hydroxyl ($HO^{\cdot}$) and perhydroxyl ($O_2H^{\cdot}$) radicals, nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^{\cdot}$) (Toivonen, 2004). These ROS are fairly stable and involved in

generally anabolic and catabolic metabolism such as photosynthesis and respiration. However, when ROS accumulate above critical threshold levels, they play a pivotal role in the precursors for the development of more destructive ROS, such as the hydroxyl radical ($OH^{\cdot}$), consequently causing lipid peroxidation, cell membrane deterioration, DNA and RNA damage, followed by biochemical and metabolic malfunctions and ultimately cell death (Mittler, 2002; Toivonen, 2004). In response to oxidative stress, plants generally generate and/or increase their own numerous enzymatic and non-enzymatic antioxidant scavenging systems. Enzymes involved in this system include superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2) (Mittler, 2002). SOD and CAT play a significant role in the production and degradation of H_2O_2, from superoxide anion ($O_2^{\cdot-}$) and to H_2O, respectively, while APX and GR are associated with ascorbate regeneration (Mittler, 2002). SOD is present in almost all cellular components, whereas CAT is in peroxisome. APX and GR in the ascorbate-glutathione cycle are found in almost all cellular components. Peroxidase (POX or POD) also scavenges H_2O_2 and thereby detoxifies into H_2O in cell wall, cytosol and vacuole (Mittler, 2002).

In addition to enzymatic antioxidant systems, ascorbic acid (vitamin C), α-tocopherol (vitamin E), β-carotene, glutathione (GSH) and other flavonoids also provide non-enzymatic scavenging systems (Larson, 1988). Ascorbic acid and glutathione, with their related enzymatic components, are a major antioxidant pathway, known as the ascorbate-glutathione cycle, which scavenges H_2O_2 to H_2O in chloroplasts, cytoplasm, mitochondria, apoplast and peroxisomes. GR, monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) are involved in the ascorbate-glutathione cycle, using electrons derived from NAD(P)H via the pentose phosphate shunt pathway. Optimum scavenging ROS in

cells is fundamentally important to be constantly adjusted a high reduced per oxidized ratio of ascorbic acid and glutathione (Mittler, 2002). In the ascorbate-glutathione cycle, ascorbic acid is oxidized to monodehydroascorbic acid (MDA) catalyzed by APX and then MDA is again reduced to ascorbic acid catalyzed by MDAR using NAD(P)H as reducing power. During that time, H_2O_2 and NAD(P)H are converted to H_2O and $NAD(P)^+$, respectively. Dehydroascorbic acid (DHA) can be reduced to ascorbic acid catalyzed by glutathione (GSH)-dependent DHAR. At the same time, DHAR can convert reduced glutathione (GSH) to oxidized glutathione (GSSG) and then GSSG can also be converted to GSH catalyzed by GR with a reducing power of NAD(P)H. These concomitantly consecutive reactions of ascorbic acid-glutathione cycle can scavenge H_2O_2 to H_2O with the consumption of NAD(P)H (Smirnoff, 2000; Mittler, 2002).

In 'Braeburn' apples stored at 1.5 kPa O_2 and 1.2 kPa CO_2 at 0 ^oC for 1.5 months, SOD activity was inversely related with BBD incidence depending on harvest time (Gong et al., 2001). SOD activity showed the biggest difference among different orchards, compared with POD and CAT activities. The activities of water and lipid soluble antioxidants also showed significant differences within fruits from different orchards. BBD incidence and antioxidant enzyme activities vary by harvest season (Gong et al., 2001).

In 'Blanquilla' pear stored in 2 kPa O_2 with 5 kPa CO_2 for 6 months, SOD, APX and GR activities were enhanced when compared with those activities at the harvest time, but CAT activity was reduced during storage regardless of the presence or absence of fruit injuries (Pintó et al., 2001). The activities of SOD, CAT and APX were slightly lower in damaged fruit than in healthy fruit. However, GR activity was inversely higher in damaged pear fruit (Pintó et al., 2001). Larrigaudiere et al. (2001) found that SOD, APX and LOX activities increased with increased CO_2

concentrations in ‘Blanquilla’ pears, whereas CAT and GR activities were unaffected. Short term (2 or 3 weeks) storage of ‘Conference’ pear in air or 2 kPa O_2 with 5 kPa CO_2 resulted in enhanced SOD activity but lower CAT activity (Larrigaudière et al., 2001b; Larrigaudière et al., 2003). APX and GR activities increased rapidly during the early stages of CA storage. Vilaplana et al. (2006) found no difference in total antioxidant, or SOD, and CAT activities between 1-MCP-treated and untreated ‘Golden Smoothee’ apple during air storage. However, POX activity gradually increased in untreated apple but not in 1-MCP-treated apples. Total antioxidant activity was continuously enhanced until 30 days of storage period and then declined nearly to the same level as the starting point of storage in both 1-MCP-treated and untreated apples.

In ‘Blanquilla’ pear, ascorbic acid concentration was reduced more during the early CA storage than in air storage and then recovered with the progress of storage time (Larrigaudiere et al., 2001). Also, less ascorbic acid accumulated during storage with higher CO_2 concentrations. Glutathione concentration was not affected by either air or CA storage but during the early storage time, glutathione concentration decreased or was maintained constant and then sharply increased at the end of storage (Larrigaudiere et al., 2001). Ascorbic acid concentrations fluctuated more in air stored ‘Conference’ than ‘Blanquilla’ pear, but fluctuated less in elevated CO_2 concentrations. In 5 kPa CO_2 storage regime, total ascorbic acid concentrations sharply reduced at the early period of storage and then remained constant (Larrigaudière et al., 2001a). In ‘Conference’ pear stored at numerous storage regimes, ascorbic acid concentrations were highest in peel tissue and lowest at core tissue, while the cortex tissue contained an intermediate concentration of ascorbic acid regardless of storage regimes (Veltman et al., 1999b). Lower ascorbic acid concentrations in CO_2 treated fruit were negatively associated with browning

incidence in pears stored in 2 kPa O_2 with 10 kPa CO_2. They suggested that browning might be initiated when ascorbic acid concentrations decreased to less than 1.3 mg level. When healthy tissues were compared with injured tissues, ascorbic acid concentrations were lower in storage injured tissues, but glutathione concentrations were twofold greater (Pintó et al., 2001). During the short term (3 weeks) storage, total ascorbic acid and glutathione concentrations rapidly declined in 'Conference' pear stored at both air and CA (2 kPa O_2 and 5 kPa CO_2) storage regimes. However, there was a significant enhancement of ascorbic acid found during the first day of both storages and the reduced form of glutathione (GSSG) was higher in the CA storage than in air storage (Larrigaudière et al., 2001b). In 'Golden Smoothee' apple treated with 1-MCP, ascorbic acid concentrations were similar to the untreated apple fruit. The concentration of ascorbic acid sharply declined and then remained constant (Vilaplana et al., 2006). The concentrations of H_2O_2 maintained lower in 1-MCP-treated than in untreated apples and then slightly increased at the end of storage.

2.3.10. GABA shunt pathway

GABA is a four-carbon non-protein amino acid. The concentrations of GABA accumulate in response to the numerous adverse environmental stresses. The GABA metabolite pathway is also known as the GABA shunt pathway because it bypasses two steps of TCA cycle from 2-OG to succinate through glutamate and GABA. After 2-OG converts into glutamate catalyzed by glutamate dehydrogenase (GDH, EC 1.4.1.3) in mitochondria, the α-decarboxylation of glutamate decarboxylase (GAD, EC 4.1.1.15) converts directly and irreversibly glutamate into GABA with the production of CO_2 in the cytosol. Then, in mitochondria, GABA transaminase (GABA-T, EC 2.6.1.19) catalyzes the reversible conversion of GABA to succinic semialdehyde using either pyruvate or α-ketoglutarate as amino acceptor, which converted into glutamate and alanine, respectively.

Finally, succinic semialdehyde can be catalyzed by succinic semialdehyde dehydrogenase (SSADH, EC 1.2.1.16), irreversibly oxidizing succinic semialdehyde to succinate. In this catalytic reaction, NAD^+ can be reduced back to NADH by SSADH catalytic reaction. GABA and GABA shunt in plants play several important roles in contribution of the C:N balance, regulation of cytosolic pH, protection against oxidative stress, defense against insects, osmoregulation and a signal molecule (Shelp et al., 1999; Bouché and Fromm, 2004; Fait et al., 2008). The concentration of GABA was increased in brown tissues of 'Conference' pear stored at 1 kPa O_2 and 10 kPa CO_2 at -1 ^oC than in sound tissue (Pedreschi et al., 2009).

2.3.11. Cell membrane lipid metabolites

Lipid peroxidation occurs during development of several metabolic disorders of fruit. During storage, ROS such as $O_2^{\cdot-}$ and H_2O_2 are produced from electron transport system in mitochondria and plasma membranes. These ROS can interact with iron species (Fe^{2+} or Fe^{3+}) in membranes causing to formation of lipid-free radicals (Shewfelt and Purvis, 1995). Lipid degradation and accumulation of free polyunsaturated fatty acids (PUFA) occurs by enzymatic oxidation of PUFA by lipoxygenase (LOX, EC 1.13.11.12). Otherwise, ROS nonenzymatically oxidizes to form lipid hydroperoxides (LOOH) in plant tissue. These lipid hydroperoxides and lipid peroxidation can contribute to loss of membrane integrity, alter the function of membrane-embedded and peripheral proteins and change the conformation of critical enzymes, resulting in cellular membrane decompartmentation and the loss of tissue structure (Dhindsa et al., 1981; Bartoli et al., 1996). These cellular damages enhance membrane permeability and may be expressed as increased ion leakage (Marangoni et al., 1996). In apples, ion leakage is also enhanced as senescence progresses, and decreased membrane viscosity is correlated with the increase of the amount of

phospholipids in membranes (Lurie et al., 1987). Ion leakage caused by the cellular membrane decompartmentation is associated with reactions between phenolic compounds accumulated at vacuole and tyrosinase located at plastids or PPO located at cytoplasm, and browning (Veltman and Peppelenbos, 2003).

2.4. 1-Methylcyclopropene (1-MCP)

1-MCP is a colorless gas with a molecular weight of 54, a formula of C_4H_6, with a double bond between carbon number one and two, and a methyl group on number one carbon (Blankenship and Dole, 2003; Watkins, 2006a). Commercially, 1-MCP can be complexed with γ-cyclodextrin to make a stable compound, and then 1-MCP is released as a gas when the 1-MCP complex is dissolved in water. 1-MCP is thought to competitively bind to the copper ion of the ethylene receptor on the ER membrane and thereby, endogenous and exogenous ethylene cannot bind to the receptors (Sisler et al., 1999; Sisler and Serek, 2003). The ethylene receptors are negative regulators of ethylene production and perception. That is, when the ethylene receptors are not bound to ethylene, these receptors actively inhibit ethylene response or ethylene signal transduction pathways. On the other hand, when the receptors are bound to ethylene, it allows ethylene signal transduction pathway for further physiological responses, like fruit ripening, or senescence (Hua and Meyerowitz, 1998; Alonso and Stepanova, 2004). 1-MCP is more actively bound to ethylene receptors than ethylene because the affinity of 1-MCP for the ethylene receptors is much greater than that of ethylene (Blankenship and Dole, 2003). Thus, all the receptors could be saturated with 1-MCP rather than ethylene. However, it has also been proposed that although 1-MCP irreversibly binds one or more binding sites of ethylene receptors (Reid and Celikel, 2008), when 1-MCP is not fully saturated on binding to all the ethylene receptors, it can keep sending a negative signal to ethylene signal transduction pathways catalyzed by mitogen-activated protein (MAP) kinase cascades

(Stepanova and Alonso, 2009). Therefore, although the majority of ethylene receptors are bound by ethylene, the activation of ethylene induced genes is still inhibited by the continuous signal transduction pathways of MAP kinase cascades from the unsaturated 1-MCP binding on ethylene receptors (Binder and Bleecker, 2003).

1-MCP reduces ethylene production, respiration and loss of fruit firmness and titratable acidity in apple fruit, thereby enhancing storage life of apple fruit (Fan et al., 1999a; Watkins et al., 2000). 1-MCP treatment combined with CA storage can further delay ripening and extend the storage life of fruit (Watkins et al., 2000; DeLong et al., 2004; Bai et al., 2005). 1-MCP affects a wide range of physiological processes associated with ripening. These include the production of volatile compounds in apple fruit (Bai et al., 2005; DeEll et al., 2005; Kondo et al., 2005). Moreover, 1-MCP reduced ester production and the activity of alcohol acyl-CoA transferase, a key enzyme in ester biosynthesis (Defilippi et al., 2005a). In air stored 'Red Delicious', 1-MCP treatment increased the peel concentrations of the anthocyanin derivatives, cyanidin 3-galactoside, cyanidin 3-rutinoside and cyanidin 3-arabinoside, but did not affect the concentrations of the flavonol derivatives, quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-rhamnoside and quercetin aglycone (MacLean et al., 2006). Furthermore, 1-MCP treatment enhanced the concentrations of epicatechin belonging to flavan-3-ols but did not influence the concentration of catechin, the derivatives of flavan-3-ols. Also, the concentration of chlorogenic acid, categorized as hydroxycinnamic acid, was increased during air storage (MacLean et al., 2006). The more advanced the fruit maturity, the less the effectiveness of 1-MCP in enhancing chlorogenic acid concentrations in the peels (MacLean et al., 2006). In addition to the responses of phenolic compounds to 1-MCP, PPO and POD activities tended to be lower in 1-MCP treated than in untreated 'Delicious' apple skin for 12 weeks air storage (Arquiza et al., 2005). This POD response is in agreement with the result of

Shaham et al. (2003) who reported that POD activity was reduced by 1-MCP treatment in peel tissues of 'Granny Smith' in air storage.

1-MCP did not affect spermidine and spermine titers of polyamine compounds in 'Orin' apple fruit but spermidine titer increased at 5 day after 1-MCP treatment and then slowly decreased, while putrescine level continuously declined (Pang et al., 2006). 1-MCP treatment reduced H_2O_2 concentration and increased POX activity and enzymatic antioxidant potential in 'Golden Smoothee' apple during cold storage (Vilaplana et al., 2006). Ascorbic acid concentration was not affected by 1-MCP treatment in 'Empire' apple during CA and air storage but its concentration declined during storage (Fawbush et al., 2009). 1-MCP reduced ATP concentration in 'Jonagold' apple but untreated apple increased ATP concentration. The ratio of ATP to ADP in 1-MCP treated fruit was higher than that in untreated ones (Xuan, 2008).

In addition to the metabolic responses, 1-MCP reduced certain storage disorders, such as superficial scald, soft scald, soggy breakdown and bitter pit (Zanella, 2003; DeEll and Ehsani-Moghaddam, 2010; Pesis et al., 2010) but increased the susceptibility of fruit to external and internal CO_2 injury (Fawbush et al., 2008) and firm flesh browning in CA stored 'Empire' apple at 3 ^{o}C (Watkins, 2008).

2.5. Summary

Although 1-MCP treatment maintains fruit firmness and delays fruit ripening by inhibiting ethylene production and perception over the long term storage period, 1-MCP can exacerbate storage disorder such as firm flesh browning in 'Empire' apple. The etiology of flesh browning has been not well characterized but the final reaction of flesh browning was well understood by enzymatic oxidation of phenolic compounds catalyzed with PPO and POX activities, when the cellular integrity is loss as a result of stress. However, these responses are the final consequence rather than the cause of browning.

Primary and secondary metabolisms in fruit prior to flesh browning development are largely unknown. In this study, two main approaches have been utilized to identify any causal responses. The first is an examination of enzymatic and non-enzymatic antioxidant metabolism, while the second is assessment of untargeted metabolomic responses by using GC-MS with PLS-DA statistical modeling system.

REFERENCES

Ackermann, J., Fischer, M., Amado, R., 1992. Changes in sugars, acids, and amino acids during ripening and storage of apples (cv. Glockenapfel). J. Agric. Food Chem. 40, 1131-1134.

Adams, D.O., Yang, S.F., 1979. Ethylene biosynthesis:identification of 1-aminocyclopropane-1-carboxylic acid as an intermediate in the conversion of methionine to ethylene. Proc. Natl. Acad. Sci. U.S.A. 76, 170-174.

Aharoni, A., Keizer, L.C.P., Bouwmeester, H.J., Sun, Z., Alvarez-Huerta, M., Verhoeven, H.A., Blaas, J., van Houwelingen, A.M.M.L., De Vos, R.C.H., van der Voet, H., Jansen, R.C., Guis, M., Mol, J., Davis, R.W., Schena, M., van Tunen, A.J., O'Connell, A.P., 2000. Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarrays. Plant Cell 12, 647-662.

Alcázar, R., Marco, F., Cuevas, J., Patron, M., Ferrando, A., Carrasco, P., Tiburcio, A., Altabella, T., 2006. Involvement of polyamines in plant response to abiotic stress. Biotechnol. Letters 28, 1867-1876.

Alonso, J., M., Stepanova, A., N., 2004. The ethylene signaling pathway. Science 306, 1513.

Altisent, R., Echeverria, G., Graell, J., Lopez, L., Lara, I., 2009. Lipoxygenase activity is involved in the regeneration of volatile ester-synthesizing capacity after ultra-low oxygen storage of 'Fuji' apple. J. Agric. Food Chem. 57, 4305-4312.

Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. 55, 373-399.

Apelbaum, A., Burgoon, A.C., Anderson, J.D., Lieberman, M., 1981. Polyamines inhibit biosynthesis of ethylene in higher plant tissue and fruit protoplasts. Plant Physiol. 68, 453-456.

Argenta, L.C., Mattheis, J.P., Fan, X., Finger, F.L., 2004. Production of volatile compounds by Fuji apples following exposure to high CO_2 or low O_2. J. Agric. Food Chem. 52, 5957-5963.

Argenta, L.C., Fan, X., Mattheis, J.P., 2007. Responses of 'Golden Delicious' apples to 1-MCP applied in air or water. HortScience 42, 1651-1655.

Arquiza, J.M.R.A., Hay, A.G., Nock, J.F., Watkins, C.B., 2005. 1-Methylcyclopropene interactions with diphenylamine on diphenylamine degradation, α-farnesene and conjugated trienol concentrations, and polyphenol oxidase and peroxidase activities in apple fruit. J. Agric. Food Chem. 53, 7565-7570.

Bagni, N., Tassoni, A., 2001. Biosynthesis, oxidation and conjugation of aliphatic polyamines in higher plants. Amino Acids 20, 301-317.

Bai, J., Baldwin, E.A., Goodner, K.L., Mattheis, J.P., Brecht, J.K., 2005. Response of four apple cultivars to 1-methylcyclopropene treatment and controlled atmosphere storage. HortScience 40, 1534-1538.

Ball, S.G., van de Wal, M.H.B.J., Visser, R.G.F., 1998. Progress in understanding the biosynthesis of amylose. Trends Plant Sci. 3, 462-467.

Ball, S.G., Morell, M.K., 2003. From bacterial glycogen to starch: Understanding the biogenesis of the plant starch granule. Annu. Rev. Plant Biol. 54, 207-233.

Bartley, I., M. , 1986. Changes in sterol and phospholipid composition of apples during storage at low temperature and low oxygen concentration. J. Sci. Food Agric. 37, 31-36.

Bartley, I.M., 1976. Changes in the glucans of ripening apples. Phytochemistry 15, 625-626.

Bartoli, C.G., Simontacchi, M., Montaldi, E., Puntarulo, S., 1996. Oxidative stress, antioxidant capacity and ethylene production during ageing of cut carnation (*Dianthus caryophyllus*) petals. J. Exp. Bot. 47, 595-601.

Beaudry, R.M., 1999. Effect of O_2 and CO_2 partial pressure on selected phenomena affecting fruit and vegetable quality. Postharvest Biol. Technol. 15, 293-303.

Beaudry, R.M., 2000. Responses of horticultural commodities to low oxygen: limits to the expanded use of modified atmosphere packaging. HortTechnology 10, 491-500.

Berüter, J., 1985. Sugar accumulation and changes in the activities of related enzymes during development of the apple fruit. J. Plant Physiol. 121, 331-342.

Berüter, J., 2004. Carbohydrate metabolism in two apple genotypes that differ in malate accumulation. J. Plant Physiol. 161, 1011-1029.

Binder, B.M., Bleecker, A.B., 2003. A model for ethylene receptor function and 1-methylcyclopropene action. Acta Hort. 628, 177-187.

Blankenship, S.M., Dole, J.M., 2003. 1-Methylcyclopropene: A review. Postharvest Biol. Technol. 28, 1-25.

Blanpied, G.D., Johnson, D.S., Lau, O.L., Lidster, P.D., Lougheed, E.C., Porritt, S.W., 1990. Apples. Agriculture Canada Publications, 7-22.

Bouché, N., Fromm, H., 2004. GABA in plants: just a metabolite? Trends Plant Sci. 9, 110-115.

Bouchereau, A., Aziz, A., Larher, F., Martin-Tanguy, J., 1999. Polyamines and environmental challenges: recent development. Plant Sci. 140, 103-125.

Bown, A.W., 1985. CO_2 and intracellular pH. Plant Cell Environment 8, 459-465.

Boyer, J., Liu, R.H., 2004. Apple phytochemicals and their health benefits. Nutr. J. 3, 1-15.

Brackmann, A., Streif, J., Bangerth, F., 1993. Relationship between a reduced aroma production and lipid metabolism of apples after long-term controlled-atmosphere storage. J. Amer. Soc. Hort. Sci. 118, 243-247.

Bramlage, W.J., 1993. Interactions of orchard factors and mineral nutrition on quality of pome fruit. Acta Hort. 326, 15-28.

Buléon, A., Colonna, P., Planchot, V., Ball, S., 1998. Starch granules: structure and biosynthesis. Int. J. Biol. Macromol. 23, 85-112.

Burg, S.P., Burg, E.A., 1965. Gas exchange in fruits. Physiol. Plant. 18, 870-884.

Camm, E.L., Towers, G.H.N., 1973. Phenylalanine ammonia lyase. Phytochemistry 12, 961-973.

Cheng, H.-t., Criner, G.K., Kezis, A.S., 1997. The impact of consumer characteristics on preferences for selected apple varieties. J. Food Products Marketing 3, 1 - 11.

Coseteng, M.Y., Lee, C.Y., 1987. Changes in apple polyphenoloxidase and polyphenol concentrations in relation to degree of browning. J. Food Sci. 52, 985-989.

DeEll, J.R., Murr, D.P., Mueller, R., Wiley, L., Porteous, M.D., 2005. Influence of 1-methylcyclopropene (1-MCP), diphenylamine (DPA), and CO_2 concentration during storage on ‘Empire’ apple quality. Postharvest Biol. Technol. 38, 1-8.

DeEll, J.R., Ayres, J.T., Murr, D.P., 2010. Commercial application of 1-methylcyclopropene affects storability and disorders of 'Empire' apples. Acta Hort. 857, 107-114.

DeEll, J.R., Ehsani-Moghaddam, B., 2010. Preharvest 1-methylcyclopropene treatment reduces soft scald in 'Honeycrisp' apples during storage. HortScience 45, 414-417.

Defilippi, B.-G., Kader, A.-A., Dandekar, A.-M., 2005a. Apple aroma: alcohol acyltransferase, a rate limiting step for ester biosynthesis, is regulated by ethylene. Plant Sci. 168, 1199-1210.

Defilippi, B.G., Dandekar, A.M., Kader, A.A., 2005b. Relationship of ethylene biosynthesis to volatile production, related enzymes, and precursor availability in apple peel and flesh tissues. J. Agric. Food Chem. 53, 3133-3141.

DeLong, J.M., Prange, R.K., Harrison, P.A., 2004. The influence of 1-methylcyclopropene on 'Cortland' and 'McIntosh' apple quality following long-term storage. HortScience 39, 1062-1065.

Derkacz, M., Elfving, D.C., Forshey, C.G., 1993. The history of the 'Empire' apple. Fruit Var. J. 47, 70-71.

Dhindsa, R.S., Plumb-Dhindsa, P., Thorpe, T.A., 1981. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J. Exp. Bot. 32, 93-101.

Dimick, P.S., Hoskin, J.C., 1983. Review of apple flavor-state of the art. Crit. Rev. Food Sci. Nutr. 18, 387-409.

Dixon, J., Hewett, E.W., 2000. Factors affecting apple aroma/flavour volatile concentration: a review. N. Z. J. Crop Hortic. Sci. 28, 155-173.

Echeverría, G., Graell, J., López , M.L., Lara, I., 2004. Volatile production, quality and aroma-related enzyme activities during maturation of 'Fuji' apples. Postharvest Biol. Technol. 31, 217-227.

El-Goorani, M.A., Sommer, N.F., 1981. Effects of modified atmospheres on postharvest pathogens of fruits and vegetables. Hort. Rev. 3, 412-461.

Engelberth, J., 2006. Secondary metabolites and plant defense. In: Taiz, L., Zeiger, E. (Eds.), Plant Physiology. Sinauer Associates, Inc. Publishers, Sunderland, Massachusetts. pp. 315-344.

Ernst, M., Matitschka, G., Chatterton, N., Harrison, P., 1999. A Quantitative Histochemical Procedure for Measurement of Starch in Apple Fruits. Histochemical J. 31, 705-710.

Fait, A., Fromm, H., Walter, D., Galili, G., Fernie, A.R., 2008. Highway or byway: the metabolic role of the GABA shunt in plants. Trends Plant Sci. 13, 14-19.

Fan, X., Mattheis, J.P., Patterson, M.E., Fellman, J.K., 1995. Changes in amylose and total starch content in `Fuji' apples during maturation. HortScience 30, 104-105.

Fan, X., Mattheis, J.P., Buchanan, D., 1998. Continuous requirement of ethylene for apple fruit volatile synthesis. J. Agric. Food Chem. 46, 1959-1963.

Fan, X., Blankenship, S.M., Mattheis, J.P., 1999a. 1-Methylcyclopropene inhibits apple ripening. J. Amer. Soc. Hort. Sci. 124, 690-695.

Fan, X., Mattheis, J.P., 1999. Impact of 1-methylcyclopropene and methyl jasmonate on apple volatile production. J. Agric. Food Chem. 47, 2847-2853.

Fan, X., Mattheis, J.P., Blankenship, S., 1999b. Development of apple superficial scald, soft scald, core flush, and greasiness is reduced by MCP. J. Agric. Food Chem. 47, 3063-3068.

Fawbush, F., Nock, J.F., Watkins, C.B., 2008. External carbon dioxide injury and 1-methylcyclopropene (1-MCP) in the 'Empire' apple. Postharvest Biol. Technol. 48, 92-98.

Fawbush, F., Nock, J.F., Watkins, C.B., 2009. Antioxidant contents and activity of 1-methylcyclopropene (1-MCP)-treated 'Empire' apples in air and controlled atmosphere storage. Postharvest Biol. Technol. 52, 30-37.

Fellman, J.K., Rudell, D.R., Mattinson, D.S., Mattheis, J.P., 2003. Relationship of harvest maturity to flavor regeneration after CA storage of `Delicious' apples. Postharvest Biol. Technol. 27, 39-51.

Ferenczi, A., Song, J., Tian, M., Vlachonasios, K., Dilley, D., Beaudry, R., 2006. Volatile ester suppression and recovery following 1-methylcyclopropene application to apple fruit. J. Amer. Soc. Hort. Sci. 131, 691-701.

Fernández-Trujillo, J.P., Nock, J.F., Watkins, C.B., 2001. Superficial scald, carbon dioxide injury, and changes of fermentation products and organic acids in 'Cortland' and 'Law Rome' apples after high carbon dioxide stress treatment. J. Amer. Soc. Hort. Sci. 126, 235-241.

Fidler, J.C., Wilkinson, B.G., Sharples, R.O., 1973. The biology of apple and pear storage. Res. Rev. 3, Commonwealth Agricultural Bureaux, Hort. Plantation Crops, East Malling, England.

Flath, R.A., Black, D.R., Guadagni, D.G., McFadden, W.H., Schultz, T.H., 1967. Identification and organoleptic evaluation of compounds in 'Delicious' apple essence. J. Agric. Food Chem. 15, 29-35.

Frenkel, C., Patterson, M.E., 1973. Effect of carbon dioxide on activity of succinic dehydrogenase in 'Bartlett' pears during cold storage. HortScience 8, 395-396.

Gaffney, J.J., 1978. Humidity: basic principles and measurement techniques. HortScience 13, 551-555.

Galvis-Sanchez, A.C., Fonseca, S.C., Gil-Izquierdo, A., Gil, M.I., Malcata, F.X., 2006. Effect of different levels of CO_2 on the antioxidant content and the polyphenol oxidase activity of 'Rocha' pears during cold storage. J. Sci. Food Agric. 86, 509-517.

Geigenberger, P., 2003. Response of plant metabolism to too little oxygen. Curr. Opin. Plant Biol. 6, 247-256.

Goffinet, M.C., Robinson, T.L., Lakso, A.N., 1995. A comparison of 'Empire' apple fruit size and anatomy in unthinned and hand-thinned trees. J. Hort. Sci. Biotech. 70, 375-387.

Gong, Y., Toivonen, P.M.A., Lau, O.L., Wiersma, P.A., 2001. Antioxidant system level in 'Braeburn' apple is related to its browning disorder. Bot. Bull. Acad. Sin. 42, 259-264.

Graham, D., Patterson, B.D., 1982. Responses of plants to low, nonfreezing temperatures: proteins, metabolism, and acclimation. Annu. Rev. Plant Physiol. 33, 347-372.

Grierson, W., Wardowski, W.F., 1978. Relative humidity effects on the postharvest life of fruits and vegetables. HortScience 13, 570-574.

Hansen, P., 1970. ^{14}C-studies on apple trees. V. Translocation of labelled compounds from leaves to fruit and their conversion within fruit. Physiol. Plant. 23, 564-573.

Harada, M., Ueda, Y., Iwata, T., 1985. Purification and some properties of alcohol acetyltransferase from banana fruit. Plant Cell Physiol. 26, 1067-1074.

Harel, K., Mayer, A.M., Shain, Y., 1964. Catechol Oxidases from Apples, their Properties, Subcellular Location and Inhibition. Physiol. Plant. 17, 921-930.

Heby, O., Persson, L., 1990. Molecular genetics of polyamine synthesis in eukaryotic cells. Trends Biochem. Sci. 15, 153-158.

Hecke, K., Herbinger, K., Veberic, R., Trobec, M., Toplak, H., Stampar, F., Keppel, H., Grill, D., 2006. Sugar-, acid- and phenol contents in apple cultivars from organic and integrated fruit cultivation. Eur. J. Clin. Nutr. 60, 1136-1140.

Hua, J., Meyerowitz, E.M., 1998. Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell 94, 261-271.

Hulme, A.C., 1956. Carbon dioxide injury and the presence of succinic acid in apples. Nature 178, 218-219.

Janovitz-Klapp, A.H., Richard, F.C., Nicolas, J., 1989. Polyphenol oxidase from apple. Partial purification and some properties. Phytochemistry 28, 2903-2907.

Johnston, J.W., Hewett, E.W., Hertog, M.L.A.T.M., 2002. Postharvest softening of apple (*Malus domestica*) fruit: a review. N. Z. J. Crop Hortic. Sci. 30, 145-160.

Jung, S.-K., Watkins, C.B., 2008. Superficial scald control after delayed treatment of apple fruit with diphenylamine (DPA) and 1-methylcyclopropene (1-MCP). Postharvest Biol. Technol. 50, 45-52.

Jung, S.-K., Watkins, C.B., 2011. Involvement of ethylene in browning development of controlled atmosphere-stored 'Empire' apple fruit. Postharvest Biol. Technol. In press.

Kader, A.A., 1986. Biochemical and physiological basis for effects of controlled and modified atmospheres on fruits and vegetables. Food Technol. 40, 99-104.

Kader, A.A., 1995. Regulation of fruit physiology by controlled/modified atmospheres. Acta Hort. 398, 59-70.

Kader, A.A., 2003. Physiology of CA treated produce. Acta Hort. 600, 349-354.

Kato-Noguchi, H., Watada, A.E., 1996. Regulation of glycolytic metabolism in fresh-cut carrots under low oxygen atmosphere. J. Amer. Soc. Hort. Sci. 121, 123-126.

Kaur-Sawhney, R., Flores, H.E., Galston, A.W., 1981. Polyamine oxidase in oat leaves: A cell wall-localized enzyme. Plant Physiol. 68, 494-498.

Kaur-Sawhney, R., Tiburcio, A.F., Altabella, T., Galston, A.W., 2003. Polyamines in plants: An overview. J. Cell Mol. Biol. 2, 1-12.

Kays, S.J., 1999. Preharvest factors affecting appearance. Postharvest Biol. Technol. 15, 233-247.

Ke, D., Mateos, M., Siriphanich, J., Li, C., Kader, A.A., 1993. Carbon dioxide action on metabolism of organic and amino acids in crisphead lettuce. Postharvest Biol. Technol. 3, 235-247.

Ke, D., Yahia, E., Mateos, M., Kader, A.A., 1994a. Ethanolic fermentation of 'Barlett' pears as influenced by ripening stage and atmospheric composition. J. Amer. Soc. Hort. Sci. 119, 976-982.

Ke, D., Zhou, L., Kader, A.A., 1994b. Mode of oxygen and carbon dioxide action on strawberry ester biosynthesis. J. Amer. Soc. Hort. Sci. 119, 971-975.

Kerbel, E.L., Kader, A.A., Romani, R.J., 1988. Effects of elevated CO_2 concentrations on glycolysis in intact `Bartlett' pear fruit. Plant Physiol. 86, 1205-1209.

Kieber, J.J., 2006. Ethylene: The gaseous hormone. In: Taiz, L., Zeiger, E. (Eds.), Plant Physiol. Sinauer Associates, Inc., Sunderland, MA. pp. 571-591.

Klages, K., Donnison, H., Wünsche, J., Boldingh, H., 2001. Diurnal changes in non-structural carbohydrates in leaves, phloem exudate and fruit in 'Braeburn' apple. Functional Plant Biol. 28, 131-139.

Knee, M., 1973. Polysaccharide changes in cell walls of ripening apples. Phytochemistry 12, 1543-1549.

Kondo, S., Setha, S., Rudell, D.R., Buchanan, D.A., Mattheis, J.P., 2005. Aroma volatile biosynthesis in apples affected by 1-MCP and methyl jasmonate. Postharvest Biol. Technol. 36, 61-68.

Kramer, G.F., Wang, C.Y., Conway, W.S., 1991. Inhibition of softening by polyamine application in `Golden Delicious' and `McIntosh' apples. J. Amer. Soc. Hort. Sci. 116, 813-817.

Kumar, A., Taylor, M., Altabella, T., Tiburcio, A.F., 1997. Recent advances in polyamine research. Trends Plant Sci. 2, 124-130.

Kupferman, E., 2003. Controlled atmosphere storage of apples and pears. Acta Hort. 600, 729-735.

Larrigaudiere, C., Pinto, E., Lentheric, L., Vendrell, M., 2001. Involvement of oxidative processes in the development of core browning in controlled-atmosphere stored pears. J. Hort. Sci. Biotechnol. 76, 157-162.

Larrigaudière, C., Lentheric, I., Pinto, E., Veltman, R.H., Jager, A.d., Streif, J., 2001a. High CO_2 level affects ascorbate metabolism in controlled-atmosphere stored pears. Acta Hort. 553, 639-642.

Larrigaudière, C., Lentheric, I., Pintó, E., Vendrell, M., 2001b. Short-term effects of air and controlled atmosphere storage on antioxidant metabolism in conference pears. J. Plant Physiol. 158, 1015-1022.

Larrigaudière, C., Pintó, E., Lentheric, I., 2003. Oxidative behavior of Conference pears stored in air and controlled-atmosphere storage. Acta Hort. 600, 355-360.

Larson, R.A., 1988. The antioxidants of higher plants. Phytochemistry 27, 969-978.

Lidster, P.D., Blanpied, G.D., Prange, R.K., 1990. Controlled-atmosphere disorders of commercial fruits and vegetables. Agriculture Canada Publication. 1847/E, Communications Branch, Agr. Can., Ottawa, Ont., Canada.

Lisec, J., Schauer, N., Kopka, J., Willmitzer, L., Fernie, A.R., 2006. Gas chromatography mass spectrometry-based metabolite profiling in plants. Nat. Protocols 1, 387-396.

Liu, R.H., 2004. Potential synergy of phytochemicals in cancer prevention: mechanism of action. J. Nutr. 134, 3479S-3485S.

Lloyd, J.R., Kossmann, J., Ritte, G., 2005. Leaf starch degradation comes out of the shadows. Trends Plant Sci. 10, 130-137.

López, M.L., Lavilla, T., Recasens, I.I., Riba, M., Vendrell, M., 1998. Influence of different oxygen and carbon dioxide concentrations during storage on

production of volatile compounds by Starking Delicious apples. J. Agric. Food Chem. 46, 634-643.

Lougheed, E.C., Ripley, B.D., Miller, S.R., 1983. Daminozide and senescent breakdown of Mcintosh apples. Plant Disease 67, 190-191.

Lurie, S., Sonego, L., Ben-Arie, R., 1987. Permeability, microviscosity and chemical changes in the plasma membrane during storage of apple fruit. Sci. Hort. 32, 73-83.

MacLean, D.D., Murr, D.P., DeEll, J.R., Horvath, C.R., 2006. Postharvest variation in apple (*Malus* x *domestica* Borkh.) flavonoids following harvest, storage, and 1-MCP treatment. J. Agric. Food Chem. 54, 870-878.

Marangoni, A.G., Palma, T., Stanley, D.W., 1996. Membrane effects in postharvest physiology. Postharvest Biol. Technol. 7, 193-217.

Martin-Tanguy, J., 2001. Metabolism and function of polyamines in plants: recent development (new approaches). Plant Growth Regulat. 34, 135-148.

Mathooko, F.M., 1996. Regulation of respiratory metabolism in fruits and vegetables by carbon dioxide. Postharvest Biol. Technol. 9, 247-264.

Mattoo, A.K., Handa, A.K., 2008. Higher polyamines restore and enhance metabolic memory in ripening fruit. Plant Sci. 174, 386-393.

Mayer, A.M., Harel, E., 1979. Polyphenol oxidases in plants. Phytochemistry 18, 193-215.

Meheriuk, M., Prange, R.K., Lidster, P.D., Porritt, S.W., 1994. Postharvest disorders of apples and pears. Agriculture Canada Publication. 1737/E.

Mihalyi, K., Vamos-Vigyazo, L., Kiss-Kutz, N., Babos-Szebenyi, E., 1978. The activites of polyphenol oxidase and peroxidase in fruits and vegetables as related to pH and temperature. Acta Alimentaria 7, 57-67.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405-410.

Nicolas, J.J., Richard-Forget, F.C., Goupy, P.M., Amiot, M.J., Aubert, S.Y., 1994. Enzymatic browning reactions in apple and apple products. Crit. Rev. Food Sci. Nutr. 34, 109-157.

Ohmiya, A., Kakiuchi, N., 1990. Quantitative and morphological studies on starch of apple fruit during development. J. Jap. Soc. Hort. Sci. 59, 417-423.

Pang, X.-M., Nada, K., Liu, J.-H., Kitashiba, H., Honda, C., Yamashita, H., Tatsuki, M., Moriguchi, T., 2006. Interrelationship between polyamine and ethylene in 1-methylcyclopropene treated apple fruits after harvest. Physiol. Plant. 128, 351-359.

Park, S.W., Song, K.J., Kim, M.Y., Hwang, J.-H., Shin, Y.U., Kim, W.-C., Chung, W.I., 2002. Molecular cloning and characterization of four cDNAs encoding the isoforms of NAD-dependent sorbitol dehydrogenase from the Fuji apple. Plant Sci. 162, 513-519.

Patterson, B.D., Hatfield, S.G.S., Knee, M., 1974. Residual effects of controlled atmosphere storage on the production of volatile compounds by two varieties of apples. J. Sci. Food Agric. 25, 843-849.

Paull, R.E., 1999. Effect of temperature and relative humidity on fresh commodity quality. Postharvest Biol. Technol. 15, 263-277.

Pedreschi, R., Franck, C., Lammertyn, J., Erban, A., Kopka, J., Hertog, M., Verlinden, B., Nicolaï, B., 2009. Metabolic profiling of 'Conference' pears under low oxygen stress. Postharvest Biol. Technol. 51, 123-130.

Pesis, E., Ebeler, S.E., de Freitas, S.T., Padda, M., Mitcham, E.J., 2010. Short anaerobiosis period prior to cold storage alleviates bitter pit and superficial scald in Granny Smith apples. J. Sci. Food Agric. 90, 2114-2123.

Pintó, E., Lentheric, I., Vendrell, M., Larrigaudière, C., 2001. Role of fermentative and antioxidant metabolisms in the induction of core browning in controlled-atmosphere stored pears. J. Sci. Food Agric. 81, 364-370.

Potter, A.L., Hassid, W.Z., Joslyn, M.A., 1949. Starch. III. Structure of apple starch. J. Amer. Chem. Soc. 71, 4075-4077.

Rawyler, A., Pavelic, D., Gianinazzi, C., Oberson, J., Braendle, R., 1999. Membrane lipid integrity relies on a threshold of ATP production rate in potato cell cultures submitted to anoxia. Plant Physiol. 120, 293-300.

Reid, M.S., Celikel, F.G., 2008. Use of 1-methylcyclopropene in ornamentals: Carnations as a model system for understanding mode of action. HortScience 43, 95-98.

Róth, E., Berna, A., Beullens, K., Yarramraju, S., Lammertyn, J., Schenk, A., Nicolaï, B., 2007. Postharvest quality of integrated and organically produced apple fruit. Postharvest Biol. Technol. 45, 11-19.

Rudell, D.R., Mattheis, J.P., Curry, E.A., 2008. Prestorage ultraviolet-white light irradiation alters apple peel metabolome. J. Agric. Food Chem. 56, 1138-1147.

Rudell, D.R., Mattheis, J.P., Hertog, M.L.A.T.M., 2009. Metabolomic change precedes apple superficial scald symptoms. J. Agric. Food Chem. 57, 8459-8466.

Saltveit, M.E., 1999. Effect of ethylene on quality of fresh fruits and vegetables. Postharvest Biol. Technol. 15, 279-292.

Sapers, G.M., Abbott, J., Massie, O., Watada, A., Finney, E.E., 1977. Volatile composition of McIntosh apple juice as a function of maturity and ripeness indices. J. Food Sci. 42, 44-47.

Sapers, G.M., 1993. Browning of foods: control by sulfites, antioxidants, and other means. Food Technol. 47, 75-84.

Saquet, A.A., Streif, J., Bangerth, F., 2000. Changes in ATP, ADP and pyridine nucleotide levels related to the incidence of physiological disorders in 'Conference' pears and 'Jonagold' apples during controlled atmosphere storage. J. Hort. Sci. Biotechnol. 75, 243-249.

Shaham, Z., Lers, A., Lurie, S., 2003. Effect of heat or 1-methylcyclopropene on antioxidative enzyme activities and antioxidants in apples in relation to superficial scald development. J. Amer. Soc. Hort. Sci. 128, 761-766.

Shalit, M., Katzir, N., Tadmor, Y., Larkov, O., Burger, Y., Shalekhet, F., Lastochkin, E., Ravid, U., Amar, O., Edelstein, M., Karchi, Z., Lewinsohn, E., 2001. Acetyl-CoA: alcohol acetyltransferase activity and aroma formation in ripening melon fruits. J. Agric. Food Chem. 49, 794-799.

Shelp, B.J., Bown, A.W., McLean, M.D., 1999. Metabolism and functions of gamma-aminobutyric acid. Trends Plant Sci. 4, 446-452.

Shewfelt, R.L., Purvis, A.C., 1995. Toward a comprehensive model for lipid peroxidation in plant tissue disorders. HortScience 30, 213-218.

Sisler, E.C., Blankenship, S.M., 1996. Methods of counteracting an ethylene response in plants. U.S.

Sisler, E.C., Serek, M., Dupille, E., Goren, R., 1999. Inhibition of ethylene responses by 1-Methylcyclopropene and 3-Methylcyclopropene. Plant Growth Regulat. 27, 105-111.

Sisler, E.C., Serek, M., 2003. Compounds interacting with the ethylene receptor in plants. Plant Biol. 5, 473-480.

Sisler, E.C., 2006. The discovery and development of compounds counteracting ethylene at the receptor level. Biotechnol. Adv. 24, 357-367.

Smagula, J.M., Bramlage, W.J., 1977. Acetaldehyde accumulation: is it a cause of physiological deterioration of fruits? HortScience 12, 200-204.

Smirnoff, N., 2000. Ascorbic acid: metabolism and functions of a multi-facetted molecule. Curr. Opin. Plant Biol. 3, 229-235.

Smith, A.M., Denyer, K., Martin, C., 1997. The synthesis of the starch granule. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 67-87.

Smith, A.M., Zeeman, S.C., Thorneycroft, D., Smith, S.M., 2003. Starch mobilization in leaves. J. Exp. Bot. 54, 577-583.

Smith, A.M., Zeeman, S.C., Smith, S.M., 2005. Starch degradation. Annu. Rev. Plant Biol. 56, 73-98.

Smith, T.A., 1985. Polyamines. Ann. Rev. Plant Physiol. 36, 117-143.

Smock, R.M., Neubert, A.M., 1950. Apples and apple products. Economic crops, II. Interscience Publishers Inc., New York.

Smock, R.M., 1979. Controlled atmosphere storage of fruits. Hort. Rev. 1, 301-336.

Song, J., Bangerth, F., 1996. The effect of harvest date on aroma compound production from 'Golden Delicious' apple fruit and relationship to respiration and ethylene production. Postharvest Biol. Technol. 8, 259-269.

Stepanova, A.N., Alonso, J.M., 2009. Ethylene signaling and response: where different regulatory modules meet. Curr. Opin. Plant Biol. 12, 548-555.

Stover, E., Fargione, M., Risio, R., Yang, X., 2002. Crop load reduction and fruit size following multi-step thinning of 'Empire' apple. HortScience 37, 130-133.

Streif, J., Bangerth, F., 1988. Production of volatile aroma substances by ‘Golden Delicious’ apple fruits after storage for various times in different CO_2 and O_2 concentrations J. Hort. Sci. 63, 193-200.

Streif, J., Saquet, A.A., Xuan, H., 2003. CA-related disorders of apples and pears. Acta Hort. 600, 223-230.

Sugimoto, N., Park, Sunchung, van Nocker, S., Beaudry, R., 2008. Gene expression associated with apple aroma biosynthesis. Acta Hort. 768, 57-64.

Suni, M., Nyman, M., Eriksson, N.-A., Björk, L., Björck, I., 2000. Carbohydrate composition and content of organic acids in fresh and stored apples. J. Sci. Food Agric. 80, 1538-1544.

Suttle, J.C., 1981. Effect of polyamines on ethylene production. Phytochemistry 20, 1477-1480.

Teo, G., Suzuki, Y., Uratsu, S.L., Lampinen, B., Ormonde, N., Hu, W.K., DeJong, T.M., Dandekar, A.M., 2006. Silencing leaf sorbitol synthesis alters long-distance partitioning and apple fruit quality. Proc. Natl. Acad. Sci. U.S.A. 103, 18842-18847.

Tisza, S., Sass, P., Molnár-Perl, I., 1994. Optimization of the simultaneous determination of acids and sugars as their trimethylsilyl(oxime) derivatives by gas chromatography-mass spectrometry and determination of the composition of six apple varieties. J. Chromatogr. A 676, 461-468.

Toivonen, P.M.A., 2004. Postharvest storage procedures and oxidative stress. HortScience 39, 938-942.

Trejo-Gonzalez, A., Soto-Valdez, H., 1991. Partial characterization of polyphenoloxidase extracted from 'Anna' apple. J. Amer. Soc. Hort. Sci. 116, 672-675.

Tsao, R., Yang, R., Young, J.C., Zhu, H., 2003. Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC). J. Agric. Food Chem. 51, 6347-6353.

Tu, K., Nicolaï, B., De Baerdemaeker, J., 2000. Effects of relative humidity on apple quality under simulated shelf temperature storage. Sci. Hort. 85, 217-229.

Vamos-Vigyazo, L., 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. Crit. Rev. Food Sci. Nutr. 15, 49-127.

Veberic, R., Zadravec, P., Stampar, F., 2007. Fruit quality of 'Fuji' apple (*Malus domestica* Borkh.) strains. J. Sci. Food Agric. 87, 593-599.

Veltman, R.H., Larrigaudière, C., Wichers, H.J., Van Schaik, A.C.R., Van der Plas, L.H.W., Oosterhaven, J., 1999a. PPO activity and polyphenol content are not limiting factors during brown core development in pears (*Pyrus communis* L. cv. Conference). J. Plant Physiol. 154, 697-702.

Veltman, R.H., Sanders, M.G., Persijn, S.T., Pemppelenbos, H., Oosterhaven, J., 1999b. Decreased ascorbic acid levels and brown core development in pears (*Pyrus communis* L. cv. Conference). Physiol. Plant. 107, 39-45.

Veltman, R.H., Lenthéric, I., Van der Plas, L.H.W., Peppelenbos, H.W., 2003. Internal browning in pear fruit (*Pyrus communis* L. cv Conference) may be a result of a limited availability of energy and antioxidants. Postharvest Biol. Technol. 28, 295-302.

Veltman, R.H., Peppelenbos, H.W., 2003. A proposed mechanism behind the development of internal browning in pears (*Pyrus communis* cv Conference). Acta Hort. 600, 247-255.

Vilaplana, R., Valentines, M.C., Toivonen, P., Larrigaudiere, C., 2006. Antioxidant potential and peroxidative state of 'Golden Smoothee' apples treated with 1-methylcyclopropene. J. Amer. Soc. Hort. Sci. 131, 104-109.

Volz, R.K., Biasi, W.V., Mitcham, E.J., 1998. Fermentative volatile production in relation to carbon dioxide–induced flesh browning in 'Fuji' apple. HortScience 33, 1231-1234.

Wang, S.Y., Faust, M., 1992. Ethylene Biosynthesis and Polyamine Accumulation in Apples with Watercore. J. Amer. Soc. Hort. Sci. 117, 133-138.

Watkins, C., Liu, F.W., 2010. Temperature and carbon dioxide interactions on quality of controlled atmosphere-stored 'Empire' apples. HortScience 45, 1708-1712.

Watkins, C.B., Hewett, E.W., Bateup, C., Gunson, A., 1989. Relationships between maturity and storage disorders in 'Cox's Orange Pippin' apples as influenced by preharvest calcium or ethephon sprays. N. Z. J. Crop Hortic. Sci. 17, 283-292.

Watkins, C.B., 2000. Responses of horticultural commodities to high carbon dioxide as related to modified atmosphere packaging. HortTechnology 10, 501-506.

Watkins, C.B., Nock, J.F., Whitaker, B.D., 2000. Responses of early, mid and late season apple cultivars to postharvest application of 1-methylcyclopropene (1-MCP) under air and controlled atmosphere storage conditions. Postharvest Biol. Technol. 19, 17-32.

Watkins, C.B., Kupferman, E.M., Rosenberger, D.A., 2002. Apple. Postharvest quality management guidelines. In: Gross, K.C., Wang, Y., Saltveit, M.E. (Eds.), The Commercial Storage of Fruits, Vegetables and Florist and Nursery Crops Agriculture Handbook no. 66. USDA.

Watkins, C.B., 2003. Postharvest physiological disorders of fresh crops. Encyclopedia of Applied Plant Science, 2. Academic press.

Watkins, C.B., Nock, J.F., 2004. SmartFresh™ (1-MCP) - The good and bad as we head into the 2004 season! New York Fruit Quart. 12, 3-8.

Watkins, C.B., Nock, J.F., Razafimbelo, F., 2005. Storage disorders of apples with and without 1-MCP (SmartFresh™). Cornell Fruit Handling and Storage Newsletter, 2-17.

Watkins, C.B., 2006a. Actions and uses of 1-methylcyclopropene (1-MCP). CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 1, 1-10.

Watkins, C.B., 2006b. The use of 1-methylcyclopropene (1-MCP) on fruits and vegetables. Biotechnol. Adv. 24, 389-409.

Watkins, C.B., 2008. Overview of 1-methylcyclopropene trials and uses for edible horticultural crops. HortScience 43, 86-94.

Webb, K.L., Burley, J.W.A., 1962. Sorbitol Translocation in Apple. Science 137, 766.

Williams, M.W., Patterson, M.E., 1964. Storage effects on fruits, nonvolatile organic acids and core breakdown of Bartlett pears. J. Agric. Food Chem. 12, 80-83.

Wills, R.B.H., Scott, K.J., McGlasson, W.B., 1970. A role for acetate in the development of low-temperature breakdown in apples. J. Sci. Food Agric. 21, 42-44.

Wills, R.B.H., Patterson, B.D., 1971. Low temperature breakdown in apples. Phytochemistry 10, 2983-2986.

Wills, R.B.H., Patterson, B.D., 1972. Anthocyanin content of Jonathan apple peel and the development of low temperature breakdown. J. Hort. Sci. 47, 555-556.

Wu, J., Gao, H., Zhao, L., Liao, X., Chen, F., Wang, Z., Hu, X., 2007. Chemical compositional characterization of some apple cultivars. Food Chem. 103, 88-93.

Xuan, H., Streif, J., 2008. Effect of 1-MCP on respiration and concentration of ATP/ADP in the fruit tissue of 'Jonagold' apple after storage. Acta Hort. 796, 129-135.

Yahia, E.M., 1994. Apple flavor. Hort. Rev. 16, 197-234.

Yamaki, S., 1984. Isolation of vacuoles from immature apple fruit flesh and compartmentation of sugars, organic acids, phenolic compounds and amino-acids. Plant Cell Physiol. 25, 151-166.

Zanella, A., 2003. Control of apple superficial scald and ripening—A comparison between 1-methylcyclopropene and diphenylamine postharvest treatments, initial low oxygen stress and ultra low oxygen storage. Postharvest Biol. Technol. 27, 69-78.

Zanella, A., Cazzanelli, P., Panarese, A., Coser, M., Chistè, C., Zeni, F., 2005. Fruit fluorecence response to low oxygen stress: modern storage technologies compared to 1-MCP treatment of apple. Acta Hort. 682, 1535-1542.

Zhou, P., Smith, N.L., Lee, C.Y., 1993. Potential purification and some properties of Monroe apple peel polyphenol oxidase. J. Agric. Food Chem. 41, 532-536.

Zhu, Y., Rudell, D.R., Mattheis, J.P., 2008. Characterization of cultivar differences in alcohol acyltransferase and 1-aminocyclopropane-1-carboxylate synthase gene expression and volatile ester emission during apple fruit maturation and ripening. Postharvest Biol. Technol. 49, 330-339.

CHAPTER 3

STORAGE TEMPERATURE EFFECT ON ANTIOXIDANT METABOLISM OF 1-MCP TREATED 'EMPIRE' APPLE

ABSTRACT

'Empire' apples [*Malus sylvestris* (L.) Mill var. *domestica* (Borkh.) Mansf.] are susceptible to development of firm flesh browning during controlled atmosphere storage. The browning is thought to be a chilling injury and therefore fruit are typically stored at 2-3 ^{o}C. However, flesh browning can be increased by 1-methylcyclopropene (1-MCP) treatment at these warmer temperatures. The objective of this work was to investigate the effect of 1-MCP on antioxidant systems of 'Empire' apple fruit stored at 2 kPa O_2/2 kPa CO_2 at either 0.5 ^{o}C or 3.3 ^{o}C for up to 40 weeks. Lightness and hue angle of flesh tissues decreased to a greater extent in 1-MCP treated than untreated fruit with longer storage periods, especially at the stem-end of the fruit. Nitroblue tetrazolium reducing activity, an indication of superoxide anion radical generation, was reduced by 1-MCP at both storage temperatures. H_2O_2 levels were inconsistently affected by 1-MCP treatment, while malondialdehyde levels, indicating lipid peroxidation, were higher in 1-MCP treated fruit at 0.5 ^{o}C than at 3.3 ^{o}C. Ascorbic acid concentrations were lower by 1-MCP at the end of storage at both temperatures but dehydroascorbic acid concentrations were lower by 1-MCP at 3.3 ^{o}C from week 15 to week 30. While glutathione concentrations of reduced and oxidized forms were not affected by 1-MCP at 0.5 ^{o}C, 1-MCP reduced the glutathione concentrations of the reduced form but increased that of the oxidized form at the end of storage at 3.3 ^{o}C. Effects of temperature and 1-MCP treatment on superoxide dismutase (SOD) activity were inconsistent, while Cu/Zn-SOD activity was reduced in 1-MCP treated fruit stored at 3.3 ^{o}C. While the catalase activity was not affected by 1-

MCP at 3.3 ^{o}C, its activity at 0.5 ^{o}C gradually increased 1-MCP treated fruit but reduced back to the initial level by the end of storage. 1-MCP reduced the activity of peroxidase at 0.5 ^{o}C but increased its activity at 3.3 ^{o}C. The activities of ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase were not affected consistently by storage temperature of 1-MCP treatment. Overall, the results do not indicate that a direct role of antioxidant metabolism during development of flesh browning in 'Empire' apples.

Keywords: Apple; Reactive oxygen species; Enzymatic antioxidant system; Non-enzymatic antioxidant system

3.1. INTRODUCTION

The 'Empire' is a major apple cultivar in the northeastern United States, especially in New York, that is grown for both domestic and export markets. However, a number of physiological disorders limit its storage periods in controlled atmosphere (CA) storage, including external CO_2 injury, flesh browning and core browning (Watkins and Liu, 2010a). External CO_2 injury can be controlled by careful attention to partial pressures of CO_2 (pCO_2) in the storage atmosphere or by treatment of fruit with the antioxidant diphenylamine (DPA) used for control of superficial scald (Watkins et al., 1997; DeEll et al., 2007; Fawbush et al., 2008), but a flesh browning can cause serious fruit losses (Watkins and Nock, 2005; DeEll et al., 2006, 2007). The disorder is similar to flesh browning as described by Meheriuk et al. (1994) in that affected tissues remain firm and juicy and therefore distinct from senescent breakdown. Firm flesh browning in 'Empire' apples typically becomes apparent in May/June, depending on the season, and is usually associated with storage

temperatures of 0 ^{o}C. Therefore, warmer storage temperatures (2-3 ^{o}C) are used during CA storage to avoid injury.

Recently, the adoption of 1-methylcyclopropene (1-MCP)-based technology by apple industries (Watkins, 2008) has impacted 'Empire' storage. Although the cultivar responds well to 1-MCP treatment resulting in firmer fruit and better quality characteristics after both air or CA storage (Watkins et al., 2000), 1-MCP treated fruit can have much higher flesh browning incidences than in untreated fruit after long term CA storage (Watkins, 2008; Watkins and Nock, in preparation). Interestingly, increased flesh browning in 1-MCP treated fruit is often associated with these warmer storage temperatures. The reasons for increased browning in treated fruit are unknown, but may result from extended stress associated with low temperature in a chilling injury sensitive cultivar. Jung and Watkins (2011) suggest that inhibition of fruit ripening in 1-MCP treated fruit, as a result of inhibited ethylene production at 4 ^{o}C, may induce stress-like conditions comparable with fruit stored at 0.5 ^{o}C, with or without 1-MCP.

Regardless of the external cause of flesh browning, the phenomenon is usually associated with enzymatic oxidation of phenolic compounds by polyphenol oxidase (PPO) to *o*-quinones, which polymerize non-enzymatically to produce heterogeneous black, brown or red pigments commonly called melanins (Tomas-Barberan and Espin, 2001). Under extended oxidative stress, phenolic compounds occupied in vacuoles can leak into the cytosol and subsequently, enzymatic browning reactions occur (Nicolas et al., 1994). Jung and Watkins (2010) found that PPO activity was higher in 1-MCP treated fruit at both 0.5 and 4 ^{o}C, but lowest in untreated fruit stored at 4 ^{o}C, where least browning was observed.

The current research into the origin of storage related browning disorders can be divided according to a focus on catabolic or anabolic processes, but ultimately is

reflected in the balance between oxidative and reductive processes (Franck et al., 2007). Damage to cellular processes can occur if reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot -}$) radical, H_2O_2, hydroxyl ($HO^{\cdot}$) radical, perhydroxyl ($O_2H^{\cdot}$), nitric oxide ($NO^{\cdot}$) and peroxynitrile ($ONOO^{\cdot}$), are not controlled by antioxidant scavenging systems. Lipid peroxidation, cell membrane deterioration, and DNA and RNA damage, followed by biochemical and metabolic malfunctions, will ultimately cause cell death (Hodges et al., 2004; Toivonen, 2004).

Enzymes associated with enzymatic antioxidant scavenging systems include superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX). SOD enzymes play a pivotal role in decomposing superoxide anion radicals into O_2 and H_2O_2, and then, APX, POX and CAT enzymes detoxify H_2O_2 into either H_2O or O_2 (Mittler, 2002). In addition, non-enzymatic antioxidant scavenging systems include ascorbic acid, glutathione, tocopherol, carotenoid and phenolic compounds (Larson, 1988; Noctor and Foyer, 1998; Apel and Hirt, 2004). The ascorbic acid and glutathione cycles play a fundamental role in a ROS scavenging system with APX, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) activities that generate reduced forms of ascorbic acid and glutathione by using electrons derived from NAD(P)H, via the pentose phosphate pathway (Noctor and Foyer, 1998; Apel and Hirt, 2004).

CA storage (1.5 kPa O_2/5 kPa CO_2) reduced ascorbic acid concentrations but slightly increased dehydroascorbic acid concentrations in 'Pink Lady' apples over 4 months. Ascorbic acid and dehydroascorbic acid were not detected at lower pCO_2 (1.5 kPa O_2/0.5 kPa CO_2) at month 4, but storage at1.5 kPa O_2/5 kPa CO_2 resulted in increased H_2O_2 levels (de Castro et al., 2008). 1-MCP treatment inconsistently affected dehydroascorbic acid and total ascorbic acid concentrations , but increased total antioxidant activity in 'Empire' apple flesh tissues stored at 0.5 or 2.2 ^{o}C in 2 or 3

kPa O_2/2 kPa CO_2 for 9 months (Fawbush et al., 2009). Interestingly, the higher incidence of flesh browning was associated with lower ascorbic acid concentrations in 'Pink Lady' flesh tissues stored at 1.5 kPa O_2/5 kPa CO_2 (de Castro et al., 2008). The incidence of flesh browning was associated with the accumulation of MDA and 4HNE levels in 'Blanquilla' pear stored at 2 kPa O_2/5 kPa CO_2 (Larrigaudière et al., 2001c). The incidence of flesh browning was associated with loss of ascorbic acid levels in CA stored 'Conference' and 'Rocha' pear (Veltman et al., 1999b; Veltman et al., 2000). Also, the levels of ascorbic acid were not evenly distributed and were lower within brown flesh tissues than healthy pear flesh tissues (Franck et al., 2003).

Little is known about the effects of 1-MCP on antioxidant systems on apple fruit, and especially in relation to internal browning disorders. The objective of this study was to investigate the influence of two storage temperatures on the activities of enzymatic and non-enzymatic antioxidant scavenging systems of 1-MCP treated 'Empire' apple fruit during CA storage for up to 40 weeks.

3.2. MATERIALS AND METHODS

3.2.1. Fruit source, treatments and storage conditions

'Empire' apple [*Malus sylvestris* (L.) Mill var. *domestica* (Borkh.) Mansf.] fruit used in these experiments were harvested on October 9, 2007 from mature trees at the Cornell University orchards at Lansing, NY. Fruit were selected for uniform size (approximately 7 cm in length and 8 cm in diameter) and randomly sorted to into 24 experimental units (bags) of 10 fruit. Ten fruit were used for harvest assessment as described below, and the remaining fruit were pre-cooled overnight at 3.3 ^{o}C. Half of the 10 fruit units were then either untreated or treated with 1 µL L^{-1} 1-MCP (SmartFresh tablets, 0.36% a.i., AgroFresh Co., Spring House, PA) for 24 h in sealed 4 m^3 (4,000 L) plastic tent. Fruit were vented overnight at either 0.5 ^{o}C or 3.3 ^{o}C and

then placed into 71 cm × 145 cm × 97 cm stainless steel chambers (Storage Control Systems, Inc., Spartan, MI) at 2 kPa O_2/2 kPa CO_2 balanced with N_2. Final atmosphere regimes were established within 48 h and were maintained within 0.2 kPa of the target partial pressures using an ICA 61 CA Control System (Storage Control Systems, Inc., Sparta MI) equipped with a GCS 610 Controller (David Bishop, England).

3.2.2. Fruit sampling

A 10 fruit sample was used at harvest, and 10 fruit samples were taken from each atmosphere and temperature regime at 5 weeks interval for 40 weeks. At harvest and during storage, each fruit was used for assessment of internal ethylene concentration (IEC), and flesh color. For storage samples, the IEC of each fruit was taken in the cold room immediately after storage containers were opened. Color measurements and browning ratings were carried out on individual fruit, ensuring that the fruit remained cold. Five of the fruit were used at 0, 5, 15, 30 and 40 weeks for tissue sampling, by vertically cutting 6 uniform pieces of fruit into liquid nitrogen. Frozen flesh tissues were peeled and then cryogenically milled to a fine powder with IKA ® A11 basic (IKA® Works, Inc. Wilmingten, NC), and stored at -80 ^{o}C prior to further analysis.

3.2.3. Internal ethylene concentration (IEC)

The IEC of each fruit was measured on 1 mL samples of internal gas from the core cavity (Watkins et al., 2000). Ethylene was measured using a Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, DE) equipped with a flame ionization detector and fitted with a stainless steel column packed with 60/80 mesh alumina F-1 (2 m × 2 mm, i.d.). Analyses were run isothermally with an oven temperature of 200 ^{o}C and injector and detector temperature of 220 and 250 ^{o}C,

respectively. The flow rates for nitrogen, hydrogen, and compressed air were 30, 30 and 230 mL min^{-1}. Samples were directly injected into the gas chromatograph. Ethylene was quantified by peak area and external standards were used for calibration.

3.2.4. Fruit color and internal browning index assessment

Flesh color was measured at the calyx-end region (1.5cm from the equatorial region), the equatorial region and the stem-end region (1.5cm from equatorial region) with six sites with a Minolta chromameter CR-300 (Osaka, Japan). The color measurements were expressed as chroma (intensity of color), hue angle (actual color) and L^* value (dark to light, on a scale of 0-100) (McGuire, 1992). All fruit were then assessed for internal browning injury. Each fruit was cut at least three times equatorially and the incidences of any internal browning disorder were assessed. Browning severity was determined using a subjective five-grade scoring system where 0=0%, 1=1-10%, 2=11-25%, 3=26-50%, and 4=51-100% browning, respectively.

3.2.5. Superoxide anion radical activity, hydrogen peroxide, and lipid peroxidation

The production of superoxide anion radical was determined according to the method of Doke (1983), based on its ability to reduce nitroblue terazolium (NBT) to formazan. Frozen ground tissue (0.3g) was added into 3 mL reaction mixture of 10 mM Na_2HPO_4-KH_2PO_4 (pH 7.8), 0.05% (w/v) NBT and 10 mM NaN_3. After incubation at room temperature for 1 h, the reaction mixtures were heated to 85 ^oC for 15 min and then cooled down rapidly on ice. The reaction mixture was filtered through 0.2 μm syringe filter and then the absorbance at 580 nm was recorded using a Genesys 5 spectrophotometer (Spectronic Instruments, Waltham, MA). The ability of the flesh

apple tissue to reduce NBT was expressed as increased OD_{580} g^{-1} h^{-1} (Chen and Cheng, 2004).

The extraction and determination of H_2O_2 was carried out according to Patterson et al. (1984). One gram of frozen ground tissue was homogenized with a pre-cooled mortar and pestle in 2 mL 5% (w/v) trichloroacetic acid (TCA) and 30 mg of activated charcoal washed with HCl, then centrifuged at 16,000 ×g at 4 ^{o}C for 20 min in Microfuge® 18 Centrifuge (Beckman Coutler Inc., Palo Alto, CA). The supernatant was neutralized to pH 8.5 with 17 M NH_4OH and then centrifuged at 16,000 ×g at 4 ^{o}C for 10 min. The supernatant was immediately used for H_2O_2 assay. Each extract was divided into two aliquots of 0.6 mL. Four units of catalase (CAT, EC 1.11.1.6) were only added to blank aliquot. Both the blank and the other aliquot (sample tube) without adding catalase were incubated at room temperature for 10 min and then 0.6 mL colorimetric reagent was added to both aliquots. The colorimetric reagent was daily made by mixing 1:1 (v/v) 0.6 mM potassium titanium oxalate and 0.6 mM 4-(2-pyridylazo) resorcinol monosodium salt. The assay mixture (1.2 mL) was incubated at 45 ^{o}C for 1 h, and then the absorbance was recorded at 508 nm using a Genesys 5 spectrophotometer by using the blank as a blank for each sample. The quantification of H_2O_2 was calculated based on the H_2O_2 standard regression curve, where H_2O_2 levels ranged from 0 to 100 μM.

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA). MDA content in frozen ground tissue was assayed according to the method of Hodges et al. (1999). Frozen ground apple flesh tissues (0.25g) were homogenized with a pre-cooled mortar and pestle in 2 mL of 80% (v/v) ice cold ethanol and 5% (w/v) insoluble polyvinylpolypyrrolidone (PVPP), then centrifuged at 3,000 ×g at 4 ^{o}C for 10 min in Microfuge® 18 Centrifuge. The supernatant was properly diluted based on the preliminary results. The diluted

supernatant was divided into two aliquots of 0.6 mL. One aliquot was mixed with 0.6 mL of without thiobarbituric acid (TBA) solution (-TBA), which consisted of 20% trichloroacetic acid (TCA) and 0.01% butylated hydroxyltoluene (BHT), while the other aliquot was mixed with 0.6 mL of with TBA solution (+TBA), which is comprised of the above with 0.65% TBA. After vigorous mixing, the sample was incubated at 95 ^{o}C for 25 min, cooled down quickly on ice and then centrifuged at 3,000 ×g at 4 ^{o}C for 10 min. The absorbance of the sample was recorded at 440, 532 and 600 nm using a Genesys 5 spectrophotometer. MDA equivalents were calculated in the following manner:

1) $[(\text{Abs } 532_{+TBA})-(\text{Abs } 600_{+TBA})-(\text{Abs } 532_{-TBA}-\text{Abs } 600_{-TBA})]=A$
2) $[(\text{Abs } 440_{+TBA}-\text{Abs } 600_{+TBA})\times 0.0571]=B$
3) MDA equivalent (nmol g^{-1} FW)=[(A-B/157,000) $\times 10^{6}\times$(adjusted sample FW) ×(buffer volume) ×(dilution factor)]

3.2.6. Antioxidant metabolites

Ascorbic acid (AsA) and dehydroascorbate (DHA) were measured according to Logan et al. (1998). One gram of frozen apple flesh tissue was homogenized with a pre-cooled mortar and pestle in 1.5 mL of 6% (v/v) ice cold perchloric acid ($HClO_4$) with 5% insoluble PVPP. The extract was centrifuged at 10,000 ×g at 4 ^{o}C for 10 min in a Microfuge® 18 Centrifuge. The supernatant was immediately used for the assay. For the AsA assay, 175 µL of supernatant was neutralized with 27 µL of 1.5 M sodium carbonate to raise the pH to 1-2. AsA was assayed at 265 nm in 200 mM sodium acetate buffer (pH 5.6), before and after a 15 min with 1.5 units of ascorbic oxidase. For total ascorbate assay, 185 µL extract was neutralized with 31 µL of 1.82 M sodium carbonate to raise the pH to 6-7 and incubated at room temperature for 30 min with equal volume (216 µL) of 20 mM glutathione in 100 mM Tricine-KOH (pH

8.5). Total AsA was assayed as above. DHA content was obtained by calculating the difference between total ascorbate and AsA.

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined spectrophotometrically, based on the enzymatic cycling method described by Griffith (1980). The frozen ground flesh tissue (0.5 g) was homogenized with a pre-cooled mortar and pestle in 1.5 mL of 5% (w/v) sulfosalicylic acid with 5% insoluble PVPP. The extract was centrifuged at 14,000 ×g at 4 ^{o}C for 10 min in Microfuge® 18 Centrifuge. For total glutathione, 20 µL of the supernatant was mixed with 200 µL of buffer A, which consisted of 0.5 M sodium phosphate buffer (pH 7.5) and 6.3 mM EDTA, 560 µL of 10 mM EDTA, 100 µL of 6 mM DTNB dissolved in buffer A and 100 µL of 2.1 mM NADPH. The reaction was initiated by adding 10 µL of 1 unit of glutathione reductase and then the increase in absorbance at 412 nm was monitored over 3 min at room temperature, using a Beckman diode array spectrophotometer (Model DU 7400, Beckman Instruments, Columbia, MD). For GSSG, 20 µL of the supernatant was mixed with 206 µL buffer A, which was consisted of 0.5 M sodium phosphate buffer with pH 7.5 and 6.3 mM EDTA. After adding 4 µL of 2-vinylpyridine, the mixture was incubated at room temperature for 30 min to remove GSH by derivatization. After then, the sample mixture was added with 200 µL buffer A, 360 µL 10 mM EDTA, 100 µL 6 mM DTNB, and 100 µL 2.1 mM NADPH. The reaction was initiated by adding 10 µL of 1 unit of glutathione reductase and then the increase in absorbance at 412 nm was monitored over 3 min at room temperature, using a Beckman diode array spectrophotometer (Model DU 7400, Beckman Instruments, Columbia, MD). Total GSH and GSSG contents were quantified by using standard regression curves, which ranged from 0 µM to 50 µM for total GSH and from 0 µM to 10 µM for GSSG. GSH was obtained by calculating the difference between total GSH and GSSG.

3.2.7. Antioxidant enzyme activity

Enzyme extraction was carried out in an extraction buffer containing 0.2 M sodium phosphate buffer (pH 7.8), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA and 5% insoluble PVPP. One gram of frozen ground tissue was homogenized with a pre-cooled mortar and pestle in 1.5 mL extraction buffer. For extraction of APX, 5 mM ascorbate was added to the extraction buffer. After homogenization, the extract was centrifuged at 14,000 ×g at 4 ^{o}C for 30 min in Microfuge® 18 Centrifuge. The supernatant was collected and immediately used for enzyme assay, or aliquoted and stored at -80 ^{o}C for subsequent protein quantification (Kochhar et al., 2003). The supernatant was used for the enzyme assays of SOD (EC 1.15.1.1), Cu/Zn-SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), POX (EC 1.11.1.7), MDHAR (EC 1.6.5.4), DHAR (EC 1.8.5.1) and GR (EC 1.6.4.2). The supernatant using the extraction buffer containing 1 mM ascorbate was only used for APX (EC 1.11.1.11) activity. All enzyme assays were carried out at 25 ^{o}C in a total volume of 1 mL, using a Beckman diode array spectrophotometer.

SOD activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), according to the method of Giannopolitis and Reis (1977). One mL of reaction mixture contained 0.1 M sodium phosphate buffer (pH 7.8), 10 mM methionine, 57 µM NBT, 1.3 µM riboflavin, 0.025% (v/v) Triton® X-100, and 0.11 µM EDTA and 20 µL enzyme extract. The reaction mixture was freshly made before the reaction. The reaction was initiated by illuminating the reaction mixture with 2 of 15W fluorescent lights. After 10 min illimination, the reaction was stopped by removing the light source. A blank tube with reaction mixture and extraction buffer was kept in the dark as a blank, while another tube with reaction mixture and extraction buffer was kept in light to serve as a control tube but sample tube with reaction mixture and enzyme extraction was kept in light. Activity of SOD

was reported as NBT reduction in light with extraction buffer (control tube) minus NBT reduction with sample extraction (sample tube). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

Cu/Zn-SOD activity was also determined by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), according to the method of Giannopolitis and Reis (1977) and Frei et al. (2010). One mL of reaction mixture contained 2 mM KCN with the above SOD reaction mixture. All procedures for the measurement of Cu/Zn-SOD activity were same with the above mentioned on the procedures of SOD.

APX activity was determined using the method as modified by Grace and Logan (1996). The reaction mixture contained 50 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA-Na_2, 0.5 mM ascorbate and 50 µL extracted sample. The reaction was initiated by adding 0.4 mM H_2O_2. The absorbance rate was monitored at 290 nm over 3 min. APX activity was determined by monitoring the decrease in absorbance at 290 nm as ascorbate was oxidized. A molar extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate activity, which was expressed as µmol ascorbate min^{-1} mg^{-1} protein.

CAT activity was determined using the method described by Aebi (1984). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0) and 10 µL extracted sample. The reaction was initiated by adding 10 mM H_2O_2. The absorbance was monitored at 240 nm over 5 min. A molar extinction coefficient of 39.4 mM^{-1} cm^{-1} was used to calculate CAT activity, which was expressed as µmol H_2O_2 min^{-1} mg^{-1} protein.

MDHAR activity was assayed by monitoring the decrease in absorbance at 340 nm over 3 min due to NADH oxidation (Miyake and Asada, 1992). The reaction mixture contained 50 mM Hepes-KOH (pH 7.6), 0.1 mM NADH, 20 µL extracted

sample and 2.5 mM ascorbate. The reaction was initiated by adding 0.5 unit of ascorbate oxidase. A molar extinction coefficient of 6.2 mM^{-1} cm^{-1} was used to calculate activity, which was expressed as μmol NADH min^{-1} mg^{-1} protein.

DHAR activity was measured using the method of Nakano and Asada (1981). The reaction mixture contained 50 mM Hepes-KOH (pH 7.6), 0.1 mM EDTA-Na_2, 100 μL extracted sample and 2.5 mM glutathione. The reaction was initiated by adding 0.2 mM dehydroascorbate. The absorbance was read by monitoring the increase in absorbance at 265 nm over 3 min due to ascorbate formation. A molar extinction coefficient of 14.0 mM^{-1} cm^{-1} was used to calculate activity, which was expressed as μmol DHA min^{-1} mg^{-1} protein.

GR activity was determined by the method described by Grace and Logan (1996) with some modification. The reaction mixture contained 100 mM Tris-HCl (pH 8.0), 1 mM EDTA-Na_2, 100 μL crude extracted sample and 1 mM GSSG (oxidized glutathione). The reaction was initiated by adding 0.2 mM NADPH. The absorbance was read by monitoring the decrease in absorbance at 340 nm over 3 min due to NADPH oxidation. A molar extinction coefficient of 6.2 mM^{-1} cm^{-1} was used to calculate activity, which was expressed as μmol NADPH min^{-1} mg^{-1} protein.

POX activity was measured by the method described by Hammerschmidt et al. (1982) using guaiacol as the hydrogen donor. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 10 mM guaiacol. The reaction was initiated by adding 10 μL crude extracted sample. The absorbance was recorded by monitoring the increase in absorbance at 470 mm over 3 min due to guaiacol oxidation. A molar extinction coefficient of 26.8 mM^{-1} cm^{-1} was used to calculate activity, which was expressed as μmol guaiacol min^{-1} mg^{-1} protein.

Protein concentrations were determined using the Bradford assay (Sigma-Aldrich Co., St. Louis, MO) according to the manufacturer's micro-assay protocol.

Protein concentration was measured by comparison to the bovine serum albumin (BSA) standard curve from 0 to 10 mg L^{-1} (Bradford, 1976).

3.2.8. Statistical analysis

The MINITAB software release 15 (Minitab Inc., State College, PA) was used for analysis of the data. All data were analyzed using General Linear Model (GLM). Experimental data was represented to means ± standard error. Pearson correlations were used to determine the relationship between browning severity and flesh color responses (lightness, hue angle and chroma), and between browning and antioxidant metabolism.

All antioxidant metabolite data were analyzed by using partial least-squares discriminant analysis (PLS-DA) (Pérez-Enciso and Tenenhaus, 2003). PLS-DA relates variations in a limited number of predictor variables (Y-variables) to the variations of a large number of predictor variables (X-variables). PLS-DA is a regression analysis technique in which the original X-data are projected onto a small number of underlying latent variables (LV), which are concurrently used for regression of the Y-data in such a way that the first LVs are most relevant for predicting the Y-variables.

The antioxidant metabolites were considered to be predictor variables, whereas treatment factors (untreated or 1-MCP-treated), storage time (duration, 0-40 weeks), and lightness (flesh tissue color) were considered to be response variables. The treatments factors were introduced as separate categorical variables (reading either 0 or 1), whereas storage time and lightness values were included as continuous variables. Both X- and Y-data were mean centered and scaled to unit variance to give all variables an equal chance to influence the model. PLS-DA was performed using The Unscrambler version 9.6 (Camo A/S, Trondheim, Norway).

3.3. RESULTS

3.3.1. Internal ethylene concentrations (IEC)

The IEC of fruit at harvest was 46.4 μL L^{-1}, and decreased when fruit were stored at either 0.5 or 3.3 ^{o}C (Figure 3.1). Changes in the IEC during storage were affected by 1-MCP treatment, storage temperature and storage time (P<0.001). At 0.5 ^{o}C, IECs continued to decline during storage and were significantly lower in the 1-MCP treated fruit for 15 weeks, while at 3.3 ^{o}C, the concentrations increased in both untreated 1-MCP treated fruit during storage before decreasing. IECs were lower in 1-MCP treated fruit than untreated fruit from 10 to 36 weeks of storage.

3.3.2. Flesh browning

At 0.5 ^{o}C, only a single fruit had flesh browning (slight severity) at weeks 20 and 30, but by week 40, three of five 1-MCP treated fruit had detectable browning (Figure 3.2). Flesh browning in untreated fruit remained low, occurring only at week 40. At 3.3 ^{o}C, flesh browning was detected in one 1-MCP treated fruit at week 20, but the incidence of the disorder increased at week 30 (Figure 3.2). Flesh browning was detected in untreated fruit only at week 40.

3.3.3. Flesh color

Color of the flesh at the stem, equatorial and calyx end of each fruit was assessed by the L^*, h^o and C^* measurements (Figure 3.3 - 3.5). The L^* values were relatively stable for 25 weeks of storage at 0.5 ^{o}C, but then declined to a greater extent in the stem-end tissues than the equatorial and calyx-end tissues (Figure 3.3); during this time, the L^* values of tissues from the 1-MCP treated fruit were generally lower than those of the untreated fruit. At 3.3 ^{o}C, differences between untreated and 1-MCP

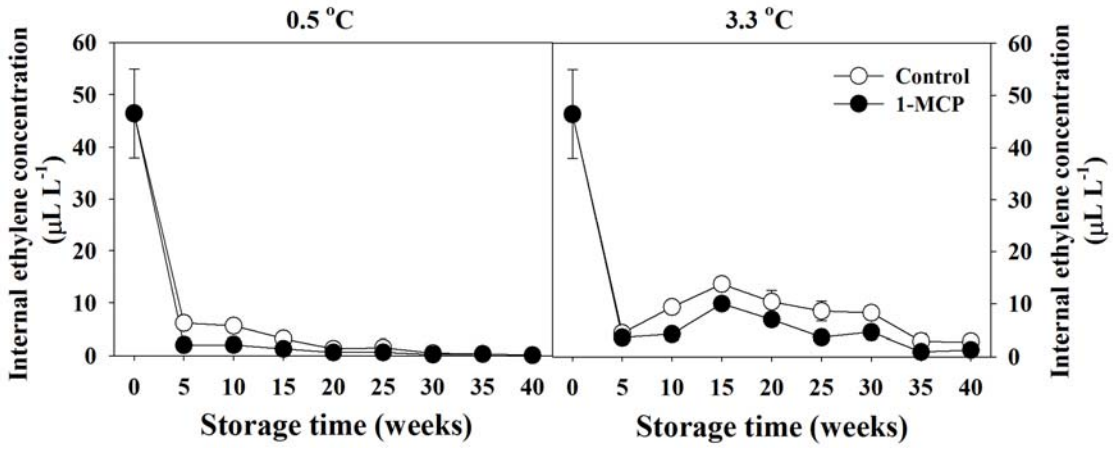

Figure 3.1. Internal ethylene concentration (IEC) of 'Empire' apple fruit untreated, or treated with 1 µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Each data point is the mean of ten replicates ± standard error, where larger than the symbols.

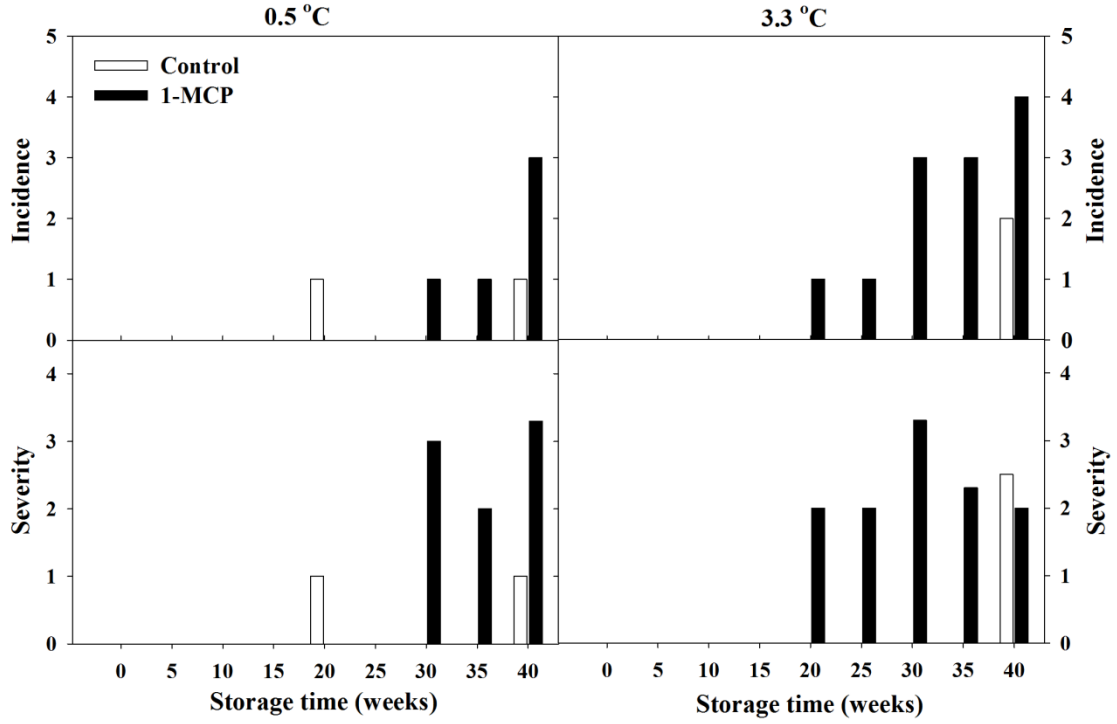

Figure 3.2. Internal browning incidence and browning severity of 'Empire' apple fruit untreated, or treated with 1 μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Incidence represents number of fruit out of five sampled that had detectable browning. Browning severity rating was subjectively evaluated where 0=0% browning, 1=1-10% browning, 2=11-25% browning, 3=26-50% browning and 4=51-100% browning coverage of fruit.

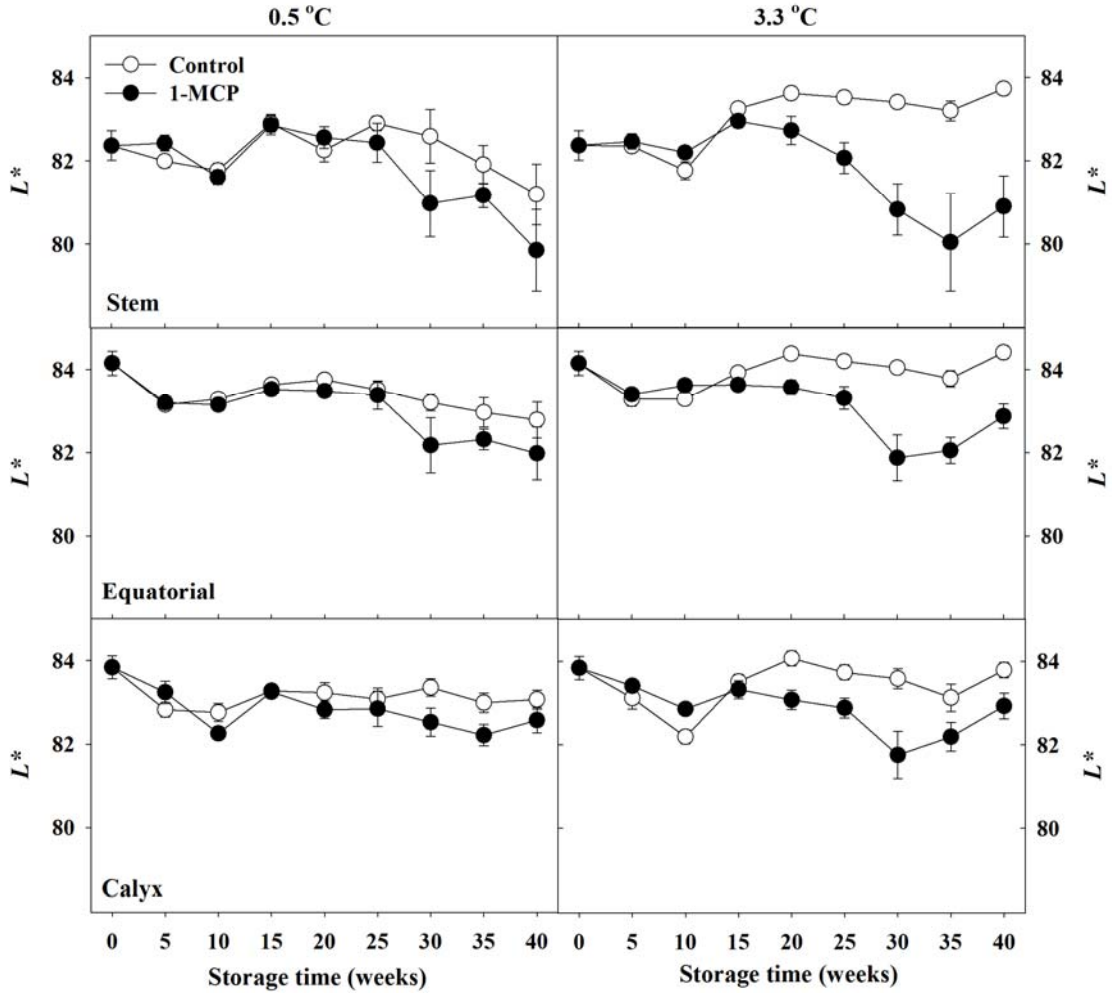

Figure 3.3. Lightness (L^*) of 'Empire' apple fruit untreated, or treated with 1 μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. The lightness was assessed at the stem-end, equatorial and calyx-end region. Each data point is the mean of ten replicates ± standard error, where larger than the symbols.

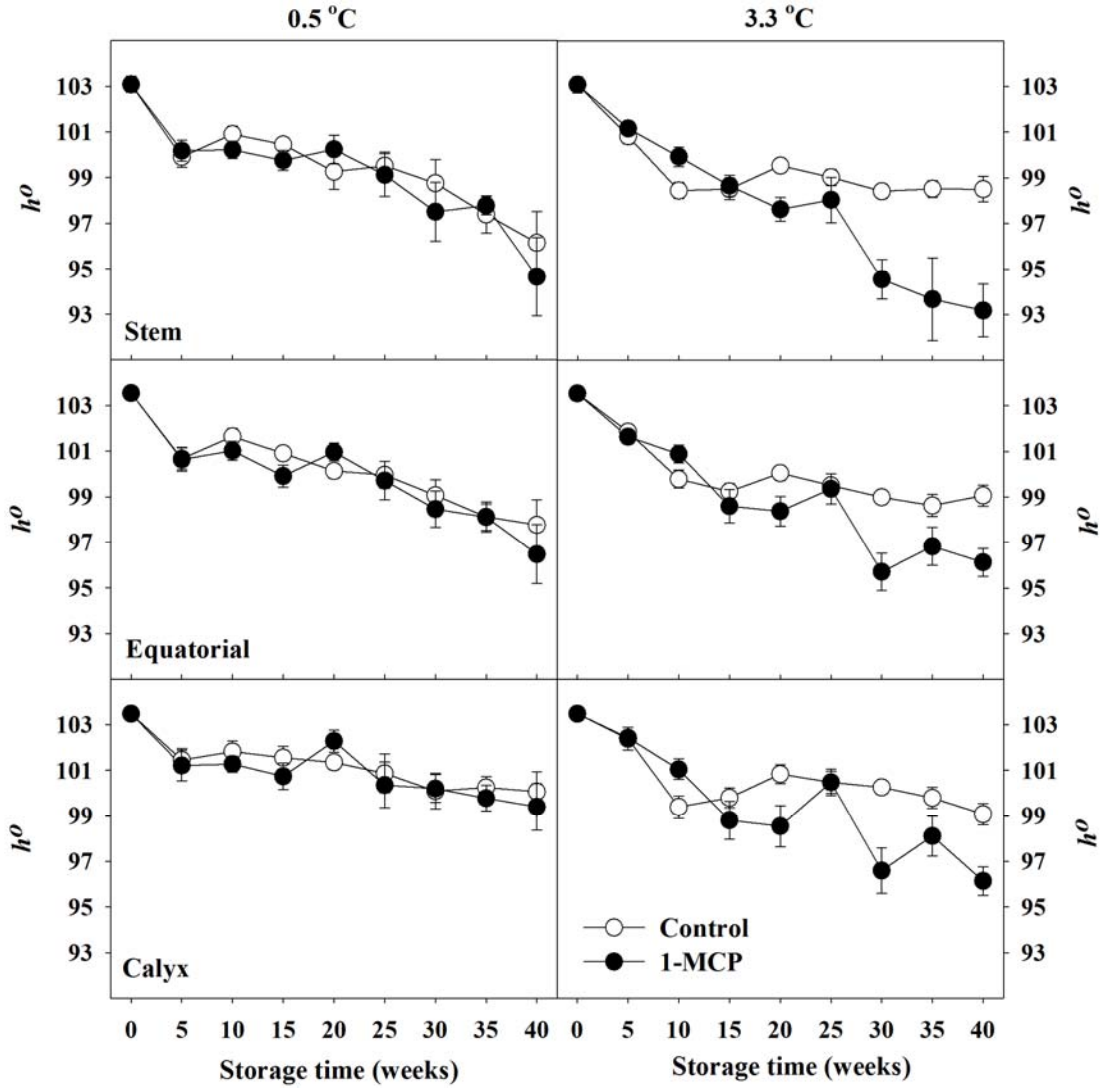

Figure 3.4. Hue angle (h^o) values of 'Empire' apple fruit untreated, or treated with 1µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 ^oC or 3.3 ^oC, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. The h^o was assessed at the stem-end, equatorial and calyx-end region. Each data point is the mean of ten replicates ± standard error, where larger than the symbols.

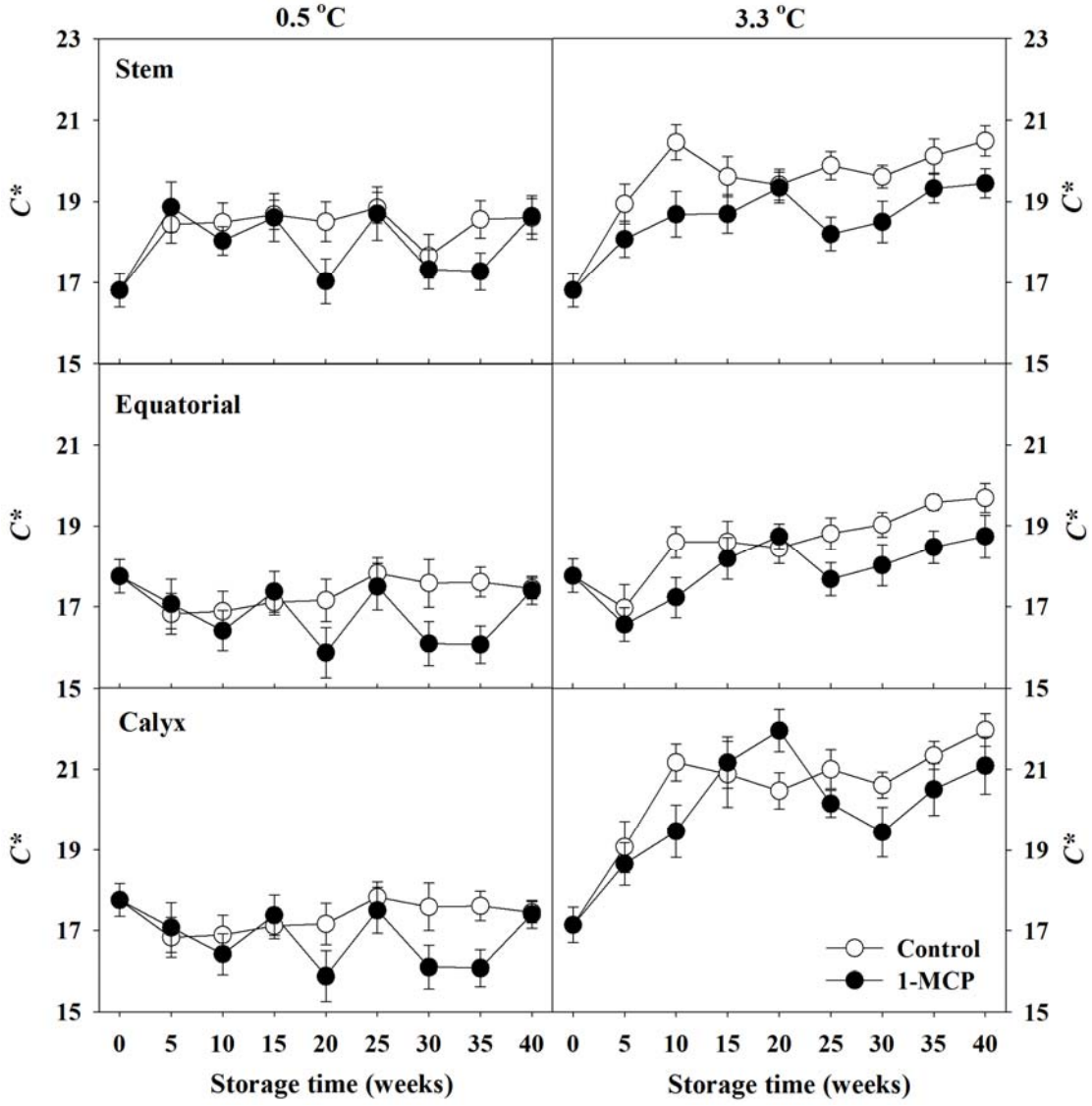

Figure 3.5. Chroma (*C**) values of 'Empire' apple fruit untreated, or treated with 1 µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2kPa CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. The *C** was assessed at the stem-end, equatorial and calyx-end region. Each data point is the mean of ten replicates ± standard error, where larger than the symbols.

treated fruit were more pronounced than at the colder storage temperature (Figure 3.3), the L^* value in all tissue zones of untreated fruit remaining stable with increasing storage, while values decreased in similar patterns in the tissues of 1-MCP treated fruit as found in untreated fruit.

The h^o values showed similar patterns as those found for the L^* values, except that there was little difference between the untreated and 1-MCP treated fruit tissues at 0.5 ^oC (Figure 3.4). In contrast, the C^* values, while higher at 3.3 ^oC than at 0.5 ^oC in stem, equatorial and calyx end tissues (P=0.017, 0.004 and 0.217, respectively), were not affected by 1-MCP treatment (Figure 3.5).

Pearson correlation coefficients for all sampling points found that the browning severity of each fruit was strongly correlated with L^* for stem, equatorial and calyx regions (r=-0.665***, -0.603*** and -0.507***, respectively), and with h^o (r=-0.759***, -0.688*** and -0.691***, respectively), but not with C^* values.

3.3.4. Superoxide anion generation, H_2O_2 concentrations and lipid peroxidation

NBT reducing activity, an indication of superoxide anion radical generation, increased over time (P<0.001) but was lower in 1-MCP treated fruit than untreated fruit (Figure 3.6.), averaging 0.13 and 0.28 OD_{580} g^{-1} h^{-1} (P<0.001), respectively. Also, NBT reducing activity was higher at 3.3 ^oC than at 0.5 ^oC (P=0.017), averaging 0.23 and 0.18 OD_{580} g^{-1} h^{-1}, respectively. However, the increase of NBT activity was greater in untreated fruit than 1-MCP treated fruit at 3.3 ^oC than at 0.5 ^oC (P=0.014).

The accumulation of H_2O_2 was not affected by 1-MCP treatment as a main effect, but the effects of 1-MCP interacted with both storage temperature and time (P<0.001). A large increase of H_2O_2 concentration occurred in untreated fruit stored at 3.3 ^oC at week 5, but patterns of change during storage were inconsistent (Figure 3.6.).

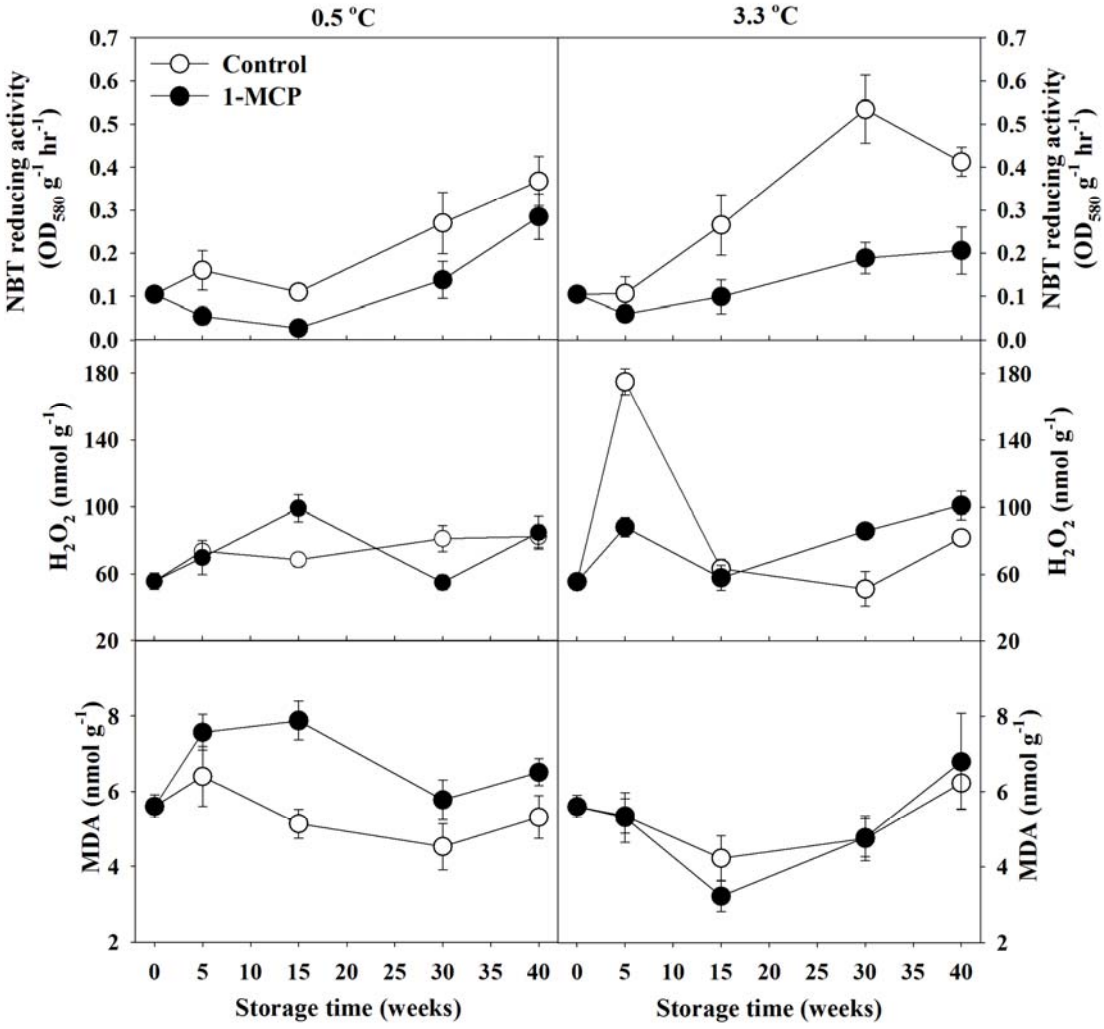

Figure 3.6. Nitroblue tetrazolium (NBT) reducing activity, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents of 'Empire' apple fruit untreated, or treated with 1µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/ 2kPa CO_2 for up to 40 weeks at 0.5 ^{o}C or 3.3 ^{o}C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

Lipid peroxidation, as indicated by MDA concentrations, was higher in 1-MCP treated fruit stored at 0.5 ^{o}C, but not at 3.3 ^{o}C (P=0.008; Figure 3.6.). Overall, MDA concentrations were lower at 3.3 ^{o}C than at 0.5 ^{o}C during the first 15 weeks of storage, but then increased (P=0.002).

3.3.5. Antioxidant metabolites

Overall, AsA concentrations were higher in untreated than 1-MCP treated fruit (P=0.035) and were higher at 0.5 ^{o}C than at 3.3 ^{o}C (P=0.002). However, the effects of 1-MCP were detected only at week 40 in fruit stored at 0.5 ^{o}C, and weeks 30 and 40 in fruit stored at 3.3 ^{o}C (P=0.028). DHA concentrations were affected by an interaction among 1-MCP treatment, storage temperature and storage time (P=0.002; Figure 3.7); an effect of 1-MCP was detected only at weeks 15 and 30, when levels were higher in untreated than 1-MCP treated fruit at 3.3 ^{o}C. The AsA/DHA ratio was affected only by storage time (data not shown).

GSH concentrations were higher in 1-MCP treated fruit than in untreated fruit at week 5 when stored at 0.5 ^{o}C, and lower in 1-MCP treated fruit at week 40 when stored at 3.3 ^{o}C (P=0.006; Figure 3. 8). GSSG concentrations were unaffected by 1-MCP treatment at 0.5 ^{o}C but at 3.3 ^{o}C, initially increased in both untreated and 1-MCP treated fruit, and decreased, but were then higher in 1-MCP treated fruit than untreated fruit at weeks 30 and 40. The GSH/GSSG ratio was unaffected by any treatment (data not shown).

3.3.6. The activities of antioxidant enzymes

APX activity was affected only by an interaction between storage time and storage temperature (P=0.019), an increase in activity occurring at week 40 at 3.3 ^{o}C,

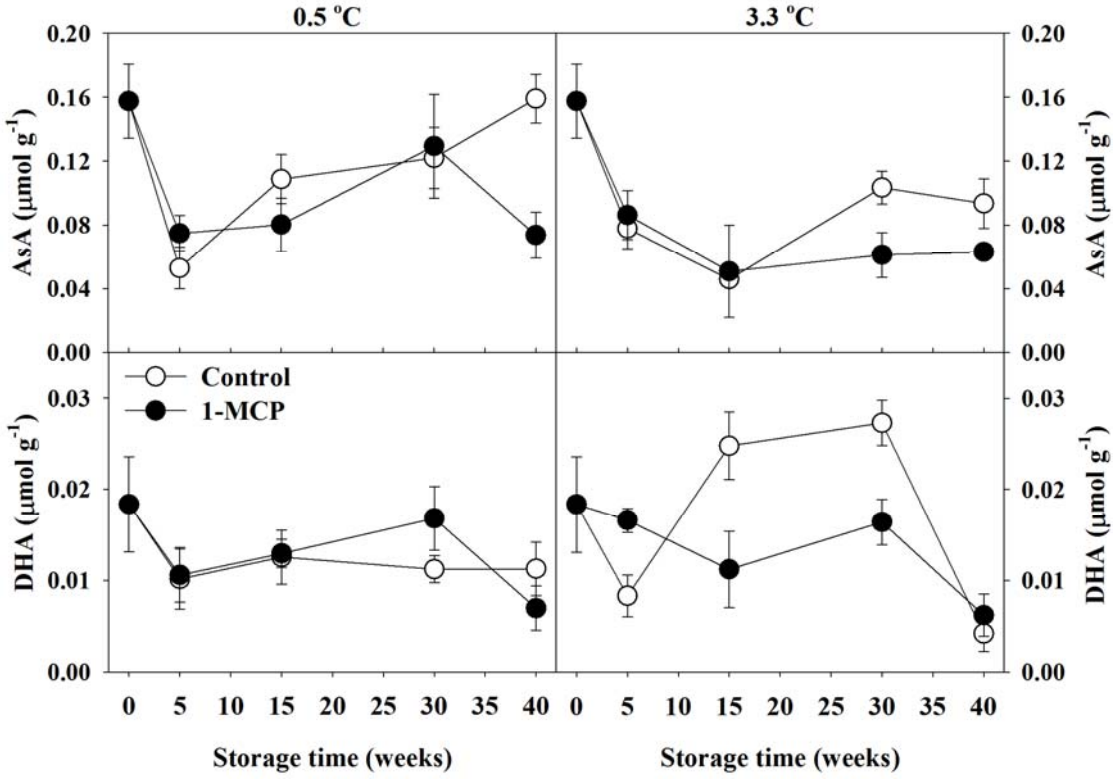

Figure 3.7. Ascorbic acid (AsA) and dehydroascorbate (DHA) of 'Empire' apple fruit untreated, or treated with 1µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

but not at 0.5 ^{o}C (Figure 3.9.). MDHAR activity was affected by an interaction between 1-MCP treatment, storage temperature and storage time (P=0.037), but no clear patterns of change are discernable (Figure 3.9). DHAR activity was lower at 0.5 ^{o}C than at 3.3 ^{o}C (P=0.003), but not affected by 1-MCP as a main effect; however, activity was lower in 1-MCP treated fruit at week 30 at 3.3 ^{o}C (Figure 3.9). GR activity was affected by an interaction among 1-MCP treatment, storage temperature and storage time (P=0.001), but changes were inconsistent (Figure 3.9).

Both SOD and Cu/Zn-SOD activities were affected by an interaction among 1-MCP, storage temperature and storage time (P=0.014 and 0.011, respectively; Figure 3.10). At 0.5 ^{o}C, activity in 1-MCP treated fruit remained relatively unchanged, while activity in untreated fruit decreased at week 30 before increasing at week 40 to levels above those of 1-MCP treated fruit. At 3.3 ^{o}C, there was little difference in SOD activity between untreated and 1-MCP treated fruit with activity remaining similar and then increasing markedly after week 30. However, activity of Cu/Zn-SOD was lower in 1-MCP treated fruit than in untreated fruit at weeks 15 and 30.

CAT activity was initially lower in 1-MCP treated fruit stored at 0.5 ^{o}C, and then activity increased by week 40 to higher rates than in untreated fruit, but there was no treatment effect in fruit stored at 3.3 ^{o}C (P=0.034; Figure 3.10).

POX activity was affected by an interaction among 1-MCP, storage temperature and storage length (P=0.014; Figure 3.10), with a greater increase in activity in untreated than 1-MCP treated fruit at 0.5 ^{o}C, but a smaller increase in 1-MCP treated fruit at 3.3 ^{o}C and no difference between untreated and 1-MCP treated fruit until week 40 at 3.3 ^{o}C.

Pearson correlation coefficients were calculated for browning of each apple at weeks 30 and 40 of storage with antioxidant metabolites and enzyme activities. Only,

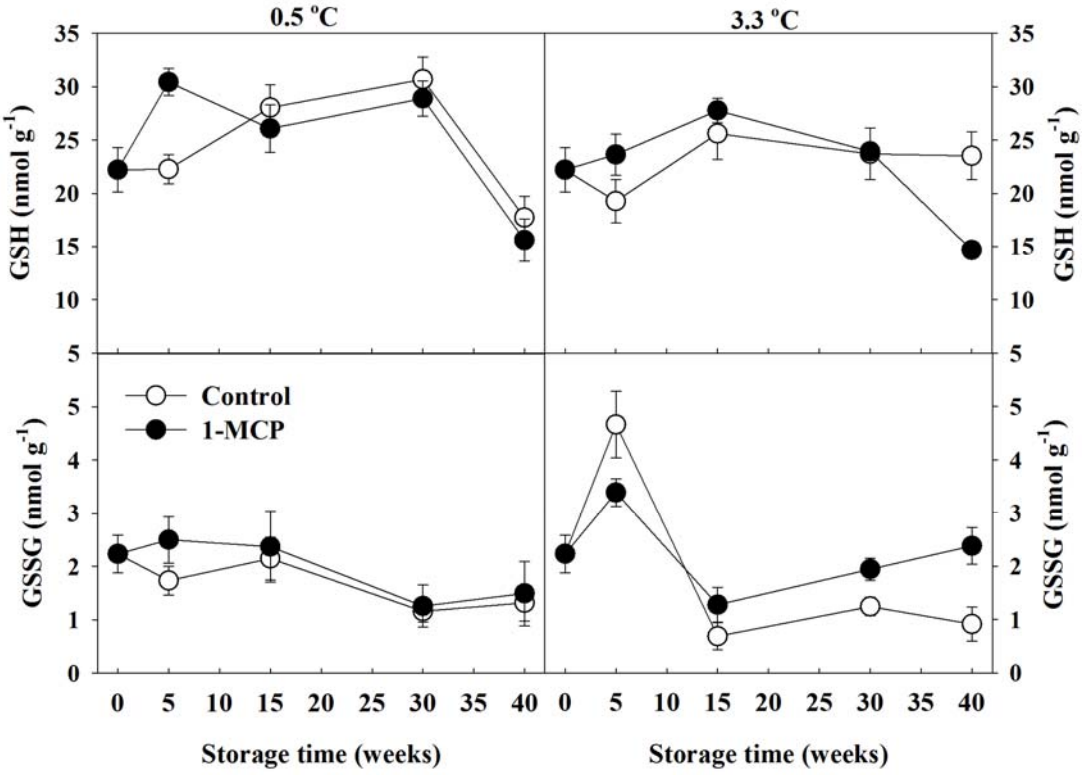

Figure 3.8. Reduced glutathione (GSH) and oxidized glutathione (GSSG) of 'Empire' apple fruit untreated, or treated with 1µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 ^{o}C or 3.3 ^{o}C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

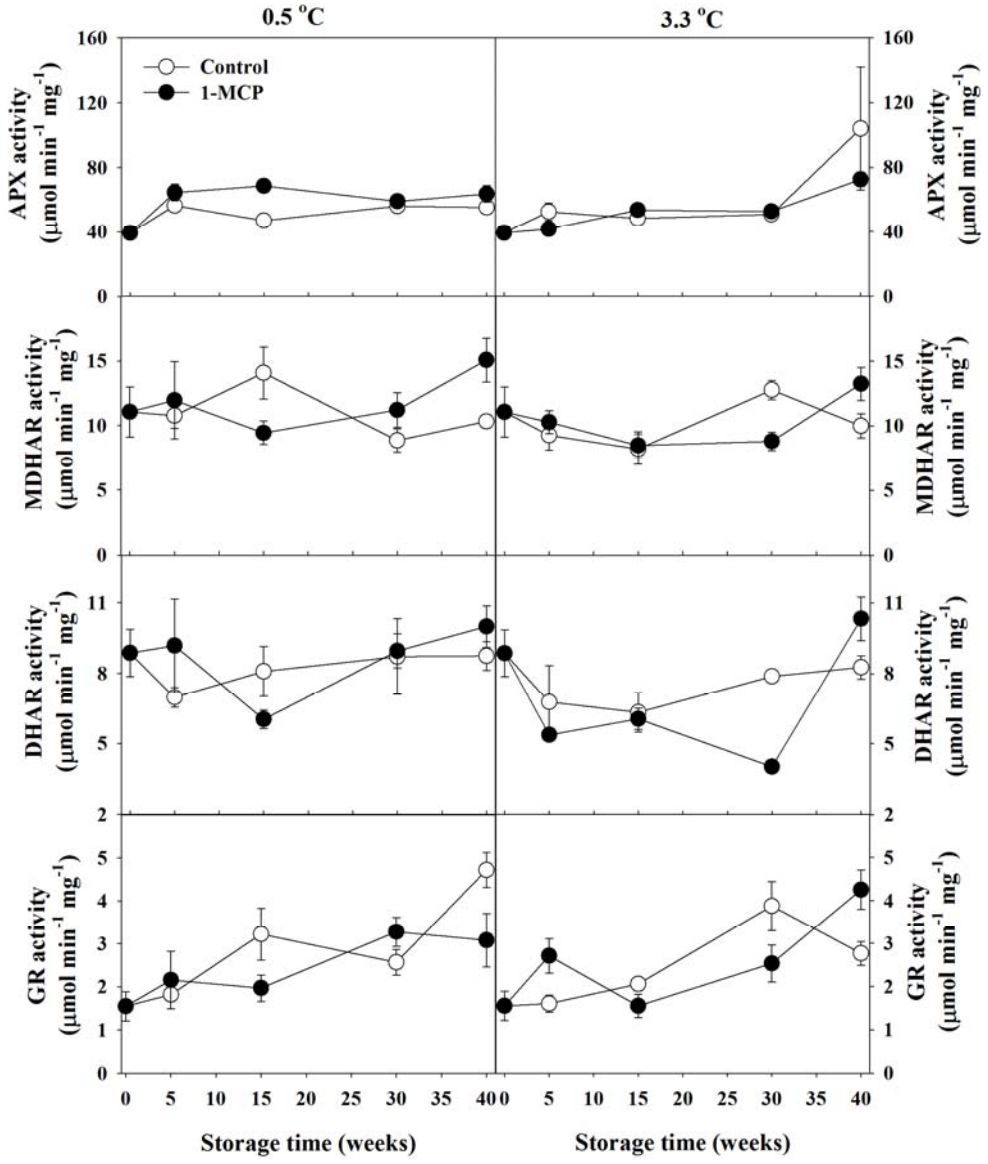

Figure 3.9. Ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) activities on a protein basis of 'Empire' apple fruit untreated, or treated with 1μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2kPa CO_2 for up to 40 weeks at 0.5 ^{o}C or 3.3 ^{o}C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

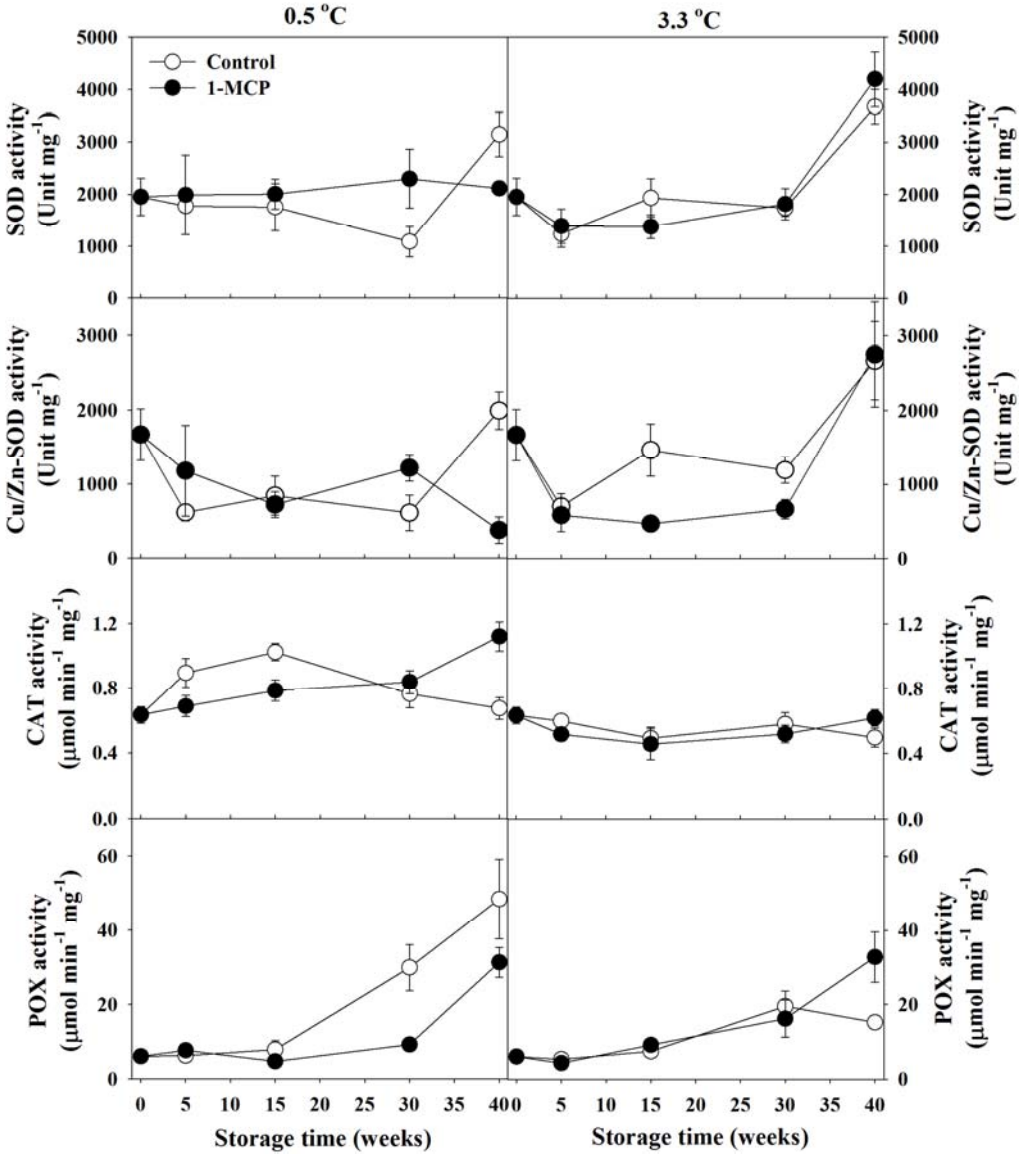

Figure 3.10. Superoxide dismutase (SOD), copper/zinc-superoxide dismutase (Cu/Zn-SOD), catalase (CAT) and guaiacol peroxidase (POX) activities on a protein basis of 'Empire' apple fruit untreated, or treated with 1μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and1-MCP treated apples, respectively. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

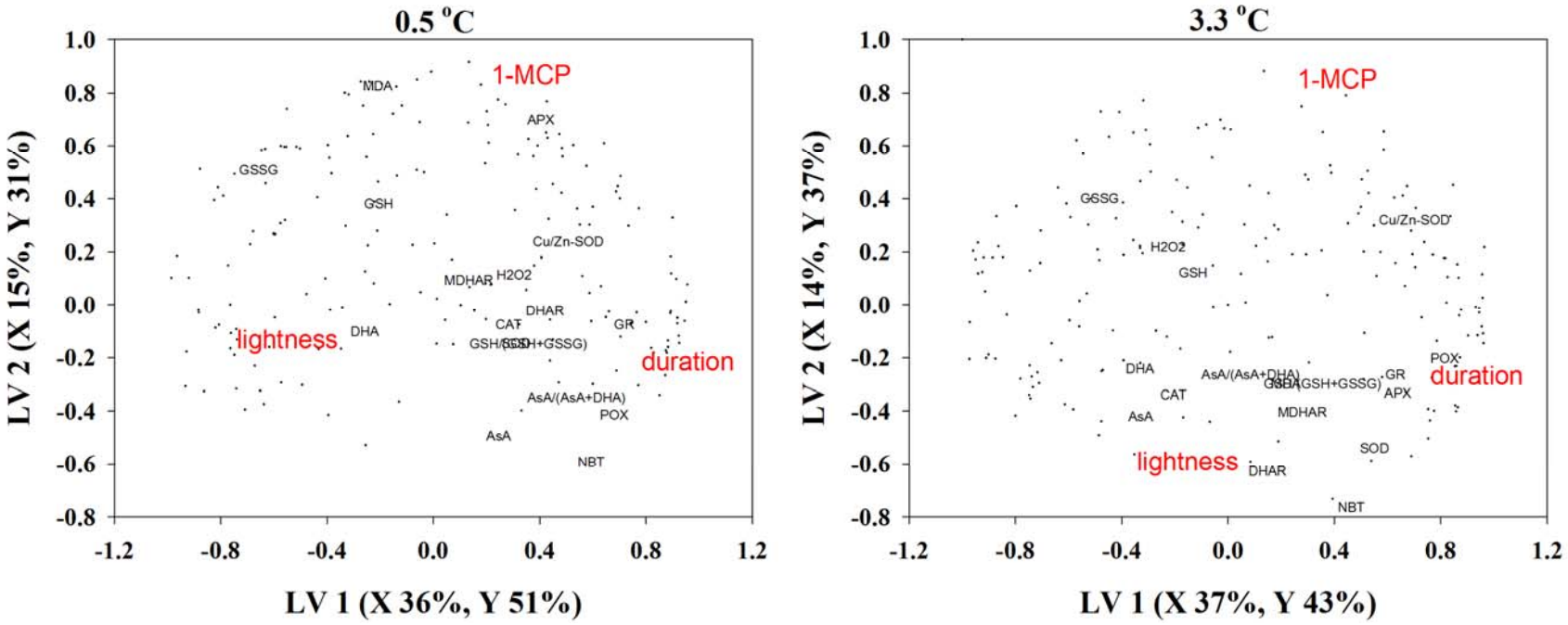

Figure 3.11. PLS-DA loading plots from models containing antioxidant metabolites and Y-variables (experimental factors; red font) loading plots within the metabolic profiles of 'Empire' apple fruit untreated, or treated with 1 μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 or 3.3 °C.

AsA, DHA, GSH and Cu/Zn-SOD were correlated, being r=-0.604***, -0.354*, -0.357* and 0.408**, respectively.

3.3.7. PLS-DA models of antioxidant metabolites

On the PLS-DA loading plots of oxidative stress metabolites, 1-MCP was associated with MDA and APX at 0.5 ^{o}C but not at 3.3 ^{o}C. The lightness value was negatively correlated with the browning scores, and therefore at the opposite place of the lightness variable on the loading plots. Therefore, the browning variable was associated with Cu/Zn-SOD at both temperatures. Furthermore, the majority of oxidative stress metabolites were localized at the positive LV1 scores at 0.5 ^{o}C but plotted at the negative LV2 scores at 3.3 ^{o}C. On the responses of all the antioxidant metabolites, MDA and APX were plotted at positive LV2 scores at 0.5 ^{o}C but most oppositely localized at the negative LV2 scores at 3.3 ^{o}C. The responses of MDA content and APX activity were differently affected by two storage temperatures (Figure 3.11).

3.4. DISCUSSION

The IEC of 1-MCP treated 'Empire' apples has been shown to be much lower than that of untreated fruit in previous studies (Watkins et al., 2000; Fawbush et al., 2008, 2009; DeEll, 2005; Jung and Watkins, 2011). In the current studies, the differences of IEC between treatments were smaller (Figure 3.1), but this is probably a feature of the rapid measurement of fruit after their removal from CA storage where the atmospheres in all fruit would be similar because of diffusion of ethylene among fruit in a closed chamber. In contrast, the IEC in fruit from earlier studies was measured after a 24 h period in air by which time the values are more reflective of endogenous ethylene production. Even where the ethylene production of 1-MCP

treated fruit was shown to increase over time, inhibition of ripening processes as indicated by flesh firmness was prolonged (Jung and Watkins, 2011).

Consistent with the inhibitory effects of 1-MCP in ripening, treated fruit had higher flesh browning incidence and/or severity at 3-4 ^{o}C, and sometimes also at 0 ^{o}C (Figure 3.2) as reported earlier (Watkins, 2008; Jung and Watkins, 2011). The L^* and h^o values indicated that greater color changes of tissues occurred in fruit kept at 0.5 ^{o}C, with or without 1-MCP (Figure 3.3 - 3.4), suggesting that changes were taking place that the naked eye could not detect. However, a clear separation between untreated and treated tissues was detected at later stages of storage at 3.3 ^{o}C. The assumption is that chilling injury, as reflected by L^* and h^o values, occurs at 0.5 ^{o}C, regardless of 1-MCP treatment, and that identical symptoms to those found at this temperature are induced by 1-MCP treatment in fruit stored at 3.3 ^{o}C.

Browning is a result of loss of membrane integrity that results in enzymatic oxidation of phenolic compounds by PPO (Nicolas et al., 1994; Tomas-Barberan and Espin, 2001). It is commonly assumed that changes to membrane integrity occur as a result of impairment of the cellular antioxidant system. The antioxidant metabolite and enzyme activity data obtained in this study were inconsistent with changes in flesh browning, however. The NBT reducing activity, an indication of superoxide anion radical generation, increased during storage and was higher at 3.3 ^{o}C than 0.5 ^{o}C, but was lower in 1-MCP treated than untreated fruit at both temperatures (Figure 3.6). Superoxide anion radicals are typically generated by electron transport systems of photosynthesis and respiration, and NADPH oxidase and NADH peroxidase at the plasma membrane (Mittler, 2002). CA storage of 1-MCP treated fruit resulted in lower respiration rates of ‘Empire’ apples (DeEll et al., 2005). Such treatments could result in suppression of alternative oxidases in electron transport systems of mitochondria, thereby reducing the generation of superoxide anion radicals. No other reports on

superoxide anion radical production in apples under CA conditions appear to be available, although postharvest treatments that result in storage life extension of several crops are associated with lower rates of production of these radicals (Li et al., 2008; Gao et al., 2009; Song et al., 2009; Jiang et al., 2010). Overall, the changes in superoxide anion radical production do not appear to be associated with flesh browning.

As ROS, high H_2O_2 concentrations are often regarded as an indication of oxidative stress, although they are also important in signal transduction involved in various processes (Apel and Hirt, 2004). 1-MCP treatment did not affect H_2O_2 concentrations consistently (Figure 3.6). Vilaplana et al. (2006) found that H_2O_2 concentrations were slightly lower at most time points in air stored 'Golden Smoothie' apples, but there was no flesh browning in the fruit used in that study. The H_2O_2 concentration was higher in CA- than air-stored 'Pink Lady' apples suggesting a response to stress, but concentrations were similar in undamaged and damaged tissues associated with CO_2-induced injury (de Castro et al. 2008). Interestingly, there was a sharp increase of H_2O_2 concentrations in untreated fruit kept at 3.3 ^oC for 5 weeks, which was a similar response to that in CA stored 'Conference' pear (Larrigaudière et al., 2001b). It is possible that H_2O_2 might play a role in a signaling molecule rather than toxic metabolite at this storage temperature, but further research is required.

As the final products of lipid peroxidation, MDA concentrations are often used as indicators of oxidative stress. However, in this study, the accumulation of MDA was not directly associated with the development of flesh browning. MDA concentrations were higher in 1-MCP treated fruit than untreated ones at 0.5 ^oC but there was no difference shown at 3.3 ^oC (Figure 3.6). MDA results were not consistent with NBT reducing activity and H_2O_2 concentrations at 0.5 ^oC. In 'Golden Smoothie' apples, the levels of MDA and 4-hydroxyalkenals (4-HNE) were unaffected by 1-

MCP treatment for the first 15 d of air storage, but were then lower in the 1-MCP treated fruit during subsequent storage (Vilaplana et al., 2006). Therefore, 1-MCP might differently contribute to ROS metabolism and oxidative stress by given storage temperatures.

Overall, while not all ROS were assessed, superoxide anion radical production and H_2O_2 concentrations are at best distinct from each other, and not indicative of higher ROS activity that would result in membrane damage.

Activity of the ascorbic acid and glutathione cycle is regarded as critical to scavenge ROS produced by stress (Noctor and Foyer, 1998). In this study, AsA, DHA, GSH and GSSG were assessed as the responses of antioxidant metabolites to 1-MCP treatment during CA storage (Figure 3.7-3.8). 1-MCP treatment reduced AsA concentrations at both temperatures but reduced DHA concentrations at 3.3 ^{o}C. However, AsA concentrations were reduced immediately after storage at both temperatures and then concentrations gradually recovered to previous concentrations at 0.5 ^{o}C, and to a slower extent at 3.3 ^{o}C in untreated fruit. DHA concentrations did not change over storage at 0.5 ^{o}C but its concentrations accumulated in untreated fruit during storage at 3.3 ^{o}C. Therefore, it is assumed that the conversion of DHA to AsA was suppressed at 3.3 ^{o}C but not by 1-MCP treatment. Furthermore, the abrupt drop of DHA concentration at week 40 might occur because DHA is degraded or converted into another byproduct metabolite rather than reduced back to AsA. The potential byproduct metabolite can be 2,3 diketo-1-gluconic acid from DHA (Washko et al., 1992). The reduction of AsA concentrations could be associated with the development of flesh browning (de Castro et al., 2008). Furthermore, a reduction of AsA concentrations in pear fruit during CA storage period was strongly correlated with the development of flesh browning (Veltman et al., 1999b; Veltman et al., 2000; Zerbini et al., 2002; Franck et al., 2003). Therefore, in this work, when considering the

incidence of flesh browning and AsA concentrations at week 40 (Figure 3.2 and 3.7), the maintenance of the threshold AsA concentrations could play a crucial role in the suppression of the development of flesh browning.

SOD functions to catalyze the dismutation of superoxide anion radical into O_2 and H_2O_2 molecules (Apel and Hirt, 2004). 1-MCP did not affect SOD activity but reduced Cu/Zn-SOD activity at 3.3 ^oC, while effects were inconsistent at 0.5 ^oC (Figure 3.10.). PLS-DA models, however, revealed that Cu/Zn-SOD activity was associated with the development of flesh browning at both temperatures. Du and Bramlage (1994) reported that senescent apple peel had much greater activities of total SOD, Cu/Zn-SOD, and Mn-SOD than healthy apple peel in 'Empire' apple stored at 0 ^oC for 24 weeks. Total SOD activity represents all activities of iron-SOD (Fe-SOD), manganese-SOD (Mn-SOD) and Cu/Zn-SOD. Fe-SOD is localized into chloroplast, Mn-SOD into mitochondria and peroxisome, and Cu/Zn-SOD into cytosol, chloroplast, peroxisome and cell wall, respectively (Alscher et al., 2002). Because superoxide anion radicals cannot diffuse across phospholipid membranes they should be decomposed within the subcellular compartments (Takahashi and Asada, 1983). As another ROS, H_2O_2 can be detoxified by APX, CAT, POX and glutathione peroxidase (GPX) (Mittler, 2002). The relationship between H_2O_2 concentration and any specific H_2O_2-detoxifying enzyme was not consistent in this study. Therefore, it might be implicated that those H_2O_2 detoxifying enzymes might play a cooperative role in scavenging H_2O_2 because H_2O_2 can be produced in several subcellular organs by numerous biochemical reactions such as the conversion of superoxide anion radicals into H_2O_2 in chloroplast and mitochondria, or glycolate oxidase and fatty acid β-oxidation in peroxisome, oxalate oxidase and amine oxidase in apoplast, and NADH peroxidases in cell wall (Mittler, 2002). However, H_2O_2 detoxifying enzyme activities were not affected by 1-MCP at 3.3 ^oC except at week 40. POX activity was highest

only at untreated fruit stored at 0.5 ^{o}C at week 40, where flesh browning was lowest. POX activity might be negatively associated with the development of flesh browning. As the indirect antioxidant scavenging enzymes, MDHAR, DHAR and GR were considered because those reduced DHA and GSSG back to AsA and GSH, respectively. These enzyme activities were not consistent with 1-MCP treatment or storage temperatures.

In conclusion, flesh browning was increased by 1-MCP treatment, and to a greater extent at 3.3 ^{o}C than at 0.5 ^{o}C, as indicated visually and by decreasing lightness and hue angle values over time. AsA concentrations were associated with the development of flesh browning, but it is not clear if this was cause or effect. However, this study did not reveal associations between flesh browning and superoxide production, H_2O_2 concentrations, and lipid peroxidation, although the PLS-DA models demonstrated that association of the lightness variable with AsA suggests that it might be candidate metabolite in flesh browning development at 3.3 ^{o}C.

REFERENCES

Aebi, H., 1984. Catalase in vitro. In: Lester, P. (Ed.), Methods Enzymol.Volume 105. Academic Press. pp. 121-126.

Alscher, R.G., Erturk, N., Heath, L.S., 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. 53, 1331-1341.

Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. 55, 373-399.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248-254.

Chen, L.-S., Cheng, L., 2004. CO_2 assimilation, carbohydrate metabolism, xanthophyll cycle, and the antioxidant system of `Honeycrisp' apple leaves with zonal chlorosis. J. Amer. Soc. Hort. Sci. 129, 729-737.

de Castro, E., Barrett, D.M., Jobling, J., Mitcham, E.J., 2008. Biochemical factors associated with a CO_2-induced flesh browning disorder of Pink Lady apples. Postharvest Biol. Technol. 48, 182-191.

DeEll, J.R., Murr, D.P., Mueller, R., Wiley, L., Porteous, M.D., 2005. Influence of 1-methylcyclopropene (1-MCP), diphenylamine (DPA), and CO_2 concentration during storage on ‘Empire’ apple quality. Postharvest Biol. Technol. 38, 1-8.

Doke, N., 1983. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. Physiol. Plant Pathol. 23, 345-357.

Du, Z., Bramlage, W.J., 1994. Superoxide dismutase activities in senescing apple fruit (*Malus domestica* Borkh.). J. Food Sci. 59, 581-584.

Fawbush, F., Nock, J.F., Watkins, C.B., 2009. Antioxidant contents and activity of 1-methylcyclopropene (1-MCP)-treated 'Empire' apples in air and controlled atmosphere storage. Postharvest Biol. Technol. 52, 30-37.

Franck, C., Baetens, M., Lammertyn, J., Scheerlinck, N., Davey, M.W., Nicolaï, B.M., 2003. Ascorbic acid mapping to study core breakdown development in `Conference' pears. Postharvest Biol. Technol. 30, 133-142.

Frei, M., Wang, Y., Ismail, A.M., Wissuwa, M., 2010. Biochemical factors conferring shoot tolerance to oxidative stress in rice grown in low zinc soil. Functional Plant Biol. 37, 74-84.

Giannopolitis, C.N., Ries, S.K., 1977. Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol. 59, 309-314.

Grace, S.C., Logan, B.A., 1996. Acclimation of foliar antioxidant systems to growth irradiance in three broad-leaved evergreen species. Plant Physiol. 112, 1631-1640.

Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. 106, 207-212.

Hammerschmidt, R., Nuckles, E.M., Kuć, J., 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. Physiol. Plant Pathol. 20, 73-82.

Hodges, D.M., DeLong, J.M., Forney, C.F., Prange, R.K., 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207, 604-611.

Hodges, D.M., Lester, G.E., Munro, K.D., Toivonen, P., M.A., 2004. Oxidative stress: importance for postharvest quality. HortScience 39, 924-929.

Jung, S.-K., Watkins, C.B., 2011. Involvement of ethylene in browning development of controlled atmosphere-stored 'Empire' apple fruit. Postharvest Biol. Technol. in press.

Kochhar, S., Watkins, C.B., Conklin, P.L., Brown, S.K., 2003. A quantitative and qualitative analysis of antioxidant enzymes in relation to susceptibility of apples to superficial scald. J. Amer. Soc. Hort. Sci. 128, 910-916.

Larrigaudière, C., Lentheric, I., Pintó, E., Vendrell, M., 2001a. Short-term effects of air and controlled atmosphere storage on antioxidant metabolism in conference pears. J. Plant Physiol. 158, 1015-1022.

Larrigaudière, C., Pinto, E., Lentheric, L., Vendrell, M., 2001b. Involvement of oxidative processes in the development of core browning in controlled-atmosphere stored pears. J. Hort. Sci. Biotech. 76, 157-162.

Larson, R.A., 1988. The antioxidants of higher plants. Phytochemistry 27, 969-978.

Logan, B.A., Grace, S.C., Adams III, W.W., Demmig-Adams, B., 1998. Seasonal differences in xanthophyll cycle characteristics and antioxidants in *Mahonia repens* growing in different light environments. Oecologia 116, 9-17.

McGuire, R.G., 1992. Reporting of objective color measurements. HortScience 27, 1254-1255.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405-410.

Miyake, C., Asada, K., 1992. Thylakoid-bound ascorbate peroxidase in spinach chloroplasts and photoreduction of its primary oxidation product monodehydroascorbate radicals in thylakoids. Plant Cell Physiol. 33, 541-553.

Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867-880.

Nicolas, J.J., Richard-Forget, F.C., Goupy, P.M., Amiot, M.J., Aubert, S.Y., 1994. Enzymatic browning reactions in apple and apple products. Crit. Rev. Food Sci. Nutr. 34, 109-157.

Noctor, G., Foyer, C.H., 1998. Ascorbate and glutathione: Keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 249-279.

Patterson, B.D., MacRae, E.A., Ferguson, I.B., 1984. Estimation of hydrogen peroxide in plant extracts using titanium(IV). Anal. Biochem. 139, 487-492.

Pérez-Enciso, M., Tenenhaus, M., 2003. Prediction of clinical outcome with microarray data: a partial least squares discriminant analysis (PLS-DA) approach. Hum. Genet. 112, 581-592.

Takahashi, M.A., Asada, K., 1983. Superoxide anion permeability of phospholipid-membranes and chloroplast thylakoids. Arch. Biochem. Biophys. 226, 558-566.

Toivonen, P.M.A., 2004. Postharvest storage procedures and oxidative stress. HortScience 39, 938-942.

Tomas-Barberan, F.A., Espin, J.C., 2001. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. J. Sci. Food Agric. 81, 853-876.

Veltman, R.H., Sanders, M.G., Persijn, S.T., Pemppelenbos, H., Oosterhaven, J., 1999. Decreased ascorbic acid levels and brown core development in pears (*Pyrus communis* L. cv. Conference). Physiol. Plant. 107, 39-45.

Veltman, R.H., Kho, R.M., van Schaik, A.C.R., Sanders, M.G., Oosterhaven, J., 2000. Ascorbic acid and tissue browning in pears (*Pyrus communis* L. cvs Rocha and Conference) under controlled atmosphere conditions. Postharvest Biol. Technol. 19, 129-137.

Washko, P.W., Welch, R.W., Dhariwal, K.R., Wang, Y., Levine, M., 1992. Ascorbic acid and dehydroascorbic acid analyses in biological samples. Anal. Biochem. 204, 1-14.

Watkins, C., Liu, F.W., 2010. Temperature and carbon dioxide interactions on quality of controlled atmosphere-stored 'Empire' apples. HortScience 45, 1708-1712.

Watkins, C.B., Nock, J.F., Whitaker, B.D., 2000. Responses of early, mid and late season apple cultivars to postharvest application of 1-methylcyclopropene (1-MCP) under air and controlled atmosphere storage conditions. Postharvest Biol. Technol. 19, 17-32.

Zerbini, P.E., Rizzolo, A., Brambilla, A., Cambiaghi, P., Grassi, M., 2002. Loss of ascorbic acid during storage of Conference pears in relation to the appearance of brown heart. J. Sci. Food Agric. 82, 1007-1013.

CHAPTER 4

METABOLIC CHANGES IN 1-MCP TREATED 'EMPIRE' APPLE AT DIFFERENT STORAGE TEMPERATURES

ABSTRACT

'Empire' apple fruit are susceptible to the development of flesh browning at 0.5 ^{o}C, and at 3.3 ^{o}C if the fruit have been treated with 1-MCP. To better understand the metabolic changes associated with flesh browning, metabolic screening using GC-MS methods have been used with untreated and 1-MCP treated 'Empire' fruit stored at 2 kPa O_2/2 kPa CO_2 at 0.5 and 3.3 ^{o}C for up to 40 weeks. Partial least squares discriminant analyses (PLS-DA) were employed to identify metabolic divergence and separation between the untreated and 1-MCP treated fruit, and storage temperature. Carbohydrates were not influenced by 1-MCP treatment and storage temperature. Malic, succinic and 2-oxoglutaric acid concentrations decreased during storage regardless of 1-MCP treatment and storage temperature. Sorbitol, tryptophan, phenylalanine, glutamate, 5-oxoproline, aspartate, homoserine, threonine, isoleucine, valine, leucine, serine, chlorogenic acid, phloridzin, and catechin increased in 1-MCP treated fruit at 3.3 ^{o}C. Most volatile compounds were strongly increased at 3.3 ^{o}C, while only done at week 5 at 0.5 ^{o}C. 1-MCP reduced the production of volatile compounds at both storage temperatures, except for (*E*)-2-hexenal. The PLS-DA loading plots revealed that sorbitol and γ-aminobutyric acid could be strongly involved in the development of flesh browning at 3.3 ^{o}C. Therefore, these results indicated that metabolic alterations occurred in response to 1-MCP treatment, especially during storage at 3.3 ^{o}C, and that these changes are associated with development of flesh browning.

Keywords: apple; metabolomics; partial least squares discriminant analysis; GC-MS; carbohydrates; organic acids; amino acids; ester; volatiles; phenolic compounds

4.1. INTRODUCTION

Metabolite concentrations of apple fruit change after harvest and during storage (Ackermann et al., 1992). These changes include loss of sucrose and accumulation of glucose and fructose (Drake and Eisele, 1999), and accumulation of sorbitol during storage (Fidler and North, 1970). Malic acid, a major organic acid in apple fruit, can be used as a substrate for the respiration and its concentrations typically decrease during storage (Ingle et al., 2000; Bai et al., 2005). Amino acids and fatty acids associated with membrane phospholipids and glycolipids also decrease during storage and ripening (Ackermann et al., 1992), and of the secondary metabolites, phenolic compounds respond differently among apple cultivars (Coseteng and Lee, 1987; Burda et al., 1990). Volatile compounds are affected by storage duration, typically declining, especially under controlled atmosphere (CA) storage conditions (Fellman et al., 1993; Echeverría et al., 2004a).

1-MCP-based technology is widely used by apple industries in the US and around the world (Watkins, 2008). 1-MCP delays ripening and extends the storage and shelf life of fruit. It can delay the loss of titratable acidity and soluble solids content (Bai et al., 2005; Larrigaudière et al., 2008) and suppress aromatic volatile production (Rupasinghe et al., 2000; Moya-Leon et al., 2007). However, little is known about the effects of 1-MCP on most metabolic processes associated with biosynthesis and degradation of primary and secondary metabolites during fruit ripening.

1-MCP can affect several storage disorders of apple fruit (Watkins, 2006, 2007). Some disorders, such as superficial scald, are inhibited by the treatment (Rupasinghe et al., 2000; Watkins et al., 2000; Tsantili et al., 2007), but the incidence of others such

as CO_2 injury can be increased (Fawbush et al., 2008). Of particular concern to the New York apple industry has been flesh browning (Watkins and Liu, 2010b), and its enhancement by 1-MCP treatment (Watkins, 2008; Jung and Watkins, 2011).

One approach to understanding the effects of 1-MCP is to characterize and identify specifically targeted metabolites, as was carried out as described in Chapter 3. An alternative approach is the use of untargeted global metabolic profiling methods. These include GC-MS methods for analysis of trimethylsilyl (oxime) derivatives in plants along with the advanced statistical modeling systems (Roessner et al., 2000; Roessner et al., 2001; Lisec et al., 2006). Rudell et al. (2008; 2009) employed global metabolic profiling systems using 'Granny Smith' apple fruit peel tissues exposed to UV-white light irradiation, and superficial scald of apple fruit.

The objective of my study was to use this analytical approach to reveal any primary and secondary metabolic alterations in 'Empire' apple that are associated with 1-MCP in relation to flesh browning. The metabolic association and separation with the given response variables was evaluated; the relative responses of individual metabolites were screened; subsequently metabolic fluctuation was characterized in untreated and 1-MCP treated fruit at different storage temperatures.

4.2. MATERIALS AND METHODS

4.2.1. Fruit source, treatments and storage conditions, and fruit sampling

Details of fruit harvest, storage and sampling are outlined in Chapter 3.

4.2.2. Metabolite extraction

Frozen flesh tissue (100 mg per replication, five replications per treatment) was weighed into liquid nitrogen-cooled 1.5 mL screw-top microcentrifuge tubes to which ~100 μL of 0.5 mm (diameter) soda lime glass beads (BioSpec Products, Inc.,

Bartlesville, OK) were added. The tissue/bead mixture remained liquid nitrogen-cooled until initiation of the extraction procedure when 1 mL of MeOH was added. Samples were then shaken vigorously using a Mini Beadbeater (BioSpec Products, Inc.) for 1 min and then floated in a 70 ^{o}C water bath for 15 min to inhibit any sample degradation resulting from 5 min at 25 ^{o}C in an ultrasonic bath followed by centrifugation for 2.5 min at 16,000 g.

4.2.4. Derivatization and GC-MS analysis

Methoxyamination and trimethylsilylation followed a procedure similar to that described by Rudell et al.(2008). Supernatant aliquots (10 or 300 µL) were placed into borosilicate glass test tubes followed by the addition of 45 µL phenyl β-D-glucopyranoside (291 ng μL^{-1} in methanol) internal standard solution. The mixture was dried under a stream of nitrogen gas and then the residue was dissolved in 125 µL of methoxyamine (20 mg mL^{-1} in anhydrous pyridine) solution and incubated for 90 min at 30 ^{o}C. Then, 125 µL of *N*,*O*-bis(trimethylsilyl)trifluoroacetaminide (BSTFA) was added and the mixture incubated for 30 min at 37 ^{o}C. Samples were then transferred into glass vials containing deactivated glass inserts and analyzed by GC-MS.

For the major carbohydrate metabolites analysis, each derivatized extract (1 µL) was injected into 5890N GC coupled with a 5971 mass selective detector (MDS) using a 7673 automatic injector (Agilent Technologies, Palo Alto, CA). Samples were volatilized in a 230 ^{o}C split (5:1) inlet lined with an unpacked 4mm internal diameter, deactivated, and taper-bottom glass liner. The GC column was a ZB 5 MB (30 m (length) × 0.25 µm (film thickness) × 0.25 mm (ID), Agilent Technologies, Palo Alto, CA). The oven initial temperature was 40 ^{o}C held for 2 min followed by an 18 ^{o}C/min increase to a final temperature 325 ^{o}C that was held for 6 min. The detector was

operated in EI mode with transfer line, source, and quadrupole temperature maintained at 250, 150, and 230 ^{o}C, respectively. Mass spectra ranging from *m/z* 30 to *m/z* 550 were recorded.

For the untargeted metabolites analysis, derivatized extract (0.5 µL) was injected into a 6890N GC coupled with a 5975B mass selective detector (MSD) using a 7683B automatic injector (Agilent Technologies, Palo Alto, CA). Samples were volatilized in a 230 ^{o}C splitless inlet lined with an unpacked 4 mm internal diameter, deactivated, tapered-bottom glass liner. Further focusing of the sample was accomplished using a pulsed injection technique that maintained a He carrier gas linear velocity of 66 cm s^{-1} for the first 0.25 min, reducing it to 40 cm s^{-1} thereafter. The GC column was a HP-5MS (30 m × 250 μm × 0.25 μm, Agilent Technolgoies, Palo Alto, CA). The initial oven temperature was held at 40 ^{o}C for 2 min followed by an 18 ^{o}C/min increase to a final temperature of 330 ^{o}C that was held for 6 min. The detector was operated in EI mode with transfer line, source, and quadrupole temperatures maintained at 250, 150, and 230 ^{o}C, respectively. Mass spectra ranging from *m/z* 30 to *m/z* 600 were recorded.

4.2.4. Volatile metabolite analysis

Frozen apple flesh tissue powder (1 g) was weighed into 20 mL glass headspace vials (Gerstel, Baltimore, MD) previously chilled in liquid nitrogen. Vials containing sample were removed from liquid nitrogen, 1 mL of saturated NaCl solution and 10 µL aqueous internal standard mixtures containing 34.4 ng μL^{-1} 1-methylethyl-butyrate and 33.4 ng μL^{-1} 5-hexanol were added, and then the vials were sealed. Vials were incubated at 22 ^{o}C for 5 min and then sonicated at 25 ^{o}C in an ultrasonic bath for 5 min prior to headspace analysis. Headspace vial temperature was maintained at -1 ^{o}C using a cooled sample tray until sampling.

Headspace vial was analyzed using an Agilent 6890N gas chromatograph coupled with a 5975B mass selective detector (Agilent Technologies, Palo Alto, CA) and an automated Gerstel multipurpose sampler (MPS) equipped with a dynamic headspace sampler (DHS). Headspace vial temperature was maintained at -1 ^{o}C using a cooled sample tray until sampling. At the beginning of the headspace sampling sequence, vials were vortexed at 1000 rpm during incubation at 30 ^{o}C for 10 min prior to sampling. Analyte was collected by sweeping 220 mL of He at 20 mL min^{-1} through the headspace and then a 60 mm (length) × 6 mm (OD) glass tube (trap) containing 90 mg of Tenax TA (60/80 mesh) and 60 mg of Carbosieve 3 (60/80 mesh) (Supelco, St. Louis, MO) maintained at 30 ^{o}C. After loading, water was removed from the trap by sweeping the sorption bed with 400 mL of He at 40 mL min^{-1} while the trap was heated.

Traps were desorbed at 20 mL min^{-1} with the inlet set in the solvent purge mode and purge pressure adjusted to the inlet pressure. The desorption temperature program started at 30 ^{o}C for 0.2 min, increasing at 72 ^{o}C min^{-1} to 300 ^{o}C and then holding for 3 min. Desorbed analyte was collected on glass bead-filled liner maintained at -145 ^{o}C for the entire desorption period.

Analyte was introduced into the GC column (HP-5MS, 30 m × 250 μm × 0.25 μm, Agilent Technolgoies, Palo Alto, CA) by heating the liner to 150 at 16 ^{o}C s^{-1} and then to a final temperature of 300 at 12 ^{o}C s^{-1}, which was held for 3 min. The He carrier linear velocity was 40 cm s^{-1}. The injection split ratio was 1:5. The oven temperature was held for 0.5 min at 30 ^{o}C and then increased to 300 ^{o}C at 12 ^{o}C min^{-1}. The detector was operated in the EI mode with transfer line, source and quadrupole temperatures maintained at 250, 150, and 230 ^{o}C, respectively. Mass spectra ranging from *m/z* 30 to *m/z* 600 were recorded.

4.2.5. Data acquisition, deconvolution and peak identification

User-defined libraries were generated using the automated mass spectral deconvolution and identification system (AMDIS; National Institute of Standards) to deconvolute GC-MS results and identify distinct chromatographic components. Retention indices (RI) were generated for each sequence by comparing the retention times of C10-C40 hydrocarbons evaluated under the same conditions as the samples with the retention times of sample components. Individual libraries made from samples from each treatment were compared and redundant components eliminated. From these libraries, mass spectral tags (MSTs) were cataloged using the RI coupled with key mass spectral features and calibration tables generated using Chemstation (G1701DA rev. D; Agilent Technologies, Palo Alto, CA). The Qedit macro was used to evaluate each compound and provide peak areas for components. Mass spectral comparison with spectral catalogues in NIST05 (National Institute of Standards) and mass spectral interpretation aided in tentative identification of many of the components. Compound identifications are based on comparison of sample compound spectra and RIs with those of authentic standards.

4.2.6. Statistical modeling and analysis

Raw peak area data were corrected by comparison of phenyl β-D-glucopyranoside for trimethylsilyl(oxime) protocol and 1-methylethyl butanoate for headspace analysis protocol in each sample with that in an external standard and then by sample fresh weight. None of the compounds used as internal standards were detected in unspiked samples.

Metabolite data were analyzed by using partial least-squares discriminant analysis (PLS-DA) (Pérez-Enciso and Tenenhaus, 2003). PLS-DA relates variations in a limited number of predictor variables (Y-variables) to the variations of a large

number of predictor variables (X-variables). PLS-DA is a regression analysis technique in which the original X-data are projected onto a small number of underlying latent variables (LV), which are concurrently used for regression of the Y-data in such a way that the first LVs are most relevant for predicting the Y-variables.

The metabolites were considered to be predictor variables, whereas treatment factors (untreated or 1-MCP-treated), storage time (duration, 0-40 weeks), and lightness (flesh tissue color) were considered to be response variables. The treatments factors were introduced as separate categorical variables (reading either 0 or 1), whereas storage time and lightness values were included as continuous variables. Both X- and Y-data were mean centered and scaled to unit variance to give all variables an equal chance to influence the model. PLS-DA was performed using The Unscrambler version 9.6 (Camo A/S, Trondheim, Norway).

4.3. RESULTS

4.3.1 Flesh browning

Flesh browning occurred earlier and was more severe at 3.3 ^{o}C than at 0.5 ^{o}C in 1-MCP treated fruit as storage time progressed (Figure 3.2). For the purposes of analyses, lightness or L* was used to quantify browning, where low and high values indicate browning or clean tissue, respectively.

4.3.2. PLS-DA models for metabolic profiles

The multivariate PLS-DA model for scores plot illustrated the considerable divergence and separation of apple flesh metabolomes with increasing storage duration and in response to 1-MCP treatment (Figure 4.1). Even, storage temperatures caused to show different metabolomic separation and divergence on the scores plots. The PLS-DA multivariate statistical models for scores and loading plots accounted for

36% and 15%, and 37% and 14% of the total X variance and 51% and 31%, and 43% and 37% of the total Y variance being explained by the two latent variables (LV1 and LV2) at 0.5 ^{o}C and 3.3 ^{o}C, respectively.

The PLS-DA multivariate statistical models for loading plots indicated that the separation and association of major carbohydrates, ester compounds, volatiles compounds (Figure 4.2), organic acids, amino acids, and major phenolic compounds (Figure 4.3) responded differently to the variables, 1-MCP treatment, storage duration, and lightness, depending on storage temperatures. As response variables, lightness plotted differentially depending on storage temperatures, while the other two variables were not affected relatively by temperature. Glucose, fructose and sorbitol were associated with duration, but sucrose and malic acid were less so at 3.3 ^{o}C. At 0.5 ^{o}C, sorbitol and fructose were less associated with storage duration, while sorbitol was more closely associated with 1-MCP treatment.

All ester compounds were localized at the positive scores of Y axis LV2 at 0.5 ^{o}C but placed at the negative scores of Y axis LV2 at 3.3 ^{o}C. For the volatile compounds, lightness was highly associated with majority of volatile compounds at 0.5 ^{o}C but less so at 3.3 ^{o}C. However, volatiles were negatively associated with storage duration (Figure 4.2).

Organic acids were more broadly distributed than amino acids at the loading plots but citric, fumaric, mucic and shikimic acids were more associated with storage duration. Succinic, pyruvic and 2-oxoglutaric acids were more associated with lightness at 0.5 ^{o}C. However, malonic acid was more associated with duration variable at 3.3 ^{o}C. Shikimic and fumaric acids were strongly associated with duration at 0.5 ^{o}C but those compounds were almost oppositely localized with duration variable at 3.3 ^{o}C. Also, malonic acid was almost oppositely localized with duration variable depending on storage temperature (Figure 4.3). Amino acids were more associated with 1-MCP

and storage duration variables rather than the lightness variable at both storage temperatures. All amino acids were more densely plotted at 3.3 ^{o}C than 0.5 ^{o}C. Threonine, leucine and glutamate were more associated with 1-MCP variable at 0.5 ^{o}C but glutamate and 5-oxoproline were more strongly associated with 1-MCP variable at 3.3 ^{o}C. Alanine was most strongly associated with storage duration variable at either storage temperature (Figure 4.3). All phenolic compounds were relatively less associated with any response variables. Of these phenolic compounds, phloridzin was the most differently plotted to the given storage temperatures (Figure 4.3).

4.3.3. Carbohydrates and sugar alcohol

Figure 4.4 illustrates the relative responses of intermediate and byproduct metabolites of glycolysis in 1-MCP treated fruit flesh. Sucrose decreased during storage but more slowly at 0.5 ^{o}C than at 3.3 ^{o}C. In contrast, fructose and glucose increased during storage at both temperatures. The effects of 1-MCP treatment on fructose and glucose were generally small, except that levels of both compounds were higher in 1-MCP treated fruit at week 15 in 3.3 ^{o}C. Sorbitol levels tended to be higher in 1-MCP treated fruit than in untreated fruit, but only after several weeks in fruit stored at 3.3 ^{o}C

Erythritol was generally higher in 1-MCP treated fruit than untreated fruit at 0.5 ^{o}C but not at 3.3 ^{o}C. *myo*-Inositol increased at the latter storage periods, and levels were not influenced by 1-MCP. Glucose 6-phosphate and fructose 6-phosphate levels increased at week 5 in fruit at both temperatures, but the effects of 1-MCP varied by temperature. While the level of these compounds increased in all 1-MCP treated fruit, the increases of glucose 6-phosphate and fructose 6-phosphate in untreated fruit were less at 0.5 than 3.3 ^{o}C at week 5. After then, the levels of glucose 6-phosphate and

fructose 6-phosphate at 3.3 ^{o}C were slightly higher in 1-MCP treated fruit than in untreated ones at weeks 30 and 40, but there was no difference shown at 0.5 ^{o}C.

Raffinose accumulated to a greater extent at 0.5 ^{o}C than at 3.3 ^{o}C, and 1-MCP increased accumulation slightly at 0.5 ^{o}C. Rhamnose was higher at 0.5 ^{o}C but lower at 3.3 ^{o}C by 1-MCP treatment at week 5. However, ribose was higher by 1-MCP at week 5 at both temperatures and then during the rest of storage, it was not affected by 1-MCP at both temperatures.

4.3.4. Organic acids

Pyruvic acid declined rapidly at both storage temperatures and was not affected by 1-MCP treatment (Figure 4.5). Citric acid levels were inconsistent during storage and not affected by 1-MCP except at week 40, when levels were lowest in 1-MCP treated fruit at both temperatures. 2-Oxoglutamic and succinic acid levels declined during storage and were not affected by 1-MCP treatment. Fumaric acid was higher in 1-MCP treated than untreated fruit and also greater at 0.5 ^{o}C than at 3.3 ^{o}C. Malic acid declined over time at either storage temperature and was not affected by 1-MCP treatment.

Threonic, maleic, and citramalic acids were inconsistently affected by storage time and 1-MCP treatment (Figure 4.4 and 4.5). Shikimic acid changed little during storage at 0.5 ^{o}C but declined at 3.3 ^{o}C. Quinic acid was not affected consistently by storage temperature and 1-MCP treatment (Figure 4.7). Malonic, phosphoric and dodecanoid acids were highest in 1-MCP treated fruit at 3.3 ^{o}C. Mucic acid was lowest in the 1-MCP treatment only at 3.3 ^{o}C (Figure 4.8).

4.3.5. Amino acids

Glutamate levels increased to a greater extent in 1-MCP treated than untreated fruit, but to a greater extent at 0.5 ^{o}C than at 3.3 ^{o}C. GABA and proline levels were not consistently affected by 1-MCP treatment at 0.5 ^{o}C, but tended to be slightly higher in treated fruit at 3.3 ^{o}C. 5-Oxoproline levels were higher in 1-MCP treated than untreated fruit, but more consistently at 3.3 ^{o}C (Figure 4.5).

Aspartate, homoserine, threonine, and isoleucine were higher at 1-MCP treated than untreated fruit at 3.3 ^{o}C, while treatment effects for asparagine and β-alanine were inconsistent. Alanine levels increased during storage but they were not affected by 1-MCP treatment. Valine and leucine levels were usually highest in 1-MCP treated fruit, though earlier in the storage period. Serine was higher at 1-MCP treated fruit than at untreated ones at 3.3 ^{o}C but effects on glycine were inconsistent (Figure 4.6).

Tryptophan and phenylalanine levels increased during storage and tended to be higher in 1-MCP treated than untreated fruit (Figure 4.7). Norvaline levels remained unchanged at 0.5 ^{o}C but they increased at 3.3 ^{o}C (Figure 4.9).

4.3.6. Fatty acid metabolites

Metabolites associated with fatty acid biosynthesis (glycerol, glycerol 3-phosphate and linoleic acid) were not affected consistently by storage temperature or 1-MCP treatment (data not shown).

4.3.7. Phenolic compounds

Changes in chlorogenic acid, phloridzin, catechin and (-) epicatechin levels were similar over time in untreated or 1-MCP treated fruit during storage at 0.5 ^{o}C. However, levels of these compounds tended to increase in untreated fruit stored in 3.3

^{o}C at week 5, before decreasing, and levels were higher at 1-MCP treated fruit than at untreated ones at weeks 20 and 30 (Figure 4.7).

4.3.8. Volatile compounds

Methanol, methyl acetate, methyl butyrate, methyl 2-methylbutyrate and methyl hexanoate metabolites were essentially absent at 3.3 ^{o}C, but accumulated greatly at 0.5 ^{o}C (Figure 4.9). Methanol levels remained high throughout the experiment, and to a slightly greater extent in the 1-MCP treated fruit. The other compounds were not affected consistently by 1-MCP.

Levels of 1-butanol, butanoic acid hexyl ester, butyl butyrate, 1-hexanol, butanoic acid propyl ester, butanoic acid 2-methyl- hexyl ester, propyl acetate, 1-pentanol, 1-propanol and butyl 2-methyl butyrate were generally high in untreated fruit during storage at 3.3 ^{o}C, while 1-MCP treated fruit had relatively rapid declines in levels. At 0.5 ^{o}C, most of these compounds peaked at week 5 in untreated fruit, but then levels declined rapidly by week 15 (Figures 4.10 and 4.11). Acetic acid butyl ester, acetic acid hexyl ester and pentyl acetate were also generally higher in untreated than at 1-MCP treated fruit at both storage temperatures. However, (*E*)-2-hexenal levels tended to be higher in 1-MCP treated than untreated fruit beyond 15 weeks of storage (Figure 4.12). 1-MCP reduced the levels of acetaldehyde, ethanol, and ethyl acetate at either temperature only at week 5. The metabolic response of these fermentative metabolites in untreated fruit was greater at 0.5 ^{o}C than at 3.3 ^{o}C. Furthermore, while these fermentative metabolites after week 5 were not changed at 3.3 ^{o}C, 1-MCP treatment slightly enhanced the relative responses of fermentative metabolites at the end of storage at 0.5 ^{o}C. Those fermentative metabolomic responses were differently reacted to storage temperature and 1-MCP treatment (Figure 4.13).

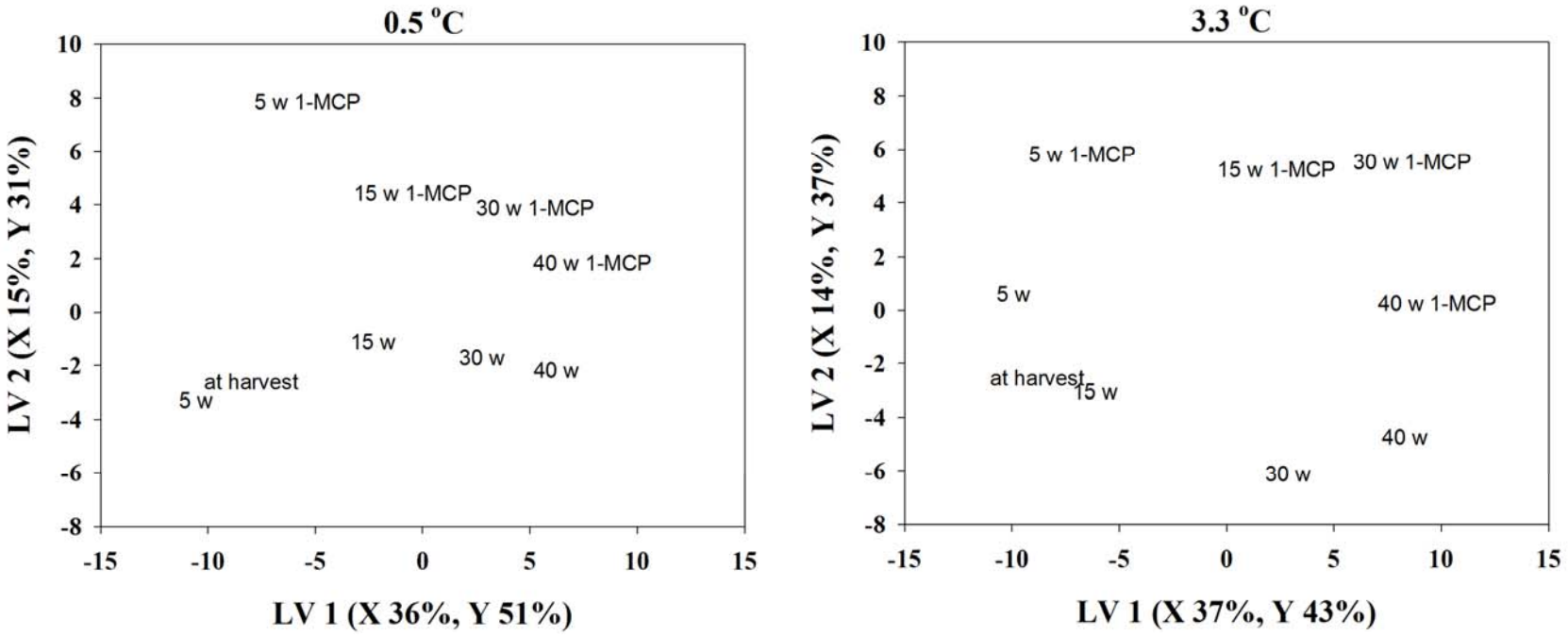

Figure 4.1. PLS-DA scoring plots of the metabolic profile of 'Empire' apple fruit untreated, or treated with 1 μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 or 3.3 °C. The first number of label on the plot represents storage duration (5, 15, 30 and 40 weeks) and w stands for weeks. 1-MCP represents 1-MCP treated apple fruit.

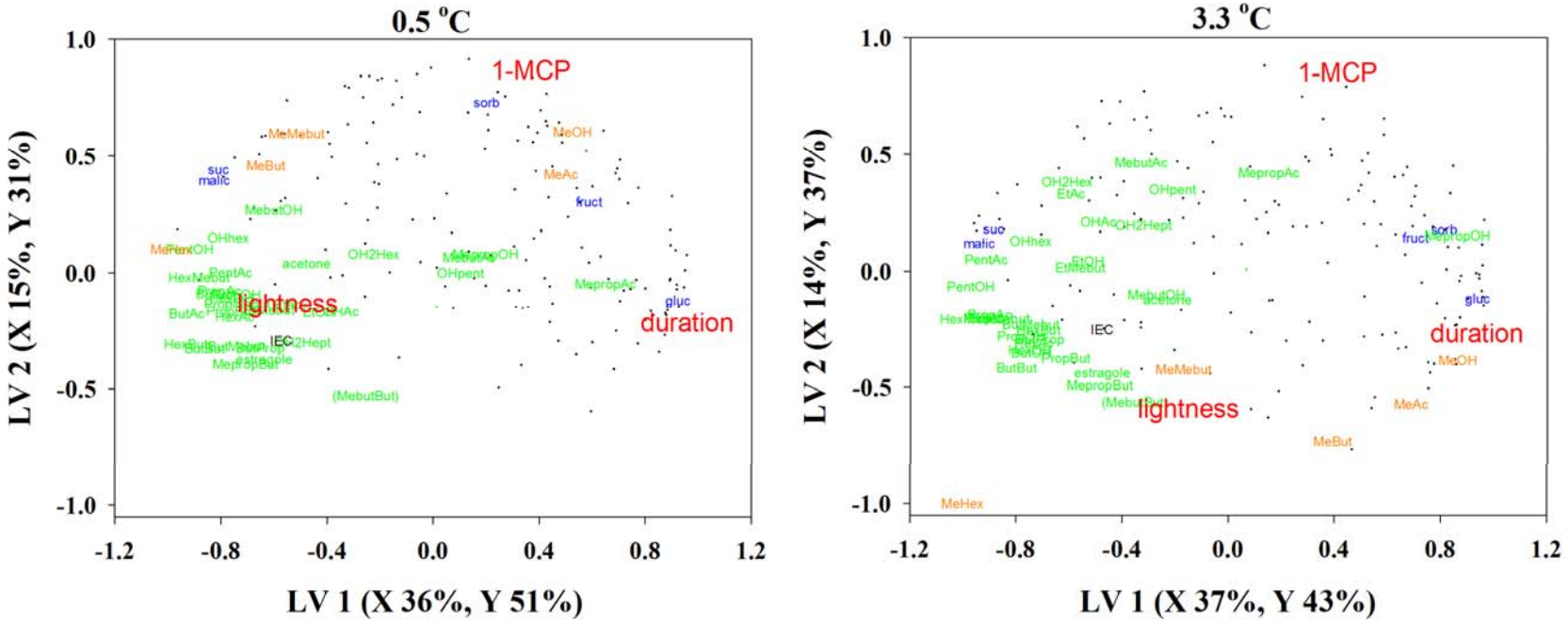

Figure 4.2. PLS-DA loading plot from models containing of ester compounds, volatiles, and major carbohydrates and Y-variables (experimental factors; red font) within the metabolic profile of 'Empire' apple fruit untreated, or treated with 1 μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 or 3.3 °C. Blue font: major carbohydrates; green font: volatile compounds; orange font: ester compounds.

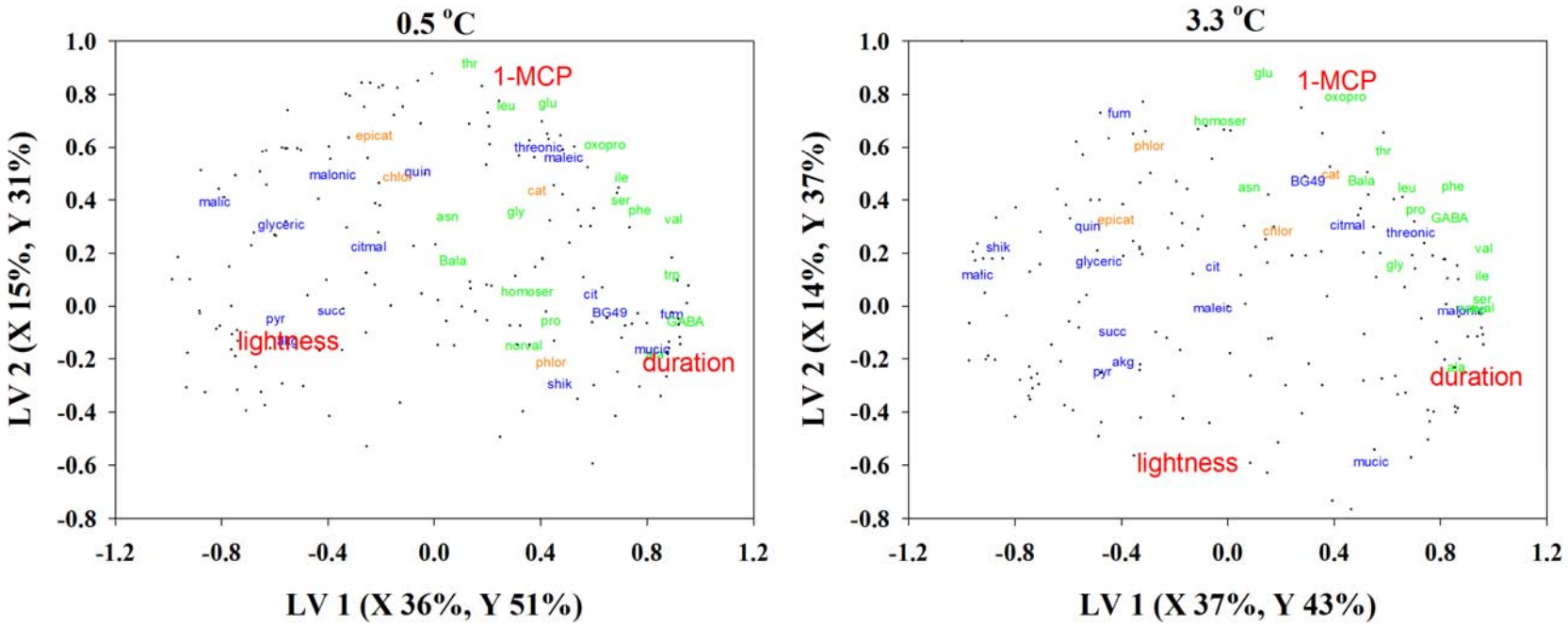

Figure 4.3. PLS-DA loading plot from models containing amino acids, organic acids and phenolic compounds and Y-variable (experimental factors; red font) loading plot within the metabolic profiles of 'Empire' apple fruit untreated, or treated with 1 µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 or 3.3 °C. Blue font: organic acids; green font: amino acids; orange font: phenolic compounds.

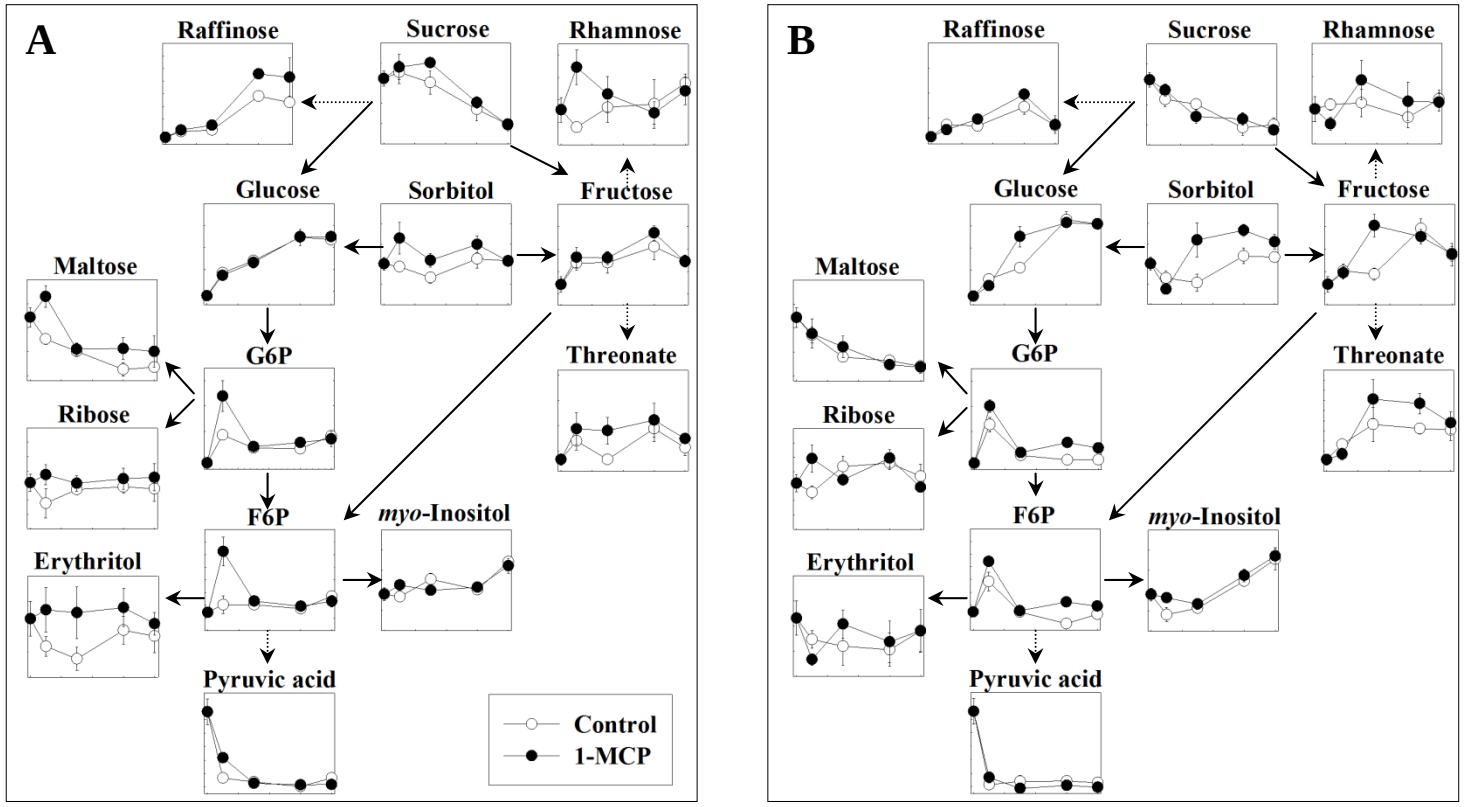

Figure 4.4. The relative responses of intermediate metabolites of glycolysis in 'Empire' apple fruit untreated, or treated with 1 µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2kPa CO_2 for up to 40 weeks at 0.5 (**A**) or 3.3 ^{o}C (**B**). The open and closed bars represent untreated and 1-MCP treated apples, respectively. Y axis represents the relative response ratio of the corresponding metabolite. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

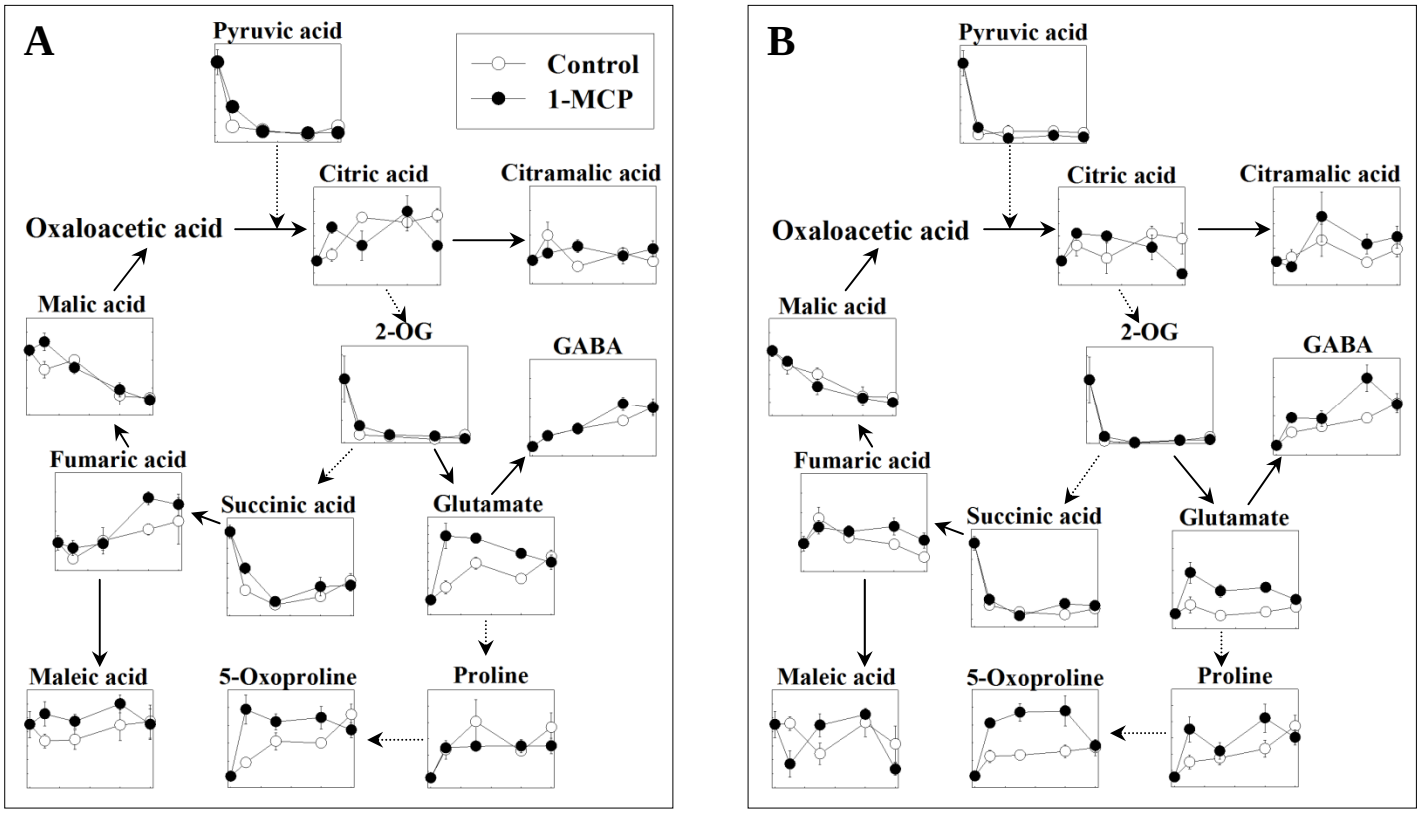

Figure 4.5. The relative responses of intermediate metabolites of TCA cycle and GABA shunt pathway in 'Empire' apple fruit untreated, or treated with 1 µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2kPa CO_2 for up to 40 weeks at 0.5 (**A**) or 3.3 ^{o}C (**B**). The open and closed bars represent untreated and 1-MCP treated apples, respectively. Y axis represents the relative response ratio of the corresponding metabolite. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

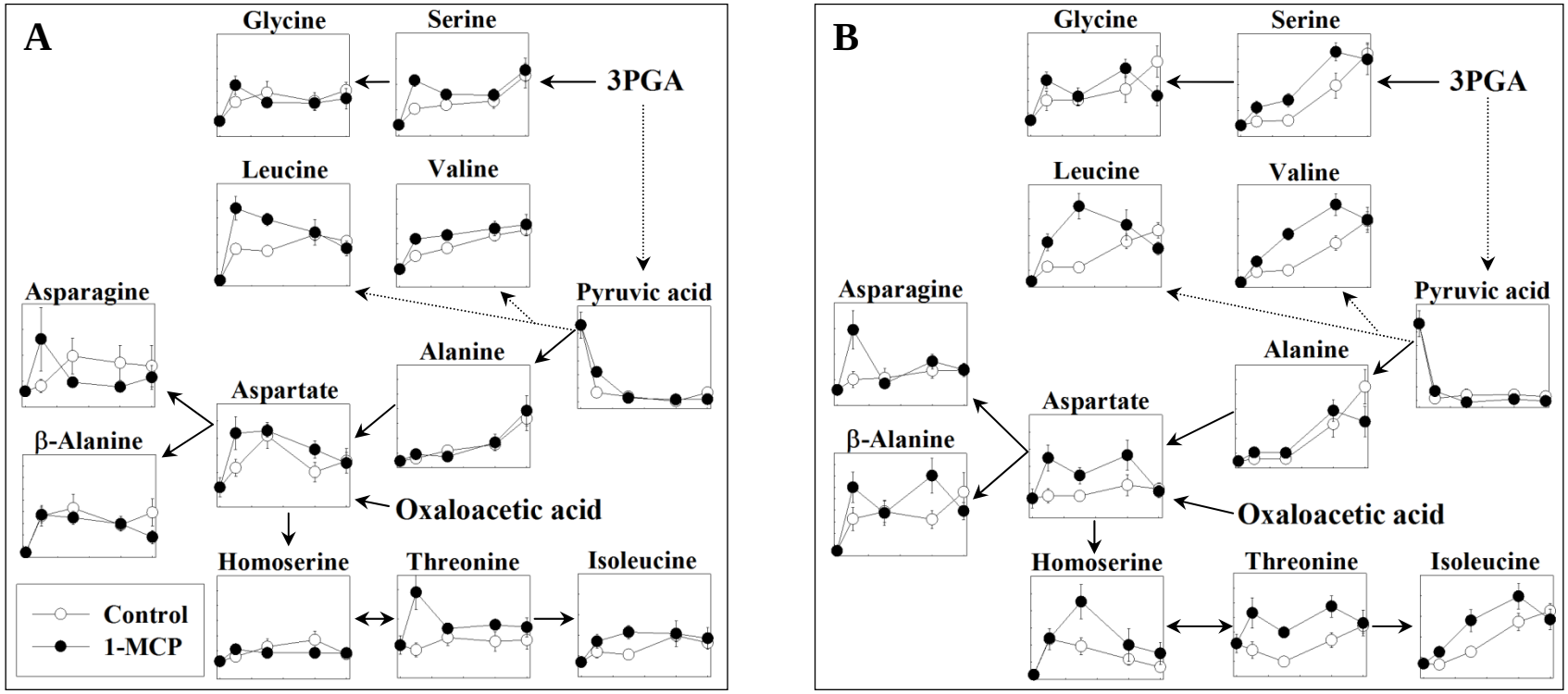

Figure 4.6. The relative responses of amino acids derived from glycolysis and TCA cycle in 'Empire' apple fruit untreated, or treated with 1 μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2kPa CO_2 for up to 40 weeks at 0.5 (**A**) or 3.3 ^{o}C (**B**). The open and closed bars represent untreated and 1-MCP treated apples, respectively. Y axis represents the relative response ratio of the corresponding metabolite. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

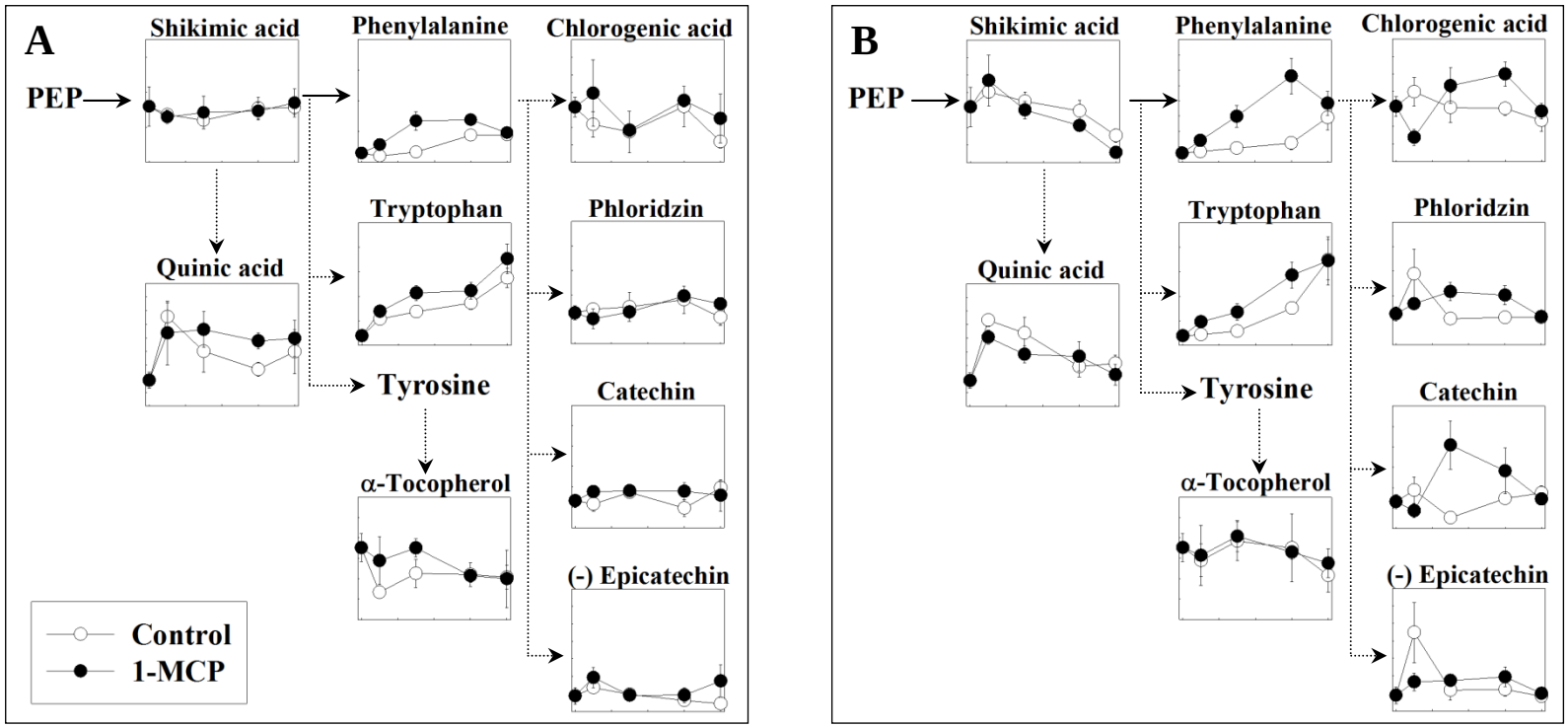

Figure 4.7. The relative responses of intermediate metabolites of aromatic amino acids and phenolic compounds biosynthetic pathways in 'Empire' apple fruit untreated, or treated with 1 µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2kPa CO_2 for up to 40 weeks at 0.5 (**A**) or 3.3 ^{o}C (**B**). The open and closed bars represent untreated and 1-MCP treated apples, respectively. Y axis represents the relative response ratio of the corresponding metabolite. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

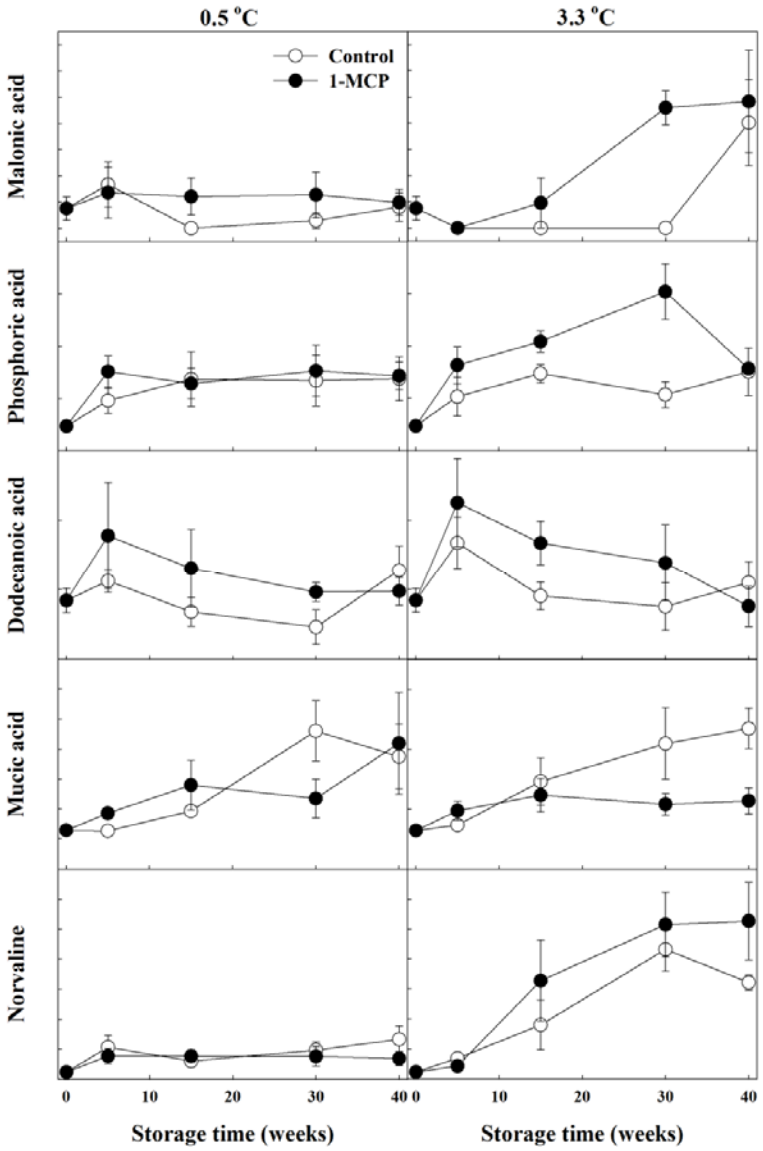

Figure 4.8. The relative responses of malonic, phosphoric, dodecanoic and mucic acids and norvaline of 'Empire' apple fruit untreated, or treated with 1 µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 or 3.3 ^{o}C. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Y axis represents the relative response ratio of the corresponding metabolite. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

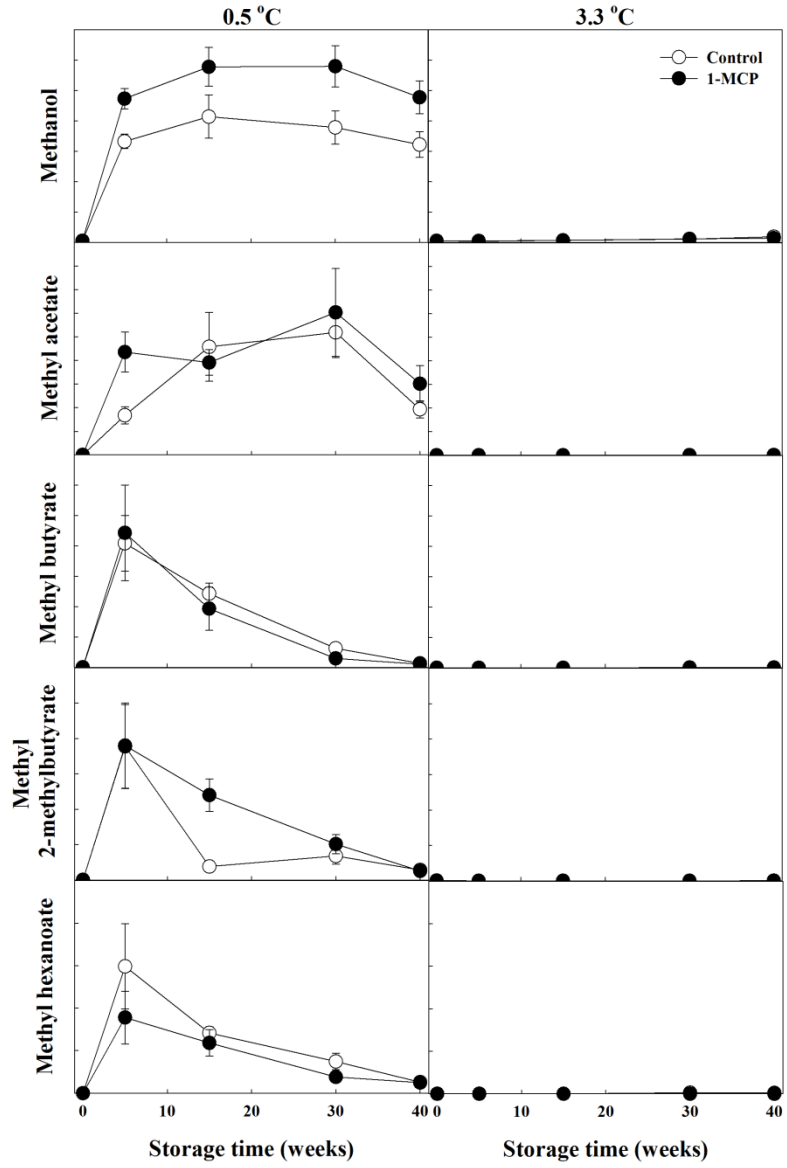

Figure 4.9. The relative responses of methanol, methyl acetate, methyl butyrate, methyl 2-methylbutyrate and methyl hexanoate of 'Empire' apple fruit untreated, or treated with 1 μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 or 3.3 °C. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Y axis represents the relative response ratio of the corresponding metabolite. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

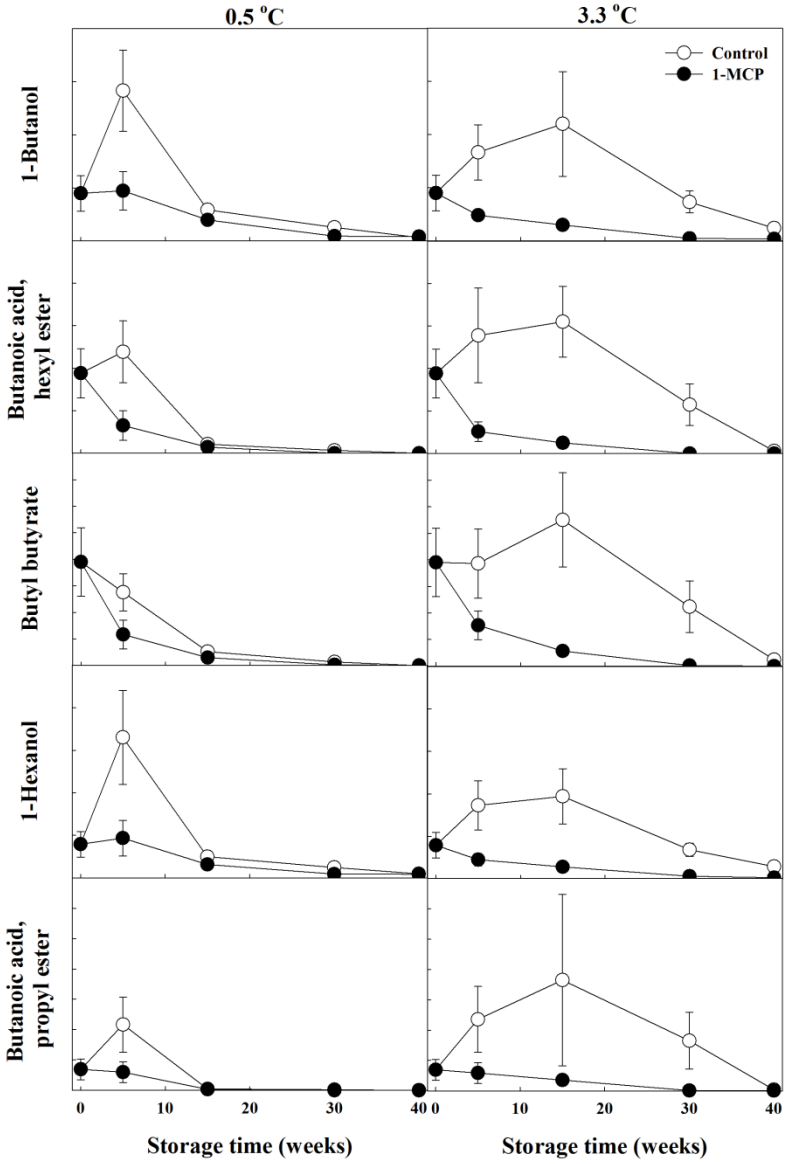

Figure 4.10. The relative responses of 1-butanol, butanoic acid hexyl ester, butyl butyrate, 1-hexanol, and butanoic acid propyl ester of 'Empire' apple fruit untreated, or treated with 1 µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 or 3.3 °C. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Y axis represents the relative response ratio of the corresponding metabolite. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

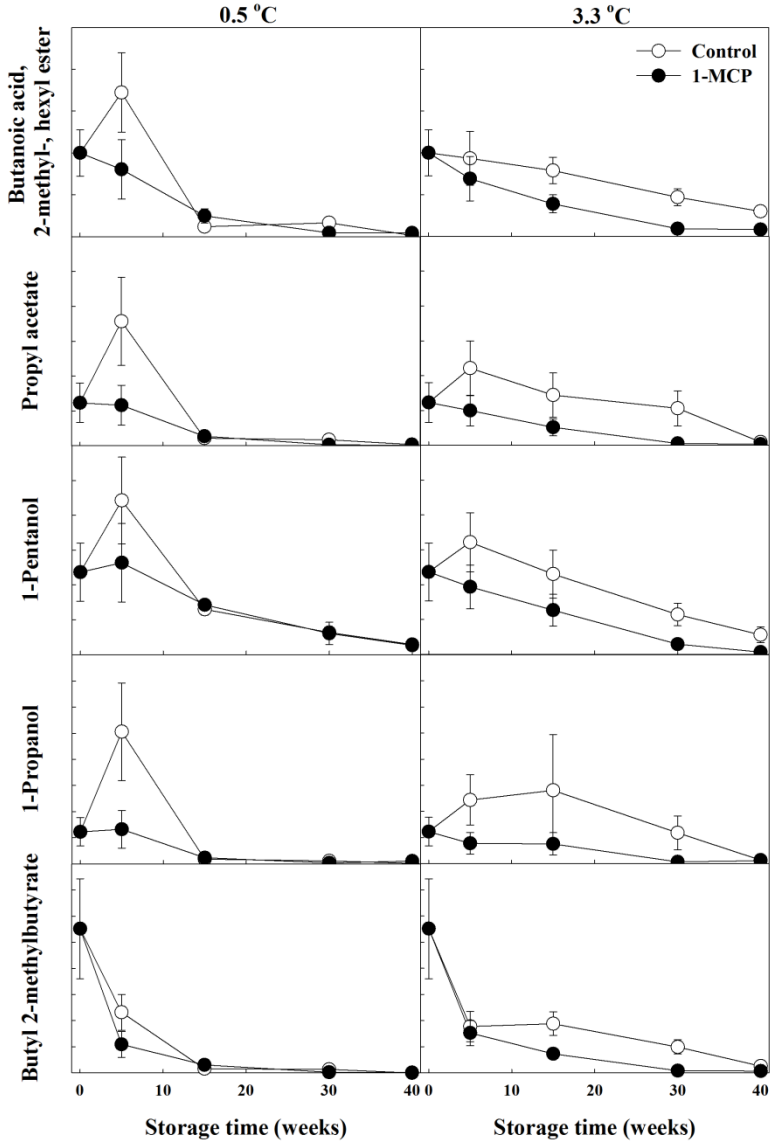

Figure 4.11. The relative responses of butanoic acid 2-methyl- hexyl ester, propyl acetate, 1-pentanol, 1-propanol, and butyl 2-methylbutyrate of 'Empire' apple fruit untreated, or treated with 1 μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 or 3.3 °C. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Y axis represents the relative response ratio of the corresponding metabolite. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

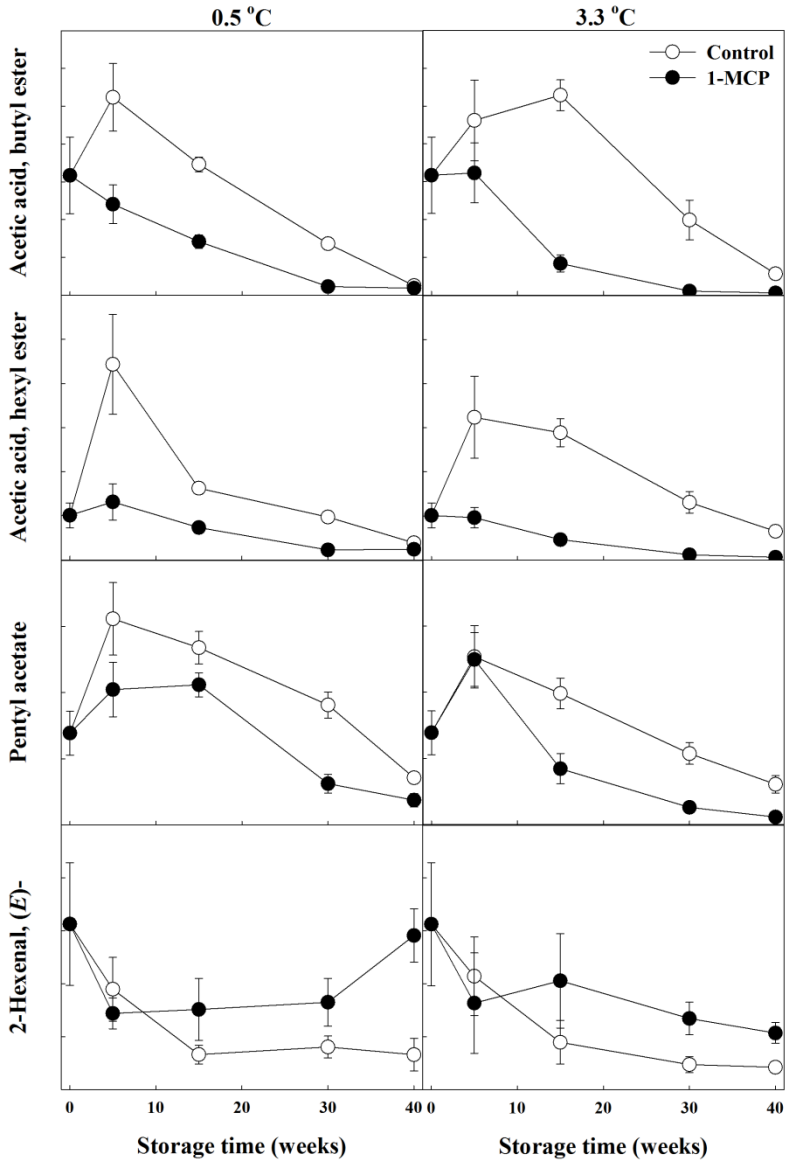

Figure 4.12. The relative responses of acetic acid butyl ester, acetic acid hexyl ester, pentyl acetate and (*E*)-2-hexenal of 'Empire' apple fruit untreated, or treated with 1 µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 or 3.3 ^{o}C. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Y axis represents the relative response ratio of the corresponding metabolite. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

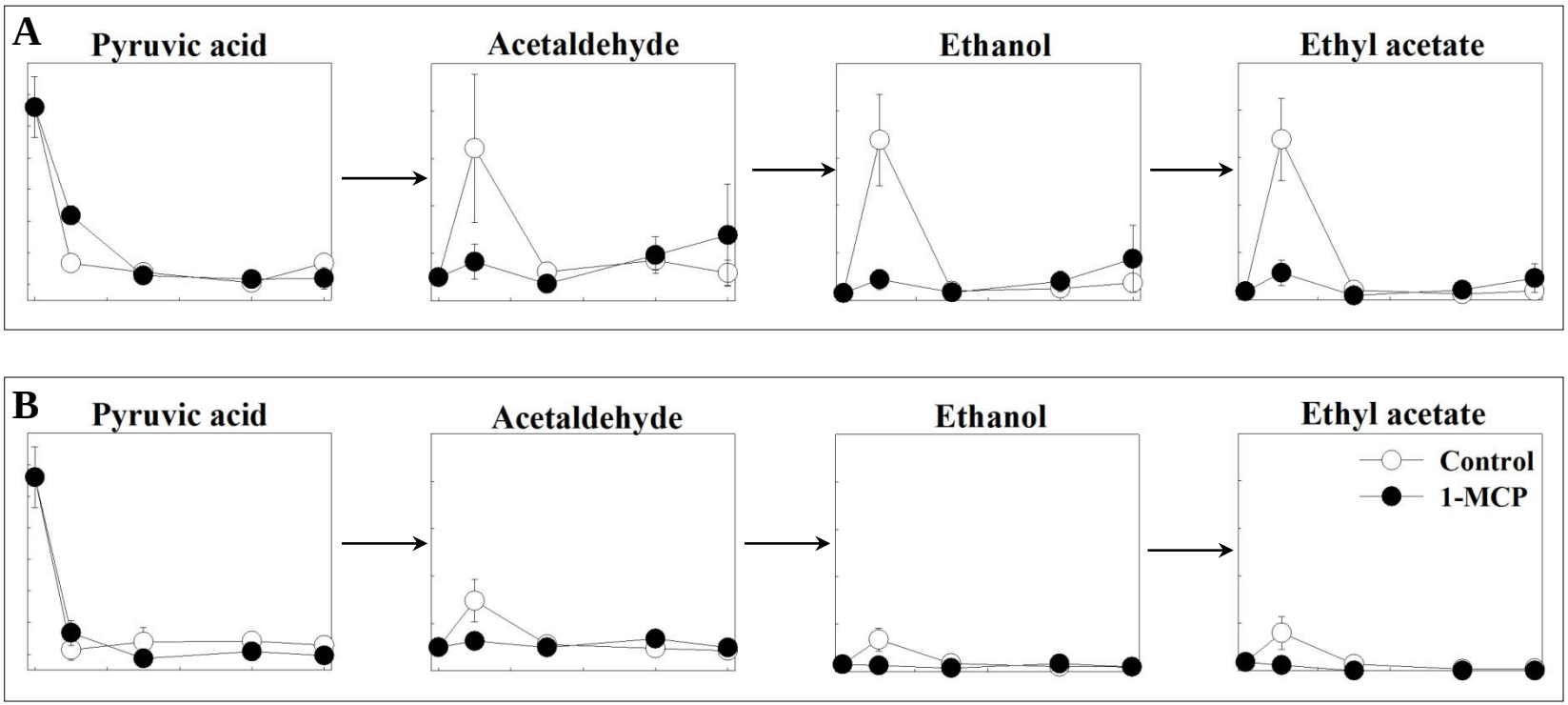

Figure 4.13. The relative responses of intermediate metabolites of alcohol fermentation pathways in 'Empire' apple fruit untreated, or treated with 1 µL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 (**A**) or 3.3 ^{o}C (**B**). The open and closed bars represent untreated and 1-MCP treated apples, respectively. Y axis represents the relative response ratio of the corresponding metabolite. Each data point is the mean of five replicates ± standard error, where larger than the symbols.

4.4. DISCUSSION

Flesh browning of 'Empire' apple was more severe and developed earlier at 3.3 ^{o}C than 0.5 ^{o}C in 1-MCP treated fruit as storage time increased (Chapter 3). During this time, extensive metabolomic separation and divergence occurred in response to 1-MCP treatment and storage temperature (Figure 4.1). Multivariate PLS-DA statistical models have been used previously to investigate metabolic alterations prior to the development of superficial scald in 'Granny Smith' apple peel (Rudell et al., 2009) and in browning tissue of 'Conference' pear (Pedreschi et al., 2009).

Sucrose decreased during storage and correspondingly, fructose and glucose levels increased irrespective of storage temperature and 1-MCP treatment (Figure 4.4). The relative responses of major carbohydrates were consistently explained by the plotted loading scores on the PLS-DA loading plot of major carbohydrates; as glucose and fructose increased over storage time, those carbohydrates were more associated with storage duration variable but as sucrose reduced over storage time, it was less associated with storage duration on the loading plots (Figure 4.2). Glucose 6-phosphate and fructose 6-phosphate sharply increased at week 5 and then dropped to initial levels, although those peaks were not detected at untreated fruit at 0.5 ^{o}C. The abrupt increase of these two compounds agreed with changes in unripe peel of 'Granny Smith'(Rudell et al., 2008). This response might also be associated with the effects of high CO_2 to increase fructose 6-phosphate level by reducing the activities of ATP and PPi phosphofructokinases and then consequently, partially suppressing respiration (Kerbel et al., 1988, 1990). The level of fructose 6-phosphate was only increased by 1-MCP treatment at week 30 and 40 at 3.3 ^{o}C. However, as the byproducts of glycolysis, rhamnose, maltose, and ribose were not consistently affected by 1-MCP treatment and storage temperature; raffinose was increased by 1-MCP treatment only at 0.5 ^{o}C (Figure 4.4).

Of the sugar alcohol metabolites, sorbitol increased more at 3.3 ^{o}C than 0.5 ^{o}C by 1-MCP treatment (Figure 4.4), consistent with the response of sorbitol to the storage duration variable on the loading plot of PLS-DA (Figure 4.2), although sorbitol was more closely associated with 1-MCP at 0.5 ^{o}C but with storage duration at 3.3 ^{o}C. Furthermore, when considering the opposite location of the lightness variable, which is inversely related with browning, an association of browning with sorbitol, methanol, and methyl acetate is apparent at 0.5 ^{o}C, but with sorbitol, fructose, and 2-methyl-1-propanol at 3.3 ^{o}C. Therefore, sorbitol could be involved in one of candidate compounds for the indication of flesh browning. However, as a diagnostic tool, the increase in sorbitol may occur too late in the storage period to be useful. Typically, the accumulation of sorbitol levels has been known to cause to develop watercore in 'Fuji' apple fruit with the advanced maturity (Bowen and Watkins, 1997). The development of watercore is associated with internal breakdown due to flooding of the intercellular spaces. The consequent anaerobic condition makes these apple tissues susceptible to develop flesh internal breakdown during storage (Smagula, 1968). Therefore, it is suggested that the increase of sorbitol levels might be involved in developing flesh browning during storage. However, *myo*-inositol were not affected by 1-MCP treatment and storage temperature as well (Figure 4.4) but the erythritol levels were relatively higher in 1-MCP treated fruits than untreated ones at week 5 and 15 at 0.5 ^{o}C

Of the organic acids of the TCA cycle, pyruvic, 2-oxoglutaric, succinic and malic acids were gradually or sharply decreased by CA storage irrespective of storage temperature and 1-MCP treatment. However, fumaric acid was enhanced by 1-MCP treatment at both storage temperatures (Figure 4.5). Malic acid as major organic acid in apple fruit is typically used as the substrate for respiration (Neal and Hulme, 1958) and thus is generally reduced over storage (Ackermann et al., 1992). 1-MCP delayed

the loss of malic acid in term of titratable acidity as a result of the reduction of respiration (Fan et al., 1999a). Furthermore, malic acid levels were considerably lower in browning tissue than in sound tissue of 'Conference' pears (Pedreschi et al., 2009). However, malic acid in this study was not affected by 1-MCP. It is uncertain why this difference occurred.

Increased succinic acid levels are associated with CO_2injury (Hulme, 1956; Fernández-Trujillo et al., 2001), but in my study, succinic acid declined during storage. This suggests that the development of flesh browning in 'Empire' apples is not a CO_2 injury. Fumaric acid tended to increase by 1-MCP treatment at both storage temperatures but it responded differently to storage temperatures, in which its level gradually increased at 0.5 ^{o}C but did not change or decrease at 3.3 ^{o}C (Figure 4.5). In 'Conference' pear, fumaric acid distinctively increased in browning tissue but in this study, its level was relatively less increased in 1-MCP treated fruit at 3.3 ^{o}C. Nevertheless, Pedreschi et al. (2009) suggested that the conversion of oxaloacetic acid from phosphoenolpyruvic acid might be enhanced when fruit were exposed to high CO_2 partial pressures during storage, and consequently, the accumulation of fumaric acid could be caused through the reversal of TCA cycle. This suggestion is quite interesting because 1-MCP tended to increase or at least delay the reduction of fumaric acid at both storage temperatures.

Glycine, β-alanine, homoserine, proline and norvaline were most differently loaded depending on storage temperatures (Figure 4.3). These amino acids were much further plotted from the origin at 3.3 ^{o}C than at 0.5 ^{o}C loading plots, suggesting that they were affected by 1-MCP treatment more at 3.3 ^{o}C than at 0.5 ^{o}C. Furthermore, this result was also supported by the relative response of these amino acids, in which those metabolites were considerably affected by either 1-MCP treatment or storage temperature. Also, Rudell et al. (2009) mentioned that proline, homoserine and

asparagines were more associated with unripe tissue but norvaline and β-alanine were more related with ripe tissue. Higher storage temperature would result in more active respiration and other metabolic activity than lower storage temperature during storage. Along with the amino acids mentioned above, glutamate, GABA, 5-oxoproline (Figure 4.5), serine, valine, leucine, aspartate, threonine, isoleucine (Figure 4.6), phenylalanine and tryptophan (Figure 4.7) accumulated more at 3.3 ^{o}C than 0.5 ^{o}C in 1-MCP treated fruit.

Typically, GABA is rapidly produced to the responses of biotic and abiotic stresses and may play numerous roles, including regulation of cytosolic pH, carbon flux into TCA cycle, nitrogen metabolism, protection against oxidative stress, redox regulation, osmoregulation and stress signaling (Bouché and Fromm, 2004; Fait et al., 2008). The increase of GABA shunt pathway metabolites was considerably elevated in browning tissue (Pedreschi et al., 2009), and they suggested that GABA could play a crucial role in the indication of tissue browning. Furthermore, the enhancement of glutamate level could be from the degradation of glutathione (GSH), following the increase of 5-oxoproline level by using the combined catalytic reactions of γ-glutamyl cyclotransferase and 5-oxoprolinase in the cytoplasm (Ohkama-Ohtsu et al., 2008). It is not well understood whether the increase of these two compounds was more associated with GABA shunt pathway or GSH degradation pathway. However, considering the relative responses, these compounds might be from the GABA shunt pathway because the response trends of these two compounds were relatively similar, compared to the GSH response (Figure 3.8). Otherwise, GSH degradation pathway might play a partial role in browning tissue because of its role in scavenging reactive oxygen species (Noctor and Foyer, 1998). The accumulation of glutamate, GABA and 5-oxoproline levels could be explained by the partial reversal of the TCA cycle (Vanlerberghe et al., 1990; Sweetlove et al., 2010). Thus, the reduction of malic and

succinic acids, and increase of fumaric and citramalic acids could be explained by this suggestion. However, the up-regulation of the partial reversal of the TCA cycle could be caused by the elevated CO_2 partial pressure during storage. Thus, it is still uncertain on this point of view.

Serine, valine, leucine, aspartate, threonine and isoleucine have been used as a precursor to produce aroma compounds. These compounds were converted into their respective α-keto acids by catalytic activity of amino acid transaminase with 2-oxoglutaric acid as the amine acceptor (Gonda et al., 2010). The increase of these amino acids was considerably influenced by the reduction of volatile compounds by 1-MCP (Figure 4.10-12). It was assumed that 1-MCP might be strongly involved in the suppression of the catalytic activity of amino acid transaminase and thereby, caused these amino acids to accumulate at 3.3 ^{o}C. The increase of aromatic amino acids such as, phenylalanine, and tryptophan, could also be associated with aromatic volatile compounds. Also, of those aromatic amino acids, phenylalanine has been known to be a precursor of phenolic compounds, which could be used as a substrate for the enzymatic browning reaction (Nicolas et al., 1994) but 1-MCP is negatively involved in the generation and production of aromatic volatile compounds (Lurie et al., 2002; Kondo et al., 2005). Therefore, it is possible that 1-MCP resulted in the accumulation of these two aromatic amino acids because of inhibition of production of these aroma volatile compounds.

Phenolic compounds accumulated in 1-MCP treated fruit to a greater extent at 3.3 ^{o}C than at 0.5 ^{o}C (Figure 4.7). On the PLS-DA loading plots phenolic compounds were relatively less associated with 1-MCP and storage duration (Figure 4.3). However, the browning variable was loaded around the region of epicatechin, chlorogenic acid, and catechin at 0.5 ^{o}C but around the regions of citramalic acid, threonic acid, proline, leucine, phenylalanine, valine and GABA at 3.3 ^{o}C. This result

indicated that the flesh browning was differently associated with phenolic compounds at different storage temperatures. Interestingly, phloridzin was only the most differently plotted compound by storage temperature, although the other two phenolic compounds responded to a similar extent at these storage temperatures. In 'Delicious' apple, 1-MCP did not increase chlorogenic acid (MacLean et al., 2006). This difference of chlorogenic acid could be explained by the difference of PAL activity, which was higher in 'Gala' than 'Red Fuji'(Shao et al., 2010). Therefore, in this work, 1-MCP increased phenylalanine levels and as a result, so did chlorogenic acid levels (Figure 4.7). Based on the coincident increase of phenylalanine and chlorogenic acid, it is assumed that the PAL activity might be up-regulated by 1-MCP to a greater extent at 3.3 ^{o}C rather than at 0.5 ^{o}C.

Ester compounds responded to lower storage temperature (0.5 ^{o}C), irrespective of 1-MCP treatment (Figure 4.9). It is different because the other volatile compounds responded less to this lower storage temperature than higher storage temperature (Figure 4.10-12). In 'Jonathan' apples, volatile compounds were associated with storage temperature (Wills and McGlasson, 1971), with higher volatile compound levels at warmer than lower storage temperatures. This differentiation was supported by the PLS-DA loading plot of volatile compounds (Figure 4.2), where the majority of volatile compounds were intensively loaded on the negative scores of LV1 and LV2 at 0.5 ^{o}C but the same compounds were much broadly separated on the negative scores of LV1 at 3.3 ^{o}C. However, isoleucine known as the precursor of many volatiles was more increased by 1-MCP at 3.3 ^{o}C (Figure 4.6) and thus inversely, the volatiles were not accumulated by 1-MCP treatment. These inverse responses suggest that isoleucine might not be incorporated into the production of volatile compounds as the amino acid transaminase was inhibited (or suppressed) by 1-MCP (Gonda et al., 2010). Otherwise, the level of the amine acceptor, 2-OG declined sharply (Figure 4.5) and thereby, the

amino acid transaminase could not react catalytically with those amino acids for the production of aroma volatiles after 1-MCP treatment. Unfortunately, it is not understood that in untreated fruit, though the levels of 2-OG as the amine acceptor decreased, certain volatile compounds were enhanced (Figure 4.12).

As the metabolites of alcohol fermentation pathway, acetaldehyde, ethanol, and ethyl acetate responded more in untreated than 1-MCP treated fruit at week 5 over storage. The relative responses of those fermentative intermediates were slightly higher at 0.5 ^{o}C than at 3.3 ^{o}C, especially at week 5 and the end of storage time. Of these fermentative metabolites, acetaldehyde could be accumulated and thereby built up to the toxic level at the cellular or subcellular organelles to cause to develop certain storage disorders and deteriorate stored fruit (Smagula and Bramlage, 1977). However, in this study, those fermentative metabolites were not built up to cause to development of flesh browning over storage time at either storage temperature, although 1-MCP may cause to increase slightly those metabolites at the end of storage at 0.5 ^{o}C. Therefore, this result indicates that the developmental mechanism of flesh browning could not be involved in the fermentative metabolites at either storage temperature.

Furthermore, the PLS-DA score plot revealed remarkable separation and considerable divergence of apple flesh metabolomes with increasing storage duration and in response to 1-MCP treatment and storage temperature. That PLS-DA scores plot model containing all observations for all treatments from harvest to 40 weeks demonstrated that the metabolic divergence already occurred at week 5 of CA storage and also, this divergence was more considerable depending on storage temperature and the separation of scoring models was more pronounced in 1-MCP treated fruit (Figure 4.1). Rudell et al. (2009) demonstrated that the metabolic alteration occurred within 1 week following storage initiation. Therefore, it is expected that the metabolic divergence could be developed much earlier than week 5. Along with the scores model,

the PLS-DA loading plot models demonstrated that how each individual compound was loaded on the plot depending on storage temperature and then how those compounds were associated with the response variables. Major carbohydrates, ester compounds, volatile compounds, amino acids, organic acids and phenolic compounds were plotted on the loading plot and then these compounds were differently or similarly loaded with 1-MCP, storage duration, and lightness, depending on storage temperatures. In those figures, we used lightness variable which was negatively correlated with browning. With respect to the lightness variable, browning was closely associated with sorbitol, methanol, methyl acetate and fructose at 0.5 ^{o}C but with sorbitol, fructose and 2-methyl-1-propanol at 3.3 ^{o}C (Figure 4.2), and with catechin, glycine, isoleucine, serine, phenylalanine and valine at 0.5 ^{o}C but with leucine, phenylalanine, proline, GABA valine, glycine, isoleucine, and threonic acid at 3.3 ^{o}C (Figure 4.3). Therefore, sorbitol, fructose, 2-methyl-1-propanol, leucine, phenylalanine, proline, GABA, valine, glycine, isoleucine and threonic acid could be the tentative metabolites closely involved in the development of flesh browning. Coincidently, the majority of those compounds were accumulated to a greater extent after 1-MCP treatment at 3.3 ^{o}C, further indicating possible involvement of these metabolites on the development of flesh browning in 1-MCP treated 'Empire' apple at 3.3 ^{o}C.

In conclusion, the PLS-DA models provide a method to simultaneously characterize and identify separation and divergence of large number of metabolites by 1-MCP treatment and storage temperature. The relative responses of several individual compounds were affected by 1-MCP and storage temperature. Sorbitol, GABA and several amino acids associated with production of aroma volatile compounds are possible candidate compounds that could be associated with the development of flesh browning at 3.3 ^{o}C. The development of flesh browning induced by 1-MCP is not

likely to be a direct CO_2 injury because of limited alcohol accumulation, reduction rather than accumulation of succinic acid, and the increase of glucose 6-phosphate and fructose 6-phosphate by 1-MCP. Overall, the development of flesh browning is likely to be a complex event with the interaction of several metabolic pathways.

REFERENCES

Ackermann, J., Fischer, M., Amado, R., 1992. Changes in sugars, acids, and amino acids during ripening and storage of apples (cv. Glockenapfel). J. Agric. Food Chem. 40, 1131-1134.

Bai, J., Baldwin, E.A., Goodner, K.L., Mattheis, J.P., Brecht, J.K., 2005. Response of four apple cultivars to 1-methylcyclopropene treatment and controlled atmosphere storage. HortScience 40, 1534-1538.

Bouché, N., Fromm, H., 2004. GABA in plants: just a metabolite? Trends Plant Sci. 9, 110-115.

Bowen, J.H., Watkins, C.B., 1997. Fruit maturity, carbohydrate and mineral content relationships with watercore in ‘Fuji’ apples. Postharvest Biol. Technol. 11, 31-38.

Burda, S., Oleszek, W., Lee, C.Y., 1990. Phenolic compounds and their changes in apples during maturation and cold storage. J. Agric. Food Chem. 38, 945-948.

Coseteng, M.Y., Lee, C.Y., 1987. Changes in apple polyphenoloxidase and polyphenol concentrations in relation to degree of browning. J. Food Sci. 52, 985-989.

Drake, S.R., Eisele, T.A., 1999. Carbohydrate and acid contents of Gala apples and Bartlett pears from regular and controlled atmosphere storage. J. Agric. Food Chem. 47, 3181-3184.

Echeverría, G., Fuentes, T., Graell, J., Lara, I., López, M.L., 2004. Aroma volatile compounds of 'Fuji' apples in relation to harvest date and cold storage

technology: A comparison of two seasons. Postharvest Biol. Technol. 32, 29-44.

Fait, A., Fromm, H., Walter, D., Galili, G., Fernie, A.R., 2008. Highway or byway: the metabolic role of the GABA shunt in plants. Trends Plant Sci. 13, 14-19.

Fan, X., Blankenship, S.M., Mattheis, J.P., 1999. 1-Methylcyclopropene inhibits apple ripening. J. Amer. Soc. Hort. Sci. 124, 690-695.

Fellman, J.K., Mattinson, D.S., Bostick, B.C., Mattheis, J.P., Patterson, M.E., 1993. Ester biosynthesis in "Rome" apples subjected to low-oxygen atmospheres. Postharvest Biol. Technol. 3, 201-214.

Fernández-Trujillo, J.P., Nock, J.F., Watkins, C.B., 2001. Superficial scald, carbon dioxide injury, and changes of fermentation products and organic acids in 'Cortland' and 'Law Rome' apples after high carbon dioxide stress treatment. J. Amer. Soc. Hort. Sci. 126, 235-241.

Fidler, J.C., North, C.J., 1970. Sorbitol in stored apples. J. Hortic. Sci. 45, 197-204.

Gonda, I., Bar, E., Portnoy, V., Lev, S., Burger, J., Schaffer, A.A., Tadmor, Y.a., Gepstein, S., Giovannoni, J.J., Katzir, N., Lewinsohn, E., 2010. Branched-chain and aromatic amino acid catabolism into aroma volatiles in *Cucumis melo* L. fruit. J. Exp. Bot. 61, 1111-1123.

Hulme, A.C., 1956. Carbon dioxide injury and the presence of succinic acid in apples. Nature 178, 218-219.

Ingle, M., D'Souza, M.C., Townsend, E.C., 2000. Fruit characteristics of 'York' apples during development and after storage. HortScience 35, 95-98.

Jung, S.-K., Watkins, C.B., 2011. Involvement of ethylene in browning development of controlled atmosphere-stored 'Empire' apple fruit. Postharvest Biol. Technol. in press.

Kerbel, E.L., Kader, A.A., Romani, R.J., 1988. Effects of elevated CO_2 concentrations on glycolysis in intact `Bartlett' pear fruit. Plant Physiol. 86, 1205-1209.

Kerbel, E.L., Kader, A.A., Romani, R.J., 1990. Respiratory and glycolytic response of suspension-cultured `Passe Crassane' pear fruit cells to elevated CO_2 concentrations. J. Amer. Soc. Hort. Sci. 115, 111-114.

Kondo, S., Setha, S., Rudell, D.R., Buchanan, D.A., Mattheis, J.P., 2005. Aroma volatile biosynthesis in apples affected by 1-MCP and methyl jasmonate. Postharvest Biol. Technol. 36, 61-68.

Larrigaudière, C., Vilaplana, R., Soria, Y., Recasens, I., 2008. Comparative study of the effects of 1-MCP treatment on apple quality by instrumental and multivariate analysis. J. Sci. Food Agric. 88, 1614-1621.

Lisec, J., Schauer, N., Kopka, J., Willmitzer, L., Fernie, A.R., 2006. Gas chromatography mass spectrometry-based metabolite profiling in plants. Nat. Protocols 1, 387-396.

Lurie, S., Pre-Aymard, C., Ravid, U., Larkov, O., Fallik, E., 2002. Effect of 1-methylcyclopropene on volatile emission and aroma in cv. Anna apples. J. Agric. Food Chem. 50, 4251-4256.

MacLean, D.D., Murr, D.P., DeEll, J.R., Horvath, C.R., 2006. Postharvest variation in apple (*Malus* x *domestica* Borkh.) flavonoids following harvest, storage, and 1-MCP treatment. J. Agric. Food Chem. 54, 870-878.

Moya-Leon, M.A., Vergara, M., Bravo, C., Pereira, M., Moggia, C., 2007. Development of aroma compounds and sensory quality of 'Royal Gala' apples during storage. J. Hortic. Sci. Biotechnol. 82, 403-413.

Neal, G.E., Hulme, A.C., 1958. The organic acid metabolism of Bramley's Seedling apple peel. J. Exp. Bot. 9, 142-157.

Nicolas, J.J., Richard-Forget, F.C., Goupy, P.M., Amiot, M.J., Aubert, S.Y., 1994. Enzymatic browning reactions in apple and apple products. Crit. Rev. Food Sci. Nutr. 34, 109-157.

Noctor, G., Foyer, C.H., 1998. Ascorbate and glutathione: Keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 249-279.

Ohkama-Ohtsu, N., Oikawa, A., Zhao, P., Xiang, C., Saito, K., Oliver, D.J., 2008. A γ-glutamyl transpeptidase-independent pathway of glutathione catabolism to glutamate via 5-oxoproline in Arabidopsis. Plant Physiol. 148, 1603-1613.

Pedreschi, R., Franck, C., Lammertyn, J., Erban, A., Kopka, J., Hertog, M., Verlinden, B., Nicolaï, B., 2009. Metabolic profiling of 'Conference' pears under low oxygen stress. Postharvest Biol. Technol. 51, 123-130.

Pérez-Enciso, M., Tenenhaus, M., 2003. Prediction of clinical outcome with microarray data: a partial least squares discriminant analysis (PLS-DA) approach. Hum. Genet. 112, 581-592.

Roessner, U., Wagner, C., Kopka, J., Trethewey, R.N., Willmitzer, L., 2000. Simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. Plant J. 23, 131-142.

Roessner, U., Luedemann, A., Brust, D., Fiehn, O., Linke, T., Willmitzer, L., Fernie, A.R., 2001. Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. Plant Cell 13, 11-29.

Rudell, D.R., Mattheis, J.P., Curry, E.A., 2008. Prestorage ultraviolet-white light irradiation alters apple peel metabolome. J. Agric. Food Chem. 56, 1138-1147.

Rudell, D.R., Mattheis, J.P., Hertog, M.L.A.T.M., 2009. Metabolomic change precedes apple superficial scald symptoms. J. Agric. Food Chem. 57, 8459-8466.

Rupasinghe, H.P.V., Murr, D.P., Paluyath, G., Skog, L., 2000. Inhibitory effect of 1-MCP on ripening and superficial scald development in 'McIntosh' and 'Delicious' apples. J. Hortic. Sci. Biotechnol. 75, 271-276.

Shao, X., Tu, K., Tu, S., Su, J., Zhao, Y., 2010. Effects of heat treatment on wound healing in Gala and Red Fuji apple fruits. J. Agric. Food Chem. 58, 4303-4309.

Smagula, J.M., Bramlage, W.J., 1977. Acetaldehyde accumulation: is it a cause of physiological deterioration of fruits? HortScience 12, 200-204.

Smagula, J.M., Bramlage, W.J., 1968. Effects of watercore on respiration and mitochondrial activity in 'Richard Delicious' apples. Proc. Amer. Soc. Hort. Sci. 93, 753-761.

Sweetlove, L.J., Beard, K.F.M., Nunes-Nesi, A., Fernie, A.R., Ratcliffe, R.G., 2010. Not just a circle: flux modes in the plant TCA cycle. Trends Plant Sci. 15, 462-470.

Vanlerberghe, G.C., Feil, R., Turpin, D.H., 1990. Anaerobic metabolism in the N-limited green alga *Selenastrum minutum*: I. Regulation of carbon metabolism and succinate as a fermentation product. Plant Physiol. 94, 1116-1123.

Watkins, C.B., 2008. Overview of 1-methylcyclopropene trials and uses for edible horticultural crops. HortScience 43, 86-94.

Watkins, C.B., Liu, F.W., 2010. Temperature and carbon dioxide interactions on quality of controlled atmosphere-stored 'Empire' apples. HortScience 45, 1708-1712.

Wills, R.B.H., McGlasson, W.B., 1971. Effect of storage temperature on apple volatiles associated with low temperature breakdown. J. Hort. Sci. 46, 115-120.

CHAPTER 5

SUMMARY AND FUTURE WORK

5.1. Summary

The ‘Empire’ apple is a major apple cultivar in New York apple industry but is susceptible to development of firm flesh browning during CA storage. This firm flesh browning is thought to be a chilling injury and thus typically stored at 2-3 ^{o}C to avoid a chilling injury. Since 1-MCP technology was approved by EPA to use edible horticultural commodity, it has been widely applied to apple storage industry. Unfortunately, 1-MCP can cause increased development of flesh browning in ‘Empire’ apple stored at higher storage temperature regimes, such as 3 ^{o}C. This study has been focused on the elucidation of any putative association of antioxidant mechanism on the development of firm flesh browning, and characterization of any primary and secondary metabolic alterations in ‘Empire’ apple during CA storage for up to 40 weeks.

In Chapter 3, I investigated the enzymatic and non-enzymatic antioxidant metabolism to scavenge ROS during CA storage in 1-MCP treated ‘Empire’ apple fruit. 1-MCP treated fruit had lower lightness and hue angle than untreated ones with longer storage periods. NBT reducing activity was reduced by 1-MCP but H_2O_2 levels were inconsistently affected by 1-MCP. Ascorbic acid levels were decreased by 1-MCP treatment at the end of storage at both storage temperatures but GSH levels were lower and GSSG levels were higher by 1-MCP treatment at the end of storage only at 3.3 ^{o}C. Cu/Zn-SOD activity was only reduced by 1-MCP treatment at weeks 15 and 30. The other enzyme activities for antioxidant metabolism were not influenced consistently by 1-MCP treatment. Therefore, these results presented that the

development of flesh browning in ‘Empire’ apple appear to be less directly associated with antioxidant scavenging system.

In Chapter 4, I evaluated qualitative and relative quantitative metabolite alterations in ‘Empire’ apple during CA storage at different temperatures and with or without 1-MCP treatment. A PLS-DA statistical model system was used to identify potential metabolites associated with the development of firm flesh browning. The PLS-DA loading plots indicated that sorbitol and GABA could be potential candidate compounds associated with the development of flesh browning at 3.3 ^{o}C. Moreover, the PLS-DA scoring plots presented different metabolic divergence and separation between untreated and 1-MCP treated fruit at both storage temperatures. Carbohydrates were not affected by 1-MCP treatmentand malic, succinic and 2-oxoglutaric acids of TCA cycle declined during storage irrespective of 1-MCP treatment and storage temperature. However, 1-MCP treatment at 3.3 ^{o}C enhanced certain concentrations of amino acids such as, tryptophan, phenylalanine, glutamine, 5-oxoproline, aspartate, homoserine, threonine, isoleucine, valine and serine, and the phenolic compounds, chlorogenic acid, phloridzin, and catechin. However, the treatment reduced the production of volatile compounds. Overall, the 1-MCP treatment results in a dramatic alteration of the primary and secondary metabolites at 3.3 ^{o}C compared with 0.5 ^{o}C.

In conclusion, the development of firm flesh browning may not be strongly involved with enzymatic and non-enzymatic antioxidant metabolism for scavenging ROS produced by a given storage regime in 1-MCP treated ‘Empire’ apple. On the other hand, these antioxidant scavenging systems might be associated with or play a fundamental role in a defense signaling molecule for the cellular or subcellular protection to the given oxidative stress. By screening these untargeted metabolites by GC-MS and PLS-DA loading plot, sorbitol and GABA could be the candidate

metabolites involved in the development of flesh browning. Sorbitol has been known to as major metabolite for the development of watercore in 'Fuji' (Bowen and Watkins, 1997). Also, GABA as another potential metabolite has been reported to be involved with flesh browning in 'Conference' pear (Pedreschi et al., 2009). Furthermore, several amino acids and phenolic compounds for the precursor of volatile compounds were enhanced by 1-MCP treatment at 3.3 ^{o}C. These responses were well correlated with the reduction of volatile compounds. The suppression of volatile compounds by 1-MCP has been reported because the volatile biosynthesis pathway is dependent on ethylene production (Defilippi et al., 2004). Therefore, 1-MCP should be involved with suppression of any ethylene dependent metabolic pathways and thereby, this cellular homeostasis could be unbalanced to cause to develop the firm flesh browning by the metabolic alteration of primary and secondary metabolic compounds in 'Empire' apple stored at 3.3 ^{o}C during CA storage.

5.2. Future work

For the evaluation of antioxidative metabolism in a given storage regime, the early storage time could be more focused on having more detail insight into how those enzymatic and non-enzymatic antioxidant metabolism respond to CA storage in apple fruit affected by 1-MCP treatment. Based on the result of Chapter 3, there was little evidence of an involvement of the antioxidative systems for the development of flesh browning after 1-MCP treatment. Therefore, the primary response of antioxidant metabolism may not parallel incidence of flesh browning but might occur prior to the development of flesh browning. Furthermore, the reduction of ascorbic acid has been directly indicated to as damaged tissue by the development of flesh browning in pear (Franck et al., 2003). Even in 'Pink Lady' apple, ascorbic acid level decreased in browning tissue, while the level of dehydroascorbic acid increased (de Castro et al.,

2008). Thus, the browning tissue may not have enough energy to reduce dehydroascorbic acid back to ascorbic acid and thus the accumulated dehydroascorbic acid could lead to its delactonization of 2,3-diketogulonate (Smirnoff et al., 2001). Understanding the anabolic and catabolic pathways of ascorbic acid metabolism could give more insight on the development of flesh browning. Along with the function of ascorbic acid on browning, glutathione peroxidase (GPX, EC 1.11.1.9) and glutathione *S*-transferase (GST, EC 2.5.1.18) are also known to have a protective activity at adverse environmental stresses such as oxidative stress (Miao et al., 2006; Öztetik, 2008; Halušková et al., 2009). Those enzymatic activities of GPX and GST might be involved in scavenging ROS in stored apple fruit.

With respect to metabolomic responses, Rudell et al (2009) reported that metabolomic alteration occurred prior to the symptoms of superficial scald. Also, in Chapter 4, PLS-DA scoring plots showed that at week 5, 1-MCP treatment already resulted in large differences, compared with untreated fruit or the harvest sample (Figure 4.1). These results indicate that 1-MCP treatment and storage temperature affects primary and secondary metabolism within 5 weeks. Therefore, for the more detail metabolomic responses, the much earlier response should be considered by sampling at week 1, 2 or 3. Of the metabolites related to fruit ripening and senescence metabolism, the polyamines are associated with senescence and fruit ripening mechanism by inhibiting ethylene biosynthesis (Apelbaum et al., 1981; Kakkar and Rai, 1993). Polyamines and ethylene are inversely related because they are same precursor which is *S*-adenosylmethionine (SAM) (Fontecave et al., 2004). Polyamine treatment delayed fruit softening in 'Golden Delicious' and McIntosh' apple and also, inhibited the development of chilling injury symptoms such as brown core with Ca treatment (Kramer et al., 1991). However, the browning tissue had lower level of putrescine as a diamine than healthy tissue of 'Conference' pear during CA storage

(Pedreschi et al., 2009). As discussed in Chapter 4, numerous amino acids and phenolic compounds, which are used to the substrate for the volatile productions, were accumulated by 1-MCP treatment at 3.3 ^{o}C. However, volatile compounds were not responded to the accumulation of these volatile precursors in parallel. Thus, 1-MCP should suppress the activity of alcohol acyltransferase (AAT) to produce more ester and volatile compounds. AAT activity is highly dependent on ethylene biosynthesis and the suppression of ethylene biosynthesis by 1-MCP treatment should consequently inhibit AAT activity (Defilippi et al., 2005b). In addition to AAT activity, the catalytic activities of alcohol dehydrogenase (ADH, EC 1.1.1.1) and lipoxygenase (LOX, EC 1.13.1.12) are also associated with the formation of volatile compounds (Echeverría et al., 2004b). Along with amino acids and phenolic compounds, fatty acids as one of major precursors are also considerably involved in the formation of aroma volatiles and thus LOX activity should play a considerable role in biosynthesis of volatile compounds (Altisent et al., 2009). Furthermore, the symptoms of flesh browning is the final result of oxidation of phenolic compounds in that cell membrane or wall components must have ruptured or collapsed in response to a given abiotic storage stress. Therefore, cell wall and membrane metabolism could be major factors to be considered on understanding the development of firm flesh browning by 1-MCP treatment.

REFERENCES

Altisent, R., Echeverria, G., Graell, J., Lopez, L., Lara, I., 2009. Lipoxygenase activity is involved in the regeneration of volatile ester-synthesizing capacity after ultra-low oxygen storage of 'Fuji' apple. J. Agric. Food Chem. 57, 4305-4312.

Apelbaum, A., Burgoon, A.C., Anderson, J.D., Lieberman, M., 1981. Polyamines inhibit biosynthesis of ethylene in higher plant tissue and fruit protoplasts. Plant Physiol. 68, 453-456.

Bowen, J.H., Watkins, C.B., 1997. Fruit maturity, carbohydrate and mineral content relationships with watercore in ‘Fuji’ apples. Postharvest Biol. Technol. 11, 31-38.

de Castro, E., Barrett, D.M., Jobling, J., Mitcham, E.J., 2008. Biochemical factors associated with a CO_2-induced flesh browning disorder of Pink Lady apples. Postharvest Biol. Technol. 48, 182-191.

Defilippi, B.G., Dandekar, A.M., Kader, A.A., 2004. Impact of suppression of ethylene action or biosynthesis on flavor metabolites in apple (*Malus domestica* Borkh) fruits. J. Agric. Food Chem. 52, 5694-5701.

Defilippi, B.G., Dandekar, A.M., Kader, A.A., 2005. Relationship of ethylene biosynthesis to volatile production, related enzymes, and precursor availability in apple peel and flesh tissues. J. Agric. Food Chem. 53, 3133-3141.

Echeverría, G., Graell, J., López , M.L., Lara, I., 2004. Volatile production, quality and aroma-related enzyme activities during maturation of 'Fuji' apples. Postharvest Biol. Technol. 31, 217-227.

Fontecave, M., Atta, M., Mulliez, E., 2004. *S*-adenosylmethionine: nothing goes to waste. Trends Biochem. Sci. 29, 243-249.

Franck, C., Baetens, M., Lammertyn, J., Scheerlinck, N., Davey, M.W., Nicolaï, B.M., 2003. Ascorbic acid mapping to study core breakdown development in `Conference' pears. Postharvest Biol. Technol. 30, 133-142.

Halušková, L.u., Valentovičová, K., Huttová, J., Mistrík, I., Tamás, L., 2009. Effect of abiotic stresses on glutathione peroxidase and glutathione S-transferase activity in barley root tips. Plant Physiol. Biochem. 47, 1069-1074.

Kakkar, R.K., Rai, V.K., 1993. Plant polyamines in flowering and fruit ripening. Phytochemistry 33, 1281-1288.

Kramer, G.F., Wang, C.Y., Conway, W.S., 1991. Inhibition of softening by polyamine application in `Golden Delicious' and `McIntosh' apples. J. Amer. Soc. Hort. Sci. 116, 813-817.

Miao, Y., Lv, D., Wang, P., Wang, X.-C., Chen, J., Miao, C., Song, C.-P., 2006. An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. Plant Cell 18, 2749-2766.

Öztetik, E., 2008. A tale of plant glutathione *S*-transferases: Since 1970. Botanical Review 74, 419-437.

Pedreschi, R., Franck, C., Lammertyn, J., Erban, A., Kopka, J., Hertog, M., Verlinden, B., Nicolaï, B., 2009. Metabolic profiling of 'Conference' pears under low oxygen stress. Postharvest Biol. Technol. 51, 123-130.

Rudell, D.R., Mattheis, J.P., Hertog, M.L.A.T.M., 2009. Metabolomic change precedes apple superficial scald symptoms. J. Agric. Food Chem. 57, 8459-8466.

Smirnoff, N., Conklin, P.L., Loewus, F., 2001. Biosynthesis of ascorbic acid in plants: a renaissance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 437-467.