THE ROLE OF OXYGEN IN ETHYLENE-INDUCED WATERSOAKING IN IMMATURE BEIT-ALPHA CUCUMBER FRUIT

By

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To my family

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Watersoaking is an ethylene-induced disorder that affects members of the Cucurbitaceae. Our understanding of the cellular mechanisms contributing to watersoaking is incomplete. This study was conducted to address the role of oxygen in watersoaking using immature beit-alpha cucumber fruit. Ethylene at 10 $\mu L \cdot L^{-1}$ induced watersoaking, and higher concentrations did not accelerate the disorder. At least 4 d of ethylene exposure (10 $\mu L \cdot L^{-1}$) induced watersoaking and accompanying symptoms including degreening, softening, and enhanced electrolyte leakage. Continuous ethylene exposure induced accumulation of reactive oxygen species (ROS) at 2-4 d and maximum levels of ethylene receptor transcripts (Cs-ERS, Cs-ETR1, and Cs-ETR2) at 1 d. Histochemical staining revealed that watersoaking appears closely associated with H_2O_2. Of three ethylene receptor genes, Cs-ETR1 in mesocarp and Cs-ERS in exocarp were the most markedly up-regulated in response to ethylene.

Watersoaking in immature cucumber fruit was initiated in hypodermal tissue, followed by ingress to mesocarp. Altered gas-exchange properties of fresh-cut slices did not affect the spatial pattern of watersoaking. The intensity of watersoaking, however, was markedly diminished in slices compared with intact fruit. In intact fruit, hyperoxia

(40 kPa O_2) accelerated ethylene-induced watersoaking while hypoxia (2 kPa O_2) suppressed these symptoms. In fresh-cut slices, ethylene-induced symptoms were negated by hypoxia but unaffected by hyperoxia.

Ethylene-mediated increases in H_2O_2 occurred 2 d earlier than incipient watersoaking under normoxia and hyperoxia, but not hypoxia. $O_2^{\cdot -}$ production decreased in ethylene-treated fruit as watersoaking developed. Antioxidant capacity of cucumber fruit increased in response to ethylene at 6 d and 4-6 d in exocarp and mesocarp, respectively. Cucumber fruit preconditioned (2 kPa O_2 for 8 d) prior to ethylene exposure under normoxia exhibited softening, ion leakage and tissue disruption, but no watersoaking. Preconditioning reduced ethylene-induced ROS and H_2O_2 generation.

The data collectively show that watersoaking is a tissue-specific ethylene response and total ROS and H_2O_2 generation capacity appears to contribute to ethylene-induced watersoaking of immature cucumber fruit as influenced by pO_2. Transcriptional regulation of ethylene receptors was noted as an early cellular response prior to incipient watersoaking. Up-regulation of ETR1-like receptors could represent a means of offsetting the delirious effects of excess ethylene.

CHAPTER 1
INTRODUCTION

Watersoaking is a major cause of postharvest losses, frequently observed in commodities following storage at chilling temperatures and in fresh-cut fruit tissues. The syndrome is characterized by acute softening, tissue translucency, enhanced electrolyte efflux, and cell wall disassembly. Watersoaking is an ethylene-induced disorder that occurs in members of the Cucurbitaceae including watermelon, cucumber, and cantaloupe melon. Application of 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception, confirmed the involvement of ethylene in the watersoaking disorder. Although both genotype and environmental factors play a role in the development of watersoaking, our understanding of the cellular mechanisms contributing to the onset and development of watersoaking is incomplete. The present study was conducted to address the role of oxygen in watersoaking development using immature beit-alpha cucumber fruit in which exogenous ethylene induces watersoaking uniformly and predictably.

Cucumber fruit is commercially harvested prior to developmental maturation. Watersoaking development of cucumber fruit is dependent on developmental maturity (Hurr et al., 2009). Immature fruit (4-6 d after anthesis) showed nearly 100% incidence of watersoaking at 6 d of continuous 10 $\mu L \cdot L^{-1}$ ethylene exposure whereas mature cucumber fruit (10-14 d after anthesis) showed a much lower incidence (about 30%). In fruit at more advanced maturity (showing color break due to accumulation of carotenoid pigments), however, ethylene exposure caused chlorophyll degradation and massive β-carotene accumulation but did not cause watersoaking. Ethylene-induced watersoaking in immature cucumber fruit was suggested to represent a form of programmed cell

death (PCD), supported by loss of cell viability, enhanced nuclease activity and DNA laddering (Hurr et al., 2010).

In this dissertation, Chapter 3 described the influence of ethylene concentration and exposure duration on ethylene responses in immature cucumber fruit. Characteristic changes in total reactive oxygen species (ROS) upon ethylene exposure were monitored. Chapter 4 addressed the tissue specificity and spatial pattern of watersoaking development. Incipient watersoaking symptoms were first evident in hypodermal tissues, followed by ingress to mesocarp tissue. Spatial patterns of watersoaking in response to altered gas-exchange properties were monitored employing intact and fresh-cut slices of cucumber fruit. Chapter 5 focused on the role of pO_2 in the ontogeny of ethylene-induced watersoaking in cucumber fruit. Of special interest was the potential involvement of total ROS, H_2O_2, and $O_2^{\cdot -}$ and antioxidant levels in watersoaking development. The effect of preconditioning under hypoxia on subsequent ethylene responses was also investigated. Chapter 6 addressed the ethylene-induced characteristic changes in expression of three ethylene receptors (Cs-ETR1, Cs-ETR2, Cs-ERS) using semi-quantitative RT-PCR.

This research contributes to the understanding of cellular and molecular events leading to the development of ethylene-induced watersoaking in immature beit-alpha cucumber fruit.

CHAPTER 2
LITERATURE REVIEW

Horticultural commodities such as fruit and vegetables are living organisms, having active processes even after harvest. Inappropriate handling between harvest and consumption can cause both quantitative and qualitative losses. Qualitative losses including reduced flavor, nutritional value, edibility, and consumer acceptability are more difficult to measure than quantitative losses (Kader, 2005). The values of postharvest loss are diverse depending on the commodity and countries. Kader (2005) estimated postharvest losses to be the range of 7-53% in developed countries and 7-70% in developing countries. In the United States, up to 23% of fruits and 25% of vegetables were lost during postharvest period (Kantor et al., 1997). Annually, U.S. potato industry loses an estimated $300 million due to bruising (Brook, 1996). Blond (1984) estimated postharvest losses in Egypt to be nearly 20% of fruit and 30% of vegetables. In Venezuela, the estimates of postharvest losses in broccoli and celery were almost 50% (Guerra et al., 1998).

The main goal of postharvest research is to maintain quality of horticultural crops via minimizing postharvest losses between harvest and consumption. Prevention of postharvest losses can contribute to the reduction of global malnutrition and conservation of natural resources. The most effective tool for maintaining fruit quality is application of proper temperature and relative humidity (RH) during postharvest handling (Kader, 2003). Maintaining cold chain throughout postharvest handling is emphasized to reduce biological deterioration (Kader, 2003). Controlled atmosphere (CA; low O_2 and high CO_2) is another postharvest application for long-term storage of fruits such as apple and pear (Saltveit, 2003). Study of edible waxing materials and

packaging technologies has been continued to reduce water loss and respiration rate (Kader, 2003). To extend postharvest life, ethylene antagonists such as silver thiosulfate and 1-methylcyclopropene (1-MCP) have been used in flowers and fruit/vegetables, respectively (Serek et al., 1994; Sisler and Serek, 1997; Sisler, 2006). Additionally, advanced biotechnology and plant breeding tools have provided new cultivars having improved flavor, shelf-life, and/or disease resistance.

Several factors induce postharvest losses of horticultural crops. The mechanical injury of fruits and vegetables has become a very important problem due to increasing use of mechanical equipment for harvesting, packing and transportation (Brusewitz and Bartsch, 1989; Marshall and Brook, 1999). Sanitation procedures are important to control postharvest diseases (Bartz and Brecht, 2002). Physiological deterioration due to enhanced respiration rate, transpiration, and ethylene production occurs depending on preharvest and postharvest conditions. Preharvest conditions include climate, water supply, soil fertility, and cultivation practice (Ferguson et al., 1999; Mattheis and Fellman, 1999). Storage conditions including temperature, relative humidity, air pressure, and atmospheric composition (mainly O_2, CO_2, and ethylene) are also able to influence crop deterioration (Kader, 2003; Kader, 2005). Commodities stored under injurious temperatures, relative humidity, air pressure, and atmospheric composition (mainly O_2, CO_2, and ethylene) exhibit physiological disorders such as rapid softening, discoloration, surface pitting, and watersoaking (Kader, 2002; Kader, 2003; Burg, 2004a).

What is Watersoaking?

Watersoaking is one of the major causes of postharvest losses, frequently observed in ripe and over-ripe fruit, in commodities stored at chilling temperature

(Jackman et al., 1992; Fernandez-Trujillo and Artes, 1998; Karakurt and Huber, 2003; Cho et al., 2008) and in fresh-cut fruit tissues (Hong and Gross, 1998; Agar et al., 1999; Aguayo et al., 2004; Jeong et al., 2004; Ergun et al., 2007; Montero-Calderon et al., 2008). Chen and Paull (2000) reported that highly translucent pineapple has flat and off flavors, and lower edible quality. In watermelon, watersoaking is characterized by an alteration of flesh texture, enhanced electrolyte efflux, degradation of pectic polymers, cell separation, and loss of cell wall rigidity (Elkashif and Huber, 1988a, 1988b). Although this disorder was not induced by anaerobic conditions in netted melon, water-soaked tissues exhibited decreased sucrose accumulation in the flesh and increased odors of fermentation (Nishizawa et al., 2002). It is often difficult to detect the disorder upon casual observation since, for example, in cantaloupe and watermelon, watersoaking is largely restricted to internal tissues (Karakurt and Huber, 2002; Madrid et al., 2004). Watersoaking in watermelon is believed to represent a stress response or disorder unrelated to normal ripening based on the report (Karakurt and Huber, 2002) that the disorder was induced by ethylene in both immature and fully ripe fruit and that watermelon fruit typically do not exhibit watersoaking during normal development.

Both genotype and environmental factors play a role in the development of watersoaking (Hiraishi, 1972); however, the cellular events leading to the disorder remain unknown. Water-soaked tissues are collapsed and have large intercellular spaces. Using scanning microscopy and analysis of water mobility via NMR imaging, du Chatenet et al. (2000) showed larger intercellular space in water-soaked mesocarp tissue of melon fruit. These observations suggest that liquid-filled intercellular space may be induced through enhanced water movement into the apoplast, possibly caused

by sugar-induced solute potential gradients between the symplast and the apoplast. The modification in the permeability of membrane and/or cell wall could play a role in watersoaking development by leading to the invasion of the intercellular space by cell liquid and solutes. Altered permeability could selectively enhance or suppress the activity of preexisting cell wall hydrolases (Karakurt and Huber, 2002; Mao et al., 2004), for example via altered apoplastic pH or ion composition (Almeida and Huber 1999; Huber et al., 2001). The increased activity and transcript abundance of polygalacturonase (PG) in ethylene-treated watermelon fruit with the onset and development of the water-soaking disorder indicates that catabolic reactions targeting the cell walls may contribute to the disorder (Karakurt and Huber, 2004). However, these changes are noted rather late during ethylene exposure, and following visible signs of the disorder (Karakurt and Huber, 2002).

Reports that electrolyte leakage increased relatively early (<48 h) in watermelon fruit in response to ethylene (Elkashif and Huber, 1988b) suggested that enzymes targeting cell membranes are involved in development of the disorder. Mao et al. (2004) reported increased lipoxygenase (LOX), phospholipase C (PLC), and phospholipase D (PLD) activities, increased phosphatidic acid (PA), and decreased phosphatidylcholine (PC) and phosphatidylinositol (PI) levels early in the development of watersoaking in ethylene-treated watermelon fruit. These trends indicate that lipid catabolism contributes to the development of the watersoaking disorder. Metabolites of lipid degradation including peroxidative products have been implicated in senescence and programmed cell death through inhibition of protein function and propagation of radical species (Hildebrand, 1989; Avdiushko et al., 1993). Membrane dysfunction and cell leakage

could also influence cell wall metabolism indirectly through ion- or pH-mediated activation or inhibition of specific hydrolases (Almeida and Huber, 1999; Huber et al., 2001).

Relationship between Ethylene and Watersoaking

The relationship between watersoaking and ethylene has been thoroughly studied in watermelon. Ethylene treatment of healthy watermelon fruit induced the development of softening and incipient watersoaking in placental tissue in as few as 3 days. (Elkashif and Huber, 1988a, 1988b; Mao et al., 2004). Application of 1-methylcyclopropene (1-MCP), a potent ethylene-action inhibitor (Sisler, 2006), confirmed the inductive role of ethylene in the watersoaking disorder. 1-MCP treatment at 5 $\mu L \cdot L^{-1}$ for 18 h completely prevented the development of watersoaking in watermelon, even in fruit challenged with continuous ethylene for 8 d (Mao et al., 2004). Jeong et al. (2004) also found that watersoaking in fresh-cut tomato was a senescence- and possibly ethylene-related response. du Chatenet et al. (2000), however, reported that watersoaking in cantaloupe melons during late ripening was not caused by ethylene based on the observation that 1-MCP did not prevent the disorder. Although vitrescence in melon appears at the beginning of the climacteric, Madrid et al. (2004) commented that there was no relationship between the disorder and ethylene based on the ethylene production trends by fruit from plants grown in both perlite and rockwool. It seems evident that watersoaking phenomena occur via different mechanisms in different commodities.

The responses of immature cucumber fruit to ethylene parallel those reported for other members of the Cucurbitaceae, most notably watermelon. It is difficult to reconcile whether tissue watersoaking in cucumber fruit is caused by or simply contributes to the extensive pectin degradation occurring in response to ethylene. Other fruits show

similar degrees of pectin breakdown but show no overt evidence of watersoaking (Huber et al., 2001). Another factor possibly contributing to the watersoaking phenomenon in cucumber fruit may involve ethylene-induced membrane permeability changes. The response of watermelon fruit to ethylene seems to be unrelated to fruit maturity (Karakurt and Huber, 2002); however, cucumber fruit of different maturity stages showed markedly different symptom upon ethylene exposure. Watersoaking was observed in immature (4-6 d after anthesis) and to a lesser extent in mature cucumber fruit (10-14 d after anthesis), while chlorophyll degradation and massive accumulation of β-carotene without this disorder were observed in fruit of more advanced maturity (Hurr et al., 2009).

Ethylene-induced watersoaking in immature cucumber fruit was suggested to represent a form of programmed cell death (PCD**)** (Hurr et al., 2010). Hallmarks of PCD such as loss of cell viability, enhanced nuclease activity and DNA laddering were observed in ethylene-treated immature cucumber fruit. Several studies have revealed an involvement of ethylene in development of plant PCD during the hypersensitive response (Ciardi et al., 2001; Trobacher, 2009), aerenchyma formation (Drew et al., 2000), and senescence and abscission processes (Chandlee, 2001; Rogers, 2006; Chaves and de Mello-Farias, 2006; Lerslerwong et al., 2008). Ethylene binding induces ethylene signaling pathways, leading to activation of PCD-related genes and accumulation of cytosolic Ca^{2+} and reactive oxygen species (ROS) to promote PCD (Trobacher, 2009).

Ethylene Sensitivity: Receptor Level

Ethylene is a gaseous phytohormone influencing diverse development processes including seed germination, root initiation, abscission, fruit ripening, sex determination,

and senescence (Abeles et al., 1992; Kieber, 1997; Lin et al., 2009). Ethylene can function in response to biotic and abiotic stresses including pathogen attack, flooding, low temperatures, light, and wounding (Abeles et al., 1992; Lin et al., 2009) and can diffuse into and throughout plant tissues (Mattoo and Suttle, 1991). The effect of ethylene is influenced by development stage, and ethylene concentration and exposure duration (Abeles et al., 1992; Saltveit, 1999).

Ethylene action initiates with binding of ethylene to a family of endoplasmic reticulum (ER)-associated receptors including ETR1-like-family and ETR2-like family (Klee 2002; Hall et al., 2007). Ethylene receptors are similar to two-component histidine kinases (HKs) found in bacteria (Schaller and Bleecker, 1995). ETR1-like receptors including ETR1 and ERS1 consist of five subdomains essential for HK activity while ETR2-like receptors lack at least one of these subdomains (Hall et al., 2007). Ethylene binding occurs at the N-terminal transmembrane domain of the receptors, and a copper co-factor is required for binding (Rodriguez et al., 1999). Ethylene receptor regulatory genes, REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1) and GREEN-RIPE (GR), were reported in Arabidopsis and tomato, respectively (Barry and Giovannoni, 2006; Resnick et al., 2006). RTE1 and GR are negative regulators of ethylene signaling. RTE1 could regulate only a subset of etr1 alleles but there was no direct correlation between rte1 suppression and ethylene binding ability of etr1 alleles, indicating a role of RTE1 in conformational changes of the ETR1 receptor upon ethylene binding (Resnick et al., 2008). GR was also capable of modulating the signal output of specific ethylene receptors in a tissue-specific manner (Barry and Giovannoni, 2006). Ethylene binding to receptors releases the negative regulator, which results in the signal transduction chain

leading to the activation of transcription factors and the ethylene-responsive genes (Bleecker et al., 1998; Chang, 2003).

CTR1 is a downstream component of receptors. In the absence of an ethylene signal, ethylene receptors activate CTR1, and then CTR1 in turn negatively regulates the ethylene signaling pathway through a MAP-kinase cascade (Kieber et al., 1993). Other downstream components in the ethylene pathway include several positive regulators (EIN2, and EIN5) and transcription factors (EIN3, EIL1, and ERF1) located in the nucleus (Chao et al., 1997; Trobacher 2009). The inhibitory effect of CTR1 on activity of EIN2, a downstream component, could be relieved by ethylene binding (Hall et al., 2007). EIN2 level is regulated by two F-box proteins, ETP1 and ETP2. Ethylene binding reduced the levels of ETP1 and 2, inhibiting EIN degradation by ETP1 and 2 (Qiao et al., 2009). EIN3, a transcription factor, binds to the promoter of ERF1 gene and activates its transcription in an ethylene-dependent manner (Solano et al., 1998). The level of EIN3 protein is controlled by ethylene via the ubiquitin/proteosome pathway mediated by two F-box proteins, EBF1 and EBF2 (Guo and Ecker, 2003). EIN5, a 5'→3'exoribonuclease, degrades EBF1 and EBF2, resulting in accumulation of EIN3 (Olmedo et al., 2006). Transcription factors ERF1 and other Ethylene Response Element Binding Protein (EREBPs) interact with the GCC box, a cis-element in the promoter of ethylene-responsive genes and activate downstream ethylene responses (Wang and Ecker, 2002). Branch points in the ethylene response pathway may lie downstream of EIN3/EIL1. EREBPs may integrate ethylene responses with developmental signals and/or other hormone signals (Guo and Ecker, 2004; Li and Guo, 2007).

Variation in ethylene sensitivity during plant tissue maturation and development (Yang, 1987; Alexander and Grierson, 2002) may depend upon the levels of these ethylene receptors and downstream components. Since ethylene receptors function as negative regulators, there is an inverse correlation between receptor levels and ethylene sensitivity; an increase in the level of receptors causes an increase in the threshold for initiating ethylene response, allowing decreased sensitivity (Klee, 2004). Based on the previously mentioned ethylene-regulation model, increased ethylene sensitivity in mature or ripening fruits could be explained by a significant decrease in receptor level. However, tomato and other climacteric fruits exhibit a marked increase in the expression of ethylene receptor genes during ripening (Sato-Nara et al., 1999; El-Sharkawy et al., 2003; Klee, 2004). This paradox could be explained by the inconsistency between RNA and protein levels. In tomato fruit, accumulation of receptor mRNAs was greatly enhanced at the onset of ripening while receptors protein levels were highest in immature fruit and decreased at the onset of ripening (Kevany et al., 2007). 26S proteasome is involved in degradation of receptor proteins, which was confirmed by inhibitory effect of MG132 (a peptide aldehyde; an inhibitor of 26 S proteasome activity) (Kevany et al., 2007). 1-MCP treatment of mature-green tomato prevented both ripening and degradation of ethylene receptor proteins (Kevany et al., 2007).

Different expression patterns of the receptor gene family can provide another explanation for changes in ethylene sensitivities in different tissues at different developmental stages (Wilkinson et al., 1995; Payton et al., 1996; Hua et al., 1998; Sato-Nara et al., 1999; Tieman and Klee, 1999). The ethylene receptors are differently

regulated by ethylene and by other developmental factors. Transcription of receptors ERS1, ERS2, and ETR2 was regulated by ethylene itself (Hua and Meyerowitz., 1998). Wilkinson et al. (1995) reported that NR mRNA is positively regulated by ethylene in a development-specific manner. The effect of different expression patterns on ethylene sensitivity could be explained by different contributions of different receptor genes to ethylene signaling. The ETR1-like group is more important than the ETR2-like group in determining ethylene responses (Wang et al., 2003; Guo and Ecker 2004; Hall et al., 2007). The loss of the primary receptors including ETR1 and ERS1 would increase ethylene sensitivity more greatly than other receptors (Clark et al., 1998). Higher signals of ERS1 were reported in young and developing tissue than in older tissues of melon and Arabidopsis (Hua et al., 1998; Sato-Nara, 1999), which reduced sensitivity to ethylene. Functional redundancy within receptor genes might be explained by interactions with downstream components. CTR1 binds more strongly to ETR1-like receptors than ETR-2 like receptors (Guo and Ecker, 2004). Compensation responses (increased transcripts of remaining receptors to compensate for missing receptors) were observed in loss-of-function mutations but there was a difference in phenotype, indicating specialized roles of receptor members in signal output (O'Malley et al., 2005).

Proteosomal degradation of transcription factors, especially by 26S, might also play a role in tissue-specific ethylene responses (Trobacher, 2009). In non-sensitive tissue, transcription factors responsible for ethylene response might be degraded by some mechanism to block the undesired ethylene response. Trobacher (2009) proposed that other plant hormones such as abscisic acid (ABA) and cytokinins could inhibit ethylene-induced PCD through enhanced degradation of transcription factors.

Interactions between ethylene and other phytohormones have been extensively reviewed by Wang et al. (2002) and Yoo et al. (2009).

Interactions between Ethylene and Oxygen

Oxygen (O_2) is the terminal electron acceptor in the oxidative phosphorylation pathway, supplying ATP for cellular metabolism by regenerating NAD from NADH (Geigenberger, 2003). O_2 is also essential in several cellular pathways such as heme, sterol, fatty-acid, and ethylene biosynthesis (Geigenberger, 2003). Intercellular O_2 concentration is determined not only by the atmospheric O_2 concentration but also O_2 consumption rate, intercellular resistance to gas diffusion, and the porosity and gas exchange resistance of tissue surface (Burg, 2004). Since plants do not have efficient systems for O_2 delivery, re-entry of O_2 into hypoxic or anoxic plant tissues leads to the formation of potentially harmful reactive oxygen species, inducing rapid oxidative damage (Crawford and Braendle, 1996; Biemelt et al., 1998).

pO_2 is an important factor controlling the postharvest physiology of commodities. Generally, hypoxic environments (lower than 21 kPa O_2) induce beneficial reactions including reductions in respiration rate, ethylene synthesis and perception, chlorophyll degradation, cell wall degradation, and phenolic oxidation (Mir and Beaudry, 2001; Burg, 2004). Reduction of respiration reduces the rate of deterioration of fruits and vegetables, extending storage life (Burton, 1974; Herner, 1987). Decreased respiration under hypoxia was observed in avocado fruit (Solomos and Kanellis, 1989; Metzidakis and Sfakiotakis, 1995), broccoli buds (Makhlouf et al., 1989), tomato fruit (Kim et al., 1999), and fresh-cut bell pepper (Conesa et al., 2007). Under 5-7 kPaO_2, ethylene production in intact fruits including apple and avocado declined by 50% (Abeles et al., 1992). Under hypoxic conditions (2.5 kPa O_2), the activities of PG, acid phosphatase,

and cellulase in banana and avocado fruit were suppressed compared with those of air-treated fruit (Kanellis et al., 1989; Metzidakis and Sfakiotakis, 1995). The effect of hyperoxic conditions (higher than 21 kPa O_2) on the respiration rate and ethylene production was various depending on the commodity, ripening stage, O_2 level, storage time and temperature (Kader and Ben-Yehoshua, 2000). Under increased pO_2, O_2 can enhance the production of ROS, inhibiting various metabolic activities and leading to deterioration of produce quality (Kader and Ben-Yehoshua, 2000). In contrast, hyperoxic conditions have been suggested as a viable decay control alternative to pesticides, as well as an improvement over traditional MA treatments that use elevated pCO_2 and/or reduced pO_2 (Day, 1996). Mycelia growth rate in strawberry fruit was decreased by increasing pO_2 (Wszelaki and Mitcham, 2000).

Interactions between O_2 and ethylene have been shown to play a role in the regulatory control of ripening (Altman and Corey, 1987). Elevated pO_2 (60 or 100 kPa O_2) hastened softening and ripening in 1-MCP-treated banana fruit (Jiang and Joyce, 2003). In grapes, hyperoxia (80 kPa) suppressed softening and reduced polygalacturonase (PG), β-galactosidase, and cellulase activities (Deng et al., 2005). Hypoxia delayed ethylene or propylene-induced ripening in avocado (Metzidakis and Sfakiotakis, 1995), banana (Hesselman and Freebain, 1969; Kanellis et al., 1989), kiwi (Stavroulakis and Sfakiotakis, 1997) and tomato fruits (Kapotis et al., 2004). Banana and avocado fruit subjected to hypoxic conditions (2.5 kPa O_2) showed lower PG, acid phosphatase, and cellulase activities compared with air-treated fruit (Kanellis et al., 1989; Metzidakis and Sfakiotakis, 1995). Low O_2 prevented the rise in acid phosphatase

activities and this suppression was not circumvented by subsequent application of 2.5 kPa O_2 containing 100 $\mu L \cdot L^{-1}$ ethylene (Kanellis et al., 1989).

The suppressing effect of low O_2 on enzymic activities might be mediated through a decrease in the rate of production of metabolic energy as a result of the decrease in respiration. However, Beaudry (1999) noted that the retarding effect of low O_2 may be explained by an attenuation of the biological efficacy of ethylene because this decrease in respiration may not reflect a restriction of the electron transport chain regarding high O_2 affinity of the cytochrome oxidase (Kanellis et al., 1989). Burg (2004) explained the decreased response to ethylene at reduced pO_2 as 'coupling activation'. This concept is supported by the binding of O_2 to iron-containing cytochrome and copper-containing oxidases, by carbon monoxide's high affinity for both the copper-containing ethylene receptor and cytochrome oxidase, and by the ability of CO_2 to inhibit the binding of O_2 to cytochrome oxidase. A report that avocado fruit soften very slowly when exposed to 130 $\mu L \cdot L^{-1}$ propylene (an ethylene analogue) in 2 pKa O_2, and negligibly at 1 pKa O_2 is consistent with a coupling activation (Burg, 2004). Burg and Burg (1967) provided evidence that for ethylene to exert its biological effects, O_2 is required. A synergistic effect of O_2 and ethylene was observed in ragweed seeds. Application of ethylene (10 $\mu L \cdot L^{-1}$) in 100 kPa O_2 gave 71.3% germination after 2 weeks of treatment, while ethylene in air or no ethylene in 100 kPa O_2 resulted in 41.3% and 11.3% germination, respectively (Brennan et al., 1978). These results are similar to other ethylene- O_2 interactions. In potato tubers, ethylene triggered a respiratory upsurge (Reid and Pratt, 1972) that was markedly enhanced by high O_2 tensions, while O_2 alone had little or no effect (Chin and Frenkel, 1977). In the non-ripening *rin* tomato mutant, ethylene was

used to initiate the synthesis of lycopene but O_2 concentration was rate limiting (Frenkel and Garrisoni, 1976). It appears that some of the processes that are initiated by ethylene and stimulated by O_2 might reflect oxygen utilization through the formation of peroxides.

Kidd and West (1934) suggested that the beneficial effects of reduced O_2 on the longevity of climacteric fruits might be related to its interference with ethylene production. Hypoxia reduced synthesis of and sensitivity to ethylene (Mir and Beaudry, 2001). Abeles et al. (1992) reported that the range of 5 to 7 kPa O_2 reduced ethylene production by 50% in several intact fruits. Reductions in the steady-state levels of mRNAs for genes involved in ethylene synthesis were also induced by low O_2 (5 kPa) (Geigenberger, 2003) and ethylene production of propylene-treated avocado fruit was delayed by low O_2 (1 and 2 kPa) (Metzidakis and Sfakiotakis, 1995). In kiwifruit, O_2 was found to control ethylene biosynthesis through altering 1-aminocyclopropane-1-carboxylic-acid (ACC) production, which appears to be the limiting factor in autocatalytic ethylene production under low O_2 atmospheres. Inhibition of ACC synthase (ACS) activity was reported in apples stored at 2-4 kPa O_2 (Bufler and Bangerth, 1983; Gorny and Kader, 1996) and kiwi fruit treated with 130 $\mu L \cdot L^{-1}$ propylene under O_2 levels of 10 kPa and lower (Stavroulakis and Sfakiotakis, 1997). However, the production of ACC is not the limiting factor in ethylene production of certain crops under low O_2. Stimulated ACC content and ACS activity were reported in tomato roots and leaves grown under low O_2 (by N_2 flush through growth solution) (Wang and Arteca, 1992). Apple fruit treated with 1.7 kPa O_2 for 6 h exhibited much higher ACC content than air-treated fruit, indicating that low O_2 inhibited the conversion of ACC to ethylene by ACC oxidase

(ACO) (Li et al., 1983). Decreased ACO activity appears to be an important cause of decreased ethylene production under hypoxia since O_2 is a co-substrate for the enzyme (Ververidis and John, 1991; Dong et al., 1992; Sairam et al., 2008). Km values for O_2 are 0.4 and 0.44~0.53 kPa in ACO purified from apple (Kuai and Dilley, 1992) and pear fruit (Vioque and Castellano, 1994; Kato and Hyodo, 1999), respectively. Storage below 5 kPa O_2 could inhibit ACO activity since internal pO_2 is much lower than external pO_2 due to gas diffusion barriers. Red-ripe tomato fruit stored under 4 kPa O_2 exhibited 0.2 kPa internal pO_2 (Berry and Sargent, 2009). Reduced ACO activity was observed in broccoli flower buds stored at 2.5 kPa O_2 (Makhlouf et al., 1989). Undetectable amounts of ACO protein were reported in apple fruit treated with 2 kPa O_2 for 2 months while air-treated fruit had large amounts of ACO protein (Gorny and Kader, 1999).

O_2 has been reported to exert an effect on ethylene perception (Burg and Burg, 1967). Beaudry (1999) proposed that the interactions between O_2 and ethylene might be consistent with an enzyme kinetic model, in which a substrate (O_2) must bind to a receptor before a dissociable activator (ethylene) can attach. Since O_2 is required for ethylene action (Beaudry, 1999), it is a reasonable assumption that the suppressing effects of low O_2 on fruit softening reflect an attenuation of ethylene action. O_2 depletion reportedly reduces ethylene sensitivity in fruits (Kidd and West, 1945), and Burg and Burg (1967) observed a similar effect with pea stem sections. Five percent O_2 apparently did not alter O_2 consumption (Eichenberger and Thimann, 1957), stem elongation, or CO_2 production (Hackett and Schneiderman, 1953) but markedly reduced ethylene effectiveness as measured with pea growth inhibition (Burg and Burg, 1967). Solomos and Kanellis (1989) mentioned that low O_2 might interfere with the action of

ethylene by affecting metabolic processes which lead to an increase in ethylene receptors. Enhanced-softening of 1-MCP-treated banana fruit in response to elevated pO_2 (60 and 100 kPa) may reflect the synthesis of new receptors (Jiang and Joyce, 2003). In maize leaf, low O_2 (3 kPa) caused a 15-fold increase in accumulation of RP-ERS1 after 24 h treatment (Sachs et al., 1996). It is unclear how the concentration of O_2 affected gene expression, but it is evident that a number of physiological changes take place in the cell under low O_2 atmosphere (Sachs et al., 1996).

O_2 might also affect plant metabolism related with ethylene. Burg (2004) proposed that binding of ethylene to its receptor occurs in the total absence of O_2. Afterward, the ethylene-receptor complex interacts with a rate-limiting reactant that imparts specificity to the ethylene-receptor complex when it elicits a biological response. O_2 may be essential for this step or later steps of transduction and response. Theologis and Laties (1982) noted that the effectiveness of high concentrations of O_2 in synergizing ethylene action might be induced by the involvement of O_2 in a high Km process other than respiration *per se* based on the response to elevated O_2 or to peeling. It seems that a bimolecular reaction, in the catalytic sense, between O_2 and ethylene might influence gene expression during fruit ripening. Low O_2 not only suppressed the induction of new polypeptides associated with normal ripening, but also induced the accumulation of unique polypeptides. The suppression of a number of other polypeptides in response to low O_2 environments may be a result of either suppression of translation due to the dissociation of polysomes, as was reported for soybeans (Lin and Key, 1967) and maize (Sachs and Ho, 1986), or repression of the expression of mRNA (Sachs and Ho, 1986;

Kanellis, 1987), or both (Laemmli, 1970). In avocado fruit, 2.5 kPa O_2 prevented the rise in total cellulase protein and its transcript during ripening (Solomos and Kanellis, 1989).

Reactive Oxygen Species

Ground state or triplet O_2 is converted to the much more reactive oxygen species (ROS) either by energy transfer or electron transfer (Klotz, 2002). The former leads to the formation of singlet forms of O_2, whereas the latter results in the sequential reduction to superoxide, hydrogen peroxide, and hydroxyl radical (Klotz, 2002). In plants, ROS are continuously produced as by-products of various metabolic pathways localized predominantly in chloroplasts, mitochondria, and peroxisomes (Foyer and Harbinson, 1994; Circu and Aw, 2010). Plasma membrane-bound NADPH oxidases, cell wall-bound peroxidases, and amine oxidases in the apoplast are involved in ROS production of plant cells (Mittler et al., 2004). Plants generate ROS via activating various oxidases and peroxidases in response to environmental changes (Bolwell et al., 2002), mainly in chloroplasts and mitochondria at the sites of electron transport (Apel and Hirt, 2004). Some of the oxidative and antioxidative activities have been detected in the apoplastic space (Ogawa et al., 1996; Duran and Bujan, 1998; Vanacker et al. 1998), suggesting that these activities in the apoplast have important roles in causing or alleviating tissue damage. Depending on the character of abiotic stresses, plants differentially enhance the production of ROS that are chemically distinct and/or are produced within different cellular compartments (Elstner, 1991).

Biological organisms attempt to maintain homeostatic equilibrium between production and scavenging of ROS. Oxidative damage could be inhibited by direct quenching of ROS or through disruption of free radical propagation reactions (Alscher and Hess, 1993). ROS could act as signals to activate antioxidant systems (Mittler,

2002). Plants have nonenzymatic and enzymatic ROS scavenging mechanisms (Apel and Hirt, 2004). Nonenzymatic antioxidants include the major cellular redox buffers ascorbate and glutathione (GSH), as well as tocopherol, flavonoids, alkaloids, and carotenoids. Enzymatic ROS scavenging mechanisms in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). Geigenberger (2003) reported that low-O_2-induced genes encoding peroxidase, monodehydroascorbate reductase, APX, GPX, and SOD are involved in detoxification of ROS. The balance between production and scavenging of ROS can be disturbed under extreme stress environments including high light, drought, temperature extremes, and mechanical stress (Malan et al., 1990; Elstner, 1991; Prasad et al., 1994; Hodges et al., 2004). The response of antioxidative systems to oxidative stress during postharvest storage was various depending on commodities and/or cultivars, indicating the complexity of antioxidant systems to oxidative stress (Hodges et al., 2004).

ROS generation occurs in cellular compartments including mitochondria, peroxisomes, or chloroplasts. ROS results in changes of the nuclear transcriptome, indicating that signals are transmitted from these organelles to the nucleus. The signaling pathway remains unidentified. There are several ways through which ROS could affect gene expression. ROS receptors could be activated to induce signaling transduction pathways that eventually interrupt gene expression (Apel and Hirt, 2004). In mammalian cells, death receptors such as Fas, TNFR (tumor necrosis factor receptor), and TRAIL(TNF-related apoptosis-inducing ligand) have been shown to mediate apoptosis (cell death) (Circu and Aw, 2010). In plants, ROS receptors remain unidentified. ROS receptors in plant might be two-component histidine kinases,

activating Calmodulin and a MAPK cascade and then regulating transcription factors (Mittler et al., 2004). Alternatively, components of ROS signaling pathways could be directly oxidized by ROS (Apel and Hirt, 2004). For example, ROS oxidized glutathione (GSH) to glutathione disulfide (GSSG), resulting in induction of cellular oxidative damage (Schafer and Buettner, 2001). Excessive ROS in mitochondria, a major ROS generator, could result in damage to mitochondrial DNA, triggering apoptosis (Circu et al., 2009; Rachek et al., 2009). Finally, ROS might alter gene expression by modifying the activity of transcription factors. The expression of several transcription factors such as WRKY, Zat, RAV, GRAS and Myb families is regulated by ROS (Mittler et al., 2004).

Oxygen radicals and hydrogen peroxide are highly reactive and destructive, shown to cause membrane lipid peroxidation and to enhance membrane leakage and tissue deterioration (Dhindsa et al., 1981; Fridovich, 1986; Moran et al., 1994; Rao et al., 2000; Overmyer et al., 2003; Circu and Aw, 2010). Excessive ROS was shown to trigger PCD in plants (Desikin et al., 2001; Rao and Davis, 2001; Mur et al., 2005). On the other hand, ROS can drive oxidative cross-linking of cell wall components and activate a range of defense mechanisms, protecting against invading micro-organisms (Lamb and Dixon, 1997; Mittler 2002; Mittler et al, 2004; Circu and Aw, 2010). It has been demonstrated in several systems that NAD(P)H oxidase is a plasma membrane-bound enzyme that generates superoxide radicals that are active in the extracellular space (Bolwell et al., 1999). It has been postulated that this activity may have important roles in mediating several important responses such as hypersensitivity to pathogen invasion, xylem thickening and programmed cell death (Levine et al., 1994; Mehdy et al., 1996; Barcelo, 1998; Mittler et al., 2004).

Reactive oxygen species (ROS) can control ethylene signaling directly or indirectly (Overmyer et al., 2003). Significant crosstalk between ROS and ethylene plays an important role in responding to biotic and abiotic stress (Overmyer et al., 2003; Kwak et al., 2006; Parent et al., 2008). ROS have been shown to trigger programmed cell death (PCD) in plants (Desikin et al., 2001; Rao and Davis, 2001; Overmyer et al., 2003; Mur et al., 2005; van Breusegem and Dat, 2006; Gadjev et al., 2008). Several studies have also revealed an involvement of ethylene in plant PCD (Ciardi et al., 2001; Chandlee, 2001; Rogers, 2006; Chaves and de Mello-Farias, 2006; Lerslerwong et al., 2008; Trobacher, 2009). The accumulation of ROS during ethylene exposure may be able to induce PCD, or vice versa.

Watersoaking is a major cause of postharvest losses, but our understanding of the cellular mechanisms contributing to the onset and development of watersoaking is incomplete. Since exogenous ethylene induces watersoaking uniformly and predictably (Lima et al., 2005; Hurr et al., 2009), immature beit-alpha cucumber fruit could be employed as a model system to study cellular events leading to watersoaking. In the present study, analyses of watersoaking were performed using beit-alpha mini-cucumber. Emphasis was on characterizing the relationships between ethylene, ethylene transcript abundance, pO_2 and ROS on the induction and development of the watersoaking.

CHAPTER 3
THE EFFECT OF EXOGENOUS ETHYLENE ON WATERSOAKING DEVELOPMENT AND ACCUMULATION OF RADICAL-OXYGEN SPECIES IN BEIT-ALPHA CUCUMBER FRUIT

Introduction

Watersoaking is the appearance of tissue translucency, vitrescence, watercore, or glassiness. This disorder is a significant cause of postharvest losses, frequently observed in commodities following storage at chilling temperatures (Fernandez-Trujillo and Artes, 1998; Karakurt and Huber, 2002; Cho et al., 2008), ethylene-treated fruit (Bernadac et al., 1996; Lima et al.; 2005; Mao et al., 2004; Hurr et al.,2009), and fresh-cut fruit tissues (Agar et al., 1999; Aguayo et al., 2004; Jeong et al., 2004; Ergun et al., 2007; Montero-Calderon et al., 2008). Characteristics of water-soaked tissues are acute softening, enhanced electrolyte efflux, loss of flavor, and cell wall disassembly (Bauchot et al., 1999; Karakurt and Huber, 2002; Jeong et al., 2004; Lima et al., 2005; Mao et al., 2004; Nishizawa et al., 2002).

Water-soaked tissues apparently arise from liquid suffusion into intercellular air spaces, resulting from modifications in the permeability of membrane and/or cell wall (Chen and Paull, 2000; du Chatenet et al., 2000).Degradation of cell wall and/or membrane has been reported to contribute to the disorder. Ethylene-treated watermelon fruit exhibited an increase in polygalacturonase (PG) activity (Karakurt and Huber, 2004) as well as increases in lipoxygenase (LOX), phospholipase C (PLC), phospholipase D (PLD) activities along with the onset and development of the watersoaking disorder (Mao et al., 2004). However, knowledge about the early cellular events leading to the disorder remains incomplete. Studying the physiological mechanisms of tissue watersoaking has been limited due to the inability to induce the

disorder experimentally in a predictable and consistent manner. Recently, our lab has demonstrated that immature beit-alpha cucumber fruit (cv. Manar) can serve as a model system to study watersoaking disorder (Hurr et al., 2009). Watersoaking can be induced uniformly and consistently in response to continuous ethylene exposure (10 $\mu L\ L^{-1}$) in immature fruit within 6 d.

Watersoaking is an ethylene-induced disorder in cucumber fruit (Lima et al., 2005; Hurr et al., 2009), and in other Cucurbitaceae including watermelon (Karakurt and Huber, 2002; Mao et al., 2004) and cantaloupe melon fruits (Bernadac et al., 1996). Application of 1-methylcyclopropene (1-MCP), an ethylene antagonist (Sisler, 2006), inhibits watersoaking of cucumber and watermelon fruit challenged with ethylene, confirming the involvement of ethylene in the disorder (Mao et al., 2004; Lima et al., 2005). Ethylene is a gaseous phytohormone influencing diverse development processes including germination, growth, flowering, ripening, senescence, and abscission (Abeles et al., 1992; Kieber, 1997; Lin et al., 2009). Ethylene effects on quality of horticultural commodities are dependent on a number of factors, with ethylene concentration and exposure time being the primary factors (Saltveit, 1999). Abscission response of Hibicscus was greater when exposed to higher ethylene concentration for longer duration (Høyer, 1995). When plants were exposed to 0.1 or 1 $\mu L \cdot L^{-1}$ ethylene for 12 h, there was no significant difference in intensity of bud abscission response for 23 d. However, exposure to 1 $\mu L \cdot L^{-1}$ ethylene for 72 h induced 75% of bud abscission at 4 d while that intensity of abscission response was seen at 16 d in treatment at 0.1 $\mu L \cdot L^{-1}$ for 72 h. When comparing 6, 12, or 24 h of 0.1 $\mu L \cdot L^{-1}$ ethylene exposure, longer exposure did not enhance abscission response. However, 24 h of 1 $\mu L \cdot L^{-1}$ ethylene exposure

caused twice as much abscission at 8 d compared to 12 h of exposure. On the other hand, Palou et al. (2003) reported that the skin color (hue angle) of 'Brooks' cherry and firmness of 'Patterson' and 'Castle-brite' apricot decreased in response to exogenous ethylene during storage at 5 ^{o}C, but was not influenced by ethylene concentration (0.01, 0.1, or 1 $\mu L \cdot L^{-1}$for cherry; 1, 10, or 100 $\mu L \cdot L^{-1}$ for apricot).

Watersoaking in watermelon is believed to represent a response not linked to normal ripening based on observations that ethylene induced this disorder in both immature and fully ripe fruit and that watermelon fruit typically do not exhibit watersoaking during normal development (Karakurt and Huber, 2002). However, cucumber fruit of different maturity stages showed different symptoms upon ethylene exposure. Watersoaking disorder was observed in immature (4-6 d after anthesis) and to a lesser extent in mature cucumber fruit (10-14 d after anthesis), while fruit of more advanced maturity exhibited chlorophyll degradation and massive accumulation of β-carotene without watersoaking (Hurr et al., 2009). Ethylene-induced watersoaking in immature cucumber fruit was suggested to represent a form of programmed cell death (PCD**)** (Hurr et al., 2010). Hallmarks of PCD such as loss of cell viability, enhanced nuclease activity and DNA laddering were observed in ethylene-treated immature cucumber fruit. Several studies have revealed an involvement of ethylene in development of plant PCD during the hypersensitive response (Ciardi et al., 2001), aerenchyma formation (Drew et al., 2000), and senescence and abscission processes (Chandlee, 2001; Rogers, 2006; Chaves and de Mello-Farias, 2006; Lerslerwong et al., 2008).

Reactive oxygen species (ROS) have been shown to regulate PCD in plants (Overmyer et al., 2003; van Breusegem and Dat, 2006, Gadjev et al., 2008). Plants produce ROS continuously as by-products of various metabolic pathways localized predominantly in chloroplasts, mitochondria, and peroxisomes (Foyer and Harbinson, 1994). ROS also can be generated by various oxidases and peroxidases in response to environmental stresses (Bolwell et al., 2002). Reactive oxygen species are highly reactive and destructive, shown to cause membrane lipid peroxidation, enhanced membrane leakage, tissue deterioration and cell death (Dhindsa et al., 1981; Fridovich, 1986; Moran et al., 1994; Rao et al., 2000; Overmyer et al., 2003).The enhanced accumulation of ROS was shown to trigger PCD in plant (Desikin et al., 2001; Rao and Davis, 2001; Mur et al., 2005). On the other hand, ROS can act as signals for activating PCD network (such as cross talk with phytohormones, cell death-related gene expression, MAPK-driven phosphorylation cascades, and posttranslational modifications) against biotic and environmental stresses and induce defense mechanisms (Lamb and Dixon, 1997; Mittler, 2002; Mittler et al., 2004).

To improve our understanding of ethylene-induced watersoaking in immature beit-alpha cucumber fruit, the present study was designed to investigate ethylene responses as influenced by ethylene concentration and exposure duration. Furthermore, we tested the hypothesis that accumulation of reactive oxygen species upon ethylene exposure is involved in initiation of watersoaking in immature cucumber fruit.

Materials and Methods

Plant Materials

Experiments were conducted with beit-alpha cucumber (*Cucumis Sativus* L.; ‘Manar’) harvested at immature stage (average fruit wt. 86 ± 3.2 g) from a commercial

greenhouse facility in Live oak, FL. Freshly harvested fruit were sorted by size, color and appearance, surface sterilized with 2.7 mM sodium hypochlorite, and air-dried.

Experiment 1. Influence of Ethylene Concentration and Exposure Duration on Watersoaking of Cucumber Fruit

Ethylene treatment

Intact fruit (n=50 per container) were placed in 20-L plastic containers at 13 ^{o}C and 95% RH. In an experiment designed to investigate ethylene responses as influenced by ethylene concentrations, containers were sealed and provided with flow-through air or atmospheres containing 10, 100, 500 or 1000 $\mu L \cdot L^{-1}$ of ethylene continuously. Flow rate was maintained at 500 $mL \cdot min^{-1}$ to avoid CO_2 accumulation, and the gas mixture was humidified by passing it through a water-filled glass jar (2 L). In an experiment designed to study the effect of duration of ethylene exposure, containers were supplied with flow-through atmosphere containing 10 $\mu L \cdot L^{-1}$ ethylene for 12 h, 2 d, or 4 d. After these time periods, each container was supplied with ethylene-free air for the duration of storage. Two controls were included in the experiment; in the negative control one container was continuously supplied with ethylene-free air while in the positive control ethylene was supplied continuously at a concentration of 10 $\mu L \cdot L^{-1}$. Continuous exposure to ethylene at this concentration has been shown to induce uniform watersoaking of cucumber fruit in 6 d (Lima et al., 2005; Hurr et al., 2009).

As parameters of ethylene responses, the incidence of watersoaking and changes in fruit firmness, surface hue angle, respiration rate and electrolyte leakage were monitored.

Respiration rate and ethylene production

Six cucumber fruit per treatment were repeatedly used for measuring respiration rate and ethylene production every other day. Two cucumber fruit were weighed and placed in 1,400 mL plastic containers (n=3) fitted with septa. To avoid off-gassing effect of the applied ethylene, the lid of each container was opened for 1 h before sealing. Respiration and ethylene measurements were conducted by sealing for 3 h at 13 °C, after which 0.5 and 1.0 mL of the headspace was removed by syringes to quantify carbon dioxide and ethylene, respectively. Respiration rate was determined using a gas chromatograph (GOW MAC, series 580; Bridgewater, NJ, USA) fitted with a thermal conductivity detector and a 1219 mm x 3.18 mm Porapac Q column [particle size 149-177 µm (80/100 mesh)]. The flow rate of the carrier gas (helium) was 0.4 $mL \cdot s^{-1}$ and oven and injector/detector were set at 40 ^{o}C and 25 ^{o}C (ambient), respectively. Respiration rate is expressed in µg $CO_2 \cdot kg^{-1} \cdot s^{-1}$.

Ethylene production was measured using a gas chromatograph (Tremetrics, Tracor 540; Austin, TX) fitted with a photoionization detector and an alumina-packed column [914 mm × 3.18 mm; particle size 149-177 µm (80/100 mesh)]. The flow rate of the carrier gas (helium) was 0.4 $mL \cdot s^{-1}$ and the oven and injector/detector were set at 50 and 100 ^{o}C, respectively.

Surface color

Surface color measurements were obtained from two equatorial regions per fruit with a Minolta Chroma Meter CR-400 (Minolta Camera Co. Ltd., Japan) which has an 8 mm-diameter aperture and illuminant C lighting condition. A white calibration plate was used for calibration (L* = 96.88, C* = 2.05, h°* = 89.4, a* = 0.02, b* = 2.05). The values were expressed by the CIE L (lightness)a*(range from green to red)b*(range from blue

to yellow) model (Mclaren, 1979). Hue angle was determined using the formula $h_{ab}=\tan^{-1}(b^*/a^*)$. The angular coordination of hue starts from 0^o for red, where 90^o = yellow, 180^o = green, and 270^o = blue. Five fruit per treatment were evaluated at each measurement interval.

Fruit firmness

Cucumber fruit firmness was determined using an Instron Universal Testing Instrument (Model 4411; Canton, MA, USA) equipped with a convex-tip probe (3 mm diameter for mesocarp and 7.5 mm diameter for intact fruit) and 0.05 kN load cell. For measurement of mesocarp firmness, each cucumber fruit was cut equatorially with a sharp, double-bladed knife into 10-mm thick slices. Each slice was immediately placed on a solid flat plate. Zero height was established between the probe and the intact fruit or mesocarp tissue. The probe was driven with a crosshead speed of 50 $mm \cdot min^{-1}$, and a distance of 2.5 mm. Maximum force (N) was recorded during compression. Two measurements were made per fruit and five individual fruit were evaluated per treatment at each measurement interval.

Electrolyte leakage

Electrolyte leakage was measured using a conductivity bridge (YSI 3100 conductivity instrument; Ohio, USA) equipped with a conductivity electrode. Five individual fruit per treatment were evaluated every other day. Mesocarp disks (n=5) of 4.5 mm diameter were excised using No. 2 Cork borer from 2 transverse slices (10 mm thickness) per fruit. Five disks were rinsed with distilled water, briefly blotted on Whatman #4 filter paper, and transferred into 25 mL of 250 mM mannitol in a 50 mL capped centrifuge tube. After each sample was shaken for 4 h, electrical conductivity was read. Samples were then stored at -20 °C for 24 h. After 24 h the samples were

thawed at room temperature, heated in a boiling water bath for 15 min and, after cooling to room temperature, the final conductivity was taken to determine total conductivity. All leakage data were expressed as a percentage of the total electrolyte conductivity, where initial conductivity was divided by total conductivity, and multiplied by 100.

Experiment 2. The Effects of Exogenous Ethylene on Accumulation of Reactive Oxygen Species

Ethylene treatment

After sorting fruit for size and sanitization with 2.7 mM sodium hypochlorite, intact fruit (n=20 per container) were placed in 20-L plastic containers and provided with flow-through atmospheres of air with or without 10 $\mu L \cdot L^{-1}$ of ethylene at 13 ^{o}C and 95% R.H.

ROS release from mesocarp disks

Total reactive oxygen species (ROS) released from cucumber tissue were determined using the 2',7'-dichlorofluorescin (DCFH) assay of Schopfer et al. (2001) with some modifications. ROS oxidize nonfluorescent DCFH to the highly fluorescent 2',7'-dichlorofluorescein (DCF) and fluorescence increase can be used to determine the amount of ROS release. DCFH-diacetate (10 mM) was dissolved in ethanol and stored as a stock solution at – 20 ^{o}C. Fifty µM DCFH-DA was prepared from stock solution with 20 mM K-phosphate (pH 6.0). Deacetylation of DCFH-DA (50 µM) was performed using 0.1 $g \cdot L^{-1}$ of esterase (EC 3.1.1.1 from porcine liver) at room temperature for 15 min. This solution was used for the assay immediately and discarded each day after use. Mesocarp disks (4.5 mm wide by 10mm thick, three disks per fruit) were prepared from cucumber slices (10 mm thickness) with a cork borer (#2). Three disks were rinsed with distilled water, briefly blotted on Whatman #4 filter paper, and incubated in a 50 mL centrifuge tube containing 10 mL of working solution in the dark. ROS release was

quantitatively determined by measuring relative fluorescence of aliquots in a fluorometer (Versafluor^{TM} fluorometer; Bio-Rad Laboratories, Inc., CA, USA) (Ex: 480 nm, Em: 520 nm). As the fluorescence increases due to H_2O_2 (fluorescence of H_2O_2 solution – fluorescence of working solution only) reached maximum at around 20 min and auto-oxidation of working solution was observed after 10 min (Appendix A), an incubation period of 15 min was used for measuring ROS release in the following experiments. Working solution without tissue was used to zero the instrument and 10 mM H_2O_2 (final concentration) to set the maximum fluorescence as 10,000. A standard curve was prepared with dilutions of H_2O_2 (final concentrations of 0, 10, 100, 1000, and 10000 µM) (Appendix B). Fluorescence was transformed into production of H_2O_2 in µmoles per disk per h using a standard curve. This analysis was conducted with 3 individual fruit stored with/without 10 $\mu L \cdot L^{-1}$ ethylene every other day of treatment. Peroxidase was also shown to cause DCFH oxidation (Keston and Brandt, 1965). However, endogenous peroxidase activity of cucumber fruit tissues was not a rate limiting factor based on the observation that the addition of peroxidase (1000 $U \cdot mL^{-1}$; E.C. 1.11.1.7 from horseradish) to working solution had no significant effect on the fluorescence measurements.

Histochemical staining

Hydrogen peroxide was detected using 3, 3'-diaminobenzidine 4 HCl (DAB) which yields a brown precipitate by reaction with H_2O_2 (Schraudner et al., 1998). One transverse fruit slice (30 mm thick) per fruit was prepared with a sharp knife, and then processed into a wedge-shaped section (approximately 35 ° of a circle). Cross-sections (300 µm thick) of this wedge-shaped piece were prepared using a sliding microtome

DK-10 (Uchida Yoko Co., LTD, Japan). Six slices (2 slices per fruit, 3 fruit per treatment) were immersed in 7 mL of staining solution consisting of 10 mM 2-(N-Morpholino) ethanesulphonic acid, pH 6.5 and 0.1% (w/v) DAB for 45 min under lab light on an orbital shaker, and then destained in boiling 95% ethanol for 5 min.

Accumulation of superoxide anion was measured using nitroblue tetrazolium (NBT) staining as described in Jabs et al. (1996). Cross-sections of cucumber fruit were prepared as for DAB staining. Six slices of cucumber fruit (2 slices per fruit, 3 fruit per treatment) were immersed in 7 mL staining solution consisting of 50 mM potassium phosphate, pH 6·4, 0.1% (w/v) NBT and 10 mM sodium azide (peroxidase inhibitor) for 30 min on the shaker in the dark. Tissue was destained in boiling 95% ethanol for 5 min.

Photographs of brown DAB staining and blue NBT staining were taken with a fluorescence stereomicroscope (Leica MZ 16F; Leica Microsystems Ltd., Switzerland) at two magnifications (1.25X and 8X).

Results

Experiment 1. The Effects of Ethylene Concentration and Exposure Duration on Watersoaking of Cucumber Fruit

The effect of ethylene concentration on watersoaking development is shown in Figure 3-1. Ethylene-induced watersoaking was initiated in the hypodermal tissue (Fig. 3-1 A), progressing into internal mesocarp tissue (Fig. 3-1 B) in immature beit-alpha cucumber fruit. Although fruit were not uniformly watersoaked, there was no significant difference in intensity of watersoaking among ethylene concentrations ranging from 10 to 1000 $\mu L \cdot L^{-1}$. Watersoaking affected about 10~25% and 30~100% of fruit cross-section at 7d and 8 d, respectively.

As ethylene has been reported to accelerate tissue deterioration and diminish shelf-life of horticultural crop, the respiration changes of cucumber fruit depending on ethylene concentration were measured. The initial respiration of beit-alpha cucumber fruit, assessed from CO_2 production, was around 10 μg $CO_2 \cdot kg^{-1} \cdot s^{-1}$ (Fig.3-2). Respiration rate of air-treated fruit changed little during 7 d of storage, and there was no clear peak of CO_2 production. Continuous ethylene treatment enhanced respiration rate, reaching a maximum at 4 d for all ethylene concentrations. There was no significant difference in CO_2 production and the time to reach maximum among different concentrations of ethylene. At 4 d, ethylene-treated fruit produced 2.5 to 2.8 times as much of CO_2 as control fruit regardless of ethylene concentration.

Ethylene production of beit alpha cucumber fruit was undetectable during 8 d of storage even for fruit challenged with exogenous ethylene (data not shown). The gas chromatograph used in these experiments has a detection limit for ethylene of 0.05 $\mu L \cdot L^{-1}$.

As a visible and early response of cucumber fruit to ethylene exposure, surface color change was used to ascertain the effect of ethylene concentration. Initial skin hue value was about 124 ^{o}, and this value was maintained in air-treated fruit (Fig.3-3). Ethylene induced a decrease in hue angle, with the sharpest declines noted after 4 d regardless of ethylene concentration. Ethylene-treated fruit exhibited a significant decrease in hue angle (2~3^{o} lower) compared with air-treated fruit at all measurement intervals after 4 d. Fruit exposed to 1000 $\mu L \cdot L^{-1}$ ethylene showed a steep decrease in hue angle during 5 through 6 d, having 4^{o} or 2^{o} lower hue angle than fruit treated with air or the lower concentrations of ethylene, respectively. On the other hand, 10 $\mu L \cdot L^{-1}$

ethylene induced less decrease in hue angle than the 1000 $\mu L \cdot L^{-1}$ ethylene. Fruit exposed to 10 $\mu L \cdot L^{-1}$ ethylene maintained hue angle values of around 121.5~122 ^{o} during 6 d through 8 d, significantly higher than the value of fruit treated with ethylene at 1000 $\mu L \cdot L^{-1}$.

Fruit firmness is an essential factor determining fruit quality and fruit softening has been reported as a significant response to ethylene exposure. Accordingly, the effect of ethylene concentration on firmness of intact fruit was assessed. Firmness of intact cucumber fruit stored under air increased significantly during storage (Fig.3-4), from an initial value of 19 N to 31 N at 8 d. In ethylene-treated fruit, firmness increased an average of 25% during the first 2 d, and then decreased. Ethylene-induced softening continued progressively after 3 d, declining on average 60% at 8 d for all ethylene concentrations. At 8 d, firmness of ethylene-treated fruit was 7-8 N and fruit were severely watersoaked. Firmness was not significantly influenced by ethylene concentration.

The experiment conducted to evaluate the influence of ethylene concentration on watersoaking development and accompanying symptoms revealed that ethylene concentration in excess of 10 $\mu L \cdot L^{-1}$ induced no further effects on watersoaking, respiration, surface color, and firmness of immature beit-alpha cucumber fruit. This means that the ethylene receptors present in immature cucumber fruit could be saturated at 10 $\mu L \cdot L^{-1}$. Subsequent experiments addressed the influence of ethylene exposure duration, wherein fruit were challenged with ethylene at 10 $\mu L \cdot L^{-1}$. Included in these analyses were the effects of exposure duration on watersoaking intensity, surface

color, mesocarp firmness, and electrolyte leakage. These parameters were examined in response to exposure to ethylene for 12 h, 2 d, 4d or continuously.

Watersoaking assessed at 6 d was not evident in fruit exposed to ethylene for 12 h, 2 d, or 4 d (Fig. 3-5 A). In contrast, continuous ethylene exposure (10 $\mu L \cdot L^{-1}$) induced incipient watersoaking at 6 d, which became evident as a darkening of the hypodermal tissue. As shown in Fig. 3-5 B, fruit treated with ethylene for 4 d followed by transfer to air exhibited incipient watersoaking at 17 d.

Figure 3-6 shows the surface color changes in response to ethylene exposure for different durations. The initial hue angle of air-treated fruit was about 124 ^{o}, and this value was maintained throughout storage (Fig. 3-6). There was no significant difference between fruit exposed to air or to 10 $\mu L \cdot L^{-1}$ ethylene for 12 h or 2 d, followed by air, during 13 d of storage. Fruit having received ethylene exposure for the initial 2 d showed a slight decline in hue angle after 13 d, and by 15 d exhibited a more yellowish appearance compared with fruit treated with air or with ethylene for 12 h. Increasing the duration of ethylene exposure to 4 d induced a significant decline in hue angle that paralleled that of fruit treated with ethylene continuously for 8 d. After 8 d, fruit treated with continuous ethylene were severely watersoaked and showed visible signs of surface fungal proliferation on fruit surface. While hue angle decline continued upon cessation of ethylene treatment at 4 d, watersoaking was not evident until 17 d. The initial hue angle values for the experiments described in Fig. 3-3 and Fig. 3-6 were similar, averaging about 124 ^{o}. However, the hue angle decline in fruit treated with ethylene for 4 d followed by air reached considerably lower values (Fig. 3-6, about 112 ^{o}) than did fruit treated with ethylene continuously (see Fig. 3-3, 3-6) although the hue

angle values for the fruit exposed to ethylene continuously represent values for only 8 d (due to acute watersoaking and fruit deterioration) versus up to 20 d for fruit receiving ethylene for only 4 d.

The increase in firmness of control fruit throughout storage and fruit of other treatments during the early period of storage (Fig. 3-4) suggested that epidermal tissues strongly influence whole fruit firmness. In the present experiment, firmness measurements were performed directly on mesocarp tissue of fruit cross sections in an effort to evaluate the influence of ethylene-exposure duration. Mesocarp firmness of beit-alpha cucumber fruit stored in air was initially around 8 N, increased to 10 N at 2 d, and then remained unchanged for the remaining period of storage for 20 d (Fig. 3-7). Firmness value of fruit exposed to 10 $\mu L \cdot L^{-1}$ ethylene for 12 h or 2 d were not statistically different from those of air-treated fruit. Cucumber fruit exposed to ethylene continuously exhibited a 38% decrease in mesocarp firmness at 6 d and 75% after 10 d compared with air-treated fruit. As was noted for hue angle values, a 4-d exposure to ethylene initiated declines in mesocarp firmness that were only partially interrupted following transfer to air, with values declining 16% and 38% at 6 d and 10 d, respectively, compared with air-treated fruit.

Since degradation of cell membranes has been reported to contribute to watersoaking development, electrolyte leakage of beit-alpha cucumber fruit was measured as an indicator of cellular membrane integrity (Fan and Sokorai, 2005) in response to ethylene exposure for different durations. Electrolyte leakage of immature cucumber fruit was initially about 8% (Fig. 3-8). Fruit stored under air or treated with ethylene for 12 h or 2 d had no difference in electrolyte leakage, showing a gradual

increase up to 20% during 10 d of storage with no change thereafter. Electrolyte leakage was significantly enhanced in fruit exposed to ethylene for 4 d or continuously, increasing to 20% and 45%, respectively, at 6 d. By 10 d, leakage increased to 52% and 95% in fruit exposed to ethylene for 4 d or continuously, respectively. Unlike the patterns of hue angle decline and mesocarp firmness of fruit treated with ethylene for 4 d, wherein ethylene-induced declines continued for the entire storage duration, electrolyte leakage stabilized at around 10 d.

Experiment 2. The Effects of Continuous Ethylene Exposure on Accumulation of Reactive Oxygen Species

Since reactive oxygen species (ROS) could play an important role in regulating watersoaking, 2', 7'-dichlorofluorescin (DCFH) assay was used to assess total ROS-generating capacity of mesocarp tissue from cucumber fruit stored under air or continuous ethylene (10 $\mu L \cdot L^{-1}$). The assay involves measuring the increase of dichlorofluorescein (DCF) fluorescence after oxidation of 2', 7'-dichlorofluorescin (DCFH). Initial ROS generation of air-treated fruit averaged to about 0.6 µmol of H_2O_2 equivalents per mesocarp disk per h, increasing to 3.9 µmol of H_2O_2 equivalents at 8 d (Fig. 3-9). ROS production was significantly enhanced in fruit challenged with ethylene, increasing nearly 23- and 11-fold at 4 and 6 d of treatment, respectively, compared with fruit stored in air. Peak ROS production at 6 d amounted to 9.8 µmol of H_2O_2 equivalents per mesocarp disk per h, declining to undetectable levels at 10 d. The decline in ROS production coincided with the onset of severe tissue watersoaking and enhanced electrolyte leakage, reflecting the general occurrence of tissue death.

Histochemical staining (DAB and NBT staining) was applied to determine the gross localization of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot -}$) in cross

sections of cucumber fruit stored in air or continuous 10 $\mu L \cdot L^{-1}$ ethylene. The sites and pattern of H_2O_2 and $O_2^{\cdot -}$ accumulation were different (Fig. 3-10 and Fig. 3-11). H_2O_2 accumulation, visualized as a brown precipitate formation, increased in response to continuous ethylene treatment, especially in hypodermal tissue. In contrast, ethylene-treated fruit had no visible $O_2^{\cdot -}$ accumulation in exocarp tissue while air-treated fruit showed increased blue NBT staining.

Discussion

The effects of exogenous ethylene on beit-alpha cucumber fruit included tissue (mesocarp) softening, skin degreening, increased respiration and electrolyte leakage, and watersoaking development. These observations parallel reports by Hurr et al. (2009) and Lima et al. (2005) for ethylene treatment of immature beit alpha cucumber fruit. Ethylene-treated cucumber fruit exhibited spatial patterns of watersoaking development, initiating in hypodermal tissue (outer layer of mesocarp) and then progressing into inner mesocarp tissue. This pattern could be explained as tissue-specific pattern. Even among members of the Cucurbitaceae, patterns of watersoaking development differ. Watermelon fruit exhibited watersoaking disorder in placental and mesocarp tissue concurrently, with minimal effects on surface tissues, as a response to continuous ethylene exposure (Karakurt and Huber, 2002). Watersoaking in cantaloupe fruit is an ethylene-independent phenomenon, which initiated in innermost mesocarp tissue and then progressed into peripheral tissues (du Chatenet et al., 2000). These observations indicate that the pattern of watersoaking disorder is a commodity-dependent and tissue-specific phenomenon. On the other hand, spatial patterns of watersoaking development in cucumber fruit could be questioned as results of different gas diffusion rate among different tissue types. Outer tissues have potentially higher

concentrations of applied gasses because of the presence of gas-diffusion barriers including epidermis, cuticle, and stoma (Burg, 2004; Laurin et al., 2006). This might explain why the incipient watersoaking symptoms are first evident in hypodermal tissues in response to exogenous ethylene.

Watersoaking disorder, shown in Fig. 3-1, was not uniformly developed, which could result from fruit maturity differences. The responses of detached cucumber fruit to exogenous ethylene are developmentally dependent (Hurr et al., 2009). In the present study, immature fruit were selected based on fruit size and surface color, whereas in Hurr et al. (2010) fruit were selected based on days post-anthesis (DPA). This suggests that DPA rather than fruit size and color is a more adequate criterion for ensuring uniform watersoaking in response to ethylene.

The present study was designed to investigate ethylene responses of cucumber fruit as influenced by ethylene concentration. Postharvest ethylene exposure has been reported to induce quality losses in nonclimacteric fruit and vegetable crops in a dose-dependent manner (Wills et al., 1999 Tian et al., 2000). In immature cucumber fruit, ethylene concentrations exceeding 10 $\mu L \cdot L^{-1}$ had no further influence on intensity of watersoaking, respiration increase and fruit softening, indicating that 10 $\mu L \cdot L^{-1}$ ethylene was saturating with respect to ethylene responses of cucumber fruit. Ethylene saturation has been reported in other horticultural crops. Ethylene concentrations exceeding 40 $\mu L \cdot L^{-1}$ did not induce further enhancement in color development (a/b ratio) of strawberry fruit (Tian et al., 2000). Cherry (‘Brooks’) and apricot (‘Patterson’ and ‘Castle-brite’) showed declines in skin color and firmness, respectively, in response to ethylene but

was not influenced by ethylene concentration (0.01 to 1 $\mu L \cdot L^{-1}$for cherry; 1 to 100 $\mu L \cdot L^{-1}$ for apricot) (Palou et al., 2003).

While ethylene concentrations exceeding 10 $\mu L \cdot L^{-1}$ had no further influence on the physiology of cucumber fruit, fruit responded differently depending on duration of ethylene exposure. Short term exposure (12 h) to 10 $\mu L \cdot L^{-1}$ ethylene showed no significant detrimental effect on quality of cucumber fruit during 20 d of storage. Fruit receiving ethylene for only 2 d exhibited significant decline of hue angle at 15 d, but no observable influence on watersoaking, electrolyte leakage and mesocarp firmness. Ethylene exposure for 4 d induced watersoaking, electrolyte leakage increase and firmness decline much more slowly compared to continuous exposure. The exposure-time threshold for skin degreening was 2 d, but 4d for watersoaking, electrolyte leakage, and softening. Lag time between ethylene application and the initiation of the ethylene response might be required for altering physiological mechanisms and gene expressions to induce the ethylene response. The different exposure-time thresholds for ethylene responses were observed in the present experiment, which has been reported in other horticultural commodities. A requirement for continuous ethylene was reported in pea seedlings (Warner and Leopold, 1971) and radish roots (Jackson, 1983), where the decline in growth rate mediated by ethylene returned to control rate upon removal from ethylene. Vase life of ‘Cordelia’ and ‘Prato’ lily was also significantly reduced by 48 h of ethylene exposure (10 and 100 $\mu L \cdot L^{-1}$, respectively), but not by 24 h of exposure (Elgar et al., 1999). The efficiency of 1-MCP (an ethylene antagonist) has also been reported to depend on exposure duration of 1-MCP or pre-treated ethylene. Extended shelf-life in response to 1-MCP treatment (300 $nL \cdot L^{-1}$ for 24 h) was observed in banana

fruit receiving 100 $\mu L \cdot L^{-1}$ ethylene pre-treatment for 30 or 40 h, but not fruit exposed to ethylene for 50 h (Moradinezhad et al., 2006). Grapefruit treated with 1-MCP (300 $nL \cdot L^{-1}$) for 24, 48, or 72 h exhibited 20-, 160-, or 1000-fold increase in ethylene production, respectively, compared to non treated fruit (McCollum and Maul, 2007).

Duration of exposure to ethylene had differential effects on watersoaking, surface color, respiration rate, electrolyte leakage, and fruit firmness. Hue angle of fruit treated with ethylene for 4 d or continuously reached around 119 ^{o} at 8 d simultaneously. In contrast, fruit receiving ethylene for only 4 d exhibited watersoaking, enhanced electrolyte leakage and firmness decline with much greater delay compared with continuously exposed fruit. Unlike the patterns of hue angle decline and mesocarp firmness of fruit receiving ethylene for only 4 d, wherein ethylene-induced declines continued for the entire storage duration, electrolyte leakage stabilized at around 10 d. These results might be explained by different ethylene thresholds for the different ethylene responses of immature cucumber fruit. Tian et al. (2000) have mentioned that ethylene responses in strawberry fruit have different ethylene threshold levels based on no further enhancement in color development (a/b ratio) and softening after exogenous ethylene concentration exceeded 40 and 0.5 $\mu L \cdot L^{-1}$, respectively. In grapefruit, 1-MCP treatment at concentrations over 75 $nL \cdot L^{-1}$ exhibited no further inhibition in ethylene-induced degreening while ethylene production was increased by 1-MCP treatment in a dose- and time-dependent manner over the range of 0 to 300 $nL \cdot L^{-1}$ (McCollum and Maul, 2007). Different ethylene responses of potato tuber also have various saturation concentrations (Daniels-Lake et al., 2005). Ethylene responses such as delay in sprouting, breaking of apical dominance, and increased formation of small sprouts were

saturated between 0.4 and 4 $\mu L \cdot L^{-1}$ ethylene, whereas inhibition of sprout elongation was not saturated until ethylene concentrations exceeded 40 $\mu L \cdot L^{-1}$. Cucumber fruit seem to have lower exposure-time threshold for degreening than for other ethylene responses. That is why ethylene exposure for 4 d is enough to induce degreening to the same extent as was caused by continuous exposure, but was much less effective in inducing watersoaking.

Fruit treated with ethylene for only 4 d exhibited incipient watersoaking at 17 d, when its hue angle reached considerably lower values (about 116 ^{o}) than that of fruit treated with ethylene continuously. Fruit exposed to ethylene continuously had hue angle of about 121.5 ^{o} when incipient watersoaking was observed (6 d). These data indicate-that the influence of ethylene on surface color change and watersoaking are not tightly linked. In addition, the effect of ethylene exposure on fruit surface color was more evident in fruit receiving ethylene for only 4 d compared to continuous exposure since the postharvest longevity of the former was greatly extended relative to fruit receiving continuous ethylene exposure. The darkening of the fruit surface accompanying watersoaking, which is not accompanied by chlorophyll increases (Hurr et al., 2009), might mask yellowing of the fruit surface evident in fruit receiving short-term ethylene exposure.

Plants produce reactive oxygen species (ROS) under a variety of biotic and abiotic stresses (Bolwell et al., 2002). Continuous ethylene exposure (10 $\mu L \cdot L^{-1}$) induced increases in total ROS-generating capacity of cucumber fruit after 2 d. This enhanced ROS-generating capacity preceded the decline of firmness and hue angle, and increased electrolyte leakage, and well in advance of incipient watersoaking, suggesting

that ROS accumulation plays an important role in watersoaking development. ROS can control ethylene signaling directly or indirectly (Overmyer et al., 2003). Also, the production of ROS during ethylene exposure can induce programmed cell death (PCD) in cucumber, or vice versa. Hurr et al. (2010) reported that cucumber fruit exposed to 10 $\mu L \cdot L^{-1}$ ethylene exhibited hallmarks of PCD including increased nuclease activities and visible DNA laddering at 3~4 d. Enhanced levels of ROS could potentially directly contribute to watersoaking through membrane lipid peroxidation and subsequent loss of membrane integrity (Dhindsa et al., 1981; Fridovich, 1986; Moran et al., 1994). Increased electrolyte leakage, an indicator of cellular membrane integrity, was observed in ethylene-treated fruit after 4 d in present study. Up-regulation of transcript abundance for ascorbate oxidase, allen oxide synthase, and hydroxyperoxide lyase in ethylene-treated watermelon indicated a possible relationship between ROS and watersoaking development (Karakurt and Huber, 2004).

Total ROS-generating capacity data can also explain the effect of ethylene exposure-time. Ethylene-treated fruit exhibited increases in total ROS-generating capacity at 4 d but not at 2 d, which was consistent with the lack of watersoaking development in cucumber fruit treated with ethylene for 2 d. ROS-generating capacity of continuously ethylene-treated fruit peaked at 6 d, which can explain why the watersoaking was significantly delayed in fruit treated with ethylene for only 4 d. Ethylene-enhanced ROS could alter gene expression related to the induction and development of watersoaking disorder. ROS affects gene expressions through modifying signaling transduction pathways (Apel and Hirt, 2004; Circu et al., 2009) and the activity of transcription factors (Mittler et al., 2004). To verify the significant role of

ROS in watersoaking, however, antioxidant systems should be studied. Oxidative damage could be inhibited by scavenging of ROS (Alscher and Hess, 1993; Apel and Hirt, 2004). ROS could act as signals to activate antioxidant systems (Mittler, 2002). The response of antioxidative systems to oxidative stress depends on commodities and/or cultivars, indicating the complexity of antioxidant systems to oxidative stress (Hodges et al., 2004).

The spatial accumulation of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot -}$) were studied to investigate the role of specific ROS in development of watersoaking. Both $O_2^{\cdot -}$ and H_2O_2 are key components of ROS signaling and the most commonly studied ROS (Overmyer et al., 2003). ROS-specific dyes (DAB for H_2O_2 and NBT for $O_2^{\cdot -}$) were used to identify sites of each ROS accumulation in cucumber fruit tissues. Spatial and quantitative correlation between hydrogen peroxide (H_2O_2) and watersoaking development was observed in cucumber fruit. Brown precipitate from DAB increased during 8 d of storage in ethylene-treated cucumber fruit, especially in the hypodermal area. However, ethylene exposure did not induce detectable accumulation of superoxide anion ($O_2^{\cdot -}$) in hypodermal tissue while there was strong blue deposition in exocarp tissue of air-treated fruit at 8 d. In beit-alpha cucumber fruit, H_2O_2 accumulation seems to contribute to watersoaking development upon ethylene exposure. These observations support the idea that different ROS species, depending on commodities, might be associated with development of certain responses under biotic and/or abiotic stresses. Ozone exposure was able to stimulate accumulation of both $O_2^{\cdot -}$ and H_2O_2 followed by lesion formation in Arabidopsis but only H_2O_2 accumulation in tobacco and tomato leaves (Wohlgemuth et al., 2002). A significant increase in total ROS level,

however, was detected after 2 d while significantly enhanced deposition of H_2O_2 was observed after 4 d. This inconsistency could reflect the different sensitivities between DCFH assay for total ROS-generating capacity and histochemical staining method for H_2O_2. On the other hand, this result seems to indicate the possible involvement of other ROS in watersoaking disorder of beit-alpha cucumber fruit. Therefore, further study of other ROS species such as hydroxyl($HO^{\cdot}$), peroxyl ($RO_2^{\cdot}$), and alkoxyl ($RO^{\cdot}$) radicals (Trobacher, 2009) will be valuable to elucidate the role of specific ROS in development of watersoaking.

Overall, the present study showed that ethylene concentrations exceeding 10 $\mu L \cdot L^{-1}$ had no further influence on the physiology of immature cucumber fruit. By contrast, ethylene exposure duration differently affect watersoaking development and accompanying symptoms including yellowing, softening, and electrolyte leakage. At least 4 d of ethylene exposure (10 $\mu L \cdot L^{-1}$) were required to induce watersoaking disorder in beit-alpha cucumber fruit. Enhanced total ROS-generation is closely associated with watersoaking in ethylene-treated cucumber fruit. Histochemical staining revealed that watersoaking appears to be closely associated with H_2O_2 but not with $O_2^{\cdot -}$.

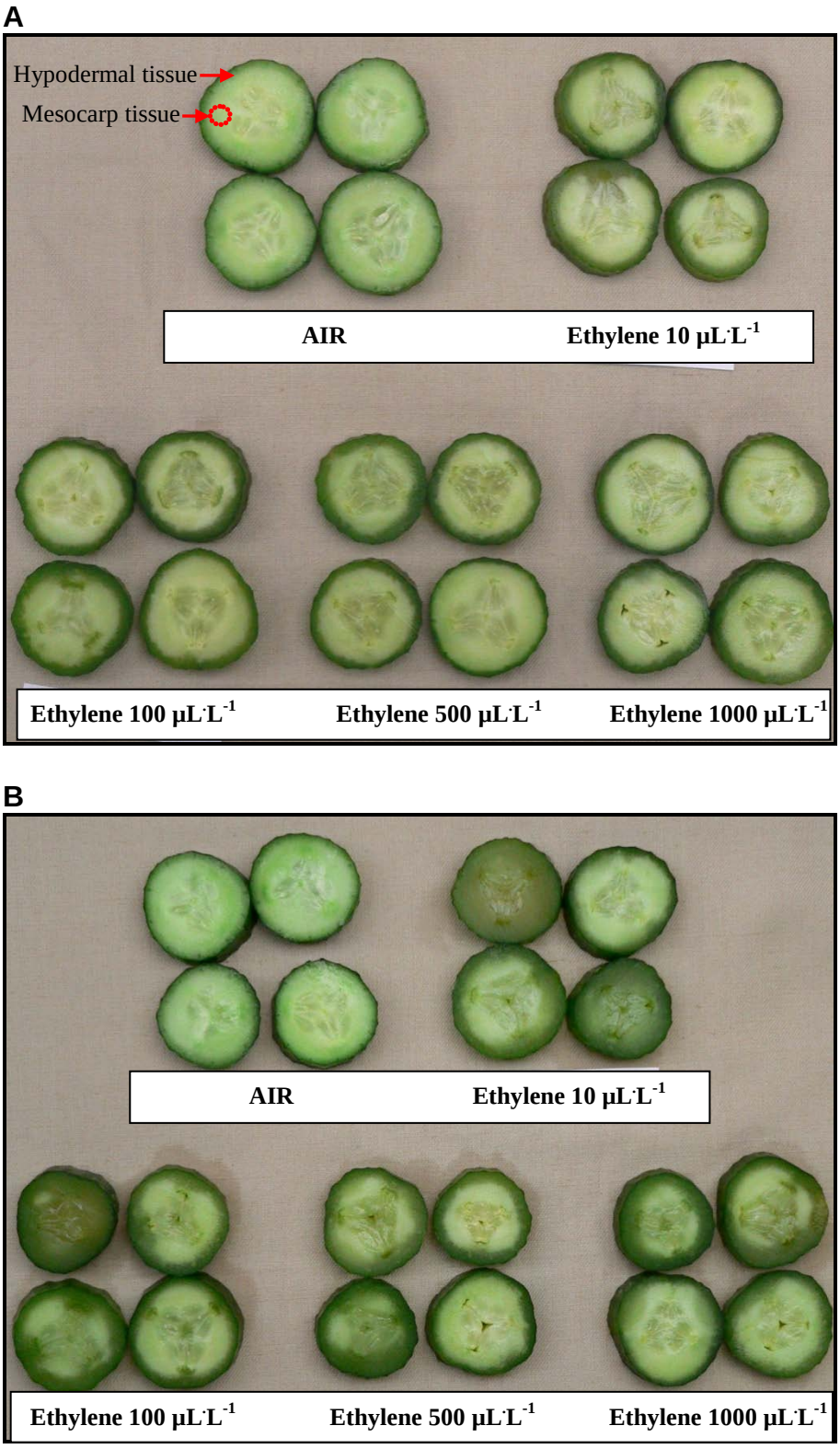


Figure 3-1. Watersoaking development of beit-alpha cucumber fruit treated with air or ethylene (10, 100, 500, or 1000 $\mu L \cdot L^{-1}$) at 13 ^{o}C. A) At 7 d. B) At 8 d.

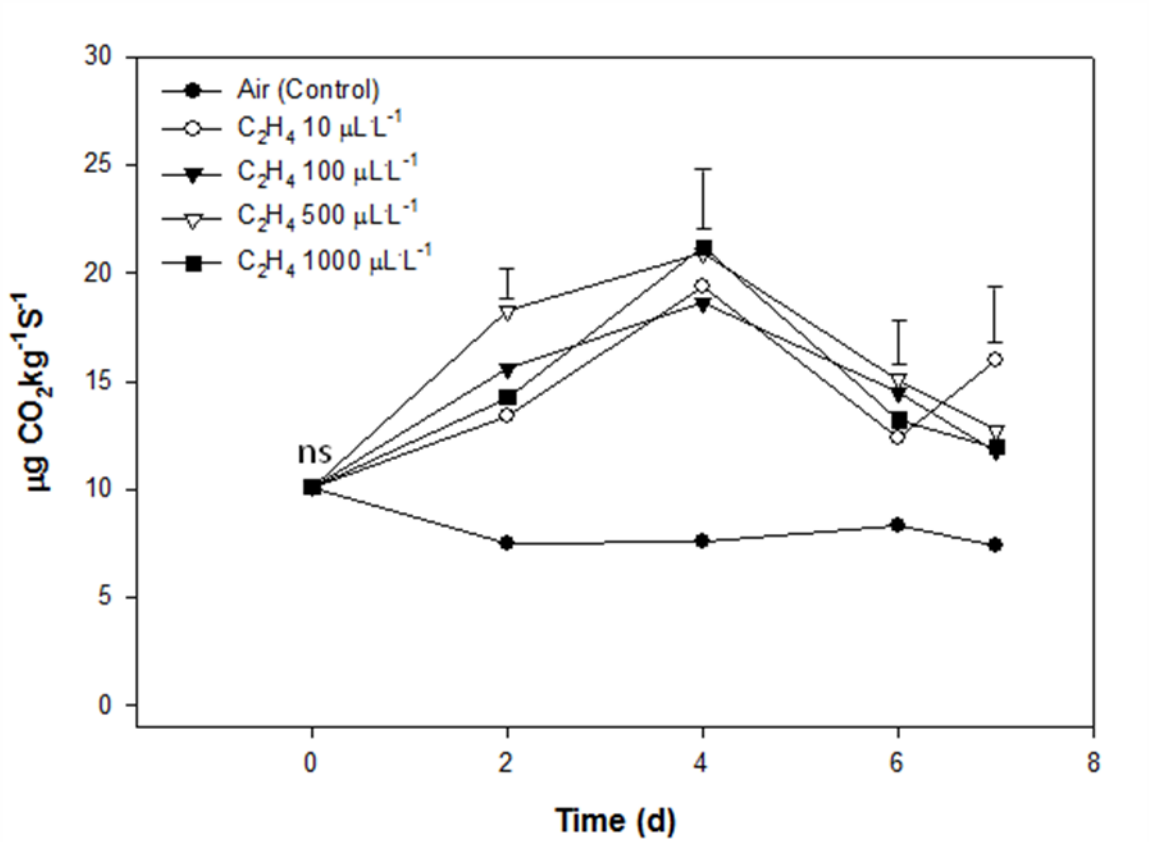


Figure 3-2. Respiration rate of beit-alpha cucumber fruit during storage at 13 ^{o}C under air or ethylene at 10, 100, 500, or 1000 µL·L^{-1}. Each point represents the mean of 6 fruit. Vertical bars represent LSD (α= 0.05) per day.

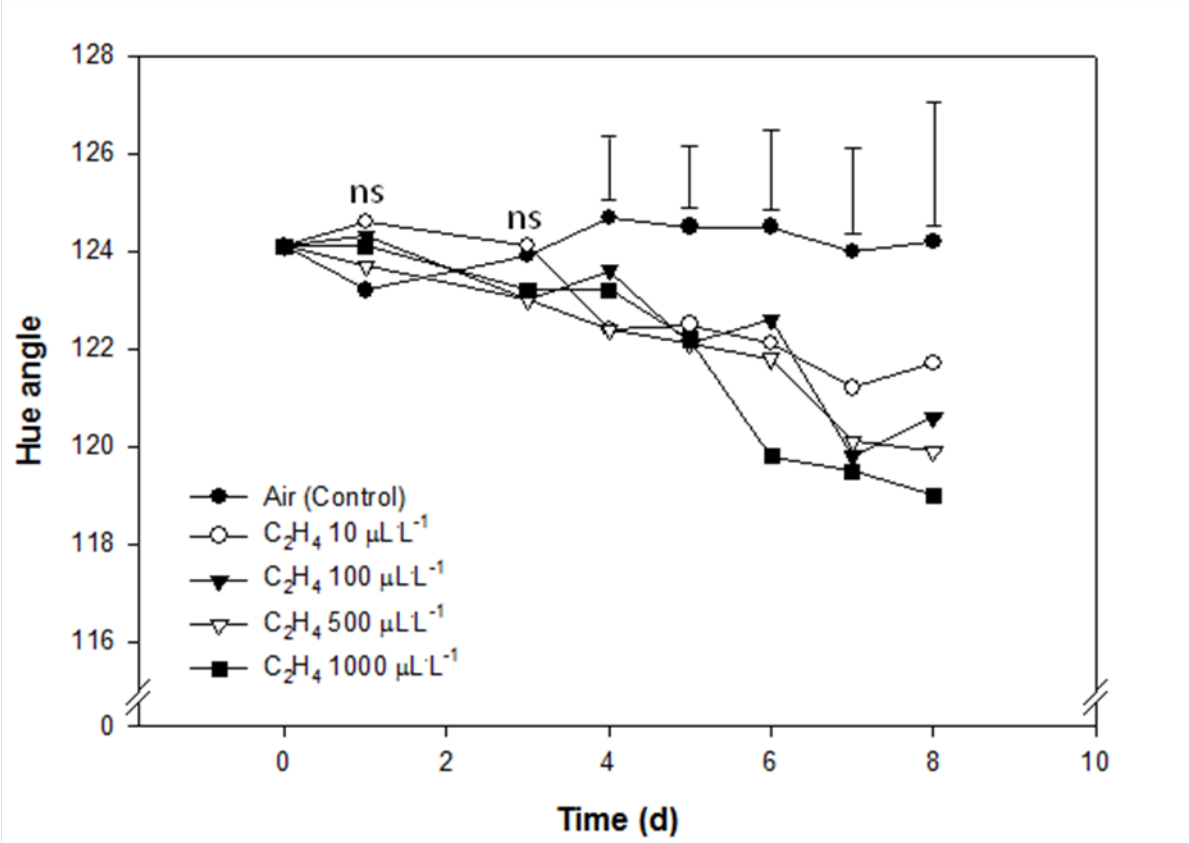


Figure 3-3. Surface hue angle of beit-alpha cucumber fruit during storage at 13 ^{o}C under air or ethylene at 10, 100, 500, or 1000 µL·L^{-1}. Each point represents the mean of 10 measurements (5 fruit, 2 measurements per fruit). Vertical bars represent LSD (α= 0.05) per day.

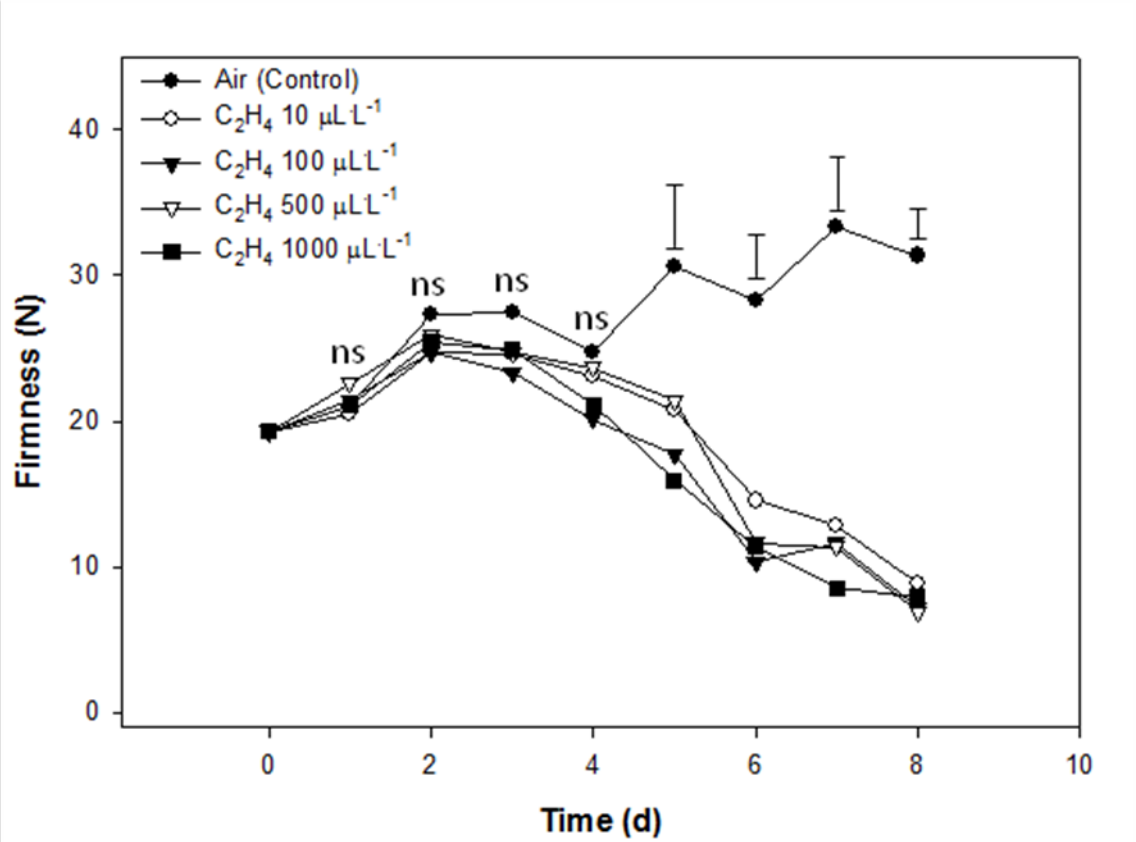
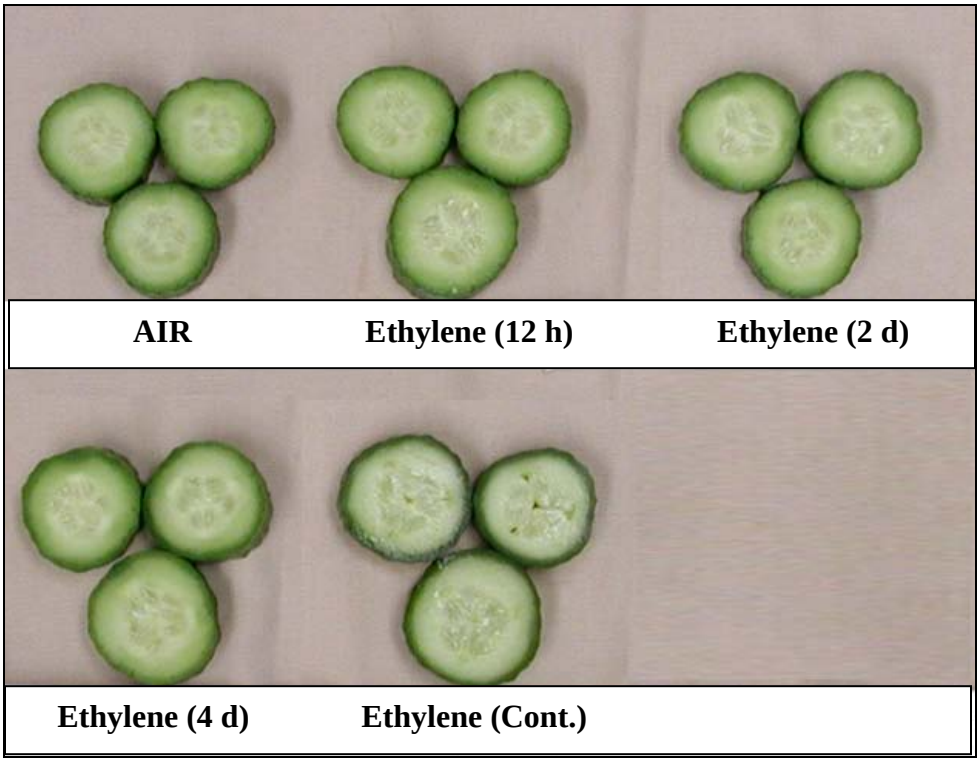


Figure 3-4. Intact fruit firmness of beit-alpha cucumber fruit during storage at 13 ^{o}C under air or ethylene at 10, 100, 500, or 1000 µL·L^{-1}. Each point indicates the mean of 10 measurements (5 fruit, 2 measurements per fruit). Vertical bars represent LSD (α= 0.05) per day.

A



B

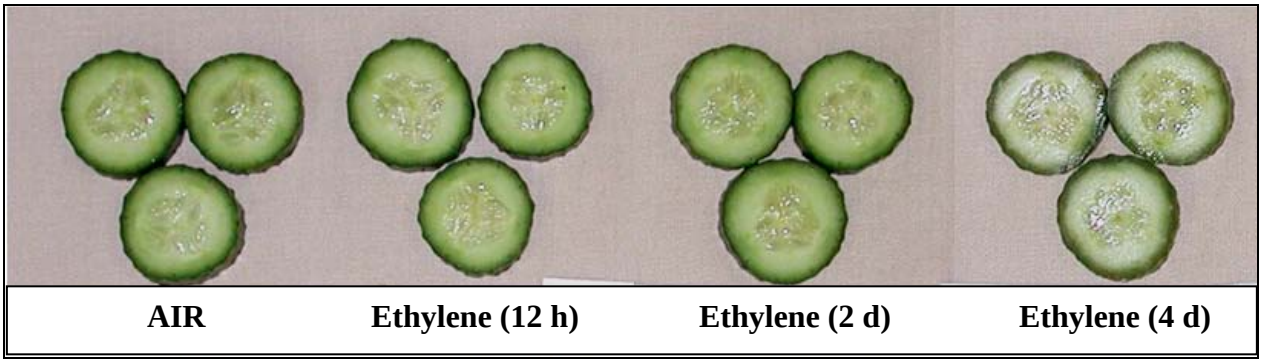


Figure 3-5. Watersoaking of beit-alpha cucumber fruit treated with air ± 10 $\mu L \cdot L^{-1}$ ethylene continuously, or stored in air after ethylene exposure for 12 h, 2 d, or 4 d. A) At 6 d. B) At 17 d.

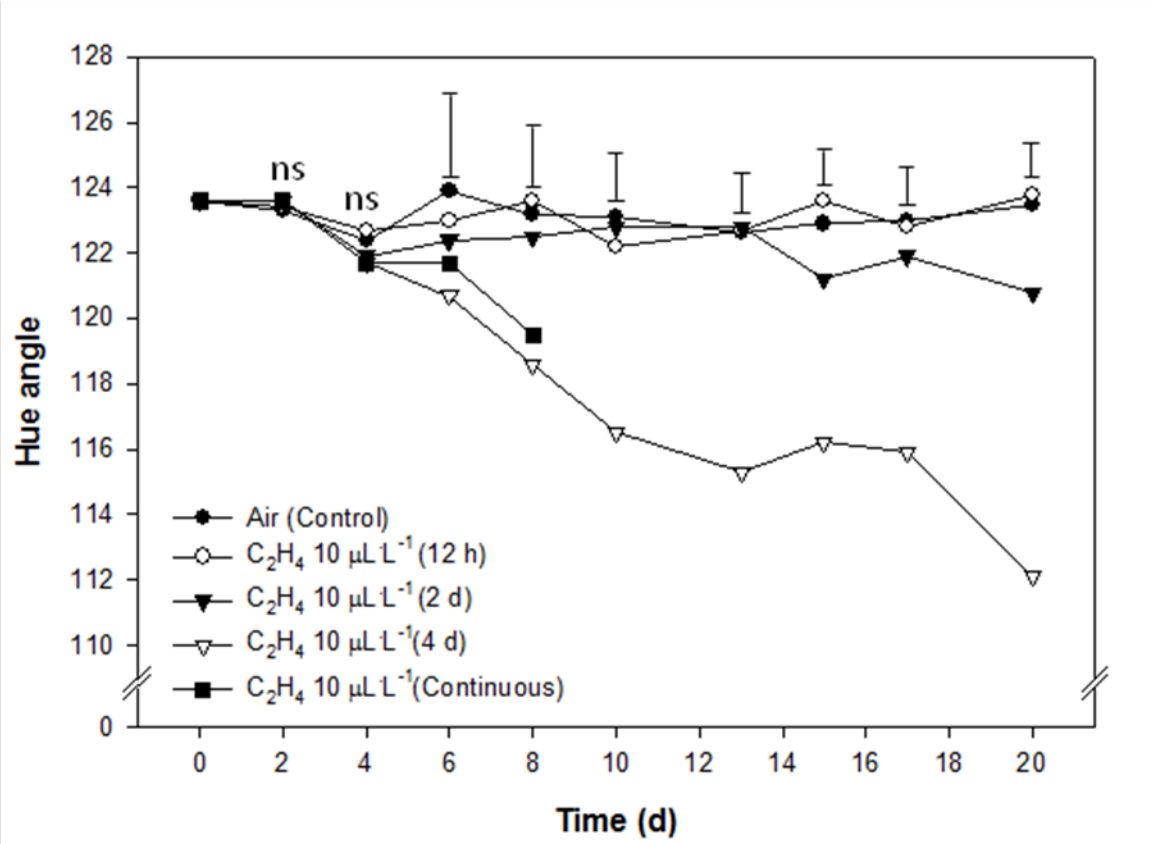


Figure 3-6. Surface hue angle of beit-alpha cucumber fruit stored in air or 10 $\mu L \cdot L^{-1}$ ethylene continuously, or stored in air after ethylene exposure for 12 h, 2 d, or 4 d. Each point indicates the mean of 10 measurements (5 fruit, 2 measurements per fruit). Vertical bars represent LSD (α= 0.05) per day.

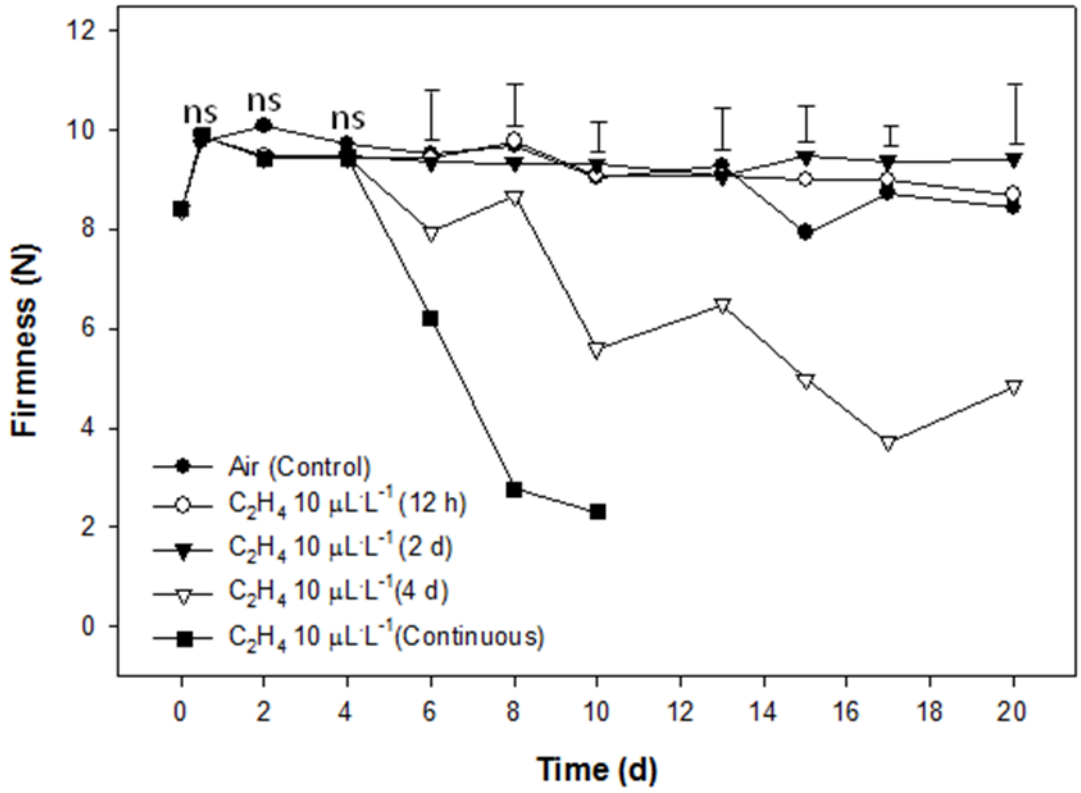


Figure 3-7. Mesocarp firmness of beit-alpha cucumber fruit stored in air or 10 $\mu L \cdot L^{-1}$ ethylene continuously, or stored in air after ethylene exposure for 12 h, 2 d, or 4 d. Each point indicates the mean of 10 measurements (5 fruit, 2 measurements per fruit). Vertical bars represent LSD (α= 0.05) per day.

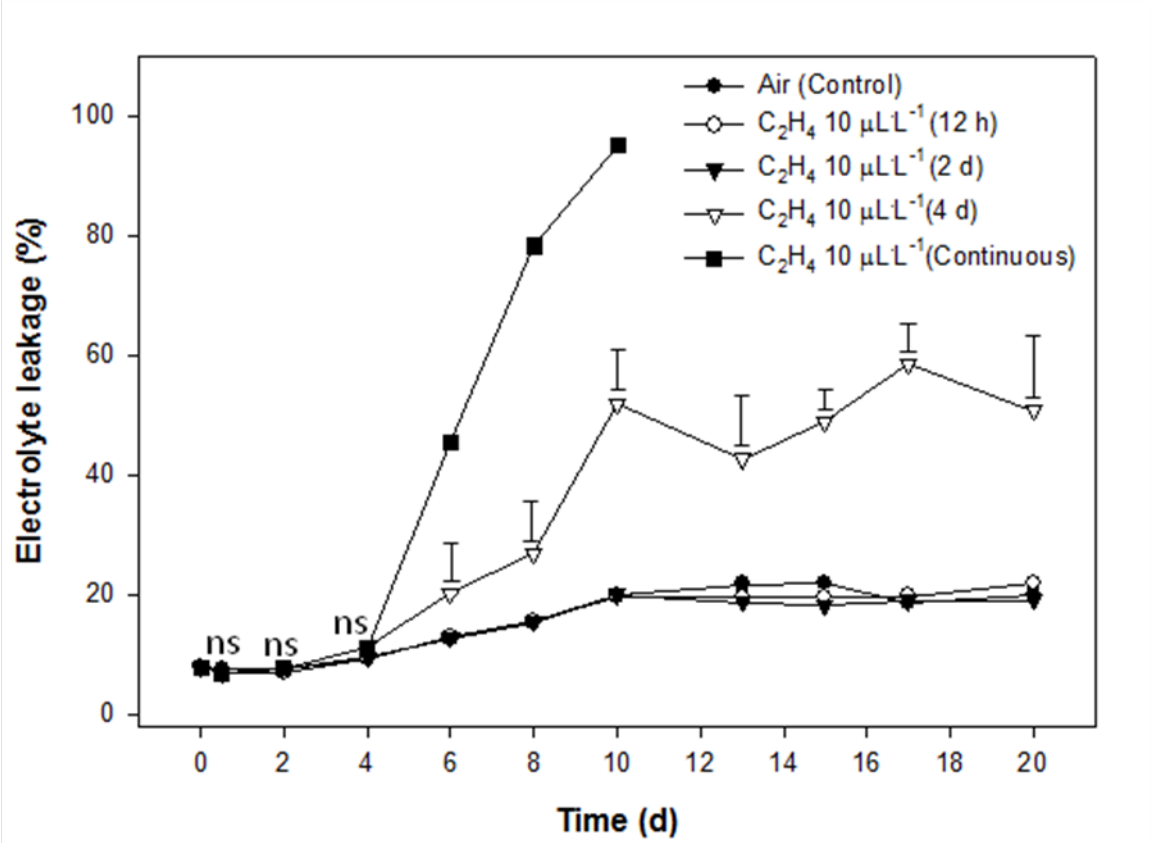


Figure 3-8. Electrolyte leakage of mesocarp tissues of beit-alpha cucumber fruit stored in air or 10 $\mu L \cdot L^{-1}$ ethylene continuously, or stored in air after ethylene exposure for 12 h, 2 d, or 4 d. Each point indicates the mean of 5 fruit. Vertical bars represent LSD (α= 0.05) per day.

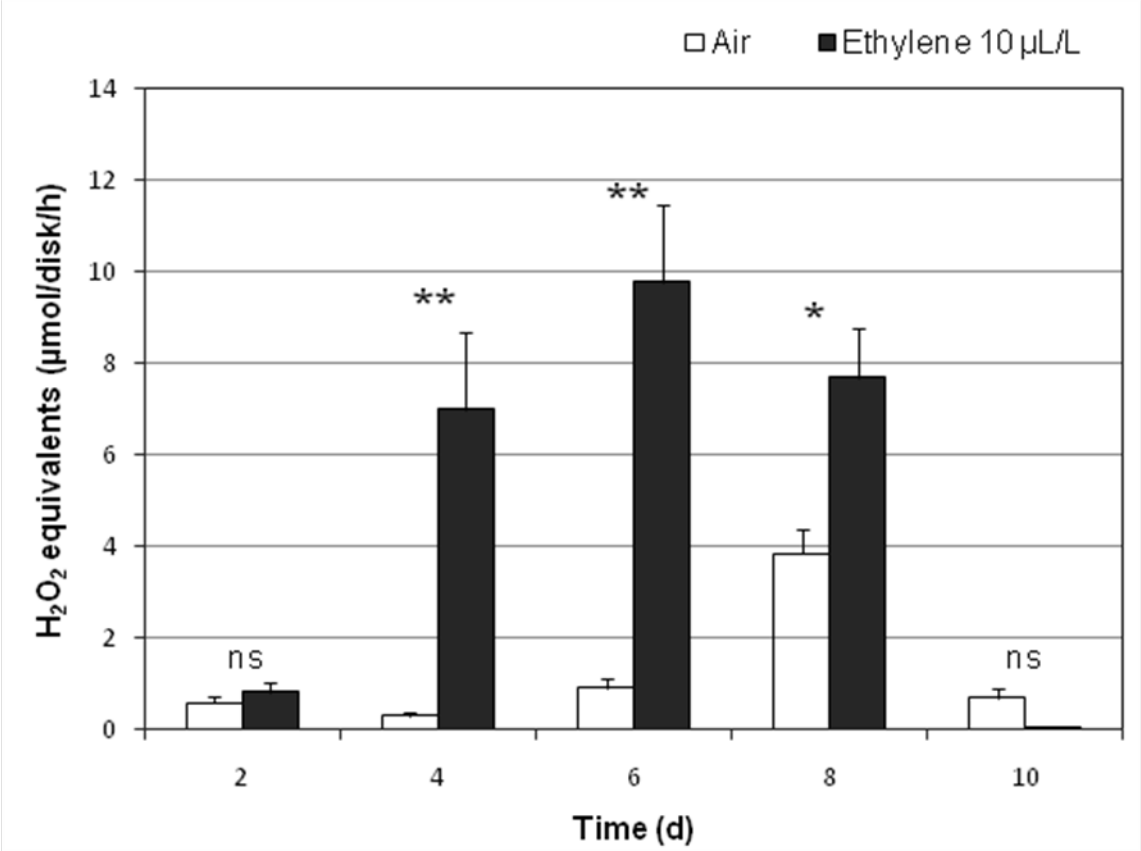


Figure 3-9. Total reactive oxygen species (ROS)-generating capacity of beit-alpha cucumber fruit stored at 13 ^{o} under air or continuous 10 $\mu L \cdot L^{-1}$ ethylene. The production of total ROS was demonstrated using the oxidation of DCFH to DCF. Relative fluorescence at 520 nm was transformed into the production of H_2O_2 in µmoles per disk per h using a standard curve. Each bar represents the mean of three fruit ± SE. ns = non-significant; *significant at $P<0.05$; **significant at $P<0.01$, according to analysis of variance.

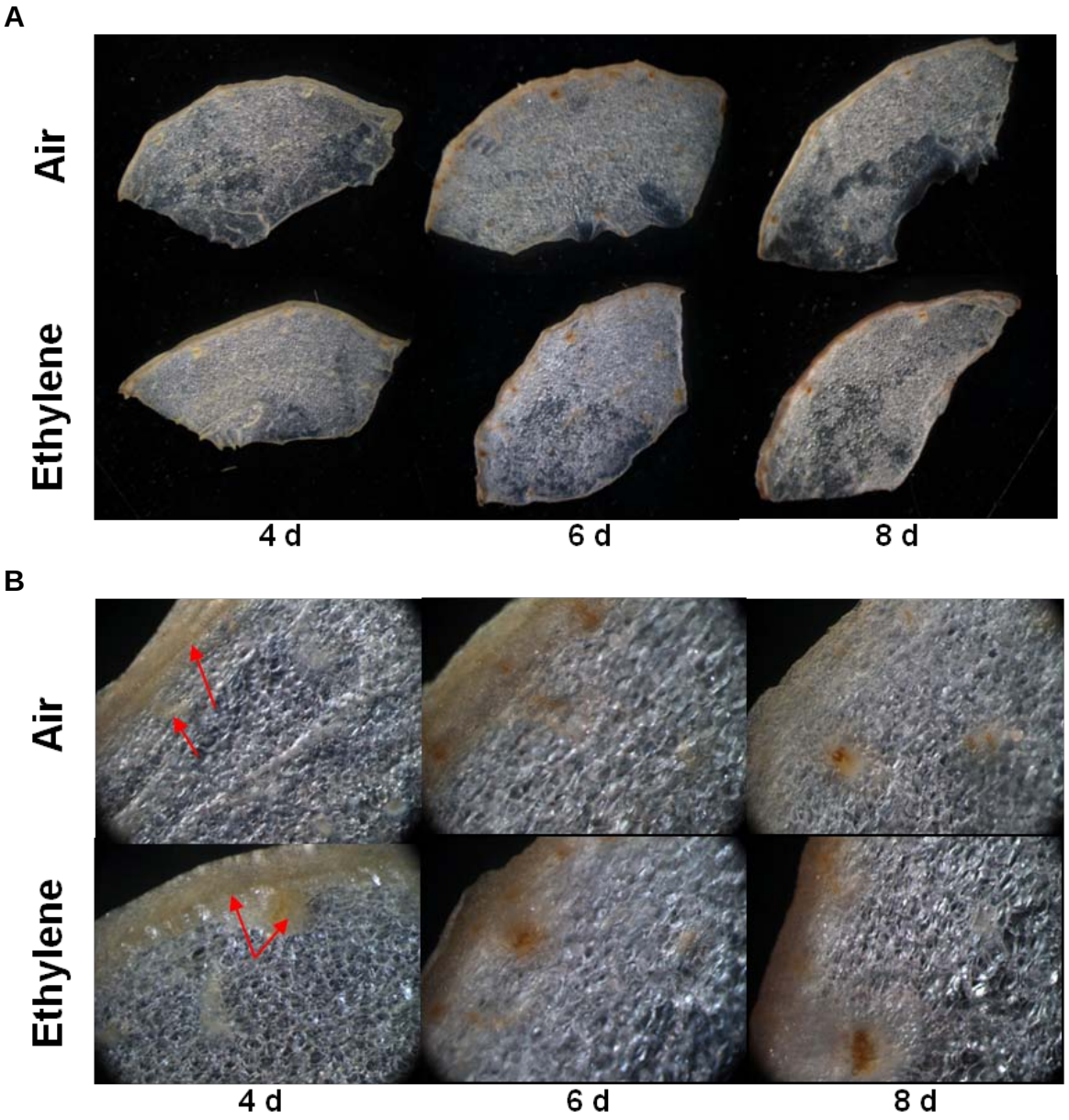


Figure 3-10. Localization of H_2O_2 in cross-section of cucumber fruit stored with/without ethylene (10 $\mu L \cdot L^{-1}$). At examining day, cross-sections (300 μm) were stained with DAB for 45 min. Photograph of brown DAB staining (red arrows) was taken with microscope at two different magnifications: A) 1.25X. B) 8X. Each photograph is one of four biological replications.

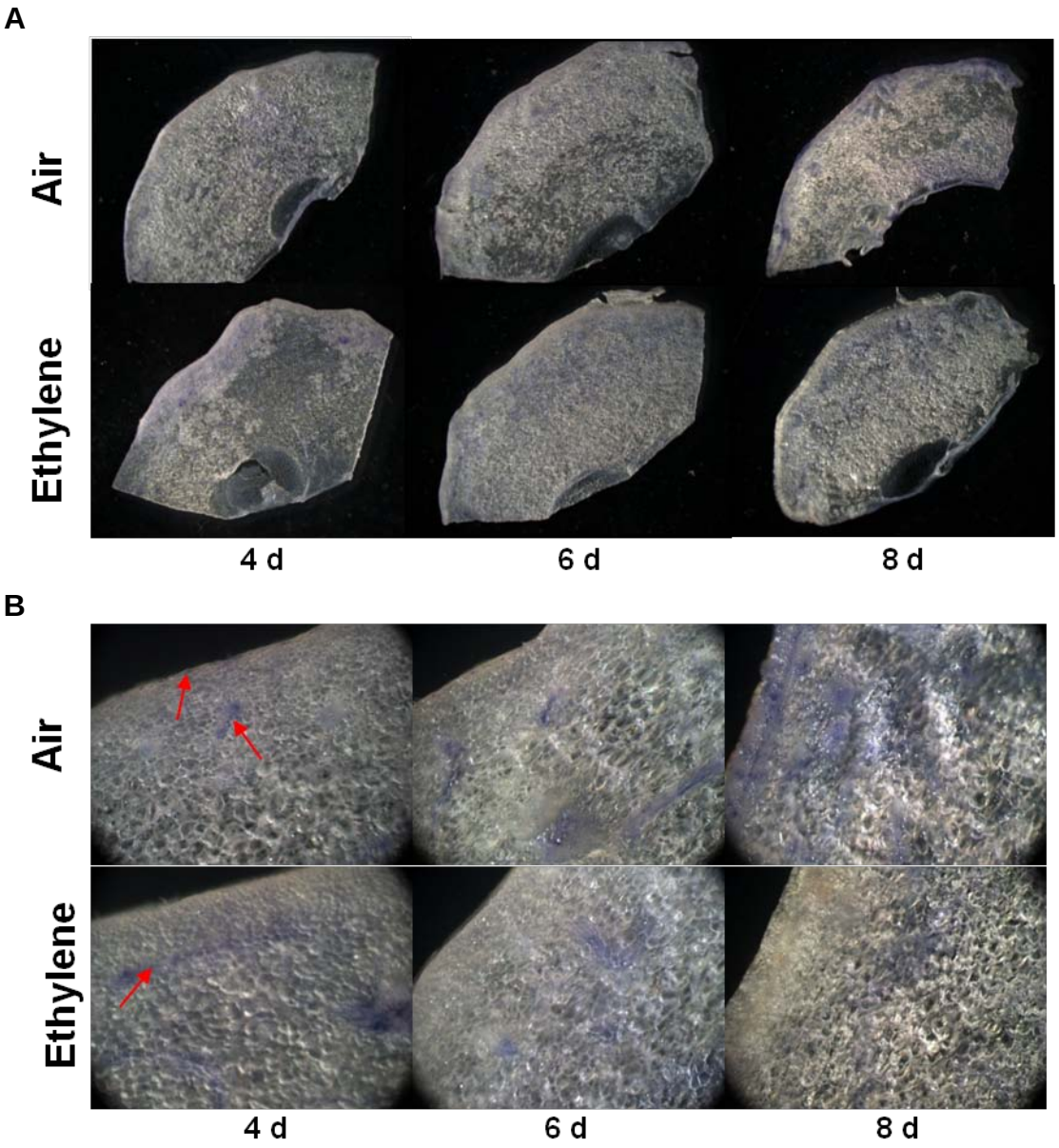


Figure 3-11. Localization of $O_2^{\cdot -}$ in cross-section of cucumber fruit stored with/without ethylene (10 $\mu L \cdot L^{-1}$). At examining day, cross-sections (300 μm) were stained with NBT for 30 min in dark. Photograph of blue NBT staining (red arrows) was taken with microscope at two different magnifications: A) 1.25X. B) 8X. Each photograph is one of four biological replications.

CHAPTER 4
DIFFERENT ETHYLENE REPONSES OF INTACT AND FRESH-CUT SLICES OF IMMATURE CUCUMBER FRUIT

Introduction

Cucumber fruit are commercially harvested prior to developmental maturation. This immature fruit produces very low levels of ethylene, but is sensitive to exogenous ethylene concentrations as low as 1 $\mu L \cdot L^{-1}$ (Villalta and Sargent, 2004). In response to exogenous ethylene, cucumber fruit exhibit increased respiration, electrolyte leakage, microbial growth, enhanced epidermal degreening, and fruit softening (Lima et al., 2005; Hurr et al., 2009; Chapter 3). Watersoaking is another characteristic ethylene response observed in immature cucumber fruit (Lima et al., 2005; Hurr et al., 2009; Chapter 3), a physiological disorder observed in other members of the Cucurbitaceae including watermelon (Karakurt and Huber, 2002; Mao et al., 2004) and cantaloupe melon (Bernadac et al., 1996). The syndrome is characterized by acute softening, subdermal tissue translucency, enhanced electrolyte efflux, and cell wall disassembly (Karakurt and Huber, 2002; Lima et al., 2005; Mao et al., 2004).

Watersoaking development appears to be affected by many factors including storage temperature, ethylene, and/or fruit maturity. Hong and Gross (2000) suggested that watersoaking is a symptom of chilling injury and membrane physical responses to low temperatures. Several studies, however, revealed that watersoaking is a wound response but not chilling injury. Watersoaking was observed in fresh-cut tomato slices stored at 10-15 ^{o}C but not in intact fruit stored at 5 ^{o}C (Bai et al., 2003; Jeong et al., 2004; Lana et al., 2006). Watersoaking dependence on ethylene as an inducer has been reported to vary from commodity to commodity. Watersoaking in cantaloupe was apparently ethylene independent, as application of 1-methylcyclopropene (1-MCP), an

ethylene antagonist (Serek et al., 1994; Sisler and Serek 1997), could not prevent watersoaking (du Chatenet et al., 2000). In contrast, 1-MCP inhibited watersoaking development of watermelon and immature cucumber fruit, indicating a causal relationship between ethylene and the disorder (Mao et al., 2004; Lima et al., 2005). The response of watermelon fruit to ethylene seems to be unrelated to fruit maturity (Karakurt and Huber, 2002) while ethylene-induced watersoaking was observed only in immature cucumber fruit but not in ripe fruit (Hurr et al., 2009).These variable responses in different commodities indicate that several mechanisms would be involved in watersoaking development.

Although study of the physiological and cellular processes leading to watersoaking remains still incomplete, degradation of cell wall and membrane were reported to contribute to watersoaking development (Karakurt and Huber, 2004; Mao et al., 2004; Lima et al., 2005). For example, ethylene-treated watermelon fruit showed increased activity and transcript abundance of polygalacturonase (PG) (Karakurt and Huber, 2004) and increased lipoxygenase (LOX), phospholipase C (PLC), phospholipase D (PLD), and phosphatidic acid (PA) (Mao et al., 2004) with the onset and development of watersoaking. In addition, reactive oxygen species (ROS) seem to be involved in watersoaking development. In immature cucumber fruit, enhanced ROS-generating capacity represented the earliest response to ethylene treatment (Chapter 3). Excess ROS production has been reported to trigger programmed cell death (PCD) in plants (Desikin et al., 2001; Rao and Davis, 2001; Mur et al., 2005), and watersoaking in immature cucumber fruit appears to represent a form of PCD (Hurr et al., 2010). These data indicate that ethylene-enhanced ROS can induce watersoaking in immature

cucumber fruit through PCD processes such as increased nuclease activity and DNA laddering.

Watersoaking in cucumber fruit is an ethylene-induced disorder (Lima et al., 2005). Ethylene is a gaseous phytohormone influencing diverse developmental processes including seed germination, abscission, fruit ripening, sex determination, and senescence (Abeles et al., 1992; Kieber, 1997). Ethylene is synthesized in response to biotic and abiotic stresses including pathogen attack, flooding, chilling, and wounding (Abeles et al., 1992) and can diffuse into and throughout plant tissues (Mattoo and Suttle, 1991). The effect of ethylene is influenced by development stage, and ethylene concentration and exposure duration (Abeles et al., 1992; Saltveit, 1999). Watersoaking development of cucumber fruit is dependent on developmental maturity (Hurr et al., 2009) and ethylene exposure duration (Chapter 3). Immature fruit (4-6 d after anthesis) showed incipient watersoaking at 6 d of continuous 10 $\mu L \cdot L^{-1}$ ethylene exposure and 100% incidence of watersoaking at 9 d whereas mature cucumber fruit (10-14 d after anthesis) showed a much lower incidence of the disorder (about 30% at 12 d). In fruit at more advanced maturity (showing color break due to accumulation of carotenoid pigments), however, ethylene exposure caused chlorophyll degradation and massive β-carotene accumulation but did not cause watersoaking. Short-duration exposure (less than 4 d) of immature cucumber to 10 $\mu L \cdot L^{-1}$ ethylene did not induce the disorder whereas fruit receiving ethylene for 4 d followed by transfer to air exhibited incipient watersoaking at 17 d (Chapter 3).

Immature cucumber fruit exhibited spatial patterns of watersoaking development. In ethylene-treated fruit, incipient watersoaking is first evident in hypodermal (outlayer of

mesocarp tissues) tissues and progresses into the inner mesocarp tissues (Chapter 3). This spatial pattern appears to be a consequence of tissue-specific ethylene responses. Even among members of the Cucurbitaceae, patterns of watersoaking development are diverse. Watersoaking initiated in innermost mesocarp tissues of cantaloupe melon fruit (du Chatenet et al., 2000) whereas ethylene-treated watermelon showed incipient watersoaking in both placental and mesocarp tissue (Karakurt and Huber, 2002). On the other hand, this spatial pattern of watersoaking in cucumber fruit (incipient watersoaking in hypodermal tissues) could be explained by differences in gas diffusion properties or gradients among different tissue types. This view is supported by the observation of Praeger and Weichmann (2001) that pO_2 profiles decreased from outer to inner tissue in cucumber fruit. The epidermis and cuticle can act as barriers to gas diffusion into internal tissues (Burg, 2004a). Stomata were also reported as an important regulator of gas exchange and transpiration in cucumber fruit (Cazier et al., 2001; Laurin et al., 2006). Therefore, it is possible that external tissues can have higher concentrations of gasses applied exogenously. This might explain why incipient watersoaking symptoms are first evident in hypodermal tissues.

In the present study, we employed intact and fresh-cut slices of cucumber fruit to address whether patterns of watersoaking differed in response to altered gas-exchange properties. Furthermore, we investigated the influence of pO_2 on watersoaking development of intact fruit and fresh-cut slices.

Materials and Methods

Plant Materials

Experiments were conducted with beit-alpha cucumber (*Cucumis Sativus* L.; ‘Manar’) harvested at immature stage (average fruit wt. 86 ± 3.2 g) from a commercial

greenhouse facility in Live oak, FL. Freshly harvested fruit were returned to Gainesville within 2 h where they were sorted by size, color and appearance, sanitized with 2.7 mM sodium hypochlorite, and air-dried. Fresh-cut slices (10 mm thick; 4 slices per fruit) were prepared from the middle portion of fruit with a double- bladed knife.

Intact fruit (n=50) were placed in a 20-L plastic container (n=4) and fresh-cut slices (4 slices per fruit from 5 fruit) were placed in a ventilated 1.5-L plastic container (FridgeSmart®, Tupperware Corp., Orlando; n=10) which in turn were placed inside a 174-L steel chamber (n=4) at 13 ^{o}C and 95% R.H. Four plastic containers holding intact fruit and four steel chambers holding fresh-cut slices were provided with flow-through atmospheres of air (21 kPa O_2) ± 10 $\mu L \cdot L^{-1}$ ethylene, 2 kPa O_2 (balance N_2) + 10 $\mu L \cdot L^{-1}$ ethylene, or 40 kPa O_2 (balance N_2) + 10 $\mu L \cdot L^{-1}$ ethylene. Flow rate was maintained at 500 $mL \cdot min^{-1}$ to avoid CO_2 accumulation and the gas mixture was humidified by passing through a water-filled glass jars (2 L).

At intervals during treatment, slices (10-mm thick) from the middle portion of the treated intact fruit were cut with a double blade knife and were evaluated along with fresh-cut slices prepared at the start of the experiment. Changes in mesocarp firmness, surface and mesocarp hue angle, electrolyte leakage, and incidence of watersoaking were evaluated as indicators of ethylene responses.

Exocarp and Mesocarp Color

Color measurement was conducted on fresh-cut slices and slices derived from intact fruit with a Minolta Chroma Meter CR-400 (Minolta Camera Co. Ltd., Japan), which has an 8 mm-diameter aperture and illuminant C lighting condition. Fruit surface and mesocarp color were measured by placing the sensor over exocarp and mesocarp tissue, respectively. Ten slices (2 slices per fruit, 5 fruit) were evaluated per treatment

and two measurements were made per slice. A white calibration plate was used for calibration (L* = 96.88, C* = 2.05, h°* = 89.4, a* = 0.02, b* = 2.05). The values were expressed by the CIE L (lightness) a*(range from green to red)b*(range from blue to yellow) model (Mclaren, 1979). Hue angle was determined using the formula $h_{ab}=\tan^{-1}(b^*/a^*)$. The angular coordination of hue is start from 0^o for red, where 90^o = yellow, 180^o = green, and 270^o = blue.

Mesocarp Firmness

Mesocarp firmness was determined on fresh-cut slices and slices prepared from intact fruit using an Instron Universal Testing Instrument (Model 4411; Canton, MA, USA) equipped with a convex-tip probe (3 mm diameter) and 0.05 kN load cell. Each slice was placed on a solid flat plate and zero height was established between the probe and the mesocarp tissue. The probe was driven with a crosshead speed of 50 $mm \cdot min^{-1}$ and the force was recorded at 2.5 mm deformation. Ten slices (2 slices per fruit, 5 fruit) were evaluated per treatment and two measurements were made per slice.

Electrolyte Leakage

Electrolyte leakage was measured using a conductivity bridge (YSI 3100 conductivity instrument; Ohio, USA) equipped with a conductivity electrode. Slices derived from intact fruit (n=5) and fresh-cut slices made from 5 fruit per treatment were evaluated every other day. Mesocarp disks (n=5) of 4.5 mm diameter were excised using No. 2 Cork borer from 2 slices (10 mm thickness) per fruit. Five disks were rinsed with distilled water, briefly blotted on Whatman #4 filter paper, and transferred into 25 mL of 250 mM mannitol (Villalta and Sargent, 2004) in a 50 mL capped centrifuge tube. After each sample was shaken for 4 h, electrical conductivity of the bathing solution was measured. Samples were then frozen at -20 °C. After 24 h the samples were thawed at

room temperature, heated in a boiling water bath for 15 min, and after cooling to room temperature, the final conductivity was determined. All leakage data were expressed as a percentage of total electrolyte conductivity, where initial conductivity was divided by total conductivity, and multiplied by 100.

ROS Release from Mesocarp Disks

Total reactive oxygen species (ROS) were determined using a 2',7'-dichlorofluorescin (DCFH) assay of Schopfer et al. (2001) with some modifications. ROS oxidize nonfluorescent DCFH to the highly fluorescent 2',7'-dichlorofluorescein (DCF) and fluorescence increase can be used to determine the amount of ROS release. DCFH-diacetate (10 mM) was dissolved in ethanol and stored at -20 ^{o}C as a stock solution. Fifty µM DCFH-DA was prepared from the stock solution with 20 mM K-phosphate (pH 6.0). Deacetylayion of DCFH-DA (50 µM) was performed using 0.1 $g \cdot L^{-1}$ of esterase (EC 3.1.1.1 from porcine liver) at room temperature for 15 min. This solution was used for the assay immediately and discarded each day after use. Mesocarp disks (4.5 mm wide by 10mm thick, three disks per fruit) were prepared from cucumber slices (10 mm thickness) with a cork borer (#2). Three disks were rinsed with distilled water, briefly blotted on Whatman #4 filter paper, and incubated in a 50 mL centrifuge tube containing 10 mL of working solution in the dark for 15 min. ROS release was quantitatively determined by measuring relative fluorescence of aliquots (2 mL) in a fluorometer (Versafluor^{TM} fluorometer; Bio-Rad Laboratories, Inc., CA, USA) (Ex: 480 nm, Em: 520 nm). Working solution without tissue was used to zero the instrument and 10 mM H_2O_2 (final concentration) to set the maximum fluorescence as 10,000. Fluorescence was transformed into production of H_2O_2 in µmoles per disk per h using a standard curve prepared with dilutions of H_2O_2 (final concentrations of 0, 10, 100, 1000,

and 10000 µM). This analysis was conducted every other day with 3 intact fruit and fresh-cut slices made from 3 fruit stored with/without 10 $\mu L \cdot L^{-1}$ ethylene. Peroxidase has been shown to cause DCFH oxidation (Keston and Brandt, 1965). However, endogenous peroxidase activity of cucumber fruit tissues was not a rate limiting factor based on the observation that the addition of peroxidase (1000 $U \cdot mL^{-1}$; E.C. 1.11.1.7 from horseradish) to the working solution had no significant effect on the fluorescence measurements.

Results

As a response to challenge with 10 $\mu L \cdot L^{-1}$ ethylene, incipient watersoaking was observed in hypodermal tissues (the outlayer of mesocarp tissues) of both intact fruit and fresh-cut slices at 7 d under normoxic and hyperoxic conditions (40 kPa O_2) (Fig. 4-1 A & B). Afterward, intact fruit exhibited more severe and rapid watersoaking development than did fresh-cut slices. As shown in Figure 4-2 A, watersoaking affected about 30~40% of mesocarp tissues of intact fruit treated with ethylene under normoxia and 80~90% incidence under hyperoxic conditions at 9 d. In contrast, 10~20% of mesocarp tissues of fresh-cut slices were watersoaked in response to ethylene under both normoxic and hyperoxic conditions at 9 d (Fig. 4-2 B). Watersoaking was not observed in intact fruit or slices exposed to normoxia without ethylene or to hypoxia (2 kPa O_2) plus ethylene. Hypoxia negated ethylene-induced watersoaking development for up to 9 d in both intact and fresh-cut slices, whereas hyperoxia accelerated ethylene-induced watersoaking in intact fruit (Fig. 4-2 A) but not in fresh-cut slices (Fig. 4-2 B). Microbial proliferation was observed in ethylene-treated fresh-cut slices (Fig. 4-2 B) as was also seen in exocarp of ethylene-treated intact fruit (data not shown).

The initial hue angle of exocarp was about 121.5 ^{o} (Fig. 4-3). This value of intact fruit treated with air changed negligibly over the initial 9 d, declining thereafter (Fig. 4-3 A). Intact fruit exposed to continuous ethylene (10 $\mu L \cdot L^{-1}$) under normoxic conditions exhibited steep declines in exocarp hue angle after 3 d. Exocarp hue angles of intact fruit treated with ethylene under normoxia were significantly lower (about 2.5 and 4 ^{o}) compared with air-treated fruit at 5 and 7 d, respectively. Ethylene-induced exocarp degreening was not accelerated by hyperoxia but negated by hypoxia. Intact fruit stored with air or ethylene under hypoxia showed similar exocarp hue angle values during storage. In contrast to intact fruit, initial exocarp hue angle of fresh-cut slices treated with air continuously decreased to about 118 ^{o} at 11 d (Fig. 4-3 B). Continuous ethylene exposure (10 $\mu L \cdot L^{-1}$) under normoxia and hyperoxia induced steep declines in exocarp hue angle of fresh-cut slices after 3 d, but there was no further acceleration by hyperoxia. At 7 d, fresh-cut slices exposed to ethylene under normoxic and hyperoxic conditions had significantly lower exocarp hue angle (about 3.5~4 ^{o}) compared to air-treated fresh-cut slices. Hypoxia reduced the rate of surface discoloration caused by exogenous ethylene and fresh-cut processing in fresh-cut slices. Fresh-cut slices exposed to ethylene under hypoxia had nearly 3 ^{o} higher exocarp hue angle compared with air-treated slices at 11 d.

Initial hue angle of mesocarp tissue was around 114.5 ^{o} (Fig. 4.4). Mesocarp hue angle of intact fruit stored under air declined slightly after 5 d, reaching around 113 ^{o} at 9 d (Fig. 4-4 A). In intact fruit, exogenous ethylene did not hasten degreening of mesocarp tissue under normoxia. However, hyperoxia with 10 $\mu L \cdot L^{-1}$ ethylene significantly enhanced mesocarp discoloration of intact fruit after 5 d, and hypoxia

reduced the rate of mesocarp degreening. At 5 d, mesocarp tissue of intact fruit treated with ethylene under hyperoxia and hypoxia had 1.5 ^{o} lower and 2 ^{o} higher hue angles, respectively, compared with air-treated fruit. Mesocarp hue angle of air-treated fresh-cut slices declined significantly after 5 d, reaching around 110 ^{o} at 9 d (Fig. 4-4 B). Fresh-cut slices stored with 10 $\mu L \cdot L^{-1}$ ethylene under normoxic or hyperoxic condition showed significantly enhanced yellowing of mesocarp tissues; 2 and 4 ^{o} lower mesocarp hue angle compared with air-treated slices at 5 and 9 d, respectively. In fresh-cut slices, the decline of mesocarp hue angle was inhibited by hypoxia during 11 d of storage as was also seen in exocarp hue angle, while hyperoxia induced no further mesocarp discoloration in fresh-cut slices.

Firmness of mesocarp tissue was initially around 9 N, a value maintained for both intact fruit and fresh-cut slices stored under air (Fig. 4-5). Intact fruit treated with 10 $\mu L \cdot L^{-1}$ ethylene under normoxia exhibited significant softening at 5 d, which was accelerated by hyperoxia (Fig. 4-5 A). At 7 d, intact fruit exhibited 40%- and 60%-declines in mesocarp firmness as a response to exogenous ethylene under normoxia and hyperoxia, respectively, compared with fruit treated with air. Mesocarp softening of intact fruit caused by exogenous ethylene was delayed by hypoxia. Mesocarp firmness of intact fruit exposed to ethylene under hypoxia reached around 4 N at 15 d, and at 9 d and 7 d in fruit treated with ethylene under normoxia and hyperoxia, respectively. Ethylene-mediated mesocarp softening was significantly delayed in fresh-cut slices compared with intact fruit (Fig. 4-5 B). Ethylene-treated intact fruit had significantly reduced mesocarp firmness compared to air-treated fruit after 3 d, whereas there was no significant difference in mesocarp firmness between air- and ethylene-treated fresh-

cut slices until 9 d. Ethylene induced a 50% decrease in mesocarp firmness of fresh-cut slices at 9 d. In fresh-cut slices, hypoxia negated ethylene-induced softening during 11 d of storage while hyperoxia showed no enhancement in softening compared with normoxia.

Both intact fruit and fresh-cut slices stored under ethylene-free atmospheres showed slight increases in electrolyte leakage during storage (Fig. 4-6). Initial electrolyte leakage was around 10%, and tissues from fresh-cut slices and intact fruit treated with air exhibited about 19% and 17% electrolyte leakage, respectively, at 11 d. Ethylene-induced electrolyte leakage in intact fruit and fresh-cut slices was differently affected by hyperoxia and hypoxia. In intact fruit, ethylene-induced increases in electrolyte leakage were significantly enhanced by hyperoxia and delayed by hypoxia (Fig. 4-6 A). At 7 d, intact fruit treated with 10 $\mu L \cdot L^{-1}$ ethylene under normoxia had 2-fold higher electrolyte leakage compared with control fruit and leakage of fruit treated with ethylene applied under hyperoxia was about 2-fold higher than leakage of fruit treated with ethylene under normoxia. Electrolyte leakage of intact fruit treated with ethylene under normoxia, hyperoxia, or hypoxia was 65%, 90%, or 35% at 11 d, respectively. In fresh-cut slices, enhanced electrolyte leakage by exogenous ethylene was negated under hypoxia while there was no acceleration by hyperoxia (Fig. 4-6 B). Fresh-cut slices stored under normoxic or hyperoxic conditions with 10 $\mu L \cdot L^{-1}$ ethylene exhibited 2-fold and 4-fold increased electrolyte leakage compared with air-treated slices at 5 d and 7 d, respectively. Increased electrolyte leakage due to ethylene exposure was greater in fresh-cut slices than in intact fruit. At 9 d, fresh-cut slices stored with ethylene under normoxia or hyperoxia exhibited around 90% electrolyte leakage while intact fruit

exposed to ethylene under normoxia or hyperoxia had 55% or 65% electrolyte leakage, respectively.

DCFH (2', 7'-dichlorofluorescin) assay was employed to measure total reactive oxygen species (ROS)-generating capacities in mesocarp tissue disks of intact fruit and fresh-cut slices stored under normoxia with or without ethylene (Fig. 4-7). ROS generation of intact fruit treated with air (IFA) amounted to about 0.6 µmol of H_2O_2 equivalents per mesocarp disk per h at 2 d. ROS-generating capacity of IFA increased to 2.6 µmol/disk/h at 4 d, reached a maximum of 3.9 µmol/disk/h at 8 d, and declined at 10 d. ROS production was significantly enhanced in intact fruit challenged with 10 $\mu L \cdot L^{-1}$ ethylene. Intact fruit exposed to ethylene (IFE) exhibited 5.0 µmol/disk/h of total ROS-generating capacity at 2 d and maximum ROS-generating capacity of 9.0 µmol/disk/h at 6 d. Total ROS-generating capacity of IFE was about 23- and 11-fold higher at 4 and 6 d, respectively, compared with IFA. Fresh-cut slices had higher total ROS-generating capacity than intact fruit during storage. Increases in ROS-generating capacity in fresh-cut slices were noted early in storage. Fresh-cut slices treated with air (FSA) produced 1.5 µmol/disk/h of total ROS at 2 d, increasing to a maximum of 6.2 µmol/disk/h at 8 d. There was an 8.5- and 3-fold increase in ROS-generating capacity of FSA compared with the value of IFA at 4 d and 6 d, respectively. When fresh-cut slices were challenged with 10 $\mu L \cdot L^{-1}$ ethylene, total ROS-generating capacity was significantly enhanced. Fresh-cut slices treated with 10 $\mu L \cdot L^{-1}$ ethylene (FSE) exhibited 3.3-fold higher total ROS-generating capacity (about 5.0 µmol/disk/h) at 2 d compared with values for FSA. Total ROS-generation of FSE was 2.6- and 4.6- fold higher than the value of FSA at 4 d and 6 d, respectively. In addition, ethylene-induced increases in total ROS-generating

capacity were significantly enhanced in fresh-cut slices (FSE) than intact fruit (IFE). Total ROS generation of FSE at 2 d was nearly 6-fold higher compared with the value of IFE. FSE produced peak total ROS production (14.2 µmol/disk/h) at 8 d; 2.3- and 1.8-fold increase compared with FSA and IFE, respectively. Both IFE and FSE showed steeper decline in total ROS production at 10 d compared with IFA and FSA.

Discussion

Watersoaking in immature beit-alpha cucumber fruit was initiated in hypodermal tissue, subsequently affecting inner mesocarp tissues. As comparing ethylene responses of intact fruit and fresh-cut slices, the hypothesis that tissue ethylene and oxygen gradients can affect this spatial pattern of watersoaking was tested. Altering tissue ethylene and oxygen gradients through the use of fresh-cut slices did not alter the spatial pattern (initial appearance in hypodermal region) of watersoaking development and in fact greatly diminished watersoaking development. These observations indicate that watersoaking in immature beit-alpha cucumber fruit is a tissue-specific phenomenon caused by exogenous ethylene. Tissue-specific responses to ethylene have been shown in other commodities. Flores et al. (2001) reported that down-regulation of ACO (1-aminocyclopropane 1-carboxylic acid oxidase) activity in melon fruit blocked autocatalytic ethylene production and suppressed ripening processes (degradation of chlorophyll, accumulation of carotenoids) in rind tissue but not in pulp tissue. In tomato, ethylene signaling mechanisms are differentially affected in a tissue-specific manner (Barry and Giovannoni, 2006). Overexpression of GR (Green-ripe) induced nonripening phenotype (Gr) fruit, but not ethylene insensitivity in tomato hypocotyls and petiole tissues.

This tissue-specific modulation of ethylene responses in immature cucumber fruit could be the consequence of different expression patterns of ethylene receptors and downstream elements among tissues. Diverse gene expressions of receptor family in different tissues have been reported in other studies. In tomato, the expression of six ethylene receptor genes was varied significantly depending on tissue types (Tieman and Klee, 1999). The outer, mid, and inner portions of melon fruit flesh exhibited slightly different patterns of CmERS1and CmETR1 expression (Sato-Nara et al., 1999), and different ETR gene expression was shown in epicarp and mesocarp tissue of plum fruit (Ferna´ndez-Otero et al., 2007). Tissue-specific expression of CTR1-like genes, the downstream element of receptors, was also reported in tomato (Adams-Phillips et al., 2004) and kiwi fruit (Yin et al., 2008). The ethylene signaling systems of different ethylene receptors could play specialized roles in determining ethylene response through different regulations by ethylene and/or different interactions with CTR1. Different response of receptor family to ethylene was reported in tomato fruit where ethylene induced an increase in NR and LeETR4 levels but a decrease in LeETR2 levels (Ciardi et al., 2000). ETR1-like receptors have been reported to bind more strongly to CTR1 than ETR-2 like receptors (Guo and Ecker, 2004). Furthermore, different combinatorial gene regulations among tissue could result in tissue specificity for ethylene. Highly conserved proteins such as GR (Green Ripe) in tomato and RTE1 (Reversion-To-Ethylene sensitivity1) in Arabidopsis have been reported to interact with specific receptors, providing an explanation for tissue-specific ethylene responses (Klee, 2006). Proteosomal degradation of transcription factors, especially by 26S, might also play a role in tissue-specific ethylene responses (Trobacher, 2009).

While the spatial pattern of watersoaking was similar in intact fruit and fresh-cut slices, fresh-cut slices exhibited much less acute ethylene-induced watersoaking compared with intact fruit. This result stands in sharp contrast to watersoaking phenomena as reported for tomato (Hong and Gross, 1998; Jeong et al., 2004), watermelon (Mao et al., 2005), papaya (Ergun et al., 2006) and ‘Galia’ (Ergun et al., 2007) and cantaloupe (Jeong et al., 2008; Luna-Guzman et al., 1999) fruits. In these fruits, quality of fresh-cut tissues even in response to storage in air is greatly limited by accelerated tissue watersoaking and juice leakage. Interestingly, in some cases, for example tomato (Jeong et al., 2004), cantaloupe (Jeong et al., 2008) and ‘Galia’(Ergun et al., 2007) fruits, watersoaking represents largely a fresh cut-specific phenomena, as the disorder occurs negligibly in intact fruit and only at very late stages of ripening. Whereas 1-MCP treatment completely prevented ethylene-induced watersoaking in intact beit-alpha cucumber fruit (Lima et al., 2005), watersoaking was reduced but not prevented in 1-MCP-treated fresh-cut tomato (Jeong et al., 2004) and papaya (Ergun and Huber, 2004). Taken together, these findings suggest that watersoaking is commodity-dependent.and that complicated and multiple mechanisms are involved in watersoaking development.

Greater resistance to watersoaking development in fresh-cut slices could be explained by reported differences in ethylene sensitivity of intact versus wounded tissues. Blocking ethylene reception by application of 1-MCP, an ethylene action inhibitor, was less beneficial or detrimental in fresh-cut products. In apple fruit, 1-MCP was more effective in intact fruit than fresh-cut slices and 1-MCP more beneficial when applied before processing than after (Jiang and Joy, 2002). 1-MCP prevented softening

of placental tissue in intact watermelon (Mao et al., 2004) but not in fresh-cut watermelon (Mao et al., 2005). Softening of persimmons was retarded when 1-MCP was applied to intact fruit prior to fresh-cut processing but not after processing (Vilas-Boas and Kader, 2007). Furthermore, 1-MCP application directly to pear slices induced even poorer quality attributes (Lu et al., 2009). Increased decay by 1-MCP treatment was reported in fresh-cut cantaloupe cubes (Jeong et al., 2004), pineapple slices (Budu and Joyce, 2003) and 'Gala' apple slices (Bai et al., 2004). This reduced efficiency of 1-MCP in fresh-cut products could be the consequence of ethylene-activated protective mechanisms in wounded tissues as reported by O'Donnell et al. (1996). In the same manner, ethylene-induced protective mechanisms seem to play a role in retarding watersoaking development in fresh-cut slices of cucumber fruit.

What makes fresh-cut slices highly resistant to watersoaking could also be the enhanced accumulation of reactive oxygen species (ROS). In present study, fresh-cut slices had higher ROS-generating capacities than intact fruit and exhibited greater increase in ROS generation when challenged with ethylene. Accumulation of ROS has been widely reported as a response to wounding stress (Hodges et al., 2004), and fresh-cut products generally exhibit 2- to 3-fold higher respiration rates than intact fruit (Cantwell and Suslow, 2002; Watada et al., 1996). Wounding accelerates a number of physiological mechanisms such as defense signaling pathways (via formation of systemin, oligogalacturonides, and oxylipins) and ion channel activation pathways at membrane (Leon et al., 2001). Accordingly, the increased respiration and metabolic demands of wounded tissues likely contributed to earlier and higher production of ROS in fresh-cut slices. In addition, excessive production of ROS could be the result of

greater water loss in fresh-cut products. Dehydration is a major factor limiting the quality of fresh-cut products (Saladíe et al., 2007; Toivonen and Brummell, 2008). Yan et al. (2003) reported that water-deficit condition enhanced ROS accumulation, especially H_2O_2, in tobacco leaves.

Wound-induced ROS in cucumber slices could act as signals to accelerate scavenging systems as reported by Mittler (2002). Plants modulate production and removal of ROS using nonenzymatic and enzymatic antioxidant mechanisms (Apel and Hirt, 2004). Increased ROS accumulation by fresh-cut processing can induce increased antioxidant levels, which might explain the reduced watersoaking in fresh-cut cucumber slices compared with intact fruit. Significant increases in antioxidant capacity by fresh-cut processing were reported in celery stem (442%), lettuce leaves (233%), and carrot roots (77%) (Reyes et al., 2007). It has been noted that wounding can enhance antioxidant capacity through increased phenolic antioxidants (Ryes et al., 2007; Heredia and Cisneros-Zevallos, 2009). Furthermore, synergetic effects of wounding and exogenous ethylene at enhancing antioxidant levels were reported in study of Heredia and Cisneros-Zevallos (2009) where ethylene exposure (1000 $\mu L \cdot L^{-1}$ for 4 d) enhanced antioxidant capacity in fresh-cut lettuce leaves and carrot roots but not in whole lettuce leaves and carrot roots. Collectively, enhancing antioxidant levels due to fresh-cut processing and exogenous ethylene might play a role in preventing watersoaking development in fresh-cut slices of beit-alpha cucumber fruit. This enhanced antioxidant system can also explain how hyperoxia enhanced softening, electrolyte leakage and watersoaking in ethylene-treated intact fruit but not in fresh-cut slices. Increased antioxidant system in fresh-cut slices could accelerate healing of tissue and decrease

susceptibility to physical disorder, which can make fresh-cut slice highly resistant to watersoaking even when challenged with ethylene under hyperoxia.

This present study demonstrated that ethylene responses in cucumber fruit are pO_2-dependent. In intact fruit, hyperoxia (40 kPa O_2) accelerated ethylene-induced watersoaking and accompanying symptoms including degreening, softening and enhanced electrolyte leakage while hypoxia (2 kPa O_2) strongly suppressed these symptoms. In fresh-cut slices, hyperoxia did not enhance watersoaking nor accompanying symptoms while hypoxia did largely prevent ethylene-induced symptoms in slices. Similarly, strong influence of pO_2 on ethylene responses (ripening and enhanced respiration) has been reported in other fruits including avocado (Metzidakis and Sfakiotakis, 1995; Burg, 2004), banana (Kanellis et al., 1989; Jiang and Joyce, 2003), kiwi (Stavroulakis and Sfakiotakis, 1997), muskmelon (Altman and Corey, 1987), and tomato (Kapotis et al., 2004).

Strong influence of pO_2 on ethylene responses might be mediated through controlling overall metabolic processes, which could be the consequence of changes in gene expression and/or enzyme activities. For example, storage under hypoxic conditions significantly suppressed gene expression of hydrolytic enzymes (Kanellis et al., 1989a, b, 1991, 1993; Loulakakis et al., 2006; Owen et al., 2004; Pasentsis et al., 2007). Identified hypoxia/ anoxia-induced genes include transcription factors (de Vetten and Ferl, 1995; Hoeren et al., 1998) and signal transduction elements (Baxter-Burrell et al., 2002; Dordas et al., 2003). The synthesis of mRNA and polypeptides associated with ethylene response could be inhibited through suppression in translation by the dissociation of polysomes (Lin and Key, 1987; Sachs and Ho, 1986) and/or in the

expression of mRNA (Kanellis, 1987, Sachs and Ho, 1986). Strong influence of pO_2 on enzymic activity or levels was also reported in several studies. Hypoxic condition (2.5 kPa O_2) suppressed the activities of polygalacturonase (PG), acid phosphatase, and cellulase in banana and avocado fruit (Kanellis et al., 1989a, 1989b; Metzidakis and Sfakiotakis, 1995). Suppressed activity of PG, β-galactosidase, and cellulose were also observed in hyperoxia (80 kPa O_2)-treated grape fruit maintaining fruit firmness (Deng et al., 2005). Since cell wall degradation has been reported to play a role in watersoaking development (Karakurt and Huber, 2004), this significant influence of pO_2 on cell wall enzymes could result in modulation of watersoaking disorder in cucumber fruit.

Another explanation for the significant influence of pO_2 on ethylene responses might be found in changes in ethylene production and/or ethylene sensitivity. This view is parallel to that of Kanellis et al. (1993) and Solomos and Kanellis (1997) mentioning that the effects of hypoxia were mediated through inhibition of ethylene biosynthesis and action. Hypoxia-delayed ethylene biosynthesis was observed in broccoli buds (Makhlouf et al., 1989) and avocado fruit (Metzidakis and Sfakiotakis, 1995). In apple, the rise in ethylene evolution was delayed when 1-MCP (1 $\mu L \cdot L^{-1}$) and hypoxia (1.52 kPa O_2) were applied together (Asif et al., 2006). pO_2 appears to influence ethylene production mainly through modulating the activity of 1-aminocyclopropane-1-carboxylic-acid oxidase (ACO) as considering O_2 is a co-substrate for this enzyme (Ververidis and John, 1991; Dong et al., 1992; Sairam et al., 2008). Km values for O_2 are 0.4 and 0.44-0.53 kPa in ACO purified from apple (Kuai and Dilley, 1992) and pear fruit (Vioque and Castellano, 1994; Kato and Hyodo, 1999), respectively. Storage below 5 kPa O_2 can

suppress ACO activity since internal pO_2 is much lower than external pO_2 due to gas diffusion barriers. For example, ripe tomato fruit stored under 4 kPa O_2 exhibited 0.2 kPa of internal pO_2 (Berry and Sargent, 2009) and pO_2 level of inner cucumber tissue was 0.3 kPa when stored at 5 kPa O_2 (Praeger and Weichmann, 2001). Under hypoxic conditions (2–2.5 pKa O_2), reduced ACO levels were observed in broccoli flower buds (Makhlouf et al., 1989) and apple fruit (Gorny and Kader, 1999). The influence of pO_2 in ethylene sensitivity has also been shown in several studies. Elongation of petioles of *Rumex* species was enhanced in response to hypoxia through an increase in ethylene sensitivity but not ethylene production (Blom et al., 1994; Voesenek et al., 1996, 1997). Up-regulation of RP-ERS1 expression in *Rumex* was highest when ethylene (5 $\mu L \cdot L^{-1}$) and hypoxia (3kPa O_2) were applied in combination (Voesenek et al., 1997; Vriezen et al., 1997). In banana fruit, 1-MCP-treated fruit softened more rapidly under hyperoxic conditions, leading to speculation that hyperoxia enhanced synthesis of new ethylene receptors (Jiang and Joyce, 2003). Taken together, O_2 might act in a rate-limiting factor in watersoaking of cucumber fruit by affecting ethylene production and/or action.

Altered responses to ethylene in response to changes in pO_2 appear to support an important role of reactive oxygen species (ROS) in watersoaking development as mentioned in Chapter 3. Continuous ethylene exposure (10 $\mu L \cdot L^{-1}$) induced marked increases in ROS-generating capacity, preceding the decline of firmness and hue angle, and increased electrolyte leakage, and well in advance of incipient watersoaking (Chapter 3 and present study). pO_2 could affect ROS levels through supplying different level of a substrate (O_2) and modulating various metabolic activities including oxidases and peroxidases. For example, hypoxia stimulated ROS generators including xanthine

oxidase, NADH and NADPH oxidase in apple fruit (Gong and Mattheis, 2003) and hypoxia-induced genes include ROS scavenging enzymes including peroxidase and superoxide dismutase in Arabidopsis (Klok et al., 2002). Living organisms respond to biotic and abiotic stress through significant crosstalk between ROS and hormones including salicylic acid, jasmonic acid, and ethylene (Overmyer et al., 2003; Kwak et al., 2006; Parent, 2008). Overmyer et al., (2003) noted that ROS could mediate ethylene signaling directly or indirectly. Therefore, we can't exclude the possibility that altered ROS level depending on pO_2 might induce significant modification in ethylene responses of cucumber fruit.

Fresh-cut cucumber slices experienced significant peel discoloration, but not enhanced softening or electrolyte leakage. Intact immature cucumber fruit, however, maintained initial peel hue value even though chlorophyll declined nearly 70% during 12 d of storage in ethylene (Hurr et al., 2009). Discoloration is a major factor limiting shelf-life and marketability of fresh-cut products. Toivonen and Brummell (2008) mentioned that Type II chlorophyll breakdown occurs in damaged cells of green plant tissues. Type II chlorophyll breakdown is mainly regulated by ROS (Brown et al., 1991). ROS are generated in the process of fatty acid degradation (by chlorophyll oxidase and/or lipoxygenase) and phenolic compounds degradation (by chlorophyll peroxidase), which can directly oxidize chlorophyll molecules and result in loss of green color (Toivonen and Brummell, 2008). This view is supported by several reports. Pheophytin accumulation was observed in discolored parsley leaves (Yamauchi and Watada, 1991) and discolored cabbage discs had increased lipoxygenase activity and fatty acid degradation (Chéour et al., 1992). Strong association between chlorophyll peroxidase,

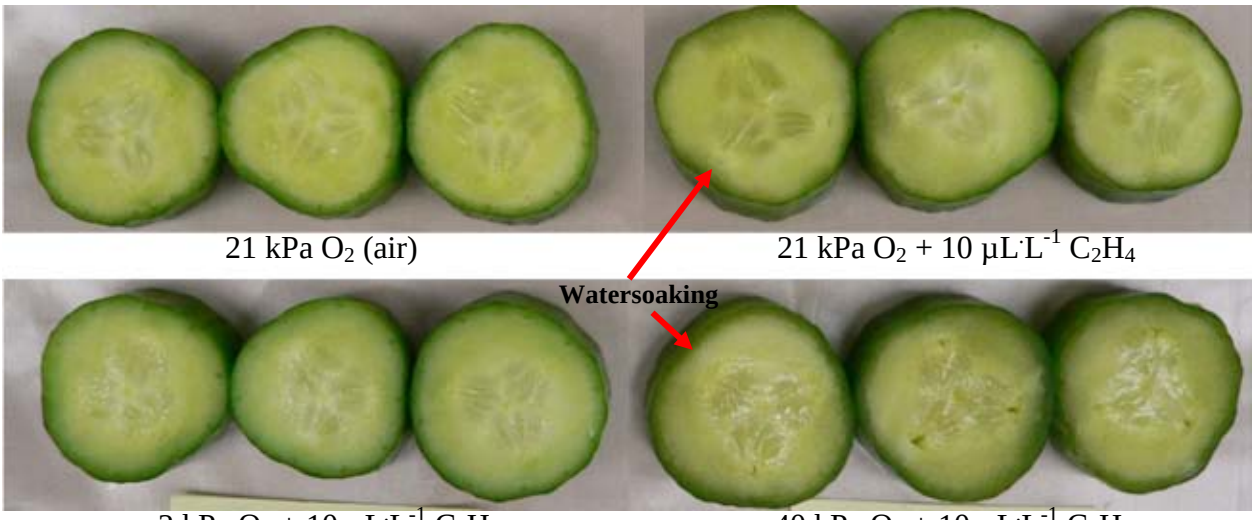
oxidase, and/or lipoxygenase activity and chlorophyll loss was observed in broccoli florets (Zhang et al., 1994; Funamoto et al., 2002, 2003; Costa et al., 2005). Based on increased ROS production by fresh-cut processing in present study, ROS-induced Type II chlorophyll oxidation might result in more significant peel discoloration in fresh-cut slices than intact fruit.

Combination of fresh processing and exogenous ethylene enhanced electrolyte leakage but not softening. This non-parallel relationship between firmness and electrolyte leakage was observed in tomato fruit (Lee et al., 2007) and 'Galia' melon fruit (Ergun et al., 2007). Observed firmness retention in fresh-cut cucumber slices could be a case of the development of hardening at the cut surface which is observed in some vegetables (Everson et al., 1992; Viña and Chaves, 2003). Retention of firmness in fresh-cut slices could be a consequence of enhanced water loss. Fresh-cut processing removes natural barriers to gas diffusion and transpiration and results in greater surface area to volume, enhancing water loss (Toivonen and Brummell, 2008). Firmness increase/retention by water loss was reported in intact immature cucumber fruit (Hurr et al., 2009). However, tissue disruption during fresh-cut processing can contribute to enhanced electrolyte leakage due to cell wall degradation. Karakurt and Huber (2003) noted that wounding induced rapid deterioration of fresh-cut papaya fruit along with increased activities of enzymes targeting cell wall and membranes including polygalacturonase, alpha- and beta-galactosidases, lipoxygenase, and phospholipase D.

In summary, the data from this study clearly demonstrated that watersoaking is a tissue-specific response. Interestingly, whole fruit exhibited more severe and rapid watersoaking than fresh-cut slices. Altered ethylene responses by pO_2 were observed in

both intact fruit and fresh-cut slices. In whole fruit, hyperoxia exacerbated watersoaking and accompanying symptoms such as yellowing, softening and enhanced electrolyte leakage while hypoxia delayed these symptoms. In fresh-cut slices, hypoxia negated the development of ethylene-induced responses and there was no acceleration by hyperoxia. Strong influence of pO_2 on watersoaking and the enhanced resistance to watersoaking in fresh-cut slices suggested that reactive oxygen species (ROS) could play an important role in mediating watersoaking development of immature cucumber fruit.

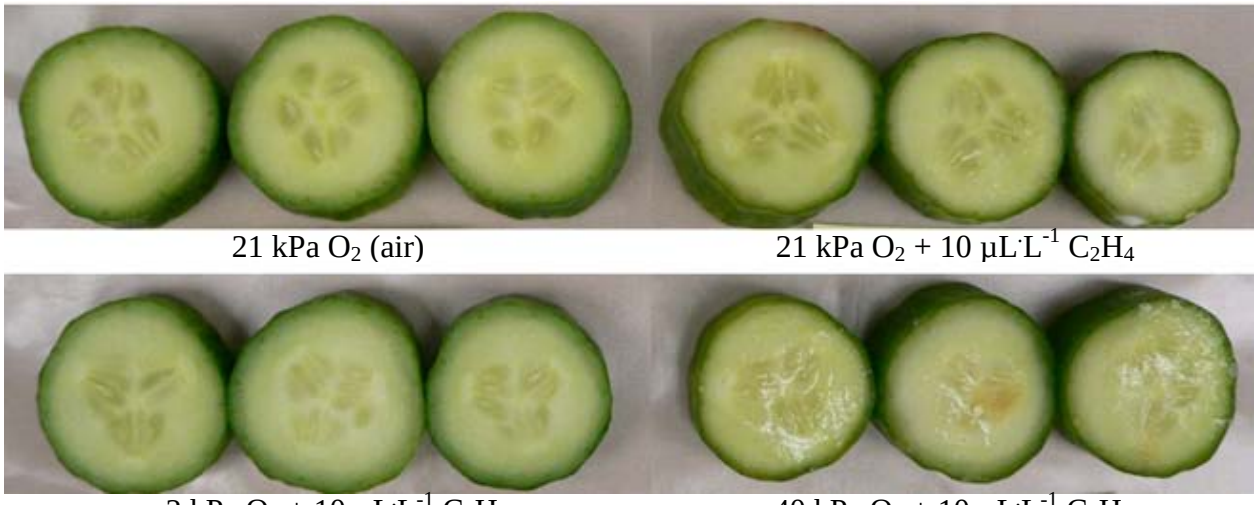
A Slices from intact fruit



21 kPa O_2 (air) | 21 kPa O_2 + 10 µL·L^{-1} C_2H_4

2 kPa O_2 + 10 µL·L^{-1} C_2H_4 | 40 kPa O_2 + 10 µL·L^{-1} C_2H_4

B Fresh-cut slices



21 kPa O_2 (air) | 21 kPa O_2 + 10 µL·L^{-1} C_2H_4

2 kPa O_2 + 10 µL·L^{-1} C_2H_4 | 40 kPa O_2 + 10 µL·L^{-1} C_2H_4

Figure 4-1. Watersoaking development of beit-alpha cucumber fruit stored at 13 ^{o}C. Intact cucumber fruit and fresh-cut slices were treated for 7 d with air or ethylene (10 µL·L^{-1}) under normoxic, hyperoxic, and hypoxic conditions. A) Slices derived from intact fruit. B) Fresh-cut slices.

A Slices from intact fruit

21 kPa O_2 (air) | 21 kPa O_2 + 10 $\mu L \cdot L^{-1}$ C_2H_4

2 kPa O_2 + 10 $\mu L \cdot L^{-1}$ C_2H_4 | 40 kPa O_2 + 10 $\mu L \cdot L^{-1}$ C_2H_4

B Fresh-cut slices

21 kPa O_2 (air) | 21 kPa O_2 + 10 $\mu L \cdot L^{-1}$ C_2H_4

2 kPa O_2 + 10 $\mu L \cdot L^{-1}$ C_2H_4 | 40 kPa O_2 + 10 $\mu L \cdot L^{-1}$ C_2H_4

Figure 4-2. Watersoaking development of beit-alpha cucumber fruit stored at 13 ^{o}C. Intact cucumber fruits and fresh-cut slices were treated for 9 d with air or ethylene (10 $\mu L \cdot L^{-1}$) under normoxic, hyperoxic, and hypoxic conditions. A) Slices derived from intact fruit. B) Fresh-cut slices.

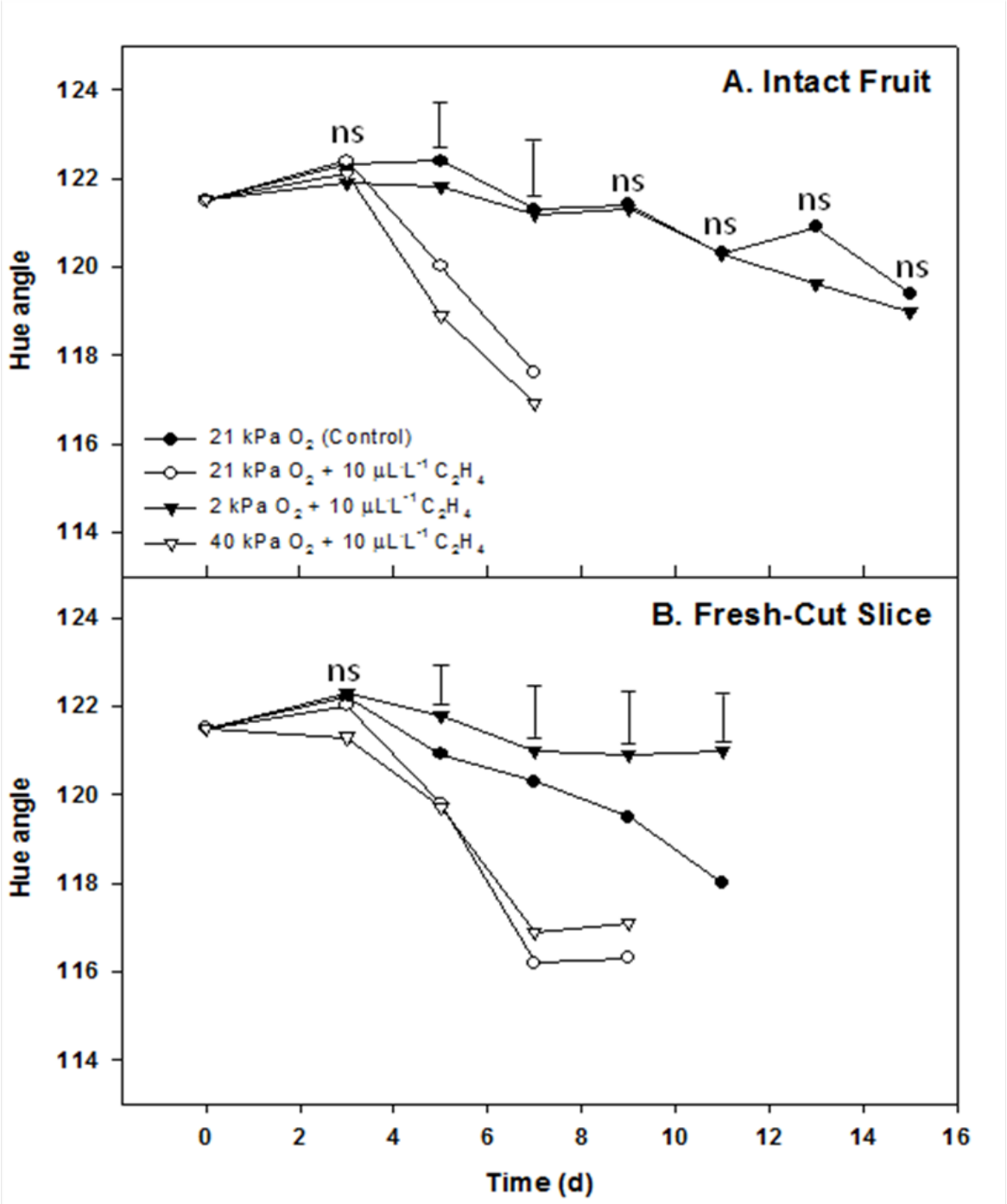


Figure 4-3. Surface hue angle of beit-alpha cucumber fruit during storage at 13 ^{o}C under air or 10 µL·L^{-1} ethylene under normoxic, hypoxic, and hyperoxic conditions. Each point represents the mean of 20 measurements (5 fruit, 2 slices per fruit, 2 measurements per slice). Vertical bars represent LSD (α= 0.05). A) Slices derived from intact fruit. B) Fresh-cut slices.

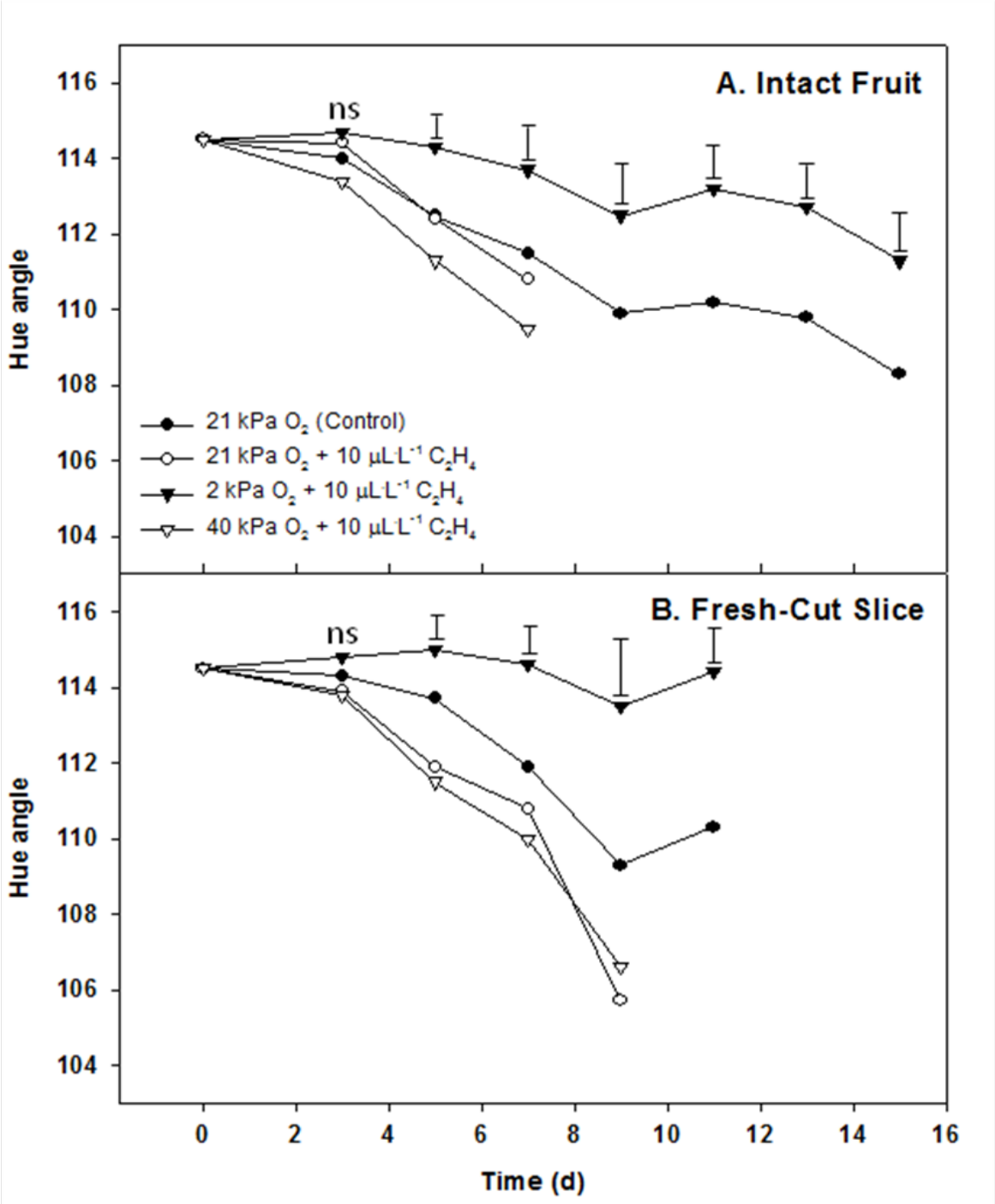


Figure 4-4. Mesocarp hue angle of beit-alpha cucumber fruit during storage at 13 ^{o}C under air or 10 µL·L^{-1} ethylene under normoxic, hypoxic, and hyperoxic conditions. Each point represents the mean of 20 measurements (5 fruit, 2 slices per fruit, 2 measurements per slice). Vertical bars represent LSD (α= 0.05). A) Slices derived from intact fruit. B) Fresh-cut slices.

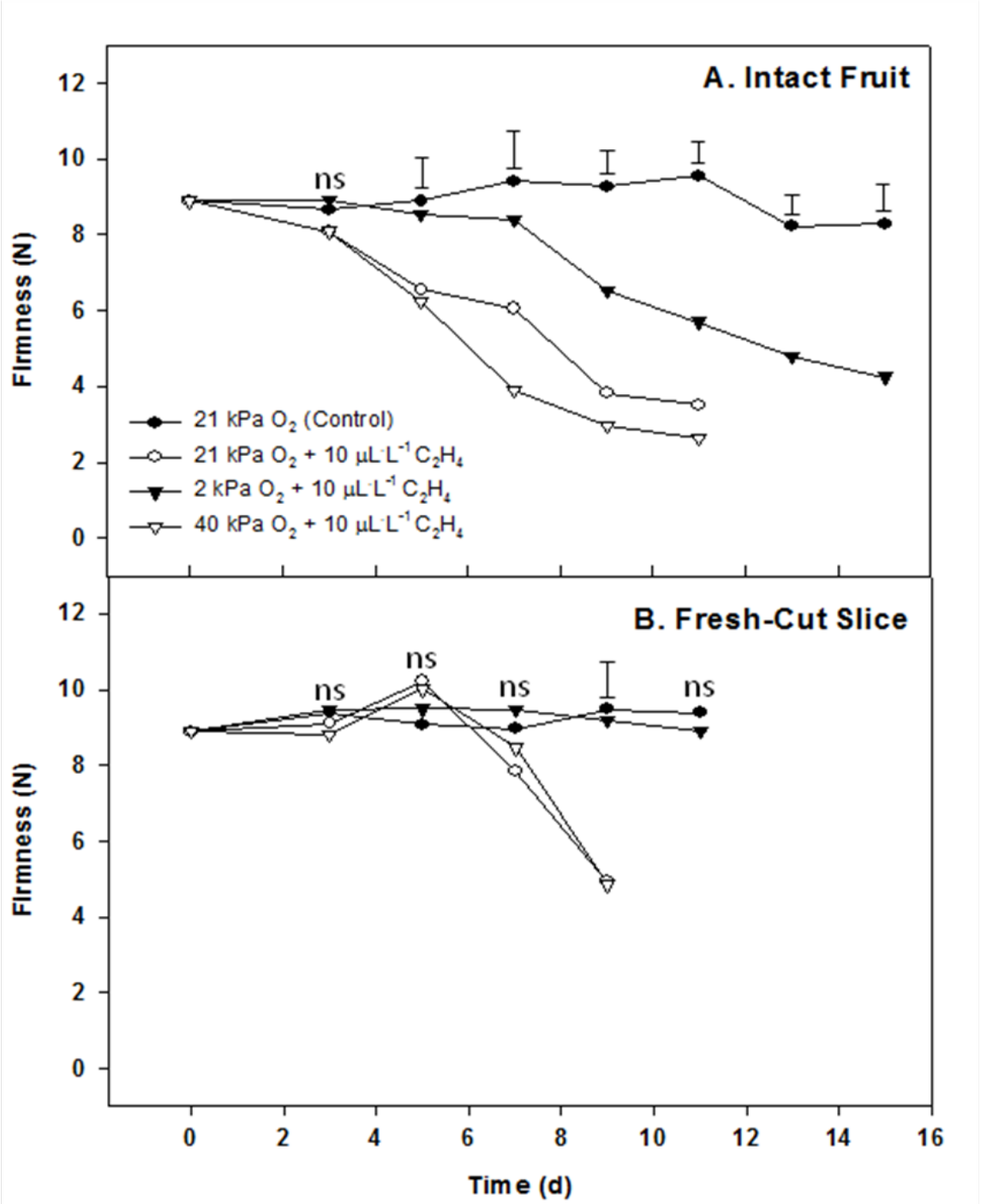


Figure 4-5. Mesocarp firmness of beit-alpha cucumber fruit during storage at 13 ^{o}C under air or 10 µL·L^{-1} ethylene under normoxic, hypoxic, and hyperoxic conditions. Each point represents the mean of 20 measurements (5 fruit, 2 slices per fruit, 2 measurements per slice). Vertical bars represent LSD (α= 0.05). A) Slices derived from intact fruit. B) Fresh-cut slices.

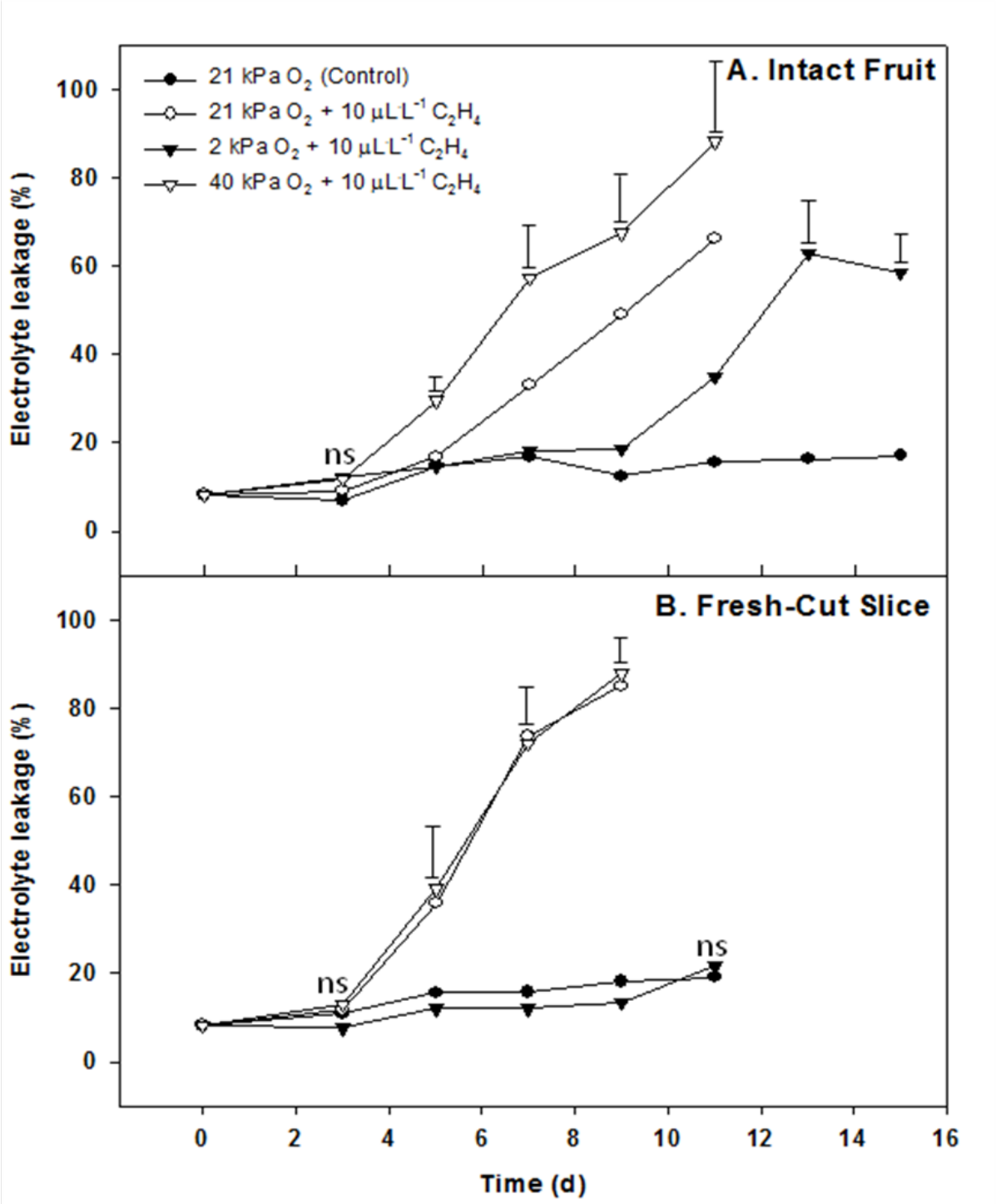


Figure 4-6. Electrolyte leakage of beit-alpha cucumber fruit during storage at 13 ^{o}C under air or 10 µL·L^{-1} ethylene under normoxic, hypoxic, and hyperoxic conditions. Each point represents the mean of 10 measurements (5 fruit, 2 measurements per fruit). Vertical bars represent LSD (α= 0.05). A) Slices derived from intact fruit. B) Fresh-cut slices.

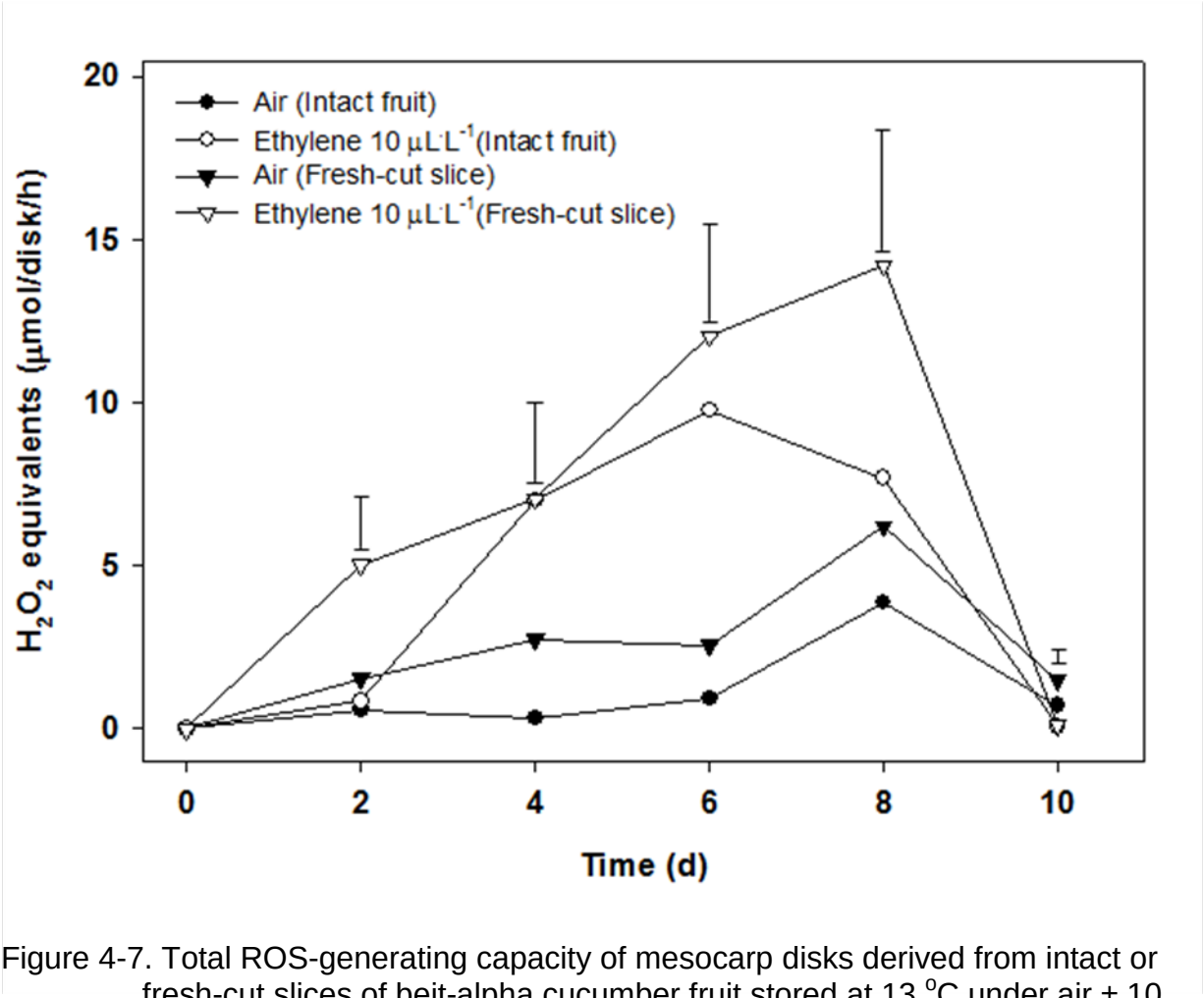


Figure 4-7. Total ROS-generating capacity of mesocarp disks derived from intact or fresh-cut slices of beit-alpha cucumber fruit stored at 13 ^{o}C under air ± 10 µL·L^{-1} ethylene. The production of total ROS was demonstrated using the oxidation of DCFH to DCF. Relative fluorescence at 520 nm was transformed into the production of H_2O_2 in µmoles per disk using a standard curve. Each point represents the mean of 3 fruit. Vertical bars represent LSD (α= 0.05).

CHAPTER 5
THE EFFECTS OF HYPOXIA AND HYPEROXIA ON ETHYLENE-INDUCED WATERSOAKING IN IMMATURE BEIT-ALPHA CUCUMBER FRUIT

Introduction

Watersoaking, the appearance of tissue translucency, is a characteristic ethylene response observed in immature cucumber fruit (Lima et al., 2005; Hurr et al., 2009; Chapter 3 and 4), as well as in other members of the Cucurbitaceae including watermelon (Karakurt and Huber, 2002; Mao et al., 2004) and cantaloupe melon (Bernadac et al., 1996). This disorder is characterized by acute softening, subdermal tissue translucency, loss of epidermal green color, enhanced electrolyte efflux, and cell wall disassembly (Karakurt and Huber, 2002; Mao et al., 2004; Lima et al., 2005). Application of 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception (Serek et al., 1994; Sisler and Serek 1997), inhibited watersoaking development of immature cucumber fruit, confirming the involvement of ethylene in the disorder (Lima et al., 2005). In ethylene-treated cucumber fruit, incipient watersoaking is first evident in hypodermal (outlayer of mesocarp tissues) tissues and then progresses into inner mesocarp tissues (Chapter 3). This spatial pattern was similar in both intact fruit and fresh-cut slices (Chapter 4), indicating that watersoaking is a tissue-specific ethylene response.

Ethylene responses of cucumber fruit were influenced by several factors. Ethylene dose dependency was shown in the report of Villalta and Sargent (2004), wherein watersoaking accompanied by enhanced respiration, softening, surface degreening were observed at ethylene levels as low as 1 $\mu L \cdot L^{-1}$. In Chapter 3, ethylene concentrations exceeding 10 $\mu L \cdot L^{-1}$ had no further effect on the physiology of cucumber fruit. Ethylene exposure duration influences watersoaking development in cucumber fruit. Short term exposure (12 h) to 10 $\mu L \cdot L^{-1}$ ethylene showed no significant detrimental

effect on quality of cucumber fruit during 20 d of storage while ethylene exposure for 4 d induced watersoaking much more slowly compared with continuous exposure (Chapter 3). Maturity of cucumber fruit is another factor affecting watersoaking disorder. Watersoaking disorder was observed in immature (4-6 d after anthesis) and to a lesser extent in mature cucumber fruit (10-14 d after anthesis), while fruit at more advanced maturity exhibited chlorophyll degradation, 'fruit' aroma evolution, and massive accumulation of β-carotene without watersoaking (Hurr et al., 2009).

Oxygen level (pO_2) can also alter ethylene responses. Ethylene responses in immature cucumber fruit were influenced by pO_2 (Chapter 4). Hyperoxia (40 kPa O_2) accelerated ethylene-induced watersoaking and accompanying symptoms including degreening, softening and enhanced electrolyte leakage in intact fruit. Storage under hypoxia (2 kPa O_2) strongly suppressed ethylene-induced symptoms in both intact fruit and fresh-cut slices. Altered ethylene responses by pO_2 were also reported in other commodities such as avocado (Burg, 2004), banana (Kanellis et al., 1989), and muskmelon (Altman and Corey, 1987). Oxygen is one of the most important factors, influencing the postharvest physiology such as respiration rate, chlorophyll degradation, cell wall degradation, and phenolic oxidation (Mir and Beaudry, 2001; Burg, 2004). pO_2 could mediate ethylene responses through modulation in ethylene perception and production and/or in the levels of active oxygen species (ROS) (Kader and Ben-Yehoshua 2000).

ROS are partially reduced forms of ground state O_2. Examples include singlet oxygen (O_2^1) formed by energy transfer and superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$) formed by sequential electron transfer (Klotz, 2002).

ROS are highly reactive and destructive, shown to cause significant tissue deterioration (Rao et al., 2000; Overmyer et al., 2003; Circu and Aw, 2010). Plants continuously generate ROS as by-products of various metabolic pathways, mainly in chloroplasts, mitochondria, and peroxisomes (Foyer and Harbinson, 1994; Apel and Hirt, 2004; Circu and Aw, 2010). Depending on the character of biotic and abiotic stresses, plants differentially enhance the generation of ROS that are chemically distinct and/or are produced within different cellular compartments (Elstner, 1991). In ethylene-treated cucumber fruit, greatly enhanced ROS generation was observed as an early cellular event before incipient watersoaking (Chapter 3 and 4). Excessive ROS was shown to trigger programmed cell death (PCD) in plants (Desikin et al., 2001; Rao and Davis, 2001; Mur et al., 2005). Watersoaking in immature cucumber fruit is a form of PCD, supported by the presence of PCD hallmarks such as loss of cell viability, enhanced nuclease activity and DNA laddering in ethylene-treated fruit (Hurr et al., 2010). Altering ROS levels could modulate the onset and development of PCD including watersoaking in immature cucumber fruit.

Enhanced ROS during stimuli can pose a threat to cells but can also serve as signals to activate antioxidant systems (Mittler, 2002). As biological organisms attempt to maintain homeostatic equilibrium between production and scavenging of ROS, oxidative damage could be inhibited by direct quenching of ROS or through disruption of ROS propagation (Alscher and Hess, 1993). Plants have nonenzymatic and enzymatic ROS scavenging mechanisms (Apel and Hirt, 2004). Nonenzymatic antioxidants include ascorbate and glutathione, tocopherol, flavonoids, alkaloids, and carotenoids. Enzymatic ROS scavenging mechanisms in plants include superoxide dismutase

(SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). The response of antioxidative systems to oxidative stress during postharvest storage varied depending on commodities and/or cultivars, indicating the complexity of antioxidant systems to oxidative stress (Hodges et al., 2004).

Previous studies (Chapter 3 and 4) suggested that ROS could play an important role in watersoaking development of immature cucumber fruit. To confirm the involvement of ROS accumulation in watersoaking, the present study investigated the change in production of ROS, especially hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot -}$), of immature cucumber fruit in response to altered pO_2. As oxidative damage could be controlled by altering antioxidant systems (Alscher and Hess, 1993; Mittler, 2002), this study also examined whether ethylene-enhanced ROS induces counter increases in antioxidant levels. The effect of preconditioning under hypoxia on subsequent ethylene responses was also investigated.

Materials and Methods

Plant Materials

Experiments were conducted with beit-alpha cucumber (*Cucumis Sativus* L.; 'Manar') harvested at immature stage (average fruit wt. 86 ± 3.2 g) from a commercial greenhouse facility in Live oak, FL. Freshly harvested fruit were returned to Gainesville within 2 h where they were sorted by size, color and appearance, sanitized with 2.7 mM sodium hypochlorite, and air-dried. Intact fruit (n=40) were stored in 20-L plastic containers provided with flow-through atmospheres of air (21 kPa O_2) ± 10 $\mu L \cdot L^{-1}$ ethylene, 2 kPa O_2 (balance N_2) ± 10 $\mu L \cdot L^{-1}$ ethylene, or 40 kPa O_2 (balance N_2) ± 10 $\mu L \cdot L^{-1}$ ethylene. Flow rate was maintained at 500 $mL \cdot min^{-1}$ to avoid CO_2 accumulation. In an experiment designed to study the effect of preconditioning under hyperoxia,

containers were connected with flow-through atmosphere of 2 kPa O_2 (balance N2) for 8 d and then reconnected with flow-through atmosphere of air (21 kPa O_2) ± 10 $\mu L \cdot L^{-1}$ ethylene for the duration of storage.

At intervals during treatments, changes in electrolyte leakage, mesocarp firmness, ROS-generating capacity, antioxidant capacity, and watersoaking incidence were monitored.

Electrolyte Leakage

Electrolyte leakage was measured using a conductivity bridge (YSI 3100 conductivity instrument; Ohio, USA) equipped with a conductivity electrode. Five individual fruits were evaluated per treatment every other day. Mesocarp disks (n=5) of 4.5 mm diameter were excised using a No. 2 Cork borer from 2 slices (10 mm thickness) per fruit. Five disks were rinsed with distilled water, briefly dried on Whatman #4 filter paper, and transferred into 25 ml of 250 mM mannitol solution (Villalta and Sargent, 2004) in a 50 ml capped centrifuge tube. Samples were shaken in an oscillating shaker (Model 5850; Eberbach, Ann Arbor, MI) at 1 cycle/s for 4 h, electrical conductivity of the bathing solution was measured. Samples then were frozen at -20 °C. After 24 h the samples were thawed at room temperature, heated in a boiling water bath for 15 min, and after cooling to room temperature, the final conductivity was determined. All leakage data were expressed as a percentage of total electrolyte conductivity, where initial conductivity was divided by total conductivity, and multiplied by 100.

Mesocarp Firmness

Mesocarp firmness was determined using an Instron Universal Testing Instrument (Model 4411; Canton, MA, USA) equipped with a convex-tip probe (3 mm diameter) and 0.05 kN load cell. Slices (10-mm thick) were prepared from intact fruit with a double

blade knife. Each slice was placed on a solid flat plate and zero height was established between the probe and the mesocarp tissue. The probe was driven with a crosshead speed of 50 mm·min^{-1} and the force was recorded at 2.5 mm deformation. This analysis was made every other day with one slice from each fruit (3 fruit per treatment) and 3 measurements were made per slice.

ROS Release from Mesocarp Disks

Total reactive oxygen species (ROS)-generating capacity was measured using the method of Schopfer et al. (2001) with some modifications. ROS oxidizes 2',7'-dichlorofluorescin (DCFH) to the highly fluorescent 2',7'-dichlorofluorescein (DCF) and fluorescence increase can be used to determine the amount of ROS release. DCFH-diacetate (10 mM) was dissolved in ethanol and stored at -20 ^{o}C as a stock solution. Fifty µM DCFH-DA was prepared from the stock solution with 20 mM K-phosphate (pH 6.0). Deacetylayion of DCFH-DA (50 µM) was performed using 0.1 g·L^{-1} of esterase (EC 3.1.1.1 from porcine liver) at room temperature for 15 min. This solution was used for the assay immediately and discarded each day after use. Mesocarp disks (4.5 mm wide by 10mm thick, three disks per fruit) were prepared from cucumber slices (10 mm thickness) with a cork borer (#2). Three disks were rinsed with distilled water, briefly blotted on Whatman #4 filter paper, and incubated in a 50 mL centrifuge tube containing 10 mL of working solution in the dark for 15 min. ROS release was quantitatively determined by measuring relative fluorescence of aliquots (2 mL) in a fluorometer (Versafluor^{TM} fluorometer; Bio-Rad Laboratories, Inc., CA, USA) (Ex: 480 nm, Em: 520 nm). Working solution without tissue was used to zero the instrument and 10 mM H_2O_2 (final concentration) to set the maximum fluorescence as 10,000. Fluorescence was transformed into production of H_2O_2 in µmoles per disk per h using a standard curve

prepared with dilutions of H_2O_2 (final concentrations of 0, 10, 100, 1000, and 10000 µM). This analysis was conducted every other day with 3 individual fruit per treatment.

H_2O_2 Release from Mesocarp Disks

For quantitative determination of hydrogen peroxide (H_2O_2) production *in vivo*, scopoletin assay was conducted as described by Schopfer et al. (2001) with some modifications. This method uses the decrease in fluorescence by H_2O_2-dependent oxidation of scopoletin. Mesocarp disks (4.5 wide by 10 mm thick, three disks from 2 slices per fruit) were taken with a cork borer (#2). Three disks were incubated in a 50 mL centrifuge tubes containing 4 mL of working solution (5 µM scopoletin and 3 µg·mL peroxidase from horseradish (EC 1.11.1.7) in 20 mM K-phosphate, pH 6) in dark. After 15 min, relative fluorescence of aliquots was read in a fluorometer (Versafluor™ fluorometer; Bio-Rad Laboratories, Inc., CA, USA) (Ex: 360 nm, Em: 460 nm). Phosphate buffer was used to set zero and working solution (without tissue) to set maximum fluorescence as 10000. A standard curve was prepared with dilutions of H_2O_2 (0, 1, 10, 100 µM). Reduction of fluorescence was transformed into production of H_2O_2 in nmoles per disk per h using a standard curve. This analysis was conducted every other day with 3 individual fruit per treatment.

$O_2^{\cdot-}$ Release from Mesocarp Disks

For quantitative determination of superoxide anion ($O_2^{\cdot-}$) production *in vivo*, XTT {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide} was used as a sensitive and physiologically compatible probe (Schopfer et al., 2001; Aktas et al., 2005). XTT forms a water-soluble and colored formazan that is not adsorbed to plant tissues, allowing a sensitive quantitative photometric determination of superoxide *in vivo*. Mesocarp disks (4.5 wide by 10 mm thick, three

disks from 2 slices per fruit) were taken with a cork borer (#2). Three disks were incubated in a 50 mL centrifuge tubes containing 4 mL of working solution (500 µM XTT in 20 mM K-phosphate, pH 6) in dark. The reaction was initiated by adding NADH to a final concentration of 200 µM. After 60 min, three aliquots (0.3 mL) per sample were taken and the absorbance at 490 nm was read in a Gen5 microplate spectrophotometer (Bio-Tec Instruments, Inc., VT, USA). Working solution (without tissue) was run in parallel to correct unspecific absorbance. The absorbance at 490 nm was transformed into production of $O_2^{\cdot -}$ in nmoles per disk per h using an extinction coefficient of $2.16 \times 10^4 \cdot M^{-1} \cdot cm^{-1}$ (Sutherland and Learmonth, 1997). This analysis was conducted every other day with 3 individual fruit per treatment.

Quantification of Antioxidant Capacity

For quantification of total antioxidants, modificed ABTS [2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] assay was conducted as described by Ozgen et al. (2006) with some modifications. ABTS is a sensitive probe abstracting an electron from antioxidants, resulting in color change. On every other day, mesocarp and exocarp tissues were excised from intact fruit and stored at -40 ^{o}C for later analysis. Partially thawed samples (1 g) were homogenized with 5 mL of distilled water using a polytron and centrifuged at 12,000 x *g* for 30 min at 4 °C. The resulting supernatants were filtered through cheesecloth and used for antioxidant assay. Working solution was prepared as follows: A solution of 7 mM ABTS in 20 mM Na-acetate (pH 4.5) was treated with potassium peroxodisulfate (final concentration of 2.45 mM), and then incubated over night in dark. This stock solution was diluted 90-95 times with 20 mM Na-acetate (pH 4.5) until the absorbance at 734 nm reached 0.70 ± 0.01, which was used as the working solution for ABTS assay. Fruit extract prepared as described

above (20 µL) was incubated with 3 mL of working solution. After 2 h, three aliquots (0.3 mL) per sample were taken and the absorbance at 734 nm was read in a Gen5 microplate spectrophotometer (Bio-Tec Instruments, Inc., VT, USA). A standard curve was prepared with dilutions of Trolox (0 - 100 µM) in working solution. Absorbance at 734 nm was expressed as trolox equivalents (TE; µmol per g tissue fresh weight) based on the standard curve. This analysis was performed every other day with 3 individual fruit per treatment.

Results

The Effect of Hyperoxia on Watersoaking Development.

Figure 5-1showed the effect of hyperoxia on watersoaking disorder. Hyperoxia (40 kPa O_2) alone did not cause watersoaking under the conditions employed in these experiments while ethylene-induced watersoaking was accelerated by hyperoxia. As a response to continuous 10 $\mu L \cdot L^{-1}$ ethylene, immature cucumber fruit showed incipient watersoaking (a darkening of the hypodermal tissues) at 6 d under both normoxic and hyperoxic conditions (Fig. 5-1 A**).** After an additional 2 d, fruit treated with ethylene under hyperoxia showed much more acute watersoaking compared with fruit stored under normoxia. At 8 d, around 75-80% of mesocarp tissue was watersoaked under hyperoxia while 30% was watersoked under normoxia (Fig. 5-1 B).

Electrolyte leakage was measured as an indicator of cellular membrane integrity (Fan and Sokorai, 2005) in response to hyperoxia since degradation of cell membrane has been reported to play a role in watersoaking development (Mao et al., 2004; Lima et al., 2005). Initial electrolyte leakage of cucumber fruit was around 15%, a value maintained in fruit stored under normoxic and hyperoxic conditions without ethylene (Fig. 5-2). Electrolyte leakage was enhanced by exogenous ethylene, but leakage was not

further affected by hyperoxia. Cucumber fruit stored under normoxic or hyperoxic conditions with 10 $\mu L \cdot L^{-1}$ ethylene exhibited no significant increase in electrolyte leakage until 4 d. Ethylene treatment under normoxia or hyperoxia induced 3-fold and 4.5-fold increases in electrolyte leakage at 6 d and 8 d, respectively, compared with air-stored fruit.

Since greatly enhanced ROS generation was observed before incipient watersoaking (Chapter 3 and 4), total ROS-generation capacity (expressed as H_2O_2 equivalents) in mesocarp tissue of cucumber fruit was measured to assess the influence of hyperoxia on watersoaking development (Fig. 5-3). Initial ROS-generating capacity of air-treated fruit was negligible. During further storage, ROS-generating capacity of air-stored fruit increased gradually through 8 d (about 16 µmol of H_2O_2 equivalents generated per disk per h). ROS production of cucumber fruit was markedly enhanced by10 $\mu L \cdot L^{-1}$ ethylene. As a response to exogenous ethylene under normoxia, cucumber fruit produced 3-fold and 3.5-fold higher ROS at 4 d and 6 d, respectively, compared with air-treated fruit. Ethylene-treated fruit exhibited maximum ROS-generating capacity of 35 µmol H_2O_2 equivalents per disk per h during 6-8 d, declining to 12 µmol/disk/h at 10 d. Hyperoxia alone induced no significant enhancement in ROS generating capacity. The ethylene-induced increase in ROS production was also not accelerated by hyperoxia. Fruit exposed to ethylene under hyperoxia produced maximum ROS of 32 µmol H_2O_2 equivalents per disk at-6 d, declining to 21 µmol/disk/h at 8 d. The decline in ROS production observed in ethylene-treated fruit was observed 2 d earlier under hyperoxia compared with normoxia, coinciding with the appearance of severe tissue watersoaking.

To investigate the role of specific ROS in development of watersoaking, generating capacity of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot -}$) were monitored during storage as both $O_2^{\cdot -}$ and H_2O_2 are key components of ROS signaling and the most commonly studied ROS (Overmyer et al., 2003). Hydrogen peroxide (H_2O_2)-generating capacity was measured by H_2O_2-dependent oxidation of scopoletin (Fig.5-4). Air-treated fruit initially produced 3.0 nmol of H_2O_2 per disk per h, increasing up to 4.6 nmol/disk/h and declining to 1.9 nmol/disk/h at 10 d. H_2O_2 generating capacity was significantly enhanced in fruit challenged with 10 $\mu L \cdot L^{-1}$ ethylene, with levels maintained at nearly 40-50% higher during 4-8 d compared with fruit stored in air. Ethylene-treated fruit produced 4.1 nmol of H_2O_2 per disk per h at 10 d, which was 2 fold higher than air-treated fruit. There was no enhancement in H_2O_2-generating capacity by hyperoxia regardless of ethylene exposure.

Generating capacity of superoxide anion ($O_2^{\cdot -}$) was measured using XTT, a sensitive probe for this radical species (Fig. 5-5). Initial production of $O_2^{\cdot -}$ in air-treated fruit was about 12.8 nmol $O_2^{\cdot -}$ per disk per h, increasing to 19.5 and 21.5 nmol/disk/h at 2 and 6 d, respectively. Ethylene exposure induced a significant decline in superoxide anion ($O_2^{\cdot -}$) generation after 6 d, when incipient watersoaking was observed. Ethylene-treated fruit produced nearly 10% and 30% less $O_2^{\cdot -}$ at 6 d and 8-10 d, respectively, compared with air-treated fruit. This decline coincided with the appearance of tissue watersoaking. Fruit stored under hyperoxia without ethylene showed slightly increased production of $O_2^{\cdot -}$ compared with air-treated fruit during storage. Ethylene-induced change in production of $O_2^{\cdot -}$ was not affected by hyperoxia.

Antioxidant systems work to detoxify ROS generated through oxidative stress (Mittler, 2002). Modified ABTS assay was conducted for quantification of total antioxidants (Fig.5-6). Exocarp tissue of air-treated fruit exhibited nearly 60 µmol TEs (trolox equivalents per g of tissue fresh weight) of antioxidant capacity, increasing slightly after 6 d (Fig.5-6 A). At 10 d, exocarp tissue of air-treated fruit had about 70 TEs (µmol/g FW) of antioxidant capacity. Ethylene exposure (10 $\mu L \cdot L^{-1}$) enhanced antioxidant capacity of exocarp tissue, resulting in 50%- and 70%-higher levels of antioxidants at 6 d and 8 d, respectively, compared with air-treated fruit. Hyperoxia (40 kPa O_2) did not affect the antioxidant levels under ethylene-free conditions. Fruit treated with ethylene under hyperoxia had similar antioxidant capacity as ethylene-treated fruit under normoxia (21 kPa O_2) through 6 d. Mesocarp tissue had lower antioxidant capacity compared with exocarp tissue (Fig.5-6 B). Initial antioxidant capacity of mesocarp tissue was about 35 TEs (µmol/g FW) and changed little during storage. Antioxidant capacity in mesocarp tissue of ethylene-treated fruit was 37% and 120% higher at 6 d and 8 d, respectively, compared with that in air-treated fruit. Hyperoxia induced a 23% increase in antioxidant levels of mesocarp tissue at 4 d compared with normoxia, but no further enhancement at the other days. Fruit treated with ethylene under hyperoxia had 30%-lower antioxidant levels in mesocarp tissue at 8 d compared with fruit treated with ethylene under normoxia, while there was no significant difference in antioxidant level of mesocarp tissue between ethylene-treated fruit under normoxic and hyperoxic conditions at 6 d.

The Effect of Hypoxia on Watersoaking Development.

Figure 5-7 shows the influence of hypoxia (2 kPa O_2) on watersoaking development. As a response to exogenous ethylene (10 $\mu L \cdot L^{-1}$) under normoxic

conditions (21 kPa O_2), incipient watersoaking was observed at 6 d (data not shown) and about 20-30% of mesocarp tissue was watersoaked at 8 d (Fig. 5-7 A). Cucumber fruit challenged with ethylene under hypoxia did not exhibit watersoaking symptoms over 14 d of storage (Fig.5-7 B).

The effect of hypoxia on electrolyte leakage is shown in Figure 5-8. Initial electrolyte leakage of cucumber fruit stored with air was around 11%, slowly increasing to 20% during 16 d of storage (Fig.5-8). Under normoxic condition (21 kPa O_2), electrolyte leakage of ethylene-treated fruit was enhanced 3.5-fold and 5.3-fold at 6 d and 8 d, respectively, compared with air-treated fruit. Storage under hypoxia (2 kPa O_2) alone did not influence electrolyte leakage of cucumber fruit. Additionally, the ethylene-mediated increase in leakage was completely suppressed through 14 d under hypoxia.

Total ROS-generation capacity in mesocarp tissue of cucumber fruit treated with /without ethylene under normoxia or hyperoxia was measured using oxidation of DCFH (Fig.5-9). Initial ROS-generating capacity of air-treated fruit was negligible, increasing thereafter to 13 and 14 µmol of H_2O_2 equivalents generated per disk per h at 8 and 10 d, respectively. Levels declined to 7 µmol of H_2O_2 equivalents/disk/h at 12 d. ROS production of cucumber fruit was significantly and rapidly enhanced by 10 $\mu L \cdot L^{-1}$ ethylene under normoxia. Ethylene exposure under normoxia induced 2.6- and 8.8-fold increases in ROS production at 2 d and 4 d, respectively. Fruit treated with ethylene under normoxia exhibited maximum ROS production (29 µmol of H_2O_2 equivalents generated per disk per h) at 8 d, declining to 6 µmol of H_2O_2 equivalents/disk/h at 10 d. Fruit stored under hypoxia without ethylene maintained slightly lower levels of ROS-generation capacity compared with air-treated fruit, but there was no significant

difference in ROS-generating capacity between air-treated and hypoxia-treated fruit. As seen in air-treated fruit, ROS levels of hypoxia-treated fruit reached a maximum of 9 µmol of H_2O_2 equivalents/disk/h) at 10 d, 35% lower compared with levels in air-treated fruit. Hypoxia negated the ethylene-induced increase in ROS–generation capacity. Fruit treated with ethylene under hypoxia did not show ethylene-enhanced ROS generation, but had similar ROS-generation capacity to fruit stored under hypoxia without ethylene through 14 d of storage.

Figure 5-10 shows the changes in hydrogen peroxide (H_2O_2)-generation capacity of cucumber mesocarp tissue. Air-treated fruit initially produced 3.8 nmol of H_2O_2 per disk per h, increasing to 5.2 nmol/disk/h at 8 d and then declining to 3.7 nmol/disk/h at 16 d. As seen in Figure 5-4, H_2O_2 generation was significantly enhanced in response to 10 $\mu L \cdot L^{-1}$ ethylene under normoxic condition. Ethylene-treated fruit produced nearly 40% and 25% higher H_2O_2 at 4 d and at 6-10 d, respectively, compared with air-treated fruit. Hypoxia (2 kPa O_2) induced a slight decline in H_2O_2 production at 10-12 d. Fruit stored under hypoxic conditions without ethylene produced about 30% less H_2O_2 at 12 d compared with air-treated fruit. Under hypoxia, ethylene-induced increases in H_2O_2 production were not observed until 8 d. Fruit treated with ethylene under hypoxia produced 4.5 nmol of H_2O_2 per disk per h at 10 d, 10% higher than fruit under hypoxia without ethylene and 22% lower than fruit treated with ethylene under normoxia. Under hypoxic conditions, H_2O_2-generation capacity in ethylene-treated fruit was 46% higher at 12 d compared with fruit not receiving ethylene.

Superoxide anion ($O_2^{\cdot -}$)-generating capacity was measured using XTT (Fig. 5-11). Initial production of $O_2^{\cdot -}$ in air-treated fruit was 21.1 nmol $O_2^{\cdot -}$ per disk per h, increasing

to 32.2 nmol/disk/h at 2 d and declining to 20.2 nmol/disk/h at 10 d. As seen in Figure 5-5, ethylene induced a decline in $O_2^{\cdot -}$ generating capacity after 6 d under normoxia. Ethylene-treated fruit produced 43% and 55% less $O_2^{\cdot -}$ at 8 d and 10 d, respectively, compared with air-treated fruit. Hypoxia significantly influenced $O_2^{\cdot -}$ generation capacity after 10 d. Fruit stored under hypoxia without ethylene showed 43% and 50% higher $O_2^{\cdot -}$ production at 14 d and 16 d , respectively, compared with air-treated fruit. Ethylene-induced changes in production of $O_2^{\cdot -}$ was negated by hypoxia. There was no significant difference in $O_2^{\cdot -}$ production between ethylene-treated and air-treated fruit under hypoxia .

Total antioxidants were measured using a modified ABTS assay (Fig.5-12). Exocarp tissue of air-treated fruit exhibited nearly 130 µmol TEs (trolox equivalents per g of tissue fresh weight) of antioxidant capacity, declining through 6 d and increasing to 159.5 TEs at 16 d (Fig.5-12A). Ethylene exposure (10 $\mu L \cdot L^{-1}$) under normoxia enhanced antioxidant capacity of exocarp tissue, resulting in 30%-increases in antioxidant levels at 6 d and 8 d compared with air-treated fruit. Hypoxia (2 kPa O_2) resulted in increased antioxidant levels during 6-10 d, when hypoxia-induced decrease in ROS production was observed (Fig. 5-9). Under hypoxic conditions, ethylene did not enhance antioxidants in exocarp tissue. There was no significant difference in antioxidant capacity of exocarp tissue between ethylene-treated and air-treated fruit under hypoxic conditions. Mesocarp tissue had lower antioxidant capacity during storage compared with exocarp tissue (Fig.5-12 B), a pattern also seen in Figure 5-6. Initial antioxidant capacity of mesocarp tissue in air-treated fruit was about 65 TEs (µmol/g FW), increasing to 87 TEs at 10 d and declining to 60 TEs at 12 d. Antioxidant capacity of

mesocarp tissue in ethylene-treated fruit was nearly 30% and 50% higher at 4 d and 6 d, respectively, compared with that in air-treated fruit. Mesocarp tissue of ethylene-treated fruit had maximum antioxidant levels of 112 TEs at 8 d, declining to 88 TEs at 10 d. Hypoxia induced an 8-9% decrease in antioxidant capacity of mesocarp tissue at 8 d compared with normoxia. Hypoxia negated ethylene-induced increases in antioxidants of mesocarp tissue that was observed under normoxia.

The Effect of Preconditioning under Hypoxia on Watersoaking Development.

Preconditioning cucumber fruit under hypoxia (2 kPa O_2) for 8 d prevented watersoaking development upon subsequent treatment with 10 $\mu L \cdot L^{-1}$ ethylene under normoxia (Fig.5-13). Other symptoms of ethylene exposure were also affected by preconditioning under hypoxia (data below).

The effect of preconditioning under hypoxia on electrolyte leakage is shown in Figure 5-14. Fruit not subjected to preconditioning and treated with continuous air (NPA), exhibited 11% initial electrolyte leakage, increasing to 20% at 16 d. Fruit not subjected to preconditioning and treated with 10 $\mu L \cdot L^{-1}$ ethylene (NPE) showed ethylene-enhanced leakage. NPE had 3.5-fold increased electrolyte leakage at 6 d compared with NPA. Storage under hypoxia (2 kPa O_2) did not significantly affect electrolyte leakage compared with normoxia. Fruit stored under continuous hypoxia (CH) averaged about 17% electrolyte leakage at 8 d, a value maintained throughout storage. After transfer from hypoxia (2 kPa O_2) to normoxia (21 kPa O_2), electrolyte leakage was not significantly changed. Fruit subjected to preconditioning and then treated with air (PA) had similar electrolyte leakage to that of NPA and CH. Ethylene-enhanced electrolyte leakage was also observed in preconditioned fruit as shown in non-preconditioned fruit. Electrolyte leakage of fruit subjected to preconditioning prior to ethylene exposure under

normoxia (PE) increased 1.5- and 4.3-fold at 12 d and 14 d (4 d and 6 d of ethylene exposure), respectively, compared with PA while ethylene-induced increase in electrolyte leakage was observed at 6 d in fruit not subjected to preconditioning (NPE).

Firmness of mesocarp tissue was initially around 6.5 N, increasing to 7.7 N at 2 d, a value maintained throughout storage for NPA (Fig. 5-15). Ethylene exposure under normoxia induced 35% and 72% decreases in mesocarp firmness of NPE at 6 d and 8 d, respectively. Hypoxia did not influence mesocarp firmness through 6 d. Thereafter, firmness slightly increased in fruit stored under hypoxia. CH had 15% higher firmness at 10 d compared with NPA. Mesocarp firmness of cucumber fruit stored under hypoxia was 7.4 N at 8 d when fruit subjected to preconditioning were transferred from hypoxia to normoxia. Firmness of PA was 8.8 N at 12 d (4 d after transferring), 14% and 18% higher compared with continuously hypoxia-treated (CH) and air-treated fruit (NPA), respectively. In fruit previously stored under hypoxia, ethylene induced a 65% decline in mesocarp firmness of PE at 12 d (4 d after transfer) compared with that of PA while an ethylene-induced decline in firmness was observed 6 d after treatment in non-preconditioned fruit (NPA).

Low initial ROS-generating capacity of air-treated fruit (NPA) increased to 3.5 µmol of H_2O_2 equivalents /disk/h at 2 d, a production rate maintained until 6 d (Fig. 5-16). Ethylene induced 2.6-fold and 8.8-fold increases in ROS generation of NPE at 2 d and 4 d, respectively. Storage under hypoxia did not significantly affect H_2O_2-generation capacity through 4 d compared with air storage. ROS-generation capacity of CH was 4.5 µmol of H_2O_2 equivalents/disk/h at 8 d, 35% of the value of NPA. Cucumber fruit subjected to hypoxia preconditioning (PA and PE) generated 12 µmol of H_2O_2

equivalents/disk/h at 10 d (2 d after transfer to normoxia) regardless of ethylene treatment. Thereafter, PA exhibited slightly lower ROS production of 8.7 µmol of H_2O_2 equivalents/disk/h at 14 d (6 d after transferring) and enhanced ROS production of 16.8 µmol of H_2O_2 equivalents/disk/h at 16 d (8 d after transferring). In preconditioned fruit, ethylene exposure induced 42% and 85% declines in ROS generation of PE at 12 (4 d after treatment) and 14 d (6 d after treatment), respectively, while ethylene significantly enhanced ROS generation at 2 d of treatment in non-conditioned fruit (NPE).

Fig.5-17 shows the hydrogen peroxide (H_2O_2)-generating capacity of cucumber fruit during storage. NPA showed initial H_2O_2-generating capacity of 3.8 nmol of H_2O_2 per disk per h, increasing to 5.2 nmol/disk/h at 8 d. Ethylene (10 $\mu L \cdot L^{-1}$) induced a 40% increase in H_2O_2-generation capacity of NPE at 4 d compared with NPA. Ethylene-mediated elevation in H_2O_2 generation (in NPE) was maintained until 10 d. Fruit stored under hypoxia (CH) produced 10% less H_2O_2 at 8 d compared with fruit stored under normoxia (NPA). Transferring from hypoxia to normoxia enhanced H_2O_2 production. Fruit transferred from hypoxia to normoxia (PA) had 20% and 200% higher H_2O_2 at 10 d and 12 d (2 d and 4 d after transferring), respectively, compared with CH. At 14 d (6 d after transferring), H_2O_2 production was not influenced by preconditioning. Ethylene did not enhance H_2O_2 production in fruit subjected to preconditioning (PE) whereas H_2O_2 production increased at 4 d in response to exogenous ethylene under continuous normoxia (NPE).

Initial superoxide anion ($O_2^{\cdot -}$) generating capacity in air-treated fruit (NPA) was 21.1 nmol of $O_2^{\cdot -}$ per disk per h, increasing to 32.2 nmol/disk/h at 2 d and declining to 20.2 nmol/disk/h at 10 d (Fig. 5-18). Ethylene-treated fruit (NPE) showed a 43%-decline

in $O_2^{\cdot-}$-generating capacity at 8 d compared with air-treated fruit (NPA). Hypoxia did not significantly influenced $O_2^{\cdot-}$ generation during 10 d of storage except at 2 d when fruit under hypoxia (CH) had 15% higher $O_2^{\cdot-}$ levels compared with NPA. Fruit stored under hypoxia for 8 d showed 28.9 nmol/disk/h of $O_2^{\cdot-}$ generation capacity. Transferring from hypoxia to normoxia attenuated an increase in $O_2^{\cdot-}$ production observed under continuous hypoxia (CH) after 12 d. PA produced 18% less $O_2^{\cdot-}$ at 14 d (6 d after transferring) compared with CH. In fruit subjected to preconditioning, ethylene induced a significant decline in $O_2^{\cdot-}$ production of PE at 14 d (6 d after treatment) while fruit treated with ethylene under continuous normoxia (NPE) exhibited a significant decline at 8 d. After preconditioning under hypoxia, ethylene-treated fruit (PE) produced 18% and 60% lower levels of $O_2^{\cdot-}$ at 14 d and 16 d (6 d and 8 d after transferring), respectively, compared with fruit not receiving ethylene (PA).

Exocarp tissue of air-treated fruit (NPA) exhibited nearly 130 µmol TEs (trolox equivalents per g of tissue fresh weight) of antioxidant capacity, declining through 6 d and thereafter increasing continuously to 159.5 TEs (µmol/g FW) through 16 d (Fig.5-19A). Ethylene exposure under normoxia induced 30% increases in antioxidant levels at 6 d and 8 d. Hypoxia did not significantly affect antioxidant capacity of exocarp tissue level during 14 d of storage except at 6 d when exocarp of fruit stored under hypoxia (CH) had nearly 20% higher antioxidant levels compared with air-treated fruit (NPA). Antioxidant capacity of exocarp of fruit subjected to hypoxia (CH) was nearly 115 TEs at 8 d, similar to the value of NPA. PA had higher antioxidant capacity of exocarp tissue, nearly 14% at both 12 d and 16 d (4 d and 6 d after transferring, respectively), compared with CH. In fruit transferred from hypoxia to normoxia, ethylene did not

enhance antioxidants level of exocarp tissue. There was no significant difference in antioxidant capacity of exocarp between PA and PE.

Mesocarp tissue of air-treated fruit (NPA) exhibited 65 TEs (µmol/g FW) of initial antioxidant-generating capacity (Fig. 5-19 B). Antioxidant generation in mesocarp tissue of NPA increased to 87 TEs at 10 d and declined to 60 TEs at 12 d. Ethylene induced sharp increases in antioxidant-generation capacity of mesocarp tissue (in NPE), nearly 30% and 50% at 4 d and 6 d, respectively, compared with NPA. Hypoxia had a negligible effect on antioxidant-generation capacity of mesocarp tissue through the first 8 d, except at 2 d. CH had slightly lower antioxidant levels after 8 d compared with NPA. Mesocarp tissue of fruit stored under hypoxia had nearly 72 TEs (µmol/g FW) of antioxidant capacity at 8 d, similar to the value for NPA. Fruit transferred from hypoxia to normoxia at 8 d (PA) exhibited a significant decline in antioxidants at 10 d (2 d after transferring), about 30% and 40% lower compared with CH and NPA, respectively. Thereafter, there were no significant differences in antioxidant capacity between CH and PA. Mesocarp tissue of fruit subjected to preconditioning (PA), however, produced slightly lower levels of antioxidants compared with continuous air-treated fruit (NPA). While ethylene enhanced antioxidant levels of mesocarp tissue at 4 d under continuous normoxia (in NPE), fruit subjected to preconditioning showed no significant enhancement in antioxidant level of mesocarp tissue in response to ethylene (in PE). In fruit subjected to hypoxia preconditioning, mesocarp tissue of PE produced 72 TEs of antioxidants at 14 d (6 d of ethylene treatment), a 1.6 fold-increase compared with PA.

Discussion

Ethylene-induced watersoaking was altered in response to different oxygen level. Watersoaking in beit-alpha cucumber fruit was initiated in hypodermal tissue after 6 d of

10 $\mu L \cdot L^{-1}$ ethylene treatment and subsequently affected mesocarp tissues, which was accelerated by hyperoxia (40 kPa O_2) but greatly negated by hypoxia (2 kPa O_2) as shown in Chapter 4. This result was parallel to previous studies. A strong influence of pO_2 on ethylene responses has been reported in other horticultural crops. Elevated pO_2 enhanced faster softening in avocado (Burg, 2004), banana (Kanellis et al., 1989; Jiang and Joyce, 2003), and muskmelon (Altman and Corey, 1987) and ethylene-induced russet spotting in lettuce (Kader and Ben-Yehoshua, 2000). Suppressing ethylene responses (such as ripening and/or degreening) by hypoxia have been reported in avocado (Metzidakis and Sfakiotakis, 1995), banana (Hesselman and Freebain, 1969; Kanellis et al., 1989), kiwi (Stavroulakis and Sfakiotakis, 1997), and tomato fruit (Kapotis et al., 2004), and in broccoli flower buds (Makhlouf et al., 1989). Hyperoxia alone, however, did not induce watersoaking in cucumber fruit, being consistent with no overt effect of hyperoxia on the quality of other commodities such as intact litchi (Duan et al., 2004) and peach fruit (Wang et al., 2005), fresh-cut peppers (Conesa et al., 2007), and pear slices (Kader and Ben-Yehosha, 2000). Taken together, these data indicate that pO_2 affects watersoaking indirectly through alterations in ethylene-associated mechanisms.

Strong influence of pO_2 on ethylene responses might be mediated through controlling overall metabolic processes, which could be the consequence of changes in gene expression and/or enzyme activities. Storage under hypoxic conditions induced significant changes in gene expression level including induction of new mRNA and protein, the suppression of other proteins, and constitutive expression of house-keeping and/or pre-existing proteins or mRNA species (Kanellis et al., 1989a, b, 1991, 1993;

Loulakakis et al., 2006; Owen et al., 2004; Pasentsis et al., 2007). Identified hypoxia/ anoxia-induced genes include transcription factors (de Vetten and Ferl, 1995; Hoeren et al., 1998) and signal transduction elements (Baxter-Burrell et al., 2002; Dordas et al., 2003) that participate in nitrogen metabolism (Mattana et al., 1994), cell wall loosening (Saab and Sachs, 1996), and fermentation (Pasentsis et al., 2007). During ripening of avocado fruit under 2.5 kPa O_2, synthesis of ripening-related mRNA and polypeptides such as cellulase was inhibited (Solomos and Kanellis, 1989; Kanellis et al., 1993). This suppression could be induced through suppression in translation by the dissociation of polysomes (Lin and Key, 1987; Sachs and Ho, 1986) and/or in the expression of mRNA (Kanellis, 1987, Sachs and Ho, 1986). Several studies also reported strong influence of pO_2 on enzymic activities**.** Modeling the effect of superatmospheric oxygen on *in vitro* mushroom PPO activity revealed that storage under hyperoxia might prevent enzymatic browning by polyphenol oxidase (PPO) (Gomez et al., 2006). Storage under high O_2 (80 kPa) delayed softening and accumulation of polygalacturonase (PG), β-galactosidase, and cellulase activities in grapes (Deng et al., 2005). Under continuous hypoxic conditions (2.5 kPa O_2), the activities of PG, acid phosphatase, and cellulase was suppressed in banana and avocado fruit compared with air-treated fruit (Kanellis et al., 1989a, 1989b; Metzidakis and Sfakiotakis, 1995). PG activity was negligible throughout storage of tomato fruit under 1 kPa O_2, regardless of exogenous ethylene treatment (Kapotis et al., 2004). Metzidakis and Sfakiotakis (1995) noted that this effect of hypoxia on enzyme activity or levels might be the consequence of a decline in metabolic energy caused by a decrease in respiration. Decreased respiration rate under hypoxia condition was observed in avocado fruit (Solomos and Kanellis, 1989; Metzidakis and Sfakiotakis,

1995), broccoli buds (Makhlouf et al., 1989), tomato fruit (Kim et al., 1999), and fresh-cut bell pepper (Conesa et al., 2007). By contrast, Solomos and Kanellis (1989) suggested that suppressed metabolic activity of the fruits under hypoxia would result in a decrease in the rate of respiration. This view was supported by the report of Srilaong and Tatsumi (2003) where the effect of pO_2 on respiration rate of cucumber fruit was influenced by storage temperature. High oxygen (100 kPa O_2) increased respiration rate at 20 ^{o}C but decreased respiration rate at 5 or 10 ^{o}C compared with air-treated fruit.

Oxygen might act as a rate-limiting factor in watersoaking of cucumber fruit by affecting ethylene production and action. This view is supported by previous reports. Kanellis et al. (1993) and Solomos and Kanellis (1997) concluded that the effects of hypoxia were through inhibition of ethylene biosynthesis and action. Hypoxia delayed ethylene biosynthesis in broccoli buds (Makhlouf et al., 1989) and avocado fruit (Metzidakis and Sfakiotakis, 1995). The rise in ethylene evolution in apple was delayed synergistically when 1-MCP (1 $\mu L \cdot L^{-1}$) and hypoxia (1.52 kPa O_2) were applied together (Asif et al., 2006). Ethylene biosynthetic genes were one of hypoxia-induced genes (Olson et al., 1995; Vriezen et al., 1999). On the other hand, elongation rate of petioles of *Rumex* species increased in response to hypoxia stress through an increase in ethylene sensitivity but not ethylene production (Blom et al., 1994; Voesenek et al., 1996, 1997). Up-regulation of RP-ERS1 expression in *Rumex* was highest when of ethylene (5 $\mu L \cdot L^{-1}$) and hypoxia (3%) were applied in combination (Voesenek et al., 1997; Vriezen et al., 1997). Elongation rates of rice coleoptiles were higher under 0.5 kPa O_2 plus ethylene (10 $\mu L \cdot L^{-1}$) than under air (21 kPa O_2) plus ethylene (Horton, 1991). Beaudry (2000) suggested that the primary benefits of hypoxic storage with

climacteric fruit are to suppress ripening through interfering with ethylene action. Changes in ethylene sensitivity by pO_2 were observed in banana fruit. 1-MCP-treated fruit softened more rapidly under high O_2 atmosphere, leading to speculation that hyperoxia enhanced synthesis of new ethylene receptors (Jiang and Joyce, 2003).

Altered ethylene responses in response to changes in pO_2 could also be explained by a significant role of reactive oxygen species (ROS) in watersoaking development. Continuous ethylene exposure (10 $\mu L \cdot L^{-1}$) induced marked increases in ROS-generating capacity after 2-4 d, preceding the decline of firmness and hue angle, and increased electrolyte leakage, and well in advance of incipient watersoaking (Chapter 3, 4 and present study). Hyperoxia (40 kPa O_2) did not increase ROS production but induced an earlier decline in ROS production concomitant with the occurrence of more severe watersoaking compared with ethylene-treated fruit under normoxia. On the other hand, ROS production was suppressed by hypoxia (2 kPa O_2), even in presence of exogenous ethylene. These data support the notion that early steps in ethylene-induced watersoaking involve ROS generation. The production of ROS during ethylene exposure might explain the induction of programmed cell death (PCD) in cucumber, or vice versa. Hurr et al. (2010) reported that cucumber fruit exposed to 10 $\mu L \cdot L^{-1}$ ethylene exhibited hallmarks of PCD including increased nuclease and protease activities and visible DNA laddering at 3-4 d, well in advance of incipient watersoaking. Enhanced levels of ROS could potentially directly contribute to watersoaking through membrane lipid peroxidation and subsequent loss of membrane integrity (Dhindsa et al., 1981; Fridovich, 1986; Moran et al., 1994). In the present study, the hypoxia-induced decline and hyperoxia-induced increase were observed in both ROS generation and electrolyte

leakage. Living organisms respond to biotic and abiotic stress through significant crosstalk between ROS and hormones including salicylic acid, jasmonic acid, and ethylene (Overmyer et al., 2003; Kwak et al., 2006; Parent, 2008). ROS could mediate ethylene signaling directly or indirectly (Overmyer et al., 2003).

Among several ROS, hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot -}$) generation was monitored during storage since both $O_2^{\cdot -}$ and H_2O_2 are key components of ROS signaling (Overmyer et al., 2003). Superoxide anion is one of abundant ROS, produced by reduction of triplet, ground-state oxygen (O_2), and superoxide is reduced to H_2O_2 either spontaneously or by superoxide dismutases (SOD) (Klotz, 2002; Halliwell, 2006). In the present study, ethylene-treated fruit had enhanced H_2O_2 production 2 d prior to incipient watersoaking under both normoxic and hyperoxic condition. The effect of hypoxia at suppressing H_2O_2 production was not affected by exogenous ethylene, paralleling the absence of watersoaking incidence under hypoxia in presence of exogenous ethylene. By contrast, $O_2^{\cdot -}$ production was decreased in ethylene-treated fruit as watersoaking developed under both normoxic and hyperoxic conditions. No significant decline in superoxide anion ($O_2^{\cdot -}$) was observed in hypoxic storage, even in presence of exogenous ethylene. These results indicate that hydrogen peroxide (H_2O_2) but not superoxide anion ($O_2^{\cdot -}$) may play a key role in ethylene-mediated watersoaking of immature cucumber fruit. This is consistent with the results from Chapter 3, where spatial and quantitative correlation between hydrogen peroxide (H_2O_2) and watersoaking development was observed in cucumber fruit by histochemical detection. In beit-alpha cucumber fruit, H_2O_2 accumulation is strongly associated with ethylene-induced watersoaking development. Significant role of certain specific ROS species in

controlling plant responses against biotic and/or abiotic stresses has been reported in other commodities. Ozone exposure accelerated accumulation of both $O_2^{\cdot -}$ and H_2O_2 followed by lesion formation in Arabidopsis but only H_2O_2 accumulation in ozone-exposed tobacco and tomato leaves (Wohlgemuth et al., 2002). A significantly enhanced deposition of H_2O_2 in ethylene-treated cucumber fruit, however, was observed after 6 d while significant increase in total ROS was detected after 2 d. In addition, the amount of H_2O_2 is much lower than the total ROS production even though different methods were applied for each measurement. These data indicate that study of other ROS species such as hydroxyl($HO^{\cdot}$), peroxyl ($RO_2^{\cdot}$), and alkoxyl ($RO^{\cdot}$) radicals (Trobacher, 2009) will be valuable to elucidate the role of specific ROS in development of watersoaking.

Watersoaking appeared to be mediated through imbalance between ROS production and scavenging. Plants try to maintain homeostatic equilibrium between production and scavenging of ROS. In the present study, antioxidant capacity of cucumber fruit was increased by exogenous ethylene at 6 d and 4-6 d in exocarp and mesocarp tissue, respectively, while ethylene-induced increases in ROS generation was observed at 2-4 d. Scavenging systems of cucumber fruit appear to be accelerated in response to the induction of ROS accumulation by ethylene. Ethylene exposure enhanced the production of ROS up to 500-700% while antioxidant capacity of ethylene-treated fruit increased up to 50-70% compared with that of air-treated fruit. This suggests that ethylene-induced ROS were not efficiently scavenged by antioxidants, and the resulting imbalance between production and scavenging of ROS could have contributed to development of watersoaking. Several studies revealed that

the balance between ROS production and scavenging can be disturbed under environmental stresses including high light, drought, low temperature, high temperature, and mechanical stress (Malan et al., 1990; Elstner, 1991; Prasad et al., 1994). Kadar and Ben-Yehoshua (2000) also similarly suggested that oxidative stress could result in physiological disorders such as lesion formation and necrosis when ROS levels exceed antioxidant capacity. Oxidative damage could be inhibited by direct quenching of ROS or disruption of the free radical propagation reaction (Alscher and Hess, 1993).

Hyperoxia (40 kPa O_2) alone did not affect antioxidant capacity of either exocarp or mesocarp tissue. Ethylene-induced increases in antioxidant levels were not accelerated by hyperoxia. On the other hand, hypoxia (2 kPa O_2) alone significantly reduced antioxidant capacity of mesocarp but not exocarp tissue. Ethylene-induced increases in antioxidant level were negated by hypoxia. These data support the idea that antioxidative systems in cucumber fruit were mediated to maintain the balance between production and scavenging of ROS in response to exogenous ethylene and modified pO_2. Activated antioxidant mechanisms were reported upon occurrence of oxidative stress in cold-stored mandarin fruit (Sala, 1998; Sala and Lafuente, 2000). Klok et al. (2002) reported that low-oxygen stress (5 kPa O_2) enhanced the expression of genes associated with antioxidant enzymes (peroxidase, ascorbate peroxidase, monodehydroascorbate reductase, glutathionereductase, and superoxide dismutase) in Arabidopsis root cultures. In blueberry fruit, antioxidant levels were significantly increased by 60-100 kPa O_2 but not 40 kPa O_2 as compared with air-treated fruit (Zheng et al., 2003). Strawberries stored under hyperoxia (40 kPa O_2) had higher antioxidant capacity compared with air-treated fruit (Ayala-Zavala, et al., 2007). The response of

antioxidative systems to oxidative stress during postharvest storage was various depending on commodities and/or cultivars, indicating the complexity of antioxidant responses to oxidative stress (Hodges et al., 2004). For a more thorough understanding of the antioxidant response to ethylene and modified pO_2, investigation of specific enzymic and non-enzymic antioxidants would be valuable. Antioxidative enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). Non-enzymic antioxidants include ascorbate and glutathione (GSH), tocopherol, flavonoids, alkaloids, and carotenoids. Since any one antioxidant assay generally does not provide complete information on antioxidant capacity of plant tissues (Ozgen et al., 2006; Thaipong et al., 2006) at least two assays for measuring total antioxidant capacity might be needed.

Preconditioning of cucumber fruit with hypoxia (2 kPa O_2) for 8 d significantly influenced subsequent ethylene responses under normoxia. In preconditioned fruit, exogenous ethylene resulted in significant softening and increased ion leakage but no watersoaking. Preconditioning with hypoxia (2-3 kPa O_2) has been frequently applied in apple fruit to suppress physiological disorders including surface scald and bitter pit during subsequent cold storage (Wang and Dilley, 2000; Zanella, 2003; Pesis et al., 2007; Val et al., 2009). Chilling symptoms in avocado fruit were reduced by preconditioning under 3 kPa O_2 for 24 h (Pesis et al., 1994). In contrast to our data, apple and avocado fruit subjected to preconditioning (2-3 kPa for 7-10 d and 3 kPa O_2 for 24 h, respectively) were firmer and exhibited less dehydration and electrolyte leakage than non-treated fruit (Pesis et al., 1994; Pesis et al., 2007; Val et al., 2009). Pre-storage under 1 kPa O_2 for 3 d delayed ripening of banana fruit during subsequent

air storage (Wills et al., 1982). The effectiveness of preconditioning with hypoxia appears to be dependent on treatment temperature and duration. One day of preconditioning (3 kPa O_2) at 17 ^oC was effective in suppressing chilling injury symptoms of avocado fruit (Pesis et al., 1994). In apple fruit, 7 d of preconditioning under hypoxia (2 kPa O_2) at 20 ^oC effectively prevented scald development while preconditioning for 24 h at 20 ^oC plus 6 d at 1 ^oC did not (Pesis et al., 2007).

Reduced watersoaking in cucumber fruit subjected to preconditioning might be explained by altered ROS generation. Preconditioning with hypoxia influenced ROS generation capacity of cucumber fruit. In cucumber fruit subjected to preconditioning, subsequent ethylene exposure did not induce increases in total ROS and H_2O_2 production (in PE) which was observed in non-preconditioned fruit (NPE). By contrast, preconditioned fruit (PE) exhibited ethylene-induced increase in electrolyte leakage and decline in firmness 2 d earlier than non-preconditioned fruit (NPE). Ethylene induced a significant decline in $O_2^{\cdot -}$ production in parallel with increased electrolyte leakage in both preconditioned (PE) and non-conditioned fruit (NPE). Preconditioning under hypoxia reduced ethylene-induced total ROS and H_2O_2 production and induced tissue softening with no visible watersoaking. These results support a role of ROS, especially H_2O_2, in watersoaking development of cucumber fruit. Preconditioning could inhibit watersoaking through enhancing scavenging system. For example, avocado fruit preconditioned under hypoxia (3 kPa O_2 for 24 h) showed significantly increased total free sulfhydryl (SH) groups in both peel and pulp tissue (Pesis et al., 1994). Free SH groups (mainly cysteine and glutathione) function as natural detoxification agents in ripening fruit including tomato and mango (Fuchs et al., 1981; Tabachnik-Ma'ayan and Fuchs, 1982).

The present study, however, showed that preconditioning (2 kPa O_2 for 8 d) alone did not enhance antioxidant capacity of either exocarp or mesocarp tissue and negated the ethylene-induced increase in antioxidant capacity. On the other hand, storage under hypoxic conditions could result in a restriction of metabolic activity, leading to changes in metabolic pathways to consume less energy and utilize oxygen more efficiently (Geigenberger, 2003). Hypoxia induces significant changes in the patterns of gene expression (Loulakakis et al., 2006; Owen et al., 2004; Pasentsis et al., 2007). Preconditioning under hypoxia could result in changes in plant metabolism, especially related to ROS production, which might disturb watersoaking development in response to subsequent ethylene exposure under normoxic environments.

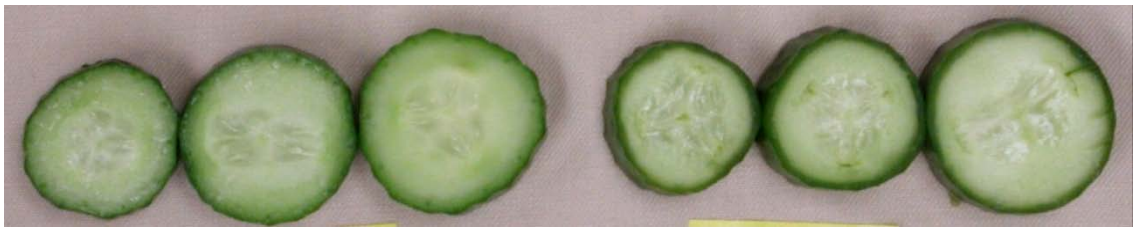
Electrolyte leakage (EL) is an indicator of cellular membrane integrity, and is commonly used as an indicator of watersoaking disorder. However, in this preconditioning experiment, ethylene exposure in preconditioned fruit induced EL decline but no watersoaking. This result indicates that electrolyte leakage alone cannot represent the sole factor contributing to watersoaking in cucumber fruit. Hurr et al (2010) noted that two distinct mechanisms of electrolyte leakage seem to operate in cucumber fruit. In their study, ethylene-induced EL in immature fruit was mechanistically different from that of more advanced fruit due to cellular structure changes. One mechanism could be developmentally regulated while the other could be affected by cellular disruption. Inconsistence between EL and watersoaking disorder in preconditioned fruit also supports the possibility of two distinctive mechanisms of EL in cucumber fruit. Ethylene exposure enhanced total ROS generation, followed by increased EL and watersoaking development in cucumber fruit not subjected to

preconditioning. By contrast, preconditioned fruit exhibited enhanced EL along with significant decline in total ROS generation and no watersoaking in response to ethylene. These results could suggest two different mechanisms of EL in cucumber fruit. One EL mechanism can be significantly related with ROS production and watersoaking development and another EL mechanism not mediated by ROS is also present in cucumber fruit.

Overall, the present study showed that ethylene-induced watersoaking in immature beit-alpha cucumber fruit was altered by pO_2. Hyperoxia (40 kPa O_2) accelerated ethylene-induced watersoaking while hypoxia (2 kPa O_2) completely negated watersoaking. Modification in ROS levels, especially H_2O_2, seems to play an important role in watersoaking development in ethylene-treated fruit. Influence of pO_2 on ethylene responses was parallel to that on ROS production and antioxidant levels. Watersoaking in cucumber fruit appeared to be mediated through imbalance between ROS production and scavenging. Cucumber fruit subjected to preconditioning under hypoxia (2 kPa O_2 for 8 d) prior to ethylene exposure under normoxia exhibited tissue softening with no visible watersoaking. Ethylene-induced increases in total ROS and H_2O_2 production were reduced by preconditioning treatment, which might inhibit watersoaking development.

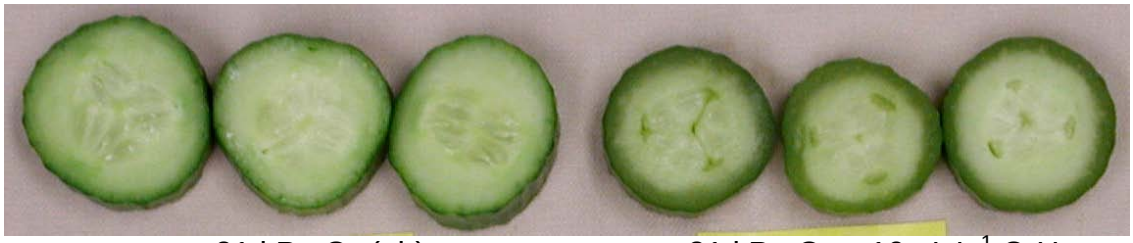
A

21 kPa O_2 (air) | 21 kPa O_2 + 10 $\mu L \cdot L^{-1}$ C_2H_4

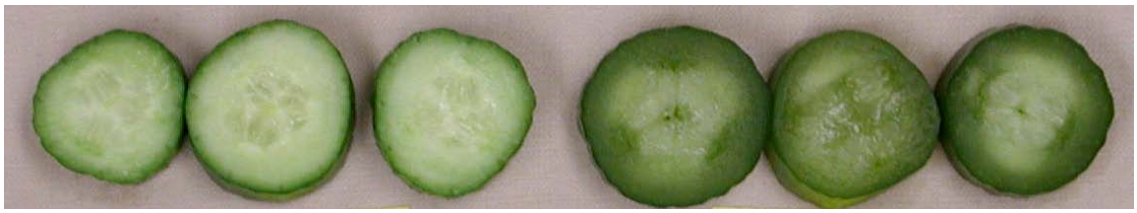


40 kPa O_2 | 40 kPa O_2 + 10 $\mu L \cdot L^{-1}$ C_2H_4

B



21 kPa O_2 (air) | 21 kPa O_2 + 10 $\mu L \cdot L^{-1}$ C_2H_4



40 kPa O_2 | 40 kPa O_2 + 10 $\mu L \cdot L^{-1}$ C_2H_4

Figure 5-1. Watersoaking development of beit-alpha cucumber fruit stored at 13 ^{o}C under normoxia (21 kPa O_2) or hyperoxia (40 kPa O_2) ± ethylene (10 $\mu L \cdot L^{-1}$). A) At 6 d. B) At 8 d.

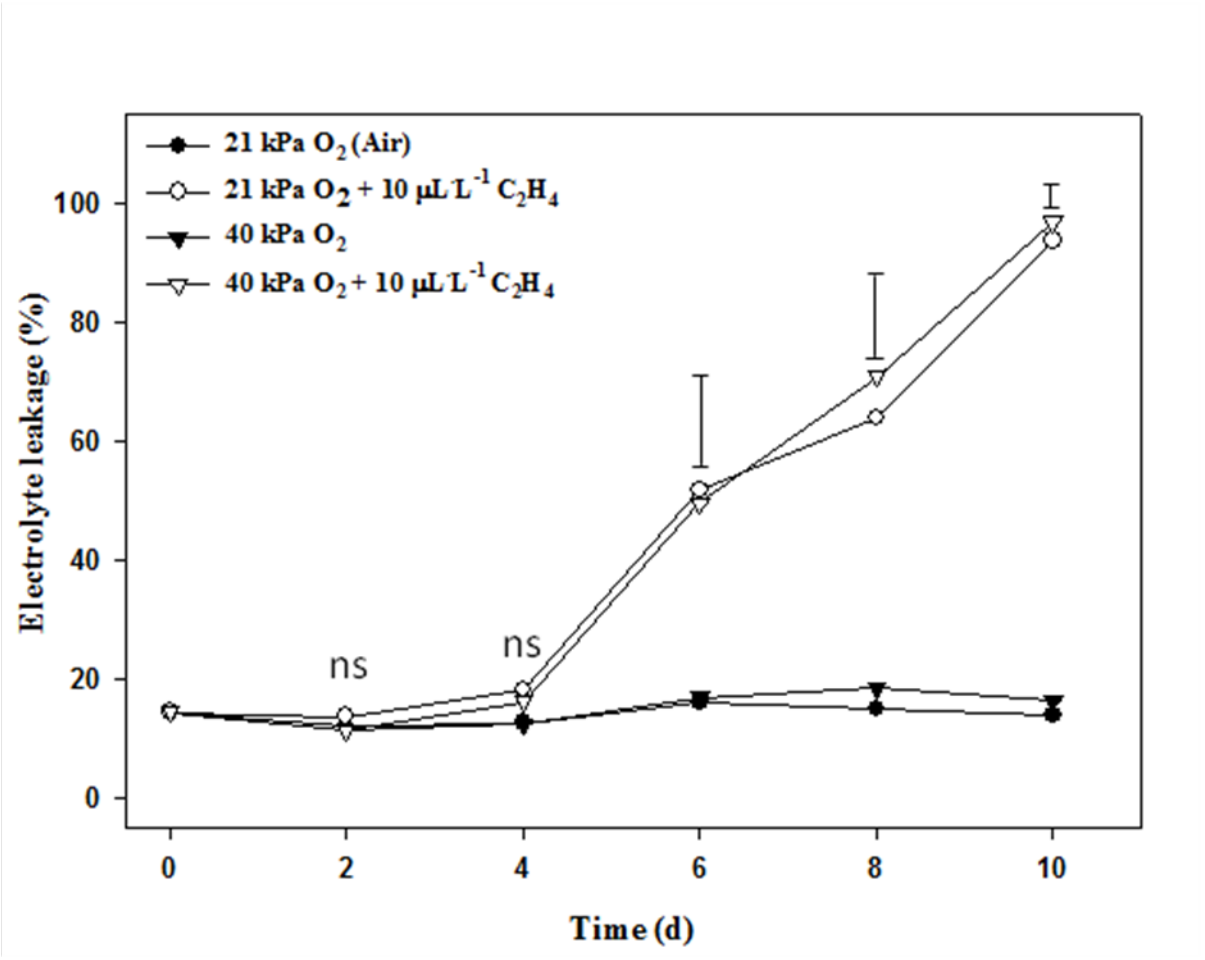


Figure 5-2. Electrolyte leakage of beit-alpha cucumber fruit stored at 13 ^{o}C under normoxia (21 kPa O_2) or hyperoxia (40 kPa O_2) ± ethylene (10 µL·L^{-1}). Each point represents the mean of five fruit. Vertical bars represent LSD (α= 0.05).

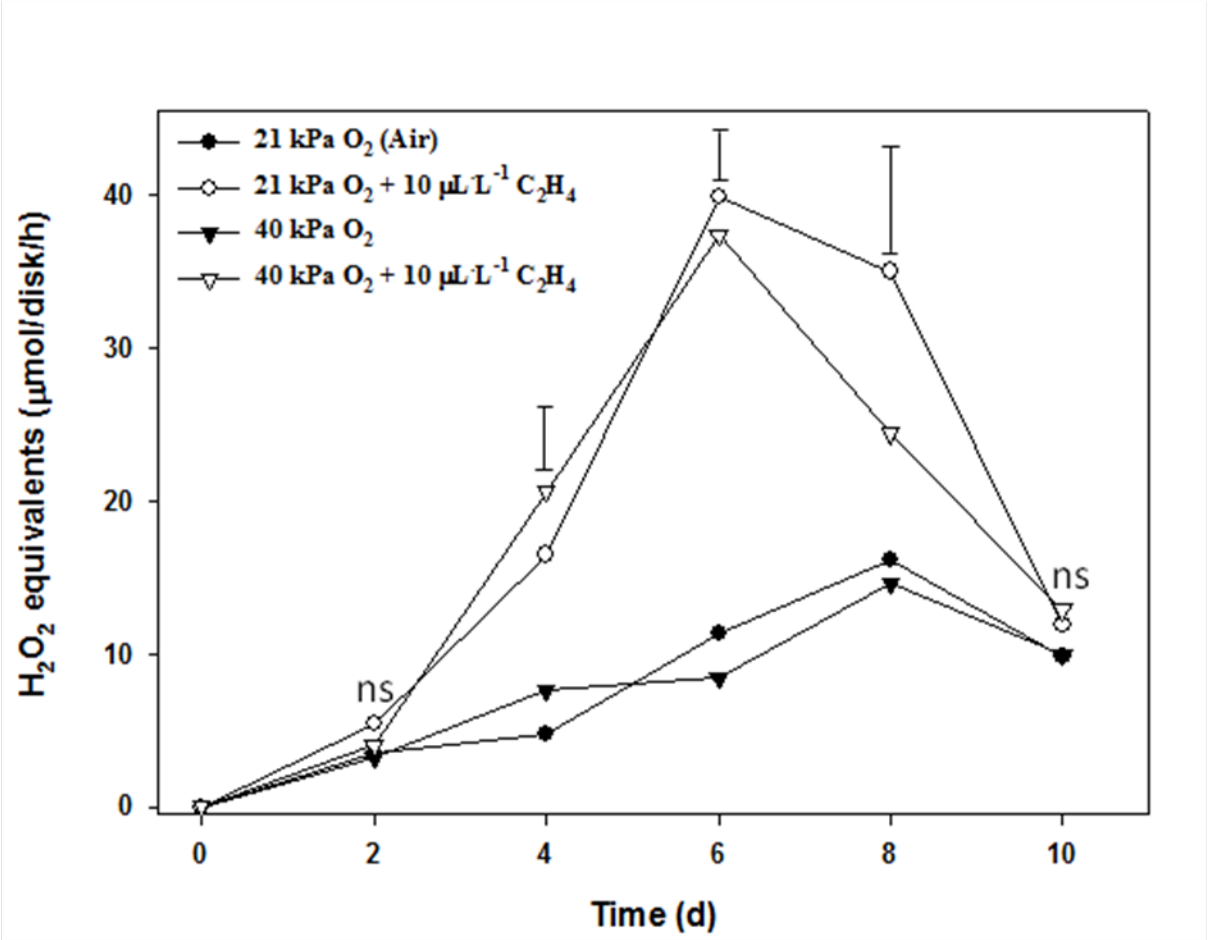


Figure 5-3. Total reactive oxygen species (ROS)-generating capacity of beit-alpha cucumber fruit stored at 13 ^{o}C under normoxia (21 kPa O_2) or hyperoxia (40 kPa O_2) ± ethylene (10 µL·L^{-1}). The production of total ROS was demonstrated using the oxidation of DCFH to DCF. Relative fluorescence at 520 nm was transformed into the production of H_2O_2 in µmoles per disk per h using a standard curve. Each point represents the mean of three fruit. Vertical bars represent LSD (α= 0.05).

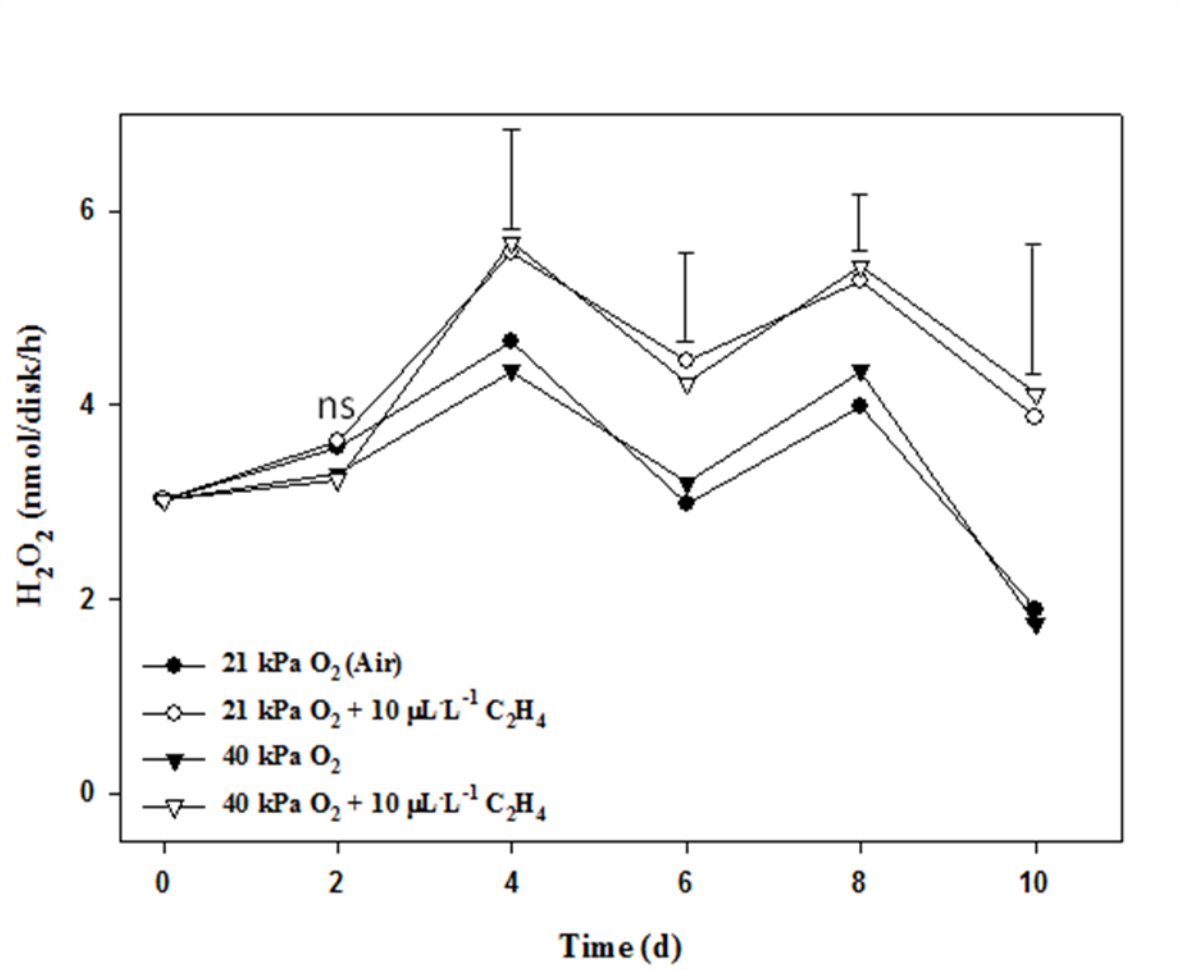


Figure 5-4. Hydrogen peroxide (H_2O_2)-generating capacity of beit-alpha cucumber fruit stored at 13 ^{o}C under normoxia (21 kPa O_2) or hyperoxia (40 kPa O_2) ± ethylene (10 µL·L^{-1}). The production of H_2O_2 was demonstrated using the oxidation of scopoletin. Relative fluorescence at 460 nm was transformed into the production of H_2O_2 in nmoles per disk per h using a standard curve. Each point represents the mean of three fruit. Vertical bars represent LSD (α= 0.05).

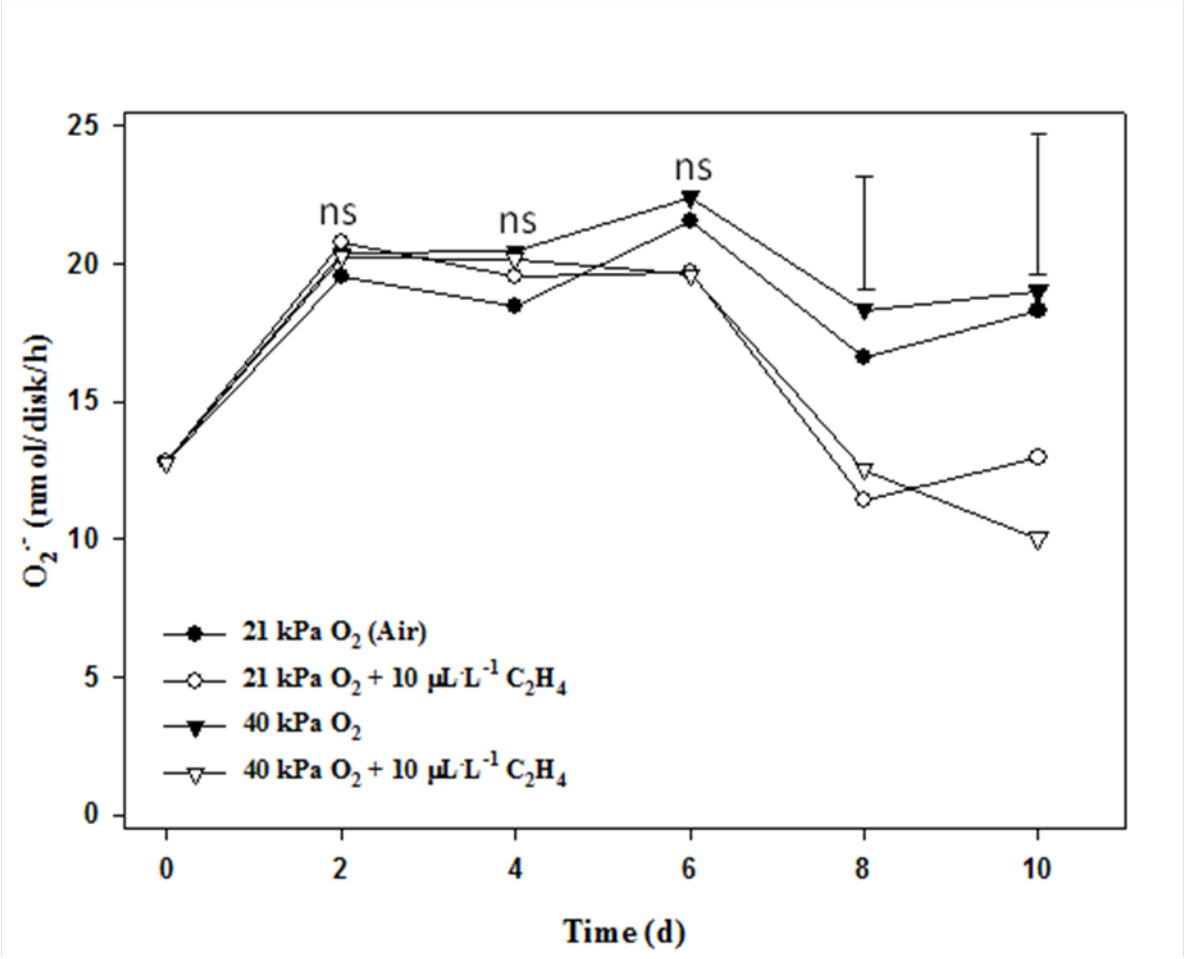


Figure 5-5. Superoxide anion ($O_2^{\cdot-}$)-generating capacity of beit-alpha cucumber fruit stored at 13 ^{o}C under normoxia (21 kPa O_2) or hyperoxia (40 kPa O_2) ± ethylene (10 µL·L^{-1}). The production of $O_2^{\cdot-}$ was demonstrated using the formazan formation of XTT. Absorbance at 490 nm was transformed into the production of $O_2^{\cdot-}$ in nmoles per disk per h. Each point represents the mean of three fruit. Vertical bars represent LSD (α= 0.05).

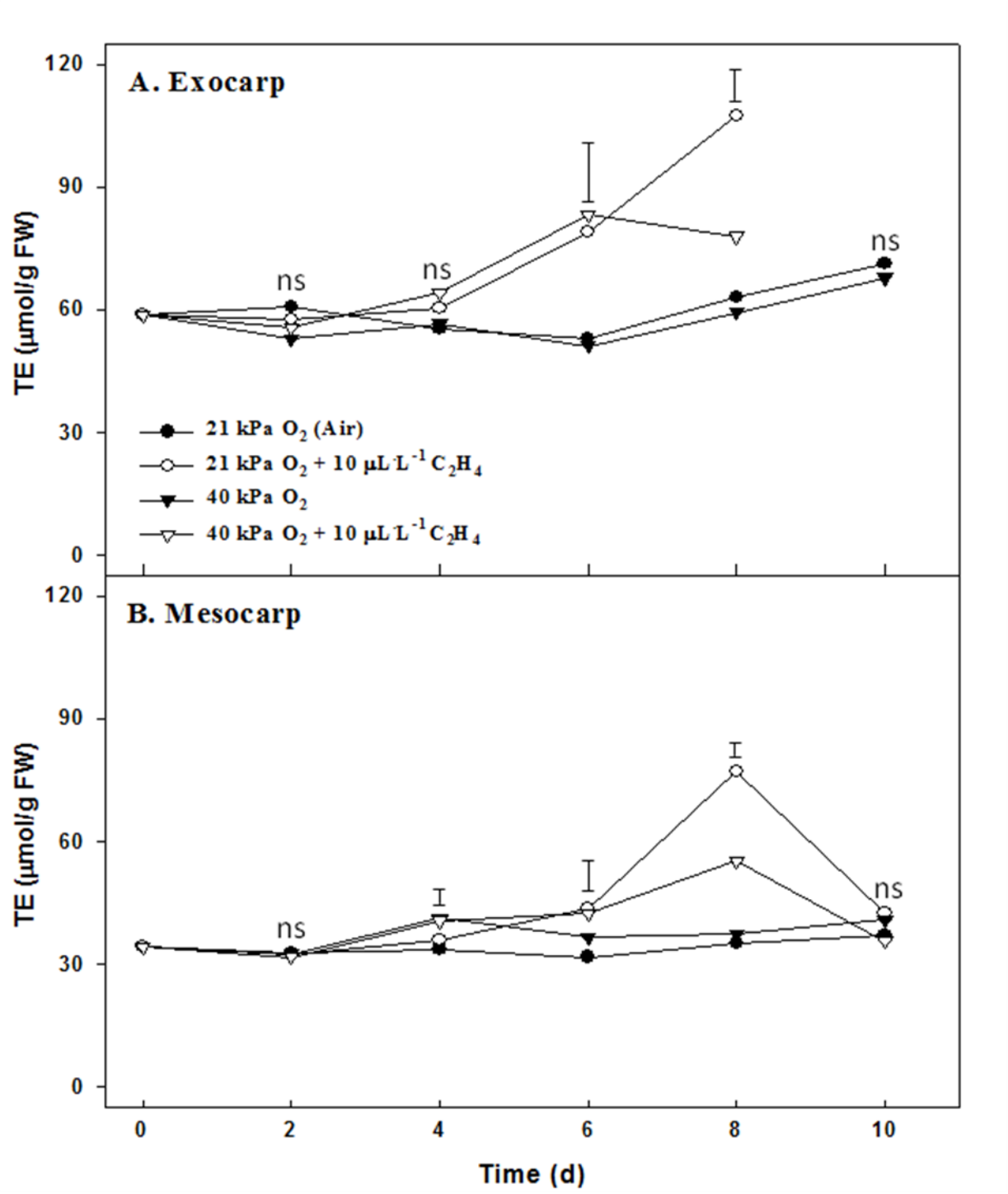
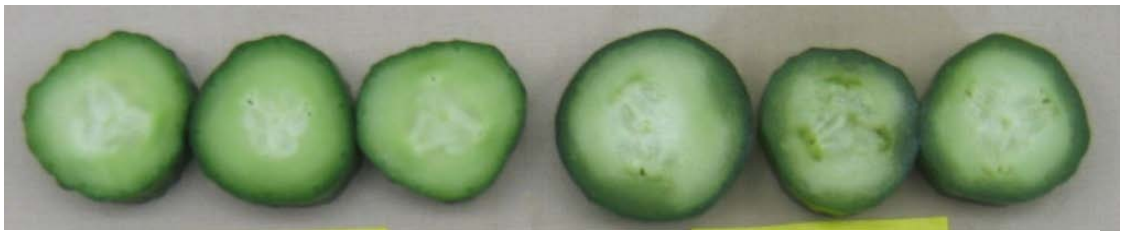
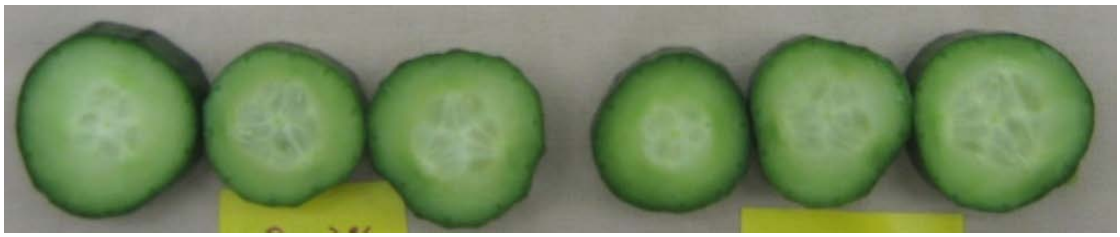


Figure 5-6. Antioxidant capacity expressed as TEs (µmol/g FW) of beit-alpha cucumber fruit during storage at 13 ^{o}C under normoxia (21 kPa O_2) or hyperoxia (40 kPa O_2) ± ethylene (10 µL·L^{-1}). A) Exocarp tissue. B) Mesocarp tissue. Each bar represents the mean of three fruit. Vertical bars represent LSD (α= 0.05).

A

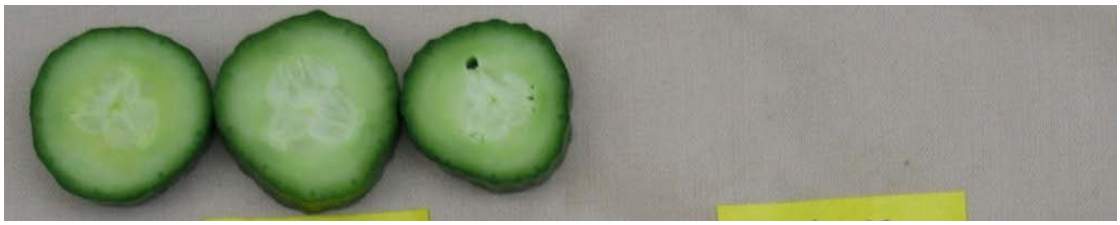


21 kPa O_2 (air) 21 kPa O_2 + 10 µL·L^{-1} C_2H_4

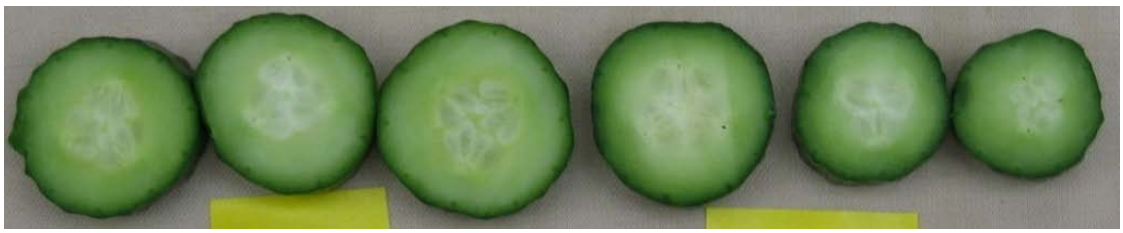


2 kPa O_2 2 kPa O_2 + 10 µL·L^{-1} C_2H_4

B



21 kPa O_2 (air)



2 kPa O_2 2 kPa O_2 + 10 µL·L^{-1} C_2H_4

Figure 5-7. Watersoaking development of beit-alpha cucumber fruit during storage at 13 ^{o}C under normoxia (21 kPa O_2) or hypoxia (2 kPa O_2) ± ethylene (10 µL·L^{-1}). A) At 8 d. B) At 14 d.

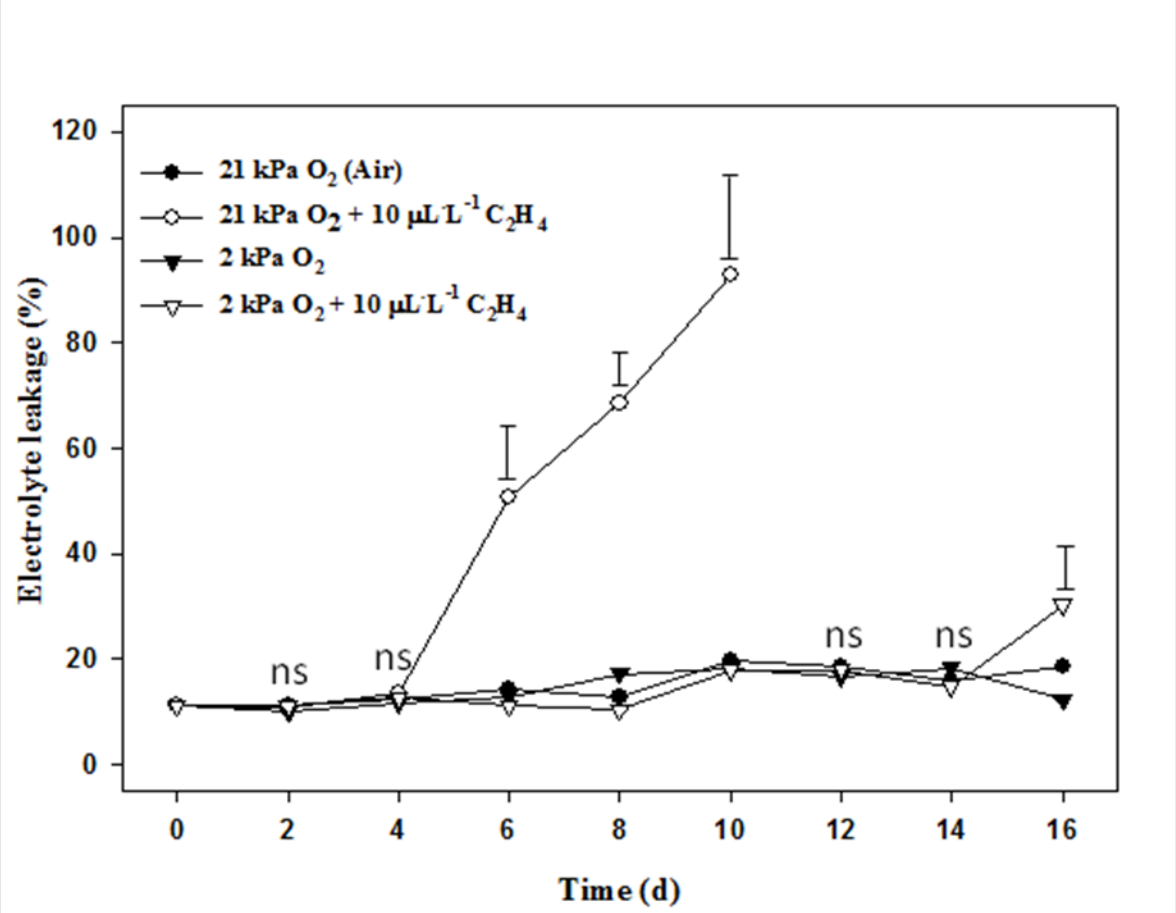


Figure 5-8. Electrolyte leakage of beit-alpha cucumber fruit during storage at 13 ^{o}C under normoxia (21 kPa O_2) or hypoxia (2 kPa O_2) ± ethylene (10 µL·L^{-1}). Each point represents the mean of five fruit. Vertical bars represent LSD (α= 0.05).

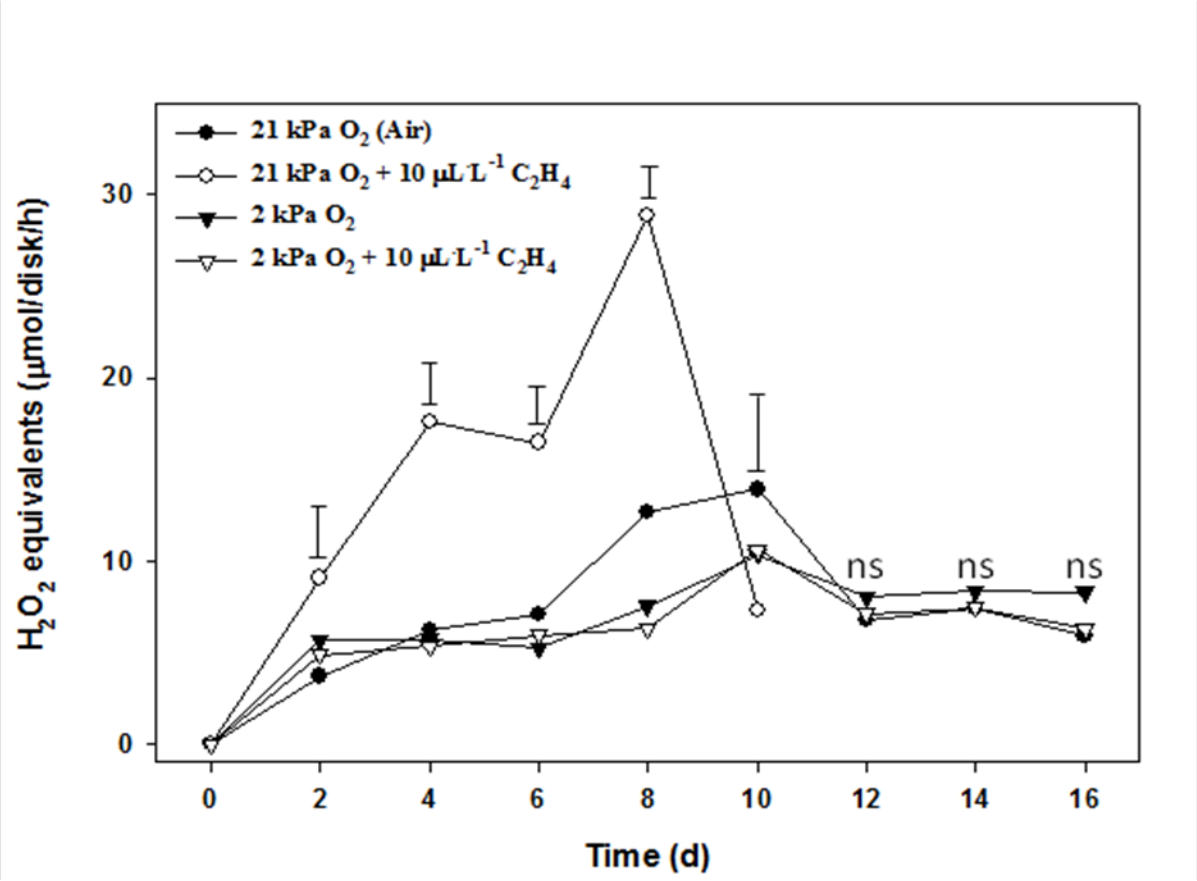


Figure 5-9. Total reactive oxygen species (ROS)-generating capacity of beit-alpha cucumber fruit stored at 13 ^{o}C under normoxia (21 kPa O_2) or hypoxia (2 kPa O_2) ± ethylene (10 µL·L^{-1}). The production of total ROS was demonstrated using the oxidation of DCFH to DCF. Relative fluorescence at 520 nm was transformed into the production of H_2O_2 in µmoles per disk per h using a standard curve. Each point represents the mean of three fruit. Vertical bars represent LSD (α= 0.05).

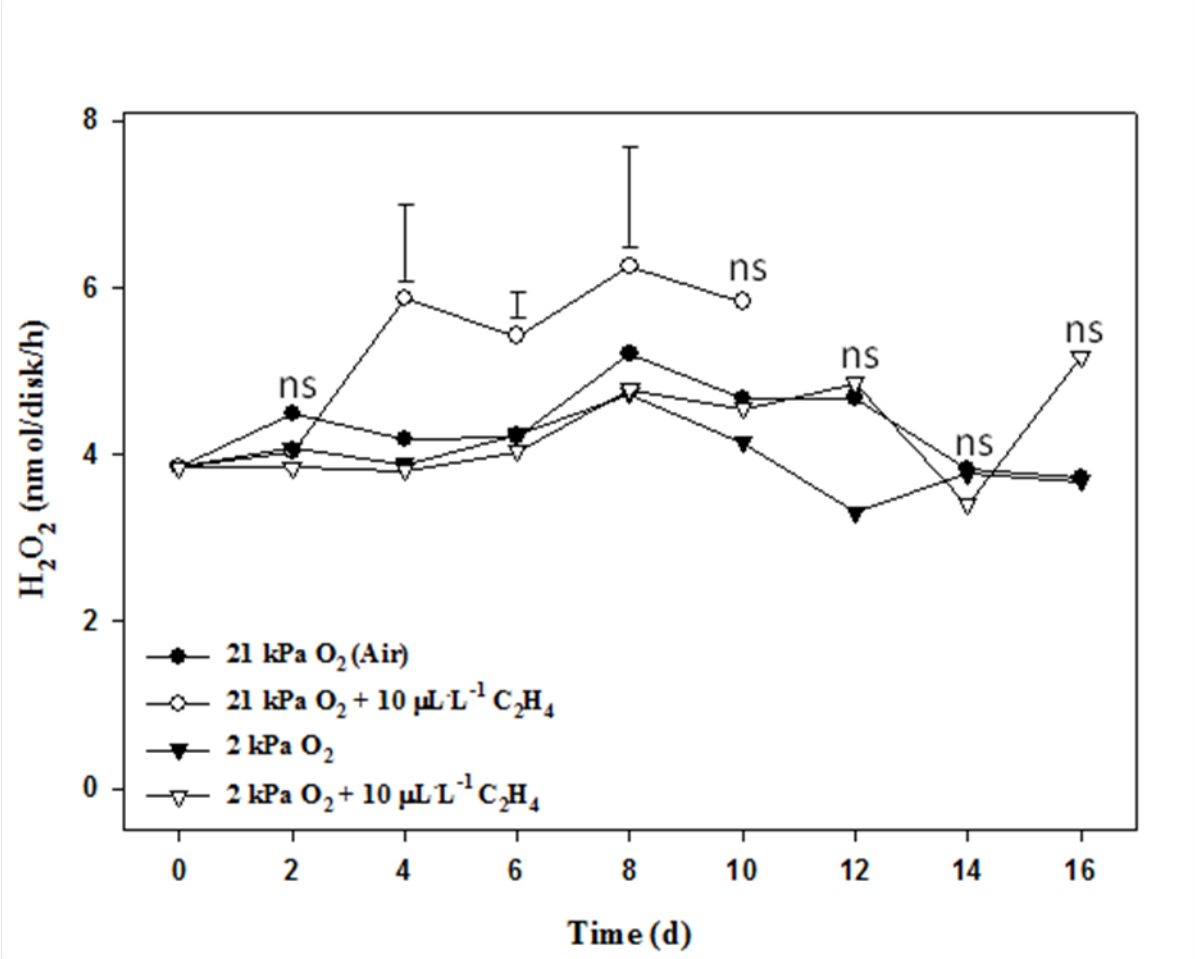


Figure 5-10. Hydrogen peroxide (H_2O_2)-generating capacity of beit-alpha cucumber fruit stored at 13 ^{o}C under normoxia (21 kPa O_2) or hypoxia (2 kPa O_2) ± ethylene (10 µL·L^{-1}). The production of H_2O_2 was demonstrated using the oxidation of scopoletin. Relative fluorescence at 460 nm was transformed into the production of H_2O_2 in nmoles per disk per h using a standard curve. Each point represents the mean of three fruit. Vertical bars represent LSD (α= 0.05).

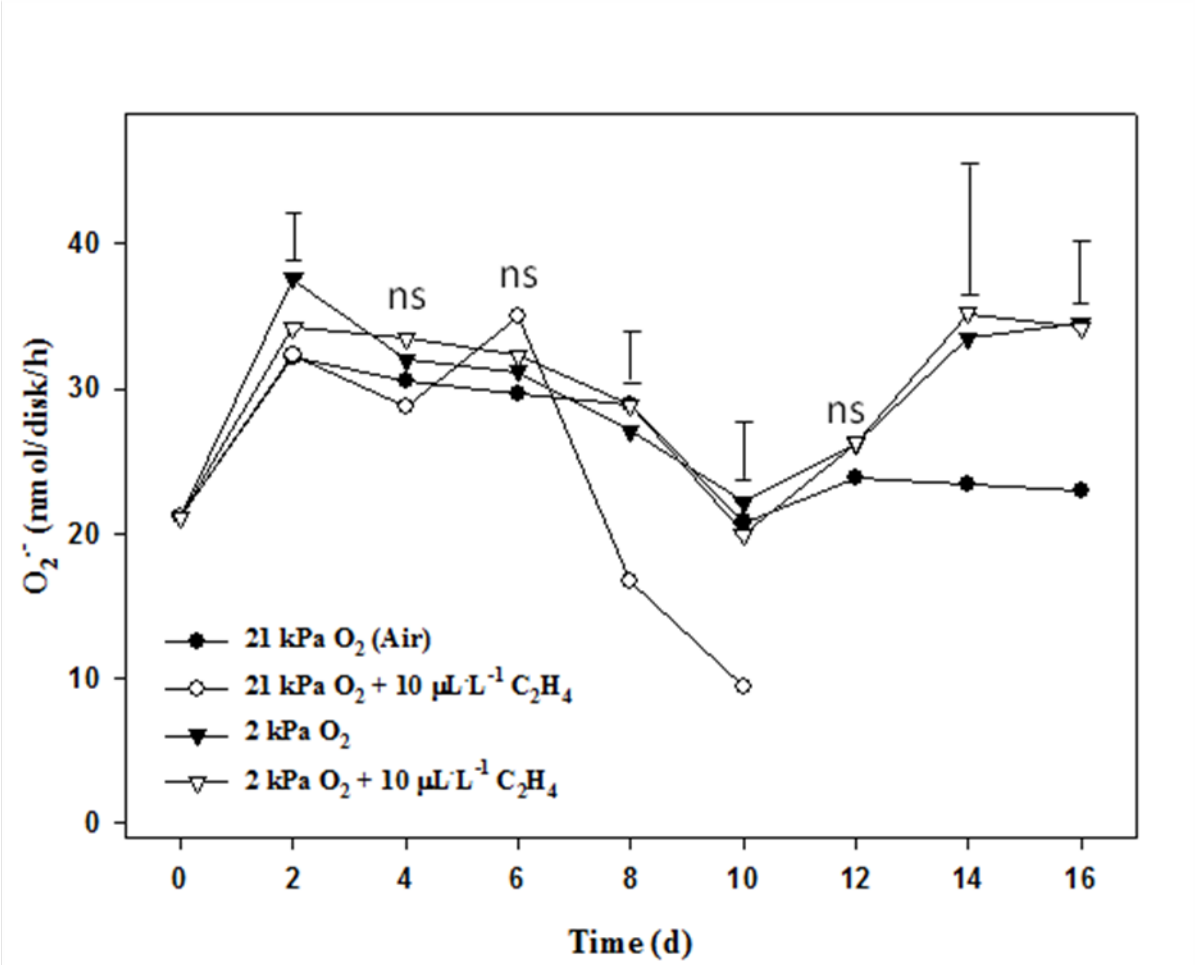


Figure 5-11. Superoxide anion ($O_2^{\cdot-}$)-generating capacity of beit-alpha cucumber fruit stored at 13 ^{o}C under normoxia (21 kPa O_2) or hypoxia (2 kPa O_2) ± ethylene (10 µL·L^{-1}). The production of $O_2^{\cdot-}$ was demonstrated using the formazan formation of XTT. Absorbance at 490 nm was transformed into the production of $O_2^{\cdot-}$ in nmoles per disk per h. Each point represents the mean of three fruit. Vertical bars represent LSD (α= 0.05).

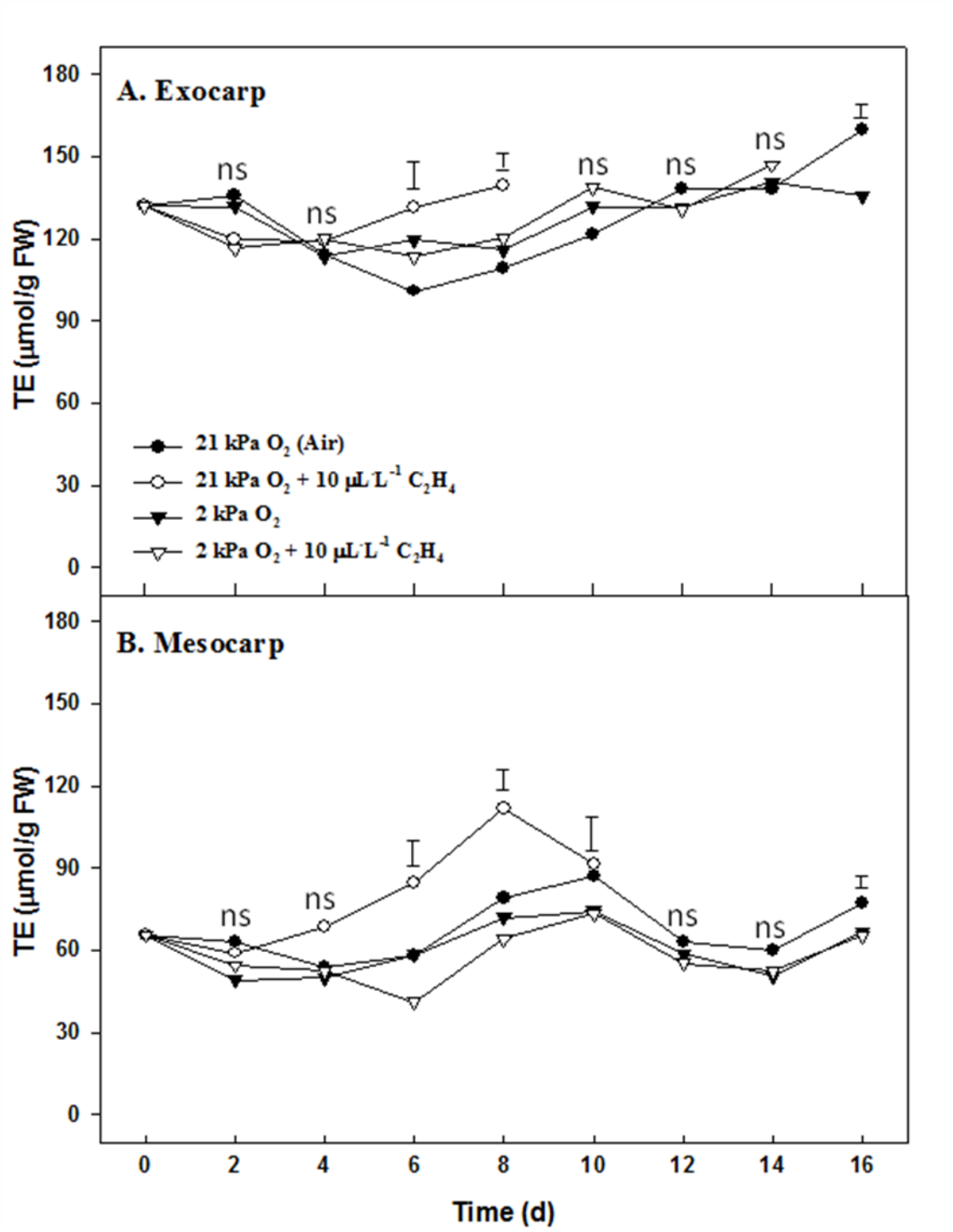
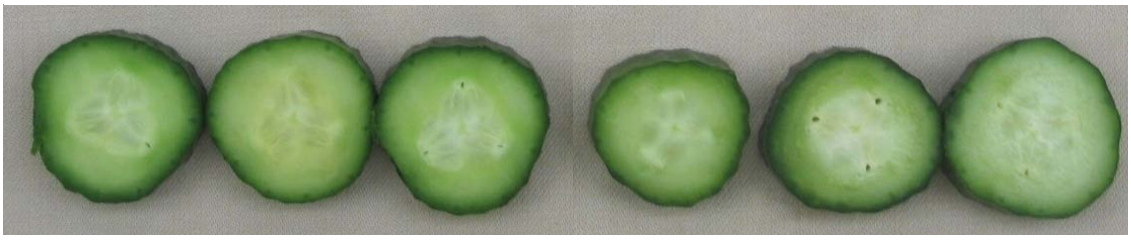


Figure 5-12. Antioxidant capacity expressed as TEs (µmol/g FW) of beit-alpha cucumber fruit during storage at 13 ^{o}C under normoxia (21 kPa O_2) or hypoxia (2 kPa O_2) ± ethylene (10 µL·L^{-1}). A) Exocarp tissue. B) Mesocarp tissue. Each point is the mean of three fruit. Vertical bar represents LSD (α= 0.05).

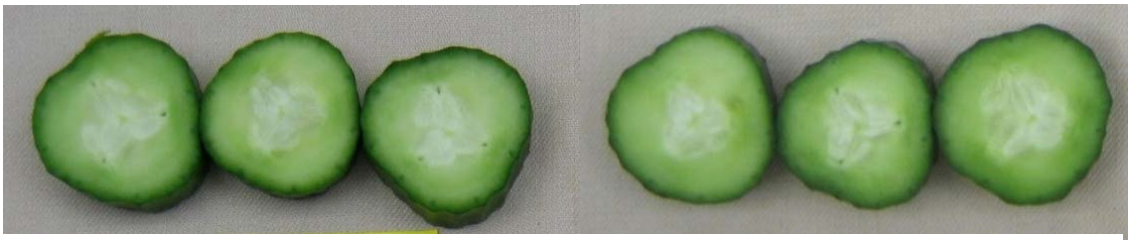
A

21 kPa O_2 (Air) 2 kPa O_2 (LO)

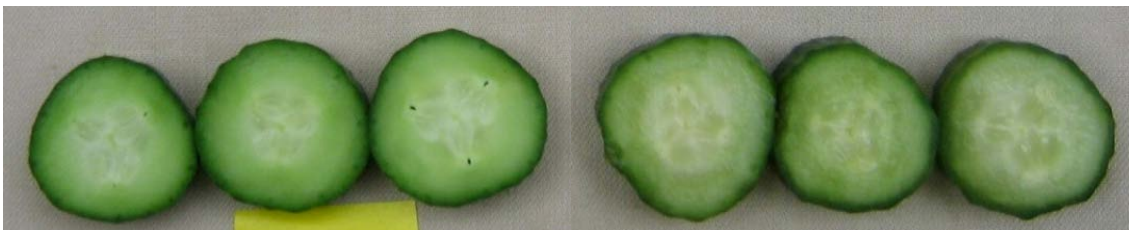


LO (8 d) + Air LO (8 d) + 10 $\mu L \cdot L^{-1}$ C_2H_4

B



21 kPa O_2 (Air) 2 kPa O_2 (LO)



LO (8 d) + Air LO (8 d) + 10 $\mu L \cdot L^{-1}$ C_2H_4

Figure 5-13. Watersoaking development of beit-alpha cucumber fruit stored at 13 ^{o}C. A) At 14 d. B) At 16 d. Fruit were treated with continuous air (21 kPa O_2), hypoxia (2 kPa O_2), or transferred to air ± 10 $\mu L \cdot L^{-1}$ ethylene after storage under hypoxia (2 kPa O_2) for 8 d. LO stands for preconditioning with low oxygen condition (hypoxic condition).

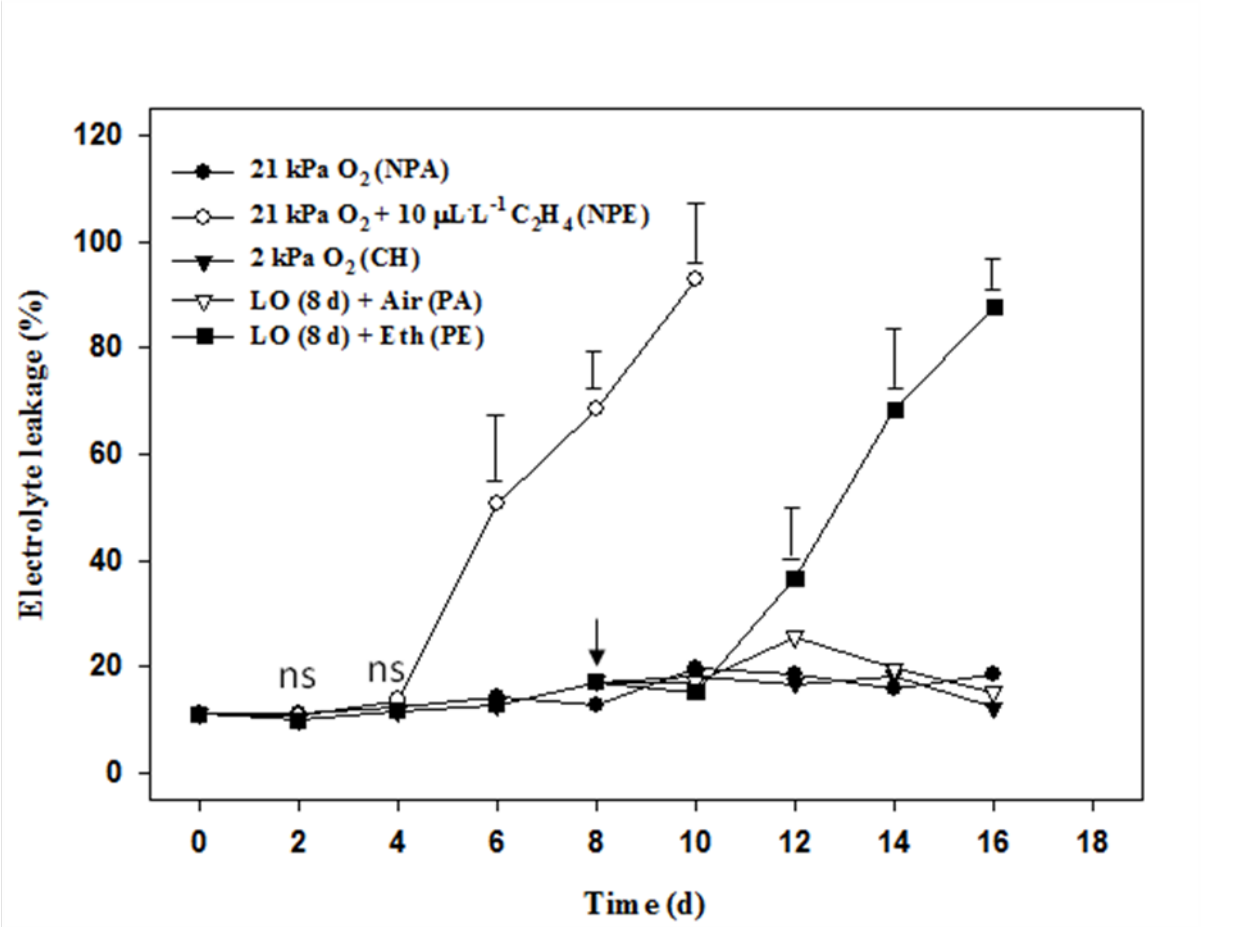


Figure 5-14. Electrolyte leakage of beit-alpha cucumber fruit stored at 13 ^{o}C. Fruit were treated with continuous air (21 kPa O_2) ± 10 µL·L^{-1} ethylene, hypoxia (2 kPa O_2), or transferred to air ± 10 µL·L^{-1} ethylene after storage under hypoxia (2 kPa O_2) for 8 d (arrow). LO stands for preconditioning with low oxygen condition (hypoxic condition). Each point represents the mean of three fruit. Vertical bars represent LSD (α= 0.05).

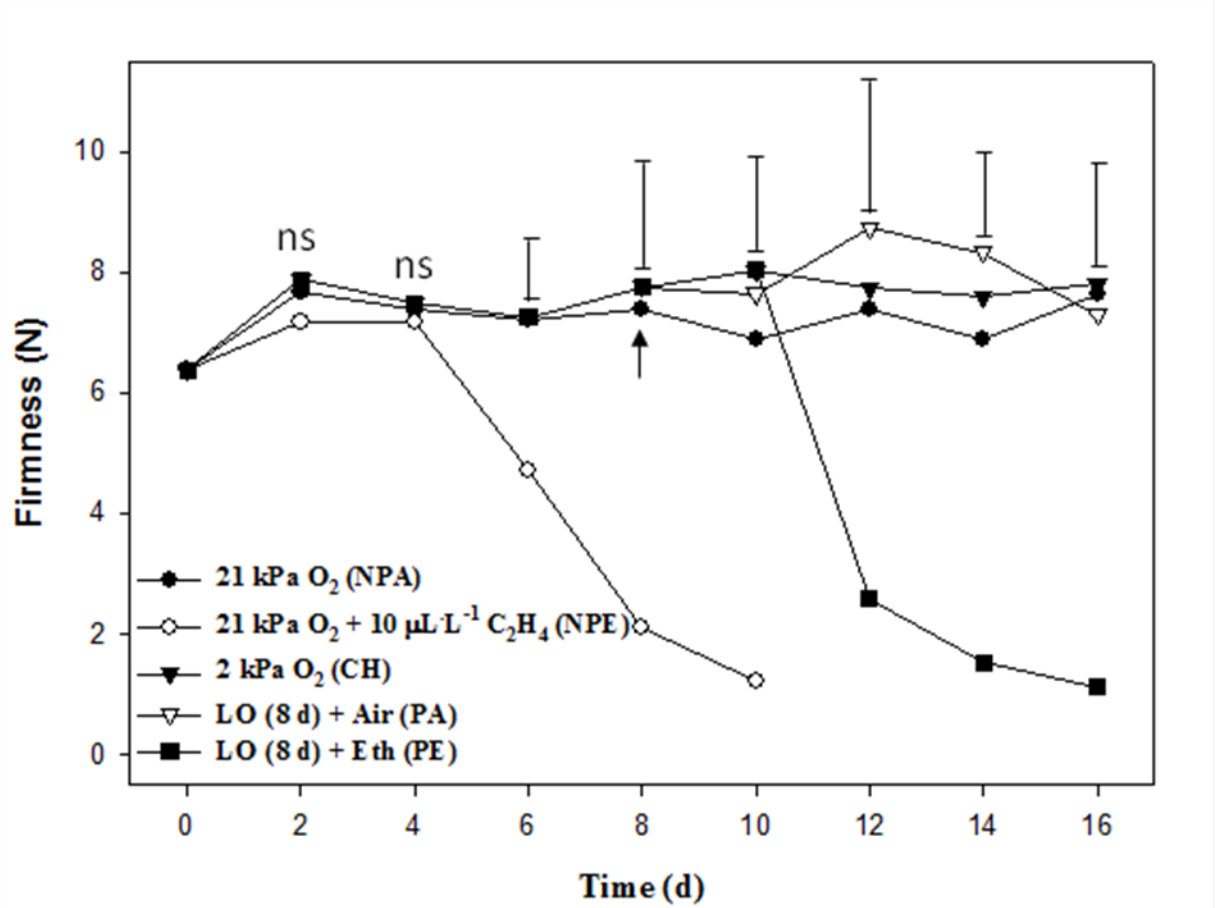


Figure 5-15. Mesocarp firmness of beit-alpha cucumber fruit stored at 13 ^{o}C. Fruit were treated with continuous air (21 kPa O_2) ± 10 µL·L^{-1} ethylene, hypoxia (2 kPa O_2), or transferred to air ± 10 µL·L^{-1} ethylene after storage under hypoxia (2 kPa O_2) for 8 d (arrow). LO stands for preconditioning with low oxygen condition (hypoxic condition). Each point represents the mean of 9 measurements (3 fruit, 3 measurements per fruit). Vertical bars represent LSD (α= 0.05).

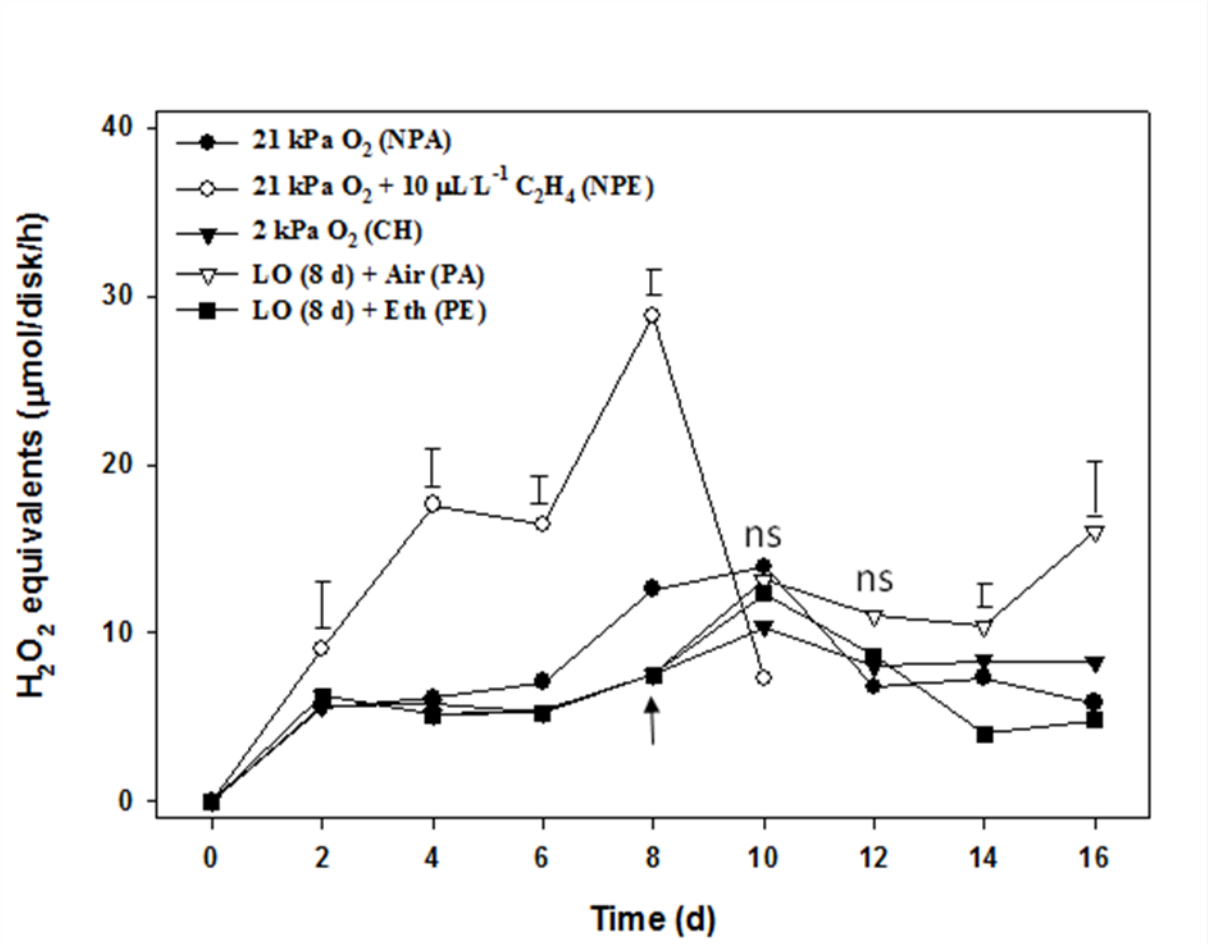


Figure 5-16. Total reactive oxygen species (ROS)-generating capacity of beit-alpha cucumber fruit stored at 13 ^{o}C. Fruit were treated with continuous air (21 kPa O_2) ± 10 µL·L^{-1} ethylene, hypoxia (2 kPa O_2), or transferred to air ± 10 µL·L^{-1} ethylene after storage under hypoxia (2 kPa O_2) for 8 d (arrow). LO stands for preconditioning with low oxygen condition (hypoxic condition). The production of total ROS was demonstrated using the oxidation of DCFH to DCF. Relative fluorescence at 520 nm was transformed into the production of H_2O_2 in µmoles per disk per h using a standard curve. Each point represents the mean of three fruit. Vertical bars represent LSD (α= 0.05).

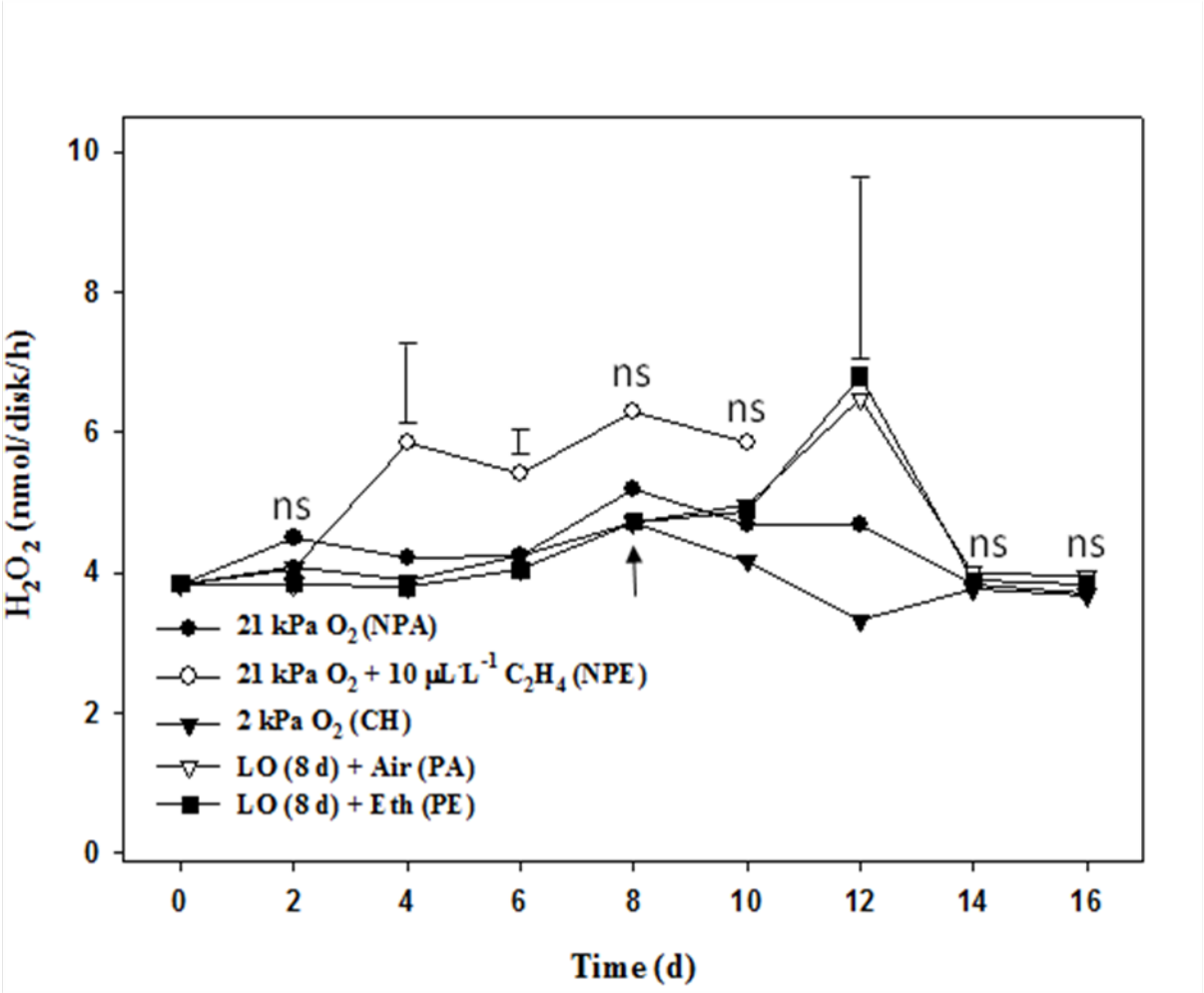


Figure 5-17. Hydrogen peroxide (H_2O_2)-generating capacity of beit-alpha cucumber fruit stored at 13 ^{o}C. Fruit was treated with continuous air (21 kPa O_2) ± 10 µL·L^{-1} ethylene, hypoxia (2 kPa O_2), or transferred to air ± 10 µL·L^{-1} ethylene after storage under hypoxia (2 kPa O_2) for 8 d (arrow). LO stands for preconditioning with low oxygen condition (hypoxic condition). The production of H_2O_2 was demonstrated using the oxidation of scopoletin. Relative fluorescence at 460 nm was transformed into the production of H_2O_2 in nmoles per disk per h using a standard curve. Each point represents the mean of three fruit. Vertical bars represent LSD (α= 0.05).

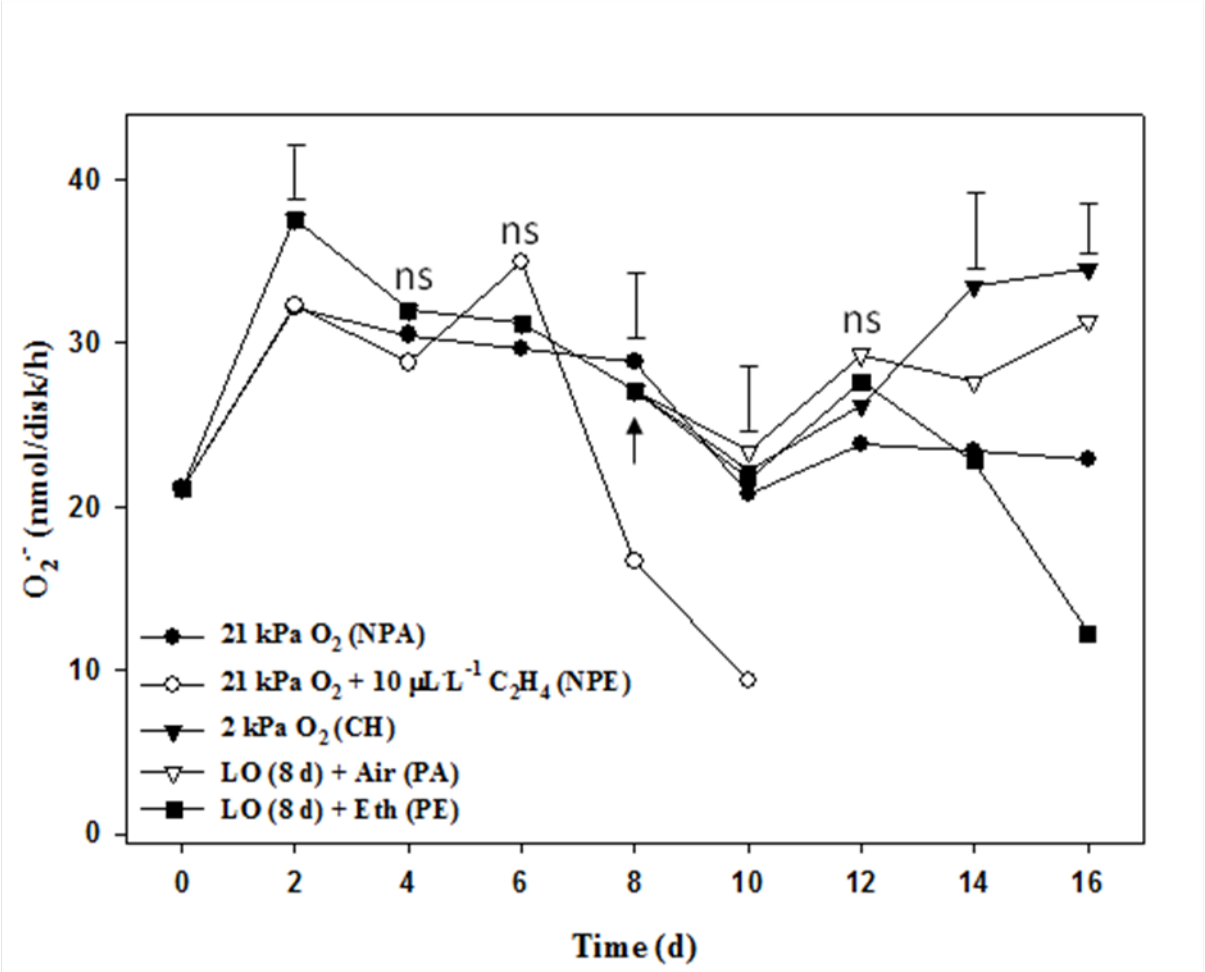


Figure 5-18. Superoxide anion ($O_2^{\cdot-}$)-generating capacity of beit-alpha cucumber fruit stored at 13 ^{o}C. Fruit was treated with continuous air (21 kPa O_2) ± 10 µL·L^{-1} ethylene and hypoxia (2 kPa O_2) or transferred to air ± 10 µL·L^{-1} ethylene after storage under hypoxia (2 kPa O_2) for 8 d (arrow). LO stands for preconditioning with low oxygen condition (hypoxic condition). The production of $O_2^{\cdot-}$ was demonstrated using the formazan formation of XTT. Abserbance at 490 nm was transformed into the production of $O_2^{\cdot-}$ in nmoles per disk per h. Each point represents the mean of three fruit. Vertical bars represent LSD (α= 0.05).

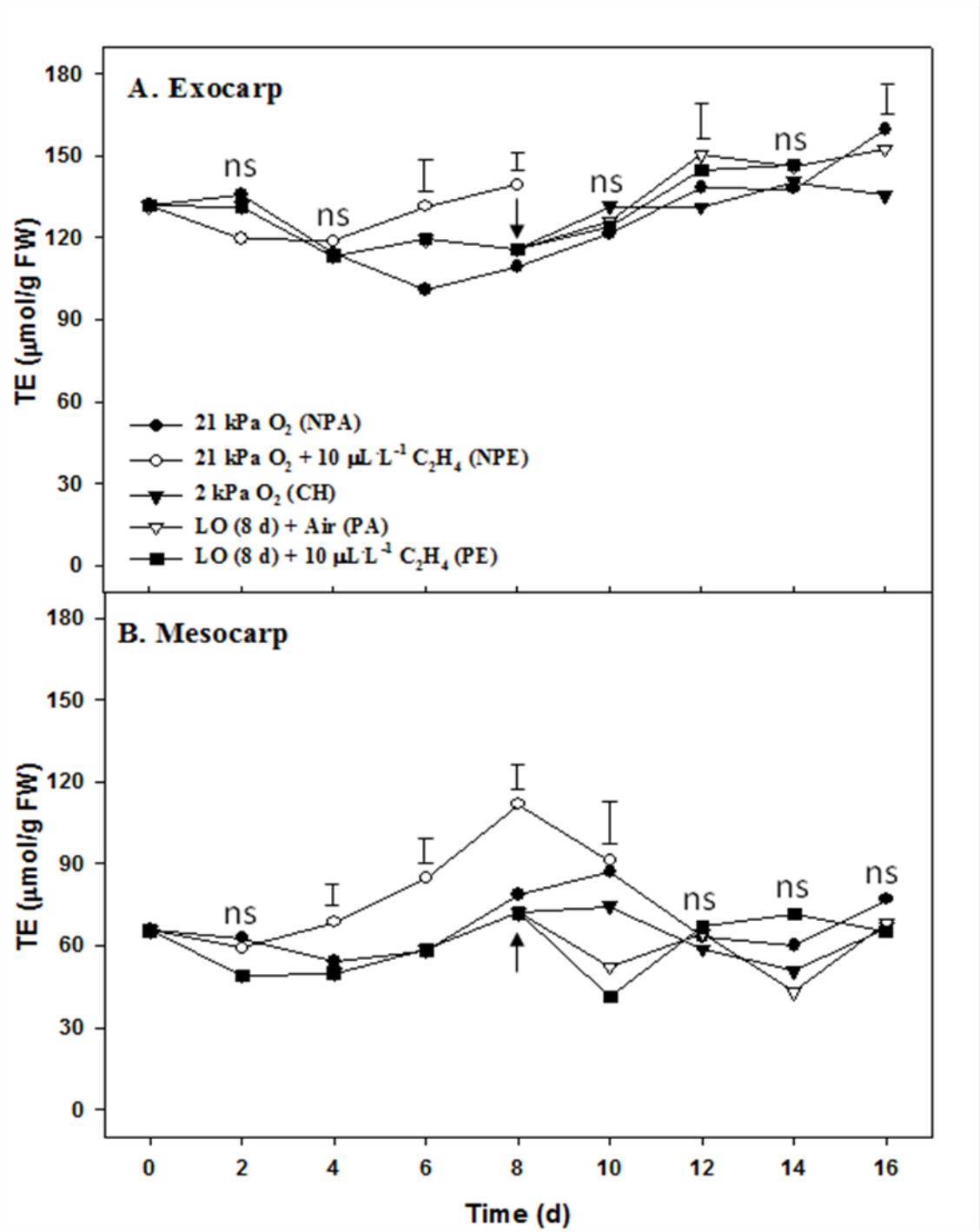


Figure 5-19. Antioxidant capacity expressed as TEs (µmol/g FW) of beit-alpha cucumber fruit during storage at 13 ^{o}C. A) Exocarp tissue. B) Mesocarp tissue. Fruit was treated with continuous air (21 kPa O_2) ± 10 µL·L^{-1} ethylene, hypoxia (2 kPa O_2), or transferred to air ± 10 µL·L^{-1} ethylene after storage under hypoxia (2 kPa O_2) for 8 d (arrow). LO stands for preconditioning with low oxygen condition (hypoxic condition). Each point represents the mean of three fruit. Vertical bars represent LSD (α= 0.05).

CHAPTER 6
THE EFFECT OF EXOGENOUS ETHYLENE ON ETHYLENE RECEPTOR TRANSCRIPTS OF IMMATURE BEIT-ALPHA CUCUMBER FRUIT

Harvested horticultural crops are highly perishable, showing physical and physiological disorders and quality losses during postharvest handling. Crop deterioration occurs in response to injurious temperature, atmospheric composition (mainly O_2, CO_2, and ethylene), air pressure, and pathogens (Kader, 2002; Kader, 2003; Burg, 2004a). Watersoaking, the appearance of tissue translucency, vitrescence, or glassiness, is one of the common physiological disorders observed in several commodities. Characteristics of water-soaked tissues are acute softening, enhanced electrolyte efflux, loss of flavor, and cell wall disassembly (Bauchot et al., 1999; Karakurt and Huber, 2002; Jeong et al., 2004; Lima et al., 2005; Mao et al., 2004; Nishizawa et al., 2002). Watersoaking was observed in fresh-cut tissues of kiwi (Agar et al., 1999), tomato (Jeong et al., 2004), 'Galia' melons (Ergun et al., 2007) and pineapple fruit (Montero-Calderon et al., 2008) and ethylene-treated daffodil flowers (Hunter et al., 2004), cucumber (Lima et al., 2005; Hurr et atl., 2009) and watermelon fruit (Karakurt and Huber, 2002; Mao et al., 2004). Chilling temperatures have also been reported to cause this disorder in peach (Fernández-Trujillo and Artés, 1998), papaya (Karakurt and Huber, 2003), cucumber fruit (Fernandez and Martinez, 2006), and green beans (Cho et al., 2008).

Watersoaking is a characteristic ethylene response observed in immature cucumber fruit, not paralleling normal senescence (Hurr et al., 2009). Application of 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception (Serek et al., 1994; Sisler and Serek 1997), inhibited watersoaking development of immature cucumber fruit, confirming the involvement of ethylene in the disorder (Lima et al., 2005). In

ethylene-treated cucumber fruit, incipient watersoaking is first evident in hypodermal (outlayer of mesocarp tissues) tissues and then progresses into inner mesocarp tissues (Chapter 3). This spatial pattern was same in both intact fruit and fresh-cut slices (Chapter 4), indicating that watersoaking is tissue-specific ethylene response. In addition, Chapter 5 revealed that hyperoxia (40 kPa O_2) alone did not induce watersoaking in immature cucumber fruit while watersoaking was accelerated by the combination of ethylene and hyperoxia.

Ethylene is a gaseous phytohormone influencing diverse aspects of plant biology including seed germination, root initiation, abscission, fruit ripening, sex determination, and senescence (Abeles et al., 1992; Kieber, 1997; Lin et al., 2009). The effect of ethylene is influenced by development stage, and ethylene concentration and exposure duration (Abeles et al., 1992; Saltveit, 1999; Hurr et al., 2009; Chapter 3). Ethylene responses rely on ethylene sensitivity (Saltveit, 1999). Several studies suggested that ethylene sensitivities are dependent on transcript abundance of the receptor gene family (Tieman et al., 2000; Cancel and Larsen, 2002; Hall and Bleecker, 2003). Ethylene action initiates with binding of ethylene to a family of endoplasmic reticulum (ER)-associated receptors such as ETR1- and ETR2-like families, which results in signal transduction leading to activation of transcription factors and ethylene-responsive genes (Bleecker et al., 1998; Klee 2002; Chang, 2003; Hall et al., 2007). Since ethylene receptors function as negative regulators, there is an inverse correlation between receptor levels and ethylene sensitivity; an increase in the level of receptor expression causes an increase in the threshold for initiating an ethylene response, allowing decreased sensitivity (Klee, 2004).

The ethylene receptors are differently regulated by ethylene. For example, transcription of receptors ERS1, ERS2, and ETR2 was regulated by ethylene itself in Arabidopsis (Hua and Meyerowitz., 1998). Wilkinson et al. (1995) also reported that NR mRNA is positively regulated by ethylene in a development-specific manner. The effect of different expression patterns on ethylene sensitivity could be explained by different contributions of different receptor genes to ethylene signaling. Ethylene receptors are controlled at transcriptional and post-transcriptional levels (Chen et al., 2007; Kevany et al., 2007). CTR (a Raf-like kinase activated by receptors) and EIN3 (transcriptional factor) are mainly regulated at post-transcriptional level (Gao et al., 2003; Guo and Ecker, 2003; Yanagisawa et al., 2003; Chen et al., 2005). At the transcriptional level, ethylene receptors are an important component in ethylene signal transduction.

The present study was designed to investigate the influence of ethylene exposure on expression of ethylene receptor genes in immature cucumber fruit. This study will help to elucidate the underlying molecular mechanisms of watersoaking and provide strategies to prevent the disorder.

Materials and Methods

Plant Materials

Experiments were conducted with beit-alpha cucumber (*Cucumis Sativus* L.; 'Manar') harvested at immature stage (average fruit wt. 86 ± 3.2 g) from a commercial greenhouse facility in Live oak, FL. Freshly harvested fruit were sorted by size, color and appearance, sanitized with 2.7 mM sodium hypochlorite, and air-dried. Afterward, intact fruit (n=25 per container) were placed in 20-L plastic containers and provided with flow-through atmospheres of air ± 10 $\mu L \cdot L^{-1}$ of ethylene at 13 ^{o}C and 95% R.H. Flow rate

was maintained at 500 $mL \cdot min^{-1}$ to avoid CO_2 accumulation, and the gas mixture was humidified by passing it through a water-filled glass jar (2 L).

RNA Extraction and Reverse Transcription

At intervals during storage (12 h, and 1, 3, and 5 d), 5 fruit per treatment were removed from treatment containers. Mesocarp and exocarp tissues were separated and cut into small pieces (approximate 0.2 g). The tissues were frozen in liquid N_2 and stored at -70 ^{o}C. Total RNA was extracted as described in Vallejos et al. (2000). Approximately 3 g of frozen tissues (composites of tissue from 5 fruit per treatment) were ground in liquid N_2 and transferred to 12 mL of extraction buffer containing 0.125 M tris (pH 7.6), 0.5 M NaCl, 6.25 mM EDTA, 2.5% sarkosyl, and 1% β-mercaptoethanol. After vortexing for 30 sec, 7.5 mL phenol/chloroform [4:1 (v/v)] was added and the samples centrifuged at 8000 x *g* for 20 min at 4 ^{o}C. The aqueous phase was re-extracted with an equal volume of chloroform/octanol [24:1 (v/v)] and centrifuged again at 8000 x *g* (4 ^{o}C, 20 min). The aqueous phase (about 10 mL) was precipitated with 1/5 vol of 12 M LiCl over night at -20 ^{o}C. After 20 min centrifugation at 8000 x *g* at 4^{o}C, the pelleted RNA was gently dispersed in 25 mL of 2 M LiCl, and then layered on top of 5 mL of 4 M LiCl. After centrifugation at 8000 x *g* (4 ^{o}C for 20 min), the pellet was resuspended in 9 mL of TE buffer (10 mM of Tris, pH 7.6, 1 mM of EDTA, pH 8.0), 1 mL of 3 M NaOAc, pH 4.5, and 25 mL of 99% EtOH at -20 ^{o}C for 2 h. The pellet recovered after centrifugation at 12000 x *g* (4 ^{o}C, 20 min) was purified by incubation with 700 µL of DEPC-treated H_2O, 600 µL of isopropanol (99.5%) and 70 µL of 3M NaOAc at -80 ^{o}C for 1 h. After centrifugation at 10500 x *g* (4 ^{o}C, 25 min), the pellet was washed with 250 µL of 75% EtOH and dried in vacuum for 20 min.

Subsequent DNase treatment was conducted following manufacturer's instructions (Promega), followed by purification with phenol/chloroform [4:1(v/v)] and 70% EtOH. RNA concentration was quantified spectrophotometrically at 260 nm. To check RNA quality and quantity (1 µg), RNA samples were electrophoresed on ethidium bromide-stained 1% agarose gels (Dal Cin et al., 2005).

cDNA was synthesized as described in Dal Cin et al. (2005). DNA-free RNA (1 µg) was reverse transcribed in RT-PCR mix containing 1 unit of RNase inhibitor, 200 units of MMLV reverse transcriptase, 2.5 µM of oligo dT_{18} primer in a final volume of 10 mL. The reaction was conducted at 37 ^{o}C for 90 min, 85 ^{o}C for 5 min, and then cooled to 10 ^{o}C. cDNA samples were stored at -80^{o}C.

Semi-Quantitative RT-PCR

Expression level of ethylene receptors was examined using semi-quantitative RT-PCR as described in Dal Cin et al. (2005). Gene-specific PCR was conducted using 1 µL of cDNA as a template in PCR mix (20 µL of total volume) containing 2 µL of 10X gold buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 15 pmol primer set, and 0.4 unit of Gold Taq DNA polymerase. Gene specific primers were designed from non-conserved sequence regions of Cs-ETR1 (AB026498), Cs-ETR2 (AB026500), and Cs-ERS (AB026499) genes using primer 3 software (http://www.genome.wi.mit.edu). Alignment of each primer was performed using Blast program (http://www.ncbi.nlm.nih.gov) to confirm its specificity. Genomic DNA contamination was controlled by designing primers on exon regions spanning at least one intron. Transcript accumulation of 18S ribosomal RNA (rRNA) gene (AF206894) was examined as an internal control. Selection of a proper internal control is a critical step since internal control is used to control experimental errors and to normalize RT-PCR data. Ideal internal control gene should be expressed

at a constant level in all samples and not affected by experimental conditions (Thellin et al., 1999). The suitable reference genes differ depending on species, tissue types, and developmental stages (Brunner et al., 2004; Czechowski et al., 2005). Most frequently used housekeeping genes in plants and animals include 18S rRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ubiquitin (UBQ), and actin (ACT) genes (Goidin et al., 2001; Kim et al., 2003; Brunner et al., 2004; Dheda et al., 2004). In the present study, the stability of expression level of 18S rRNA (AF206894) and ubiquitin (AF104391) genes were tested in the set of cucumber samples. Since 18S rRNA exhibited more stable expression than ubiquitin, 18S r RNA was used as internal control. Gene specific primers are as follows: 18S-F, 5'-CGGAGAGGGAGCCTGAGAA-3'; 18S-R, 5'-CCCGTGTTAGGATTGGGTAATTT-3'; ERS-F, 5'-CCCTACTGAATTCTATCCAATGC-3'; ERS-R, 5'- AAGTCCCACGCCACTATTTG-3'; ETR1-F, 5'- GTACATCTTGGATGCGAAGTA-3'; ETR1-R, 5'-GACGCTCTATAAGTTCCGAC-3'; ETR2-F, 5'-GGATTTACACGAAGCATGG-3'; ETR2-R, 5'- CAATCTGCACGCATCTCTC-3'. PCR was performed under the following conditions, step 1: denaturization at 95 ^{o}C for 5 min, step 2: denaturization at 95 °C for 30 s, annealing at 58 °C (18S, ERS, ETR2) or 60 ^{o}C (ETR1) for 1 min, and extension at 72 °C for 30 s (33, 38, or 39 cycles for 18S, ETR1 and 2, or ERS, respectively), and step 3: extension at 72 ^{o}C for 7 min. The PCR products were run in 2% agarose gel containing ethidium bromide. DNA mass ladder (Invitrogen) was also run to confirm the expected molecular weight of the amplified products. The densitometry value of each target band on a 2 % agarose gel with ethidium bromide staining was measured via Image J software (http://rsbweb.nih.gov/ij/) three times independently to reduce

measuring error. Each gene transcript level was normalized with the 18 S (internal standard) signal. Transcript level of ethylene-treated fruit was expressed relatively to each value of air-treated fruit at selected examine day (12 h, and 1, 3, and 5 d). Value of initial day was set as 1. Three replicates of semi-quantitative RT-PCR were conducted for each gene. Average of normalized values of three independent PCR products per treatment was used to express the transcript level of each receptor gene.

Results

The changes in expression of three ethylene receptor genes (Cs-ERS, Cs-ETR1, and Cs-ETR2) in mesocarp and exocarp tissues from fruit treated with air or continuous ethylene (10 $\mu L \cdot L^{-1}$) were monitored during storage. In mesocarp tissue, Cs-ERS, Cs-ETR1, and Cs-ETR2 transcripts increased in response to ethylene (Fig. 6-1). Half day of continuous ethylene exposure enhanced Cs-ETR 2 transcript about 2-fold, whereas Cs-ERS and Cs-ETR1 were unaffected. At 1 d, a 4-fold increase in transcript level of Cs-ETR1 was noted. Cs-ERS transcript level was less affected by exogenous ethylene than were Cs-ETR1 and Cs-ETR2. At 5 d of continuous ethylene exposure, transcript levels of Cs-ERS, Cs-ETR1, and Cs-ETR2 genes decreased 75%, 15%, and 55%, respectively, relative to levels present in air-treated fruit.

Cs-ERS, Cs-ETR1, and Cs-ETR2 transcripts also increased in exocarp tissue following exposure of fruit to ethylene (Fig. 6-2). Cucumber fruit exposed to 10 $\mu L \cdot L^{-1}$ ethylene for half day (12 h) showed 100% and 50% increases in exocarp transcript levels of Cs-ERS and Cs-ETR 2, respectively, but no change in Cs-ETR1 transcript. The greatest ethylene enhancement of transcript level, a 4-fold increase in Cs-ERS and 2-fold increases in Cs-ETR1 and Cs-ETR2, was noted at 1 d. Exocarp tissues of ethylene-treated fruit had higher transcript level of ERS and ETR2 gene over control

during 5 d of storage, while Cs-ETR1 transcript level in ethylene-treated fruit was about 65% of that in air-treated fruit at 5 d.

Discussion

Although both genotype and environmental factors play a role in the development of watersoaking (Hiraishi, 1972), the cellular events leading to the disorder remain unknown. To investigate the role of ethylene receptors (Cs-ERS, Cs-ETR1, and Cs-ETR2) on watersoaking of beit-alpha cucumber fruit, semi-quantitative RT-PCR analysis was conducted. Results indicated that all three receptor genes are ethylene-inducible in both mesocarp and exocarp tissues. Cucumber fruit stored under continuous ethylene exposure exhibited the highest transcript level of receptor genes around 1 d while incipient watersoaking occurred at 6 d. This indicates that ethylene responses in cucumber fruit included transcriptional regulation of ethylene receptors. Transcriptional regulation at receptor level may possibly act as an important key in controlling ethylene responses in cucumber fruit. Rapid regulations in expression level of receptor genes as a response to ethylene exposure have been reported in other studies. Expression level of Rh-ETR1 and Rh-ETR3 in petals of cut rose was significantly increased in response to 10 $\mu L \cdot L^{-1}$ ethylene within 18 h and 6 h, respectively (Ma et al., 2006). In tomato fruit, ethylene exposure (10 $\mu L \cdot L^{-1}$) enhanced the accumulation of NR, Le-ETR4, and Le-ETR6 mRNA within 2 h, while expression level of these receptor genes was reduced to pre-treatment level within 24 h after discontinuing ethylene treatment (Kevany et al., 2007). Fa-ETR1, Fa-ETR2 and Fa-ERS1 in strawberry fruit were also ethylene-inducible (100 $\mu L \cdot L^{-1}$for 24 and/or 48 h) (Trainotti et al., 2005) and expression levels of Ad-ERS1a, Ad-ETR2, and Ad-ETR3 in kiwi fruit were up-regulated by exogenous ethylene (100 $\mu L \cdot L^{-1}$for 24 h) within 1 d (Yin et al., 2008). 1-MCP treatment (5 $\mu L \cdot L^{-1}$ for

24 h every other day) of avocado fruit reduced transcripts level of Pa-ERS1 whereas termination of 1-MCP treatment enhanced expression levels to those observed at the climacteric peak of control fruit (Owino et al., 2002).

In ethylene-treated cucumber fruit, different expression patterns of ethylene receptors were observed between mesocarp and exocarp tissues. Of three ethylene receptor genes, Cs-ETR1 in mesocarp tissue and Cs-ERS in exocarp tissue were the most significantly up-regulated in response to exogenous ethylene. Transcript levels of Cs-ERS were most abundant in shoot apices of cucumber plant, Cs-ERS gene was the most significantly up-regulated in response to ethrel treatment (Yamasaki et al., 2000). The response of Cs-ERS in both cucumber exocarp and shoot apices suggests that exocarp responses may be more vegetative in nature than those occurring in mesocarp tissue. This tissue-specific expression of receptor genes is parallel to previous reports. In strawberry, exogenous ethylene induced an increase in expression of Fa-ETR2 in white fruit and Fa-ERS1 in red fruit (Trainotti et al., 2005). Melon fruit exhibited slightly different expression patterns among outer, middle, and inner flesh tissues (Sato-Nara et al., 1999) and ETR gene of plum fruit was differentially expressed in exocarp and mesocarp (Fernández-Otero et al., 2007). By contrast, similar expression patterns of receptors (Ad-ERS1, Ad-ETR1, Ad-ETR2, and Ad-ETR3) were observed in both flesh and core tissues of kiwi fruit (Yin et al., 2008). Different expression patterns of ethylene receptors could affect tissue ethylene sensitivity by different contributions of different receptor genes to ethylene signaling.

Cs-ETR1, Cs-ETR2, and Cs-ERS had 90%, 71%, and 79% amino acid sequence similarities to Arabidopsis ETR1, ETR2 and ERS1, respectively (Yamasaki et al., 2000).

Cs-ETR1 and Cs-ERS belong to the ETR1-like group. As concerning their significant structural similarity to Arabidopsis and greatest increase in Cs-ETR1 and Cs-ERS expression by exogenous ethylene, ETR1-like receptors might play a dominant role in regulating ethylene responses in beit-alpha cucumber fruit. Wang et al. (2003) had already noted functional redundancy within receptor genes and indicated that the ETR1-like group is more important than the ETR2-like group in determining ethylene responses in Arabidopsis.

Generally, living organisms try to maintain homeostatic equilibrium in response to biotic and abiotic stresses. When plants are subject to hormonal changes, for example, they can control it by modification in hormone synthesis, catabolism and/or sensitivity (Klee, 2004). Beit-alpha cucumber fruit produced no detectable ethylene, even upon ethylene exposure, and there is no known biochemical pathway for catabolism of ethylene. Therefore, alteration in ethylene sensitivity would appear to represent the primary defense to challenge with excess ethylene. Klee (2004) speculated that ethylene-induced increases in receptor expression can play a role in repressing ethylene responses. The data presented for cucumber fruit are consistent with this view. Postharvest ethylene exposure enhanced accumulation of receptor genes after 12 h or 1 d in both mesocarp and exocarp tissue. Receptor transcripts in mesocarp tissue, but not in exocarp tissue, decreased significantly 1 d before development of visible symptoms of watersoaking, which might reflect a loss in capacity to ward off deleterious effects of ethylene. It was reported that decreased receptor content enhanced sensitivity of plant tissues to ethylene (Hall and Bleecker, 2003).

The present study was limited to ethylene effects on ethylene receptor transcripts. However, it is clear that hormone pathways can be regulated post-translationally. Kavany et al. (2007) reported that tomato fruit treated with exogenous ethylene (10 $\mu L \cdot L^{-1}$ for 8 h) showed a rapid degradation (about 50%) in receptor (NR, ETR4, and ETR6) proteins within 2 h with patterns independent of transcript levels. This is in contrast to the results of O'Malley et al. (2005) where Arabidopsis exhibited a positive correlation between levels of ethylene receptor transcript and functional ethylene-binding activity. Therefore, further study in ethylene receptor protein levels will be needed to elucidate the role of ethylene receptor genes in ethylene-induced watersoaking of beit-alpha cucumber fruit.

Overall, the present study revealed that all three receptor genes are ethylene-inducible in both mesocarp and exocarp tissues. Modification in expression level of ethylene receptors is an early cellular response prior to the occurrence of watersoaking disorder. Among different receptors, ETR1-like receptors seem to play a role in regulating ethylene responses in beit-alpha cucumber fruit.

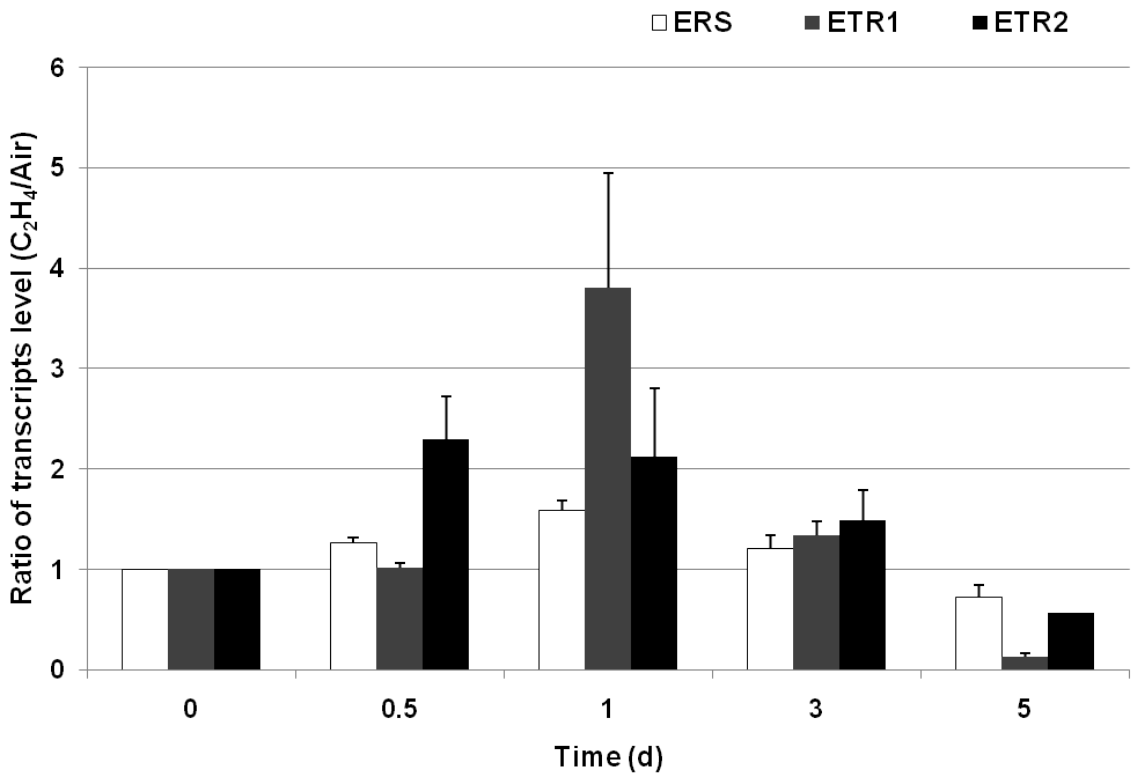


Figure 6-1. Gene expression level of ethylene receptors (Cs-ERS, Cs-ETR1, and Cs-ETR2) in mesocarp tissue of immature cucumber fruit stored with air ± 10 $\mu L \cdot L^{-1}$ ethylene continuously at 13 ^{o}C. The expression level of ethylene receptors was normalized to 18S rRNA internal control, and then transcript content of ethylene-treated fruit was expressed as a ratio to one of air-treated fruit at each day. Value of initial day was set as 1. Each bar represents the mean of three replications (three individual PCR products with composites of five fruit) ± S.E.

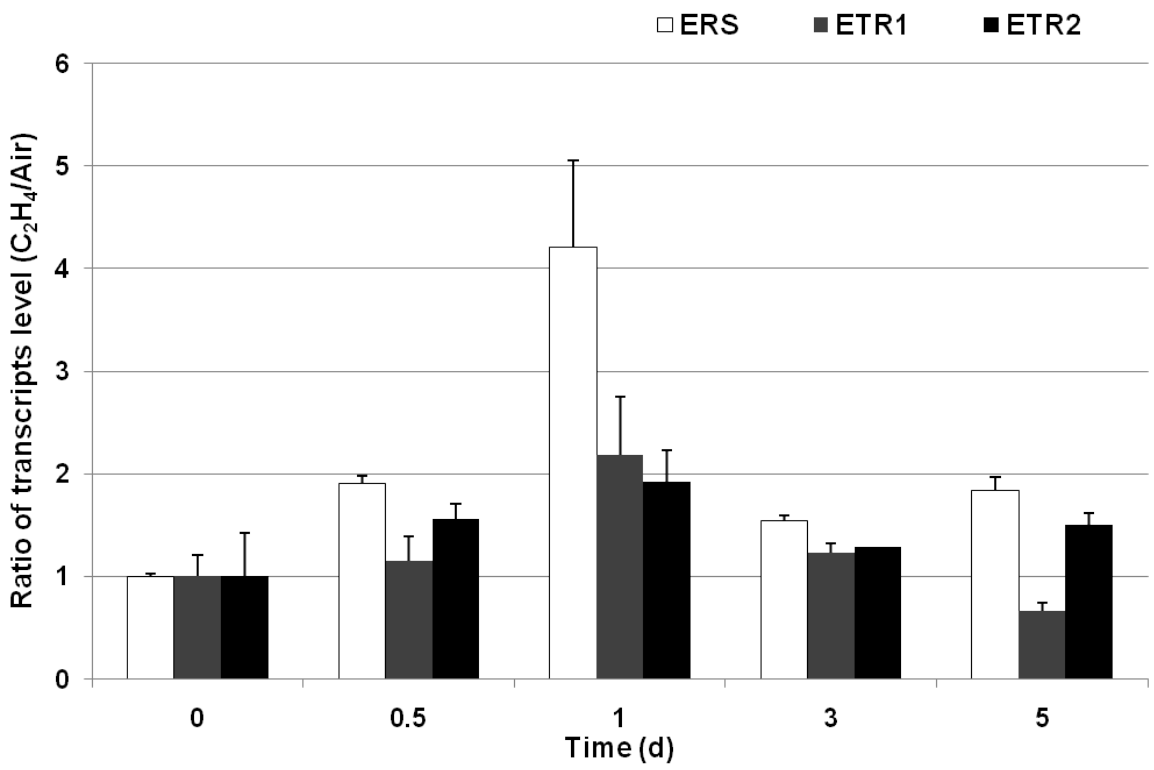


Figure 6-2. Gene expression level of ethylene receptors (Cs-ERS, Cs-ETR1, and Cs-ETR2) in exocarp tissue of immature cucumber fruit stored with air ± 10 $\mu L \cdot L^{-1}$ ethylene continuously at 13 ^{o}C. The expression level of ethylene receptors was normalized to 18S rRNA internal control, and then transcript content of ethylene-treated fruit was expressed as a ratio to one of air-treated fruit at each day. Value of initial day was set as 1. Each bar represents the mean of three replications (three individual PCR products with composites of five fruit) ± S.E.

CHAPTER 7
CONCLUSIONS

The present study was conducted to address the role of oxygen in watersoaking development using immature beit-alpha cucumber fruit in which exogenous ethylene induces watersoaking uniformly and predictably.

The influence of ethylene concentration and exposure duration on ethylene responses was addressed. Ethylene at 10 $\mu L \cdot L^{-1}$ induced tissue softening followed by watersoaking, skin discoloration, and increased respiration and electrolyte leakage. Higher concentrations did not accelerate ethylene responses, indicating 10 $\mu L \cdot L^{-1}$ ethylene was saturating with respect to ethylene responses of cucumber fruit. By contrast, duration of ethylene exposure differentially affected physiological responses. Short-term exposure (12 h) to 10 $\mu L \cdot L^{-1}$ ethylene elicited no detrimental effect on quality of cucumber fruit. Fruit receiving ethylene for only 2 d exhibited a significant decline of hue angle only at 15 d. Ethylene exposure for only 4 d induced watersoaking, electrolyte leakage increase and firmness decline much more slowly compared to continuous exposure, but was without significant effect on hue angle. Hue angle of fruit treated with ethylene for 4 d or continuously reached around 119 ^{o} at 8 d. These results might be explained by different ethylene thresholds for the different ethylene responses of immature cucumber fruit.

Accumulation of reactive oxygen species (ROS) and ethylene receptor transcripts was observed in response to ethylene. Both responses occurred rapidly in response to ethylene and well in advance of incipient watersoaking and accompanying symptoms. Continuous ethylene exposure (10 $\mu L \cdot L^{-1}$) induced noticeable increases in ROS-generating capacity after 2 d while there was no detectable ROS generation until 8 d in

air-treated fruit. Histochemical staining (DAB and NBT staining for H_2O_2 and NBT for $O_2^{\cdot -}$, respectively) was performed to identify spatial and quantitative correlation between H_2O_2/$O_2^{\cdot -}$ and watersoaking in cucumber fruit tissues. Brown precipitate from DAB increased during 8 d of storage in ethylene-treated cucumber fruit, especially in the exocarp. However, ethylene exposure did not induce detectable accumulation of superoxide anion ($O_2^{\cdot -}$) in exocarp tissue while there was strong blue deposition in exocarp tissue of air-treated fruit at 8 d. ROS accumulation, especially H_2O_2, appears to play an important role in watersoaking development. In addition, semi-quantitative RT-PCR revealed that transcript abundance for three receptor genes (Cs-ERS, Cs-ETR1, and Cs-ETR2) increased in both mesocarp and exocarp tissues in response to ethylene. Transcript levels of receptor genes were most significantly increased (2 to 4-fold) around 1 d in response to continuous ethylene exposure (10 $\mu L \cdot L^{-1}$). Of three ethylene receptor genes, Cs-ETR1 in mesocarp tissue and Cs-ERS in exocarp tissue were most significantly up-regulated by exogenous ethylene, indicating ETR1-like receptors might play a dominant role in regulating ethylene responses in beit-alpha cucumber fruit.

Watersoaking appears to be a tissue-specific response since altering tissue ethylene and oxygen gradients through the use of fresh-cut slices did not affect the spatial pattern of watersoaking development. In the present study, intact and fresh-cut slices of cucumber fruit were employed to address whether patterns of watersoaking differed in response to altered gas-exchange properties. Incipient watersoaking of cucumber fruit was first evident in hypodermal (outlayer of mesocarp tissues) tissues and progressed into inner mesocarp tissue in both intact and fresh-cut slices.

Fresh-cut slices of cucumber fruit, however, exhibited altered ethylene responses compared with intact fruit. Fresh-cut cucumber slices treated with continuous ethylene exhibited peel discoloration, but not enhanced softening or electrolyte leakage. Fresh-cut slices were highly resistant to watersoaking, even when challenged with ethylene under hyperoxic conditions. In intact fruit, hyperoxia (40 kPa O_2) accelerated ethylene-induced watersoaking and accompanying symptoms including degreening, softening and enhanced electrolyte leakage while hypoxia (2 kPa O_2) strongly suppressed these symptoms. In fresh-cut slices, hyperoxia did not enhance watersoaking nor accelerate softening, hue angle decline, and electrolyte leakage. Hypoxia, however, negated ethylene-induced symptoms in slices. Fresh-cut slices had higher ROS-generating capacities than intact fruit regardless of ethylene treatment. Increased ROS accumulation by fresh-cut processing could enhance antioxidant levels (Reyes et al., 2007; Heredia and Cisneros-Zevallos, 2009), which might result in reduced watersoaking in fresh-cut slices compared with intact fruit.

Altered pO_2 markedly affected the course of watersoaking development of cucumber. Hyperoxia (40 kPa O_2) accelerated watersoaking development while hypoxia (2 kPa O_2) negated watersoaking. The effects of altered pO_2 appeared to be mediated through altered ROS balance. Ethylene-treated fruit had enhanced ROS-generating capacity and H_2O_2 production at 4 d and 2d, respectively, earlier than incipient watersoaking under both normoxic and hyperoxic conditions. By contrast, $O_2^{\cdot -}$ production declined in ethylene-treated fruit as watersoaking initiated and developed under both normoxic and hyperoxic conditions. Neither significant increases in ROS and H_2O_2 production nor decline in superoxide anion ($O_2^{\cdot -}$) was observed in hypoxic storage

even in presence of exogenous ethylene. Antioxidant capacity of cucumber fruit increased in response to exogenous ethylene at 6 d and 4-6 d in exocarp and mesocarp tissues, respectively. ROS production of ethylene-treated fruit increased up to 500-700% while antioxidant capacity of ethylene-treated fruit increased up to 50-70% compared with those of air-treated fruit. These results indicate that ethylene-induced ROS might not be adequately scavenged by antioxidants, resulting in imbalances between ROS production and scavenging and leading to watersoaking.

Preconditioning treatment (2 kPa O_2 for 1 wk) altered total ROS and H_2O_2 production and strongly suppressed watersoaking in response to subsequent ethylene exposure. Cucumber fruit subjected to preconditioning (2 kPa O_2 for 1 wk) prior to ethylene exposure (10 $\mu L \cdot L^{-1}$) under normoxia exhibited significant softening, ion leakage and tissue disruption, but no watersoaking. Preconditioning treatment reduced ethylene-induced increases in total ROS production and H_2O_2 levels. Altered ROS generation could disturb watersoaking development in response to subsequent ethylene exposure under normoxic environments.

The possible mechanisms of watersoaking development in immature cucumber fruit was proposed in Figure 7-1 based on the present study and previous reports. It is the simplified mechanisms and would not be applied to all fruit systems.

The work presented here could significantly help our understanding of how ethylene induces watersoaking disorder in immature cucumber fruit. Elucidating early cellular events could greatly enhance our ability to reduce the quality losses caused by watersoaking disorder.

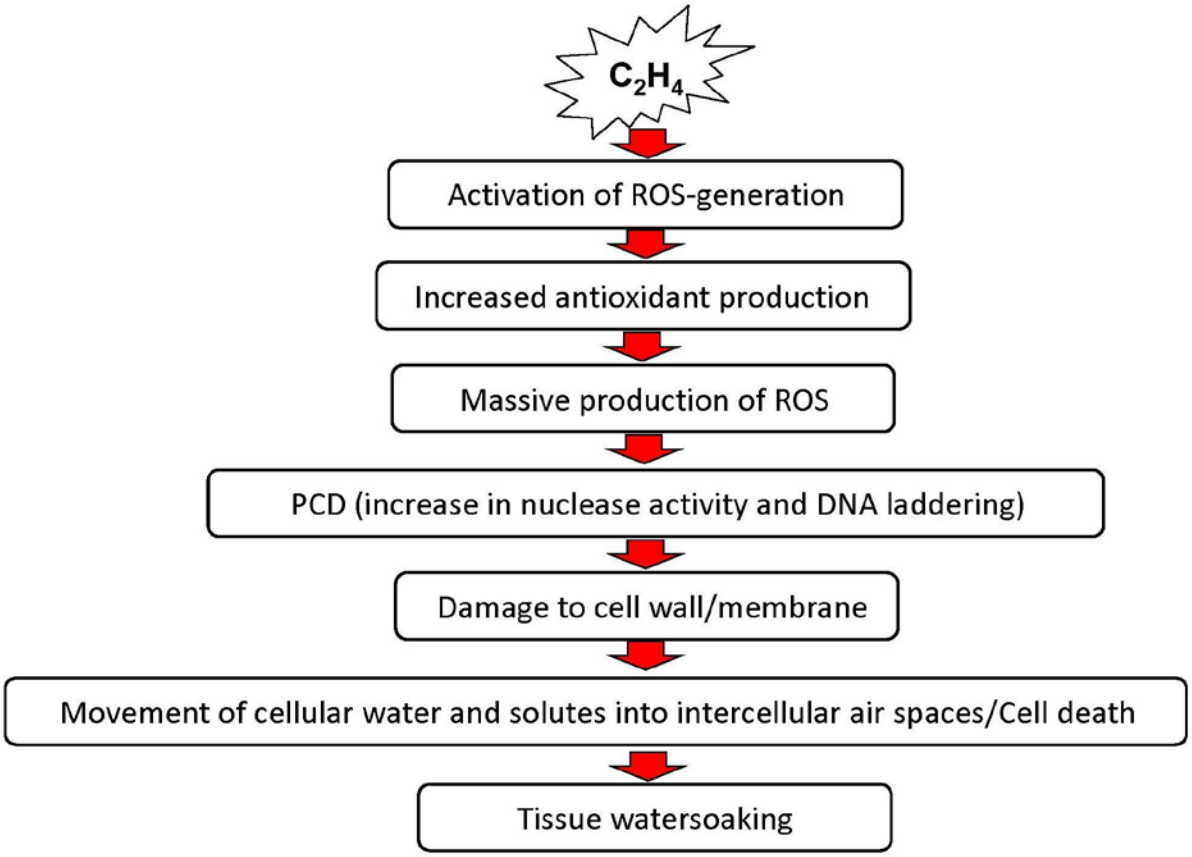


Figure 7-1. Proposed model for watersoaking development in immature beit-alpha cucumber fruit.

APPENDIX A
TIME COURSE OF DCFH OXIDATION

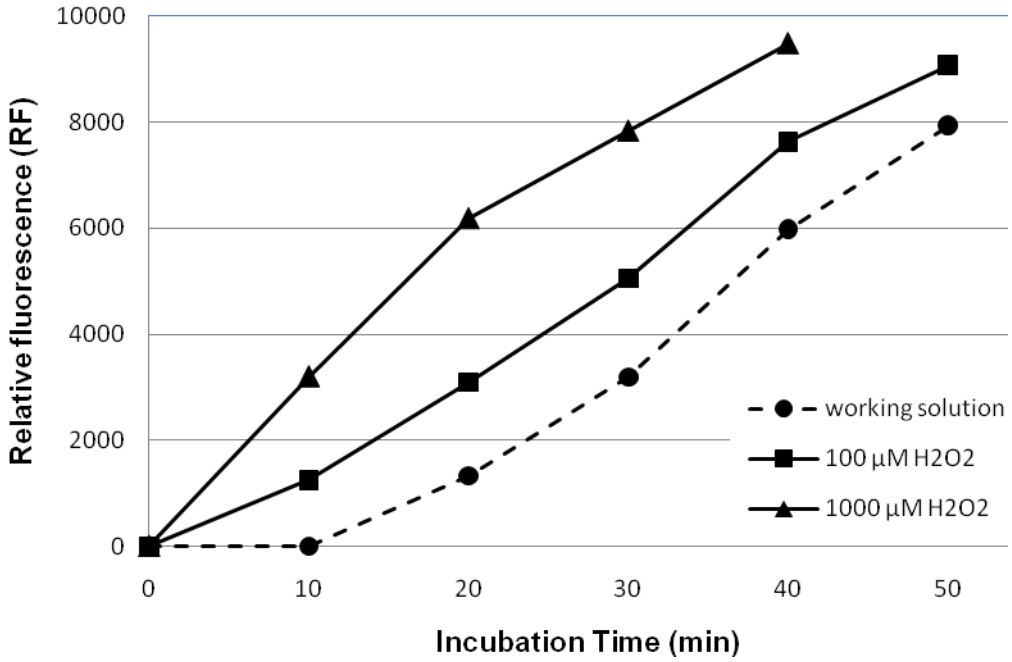


Figure A-1. Time course of DCFH oxidation by H_2O_2 (100 and 1000 µM). Relative fluorescence of incubating medium was measured with a fluorometer (Ex: 480 nm, Em: 520 nm). Working solution without tissue was used to set zero and 10,000 µM H_2O_2 to set maximum fluorescence as 10,000.

APPENDIX B
STANDARD CURVE FOR DCFH ASSAY

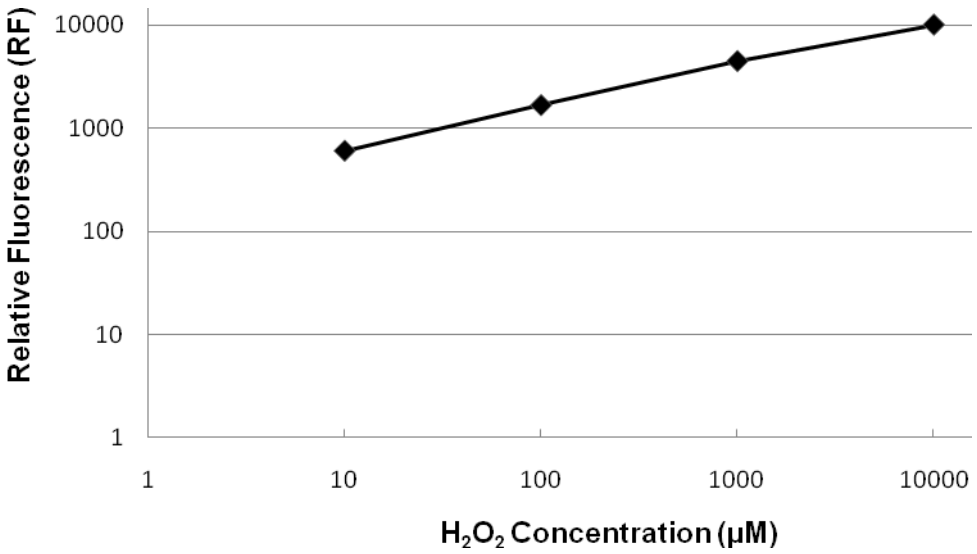


Figure A-2. A standard curve for DCFH assay was prepared with dilutions of H_2O_2 (final concentrations of 10, 100, 1000, and 10000 µM). Relative fluorescence of incubating medium was measured with a fluorometer (Ex: 480 nm, Em: 520 nm). Working solution without tissue was used to set zero and 10,000 µM H_2O_2 to set maximum fluorescence as 10,000.

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BIOGRAPHICAL SKETCH

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