THE ENHANCEMENT OF FRESH PRODUCE ANTIOXIDANT CAPACITY BY WOUNDING STRESS AND PHYTOHORMONES

A Dissertation

by

JOSE BASILIO HEREDIA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2006

Major Subject: Food Science and Technology

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ABSTRACT

The Enhancement of Fresh Produce Antioxidant Capacity
by Wounding Stress and Phytohormones. (May 2006)
Jose Basilio Heredia, B.S., Instituto Tecnológico de Culiacán;
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The use of postharvest abiotic stresses, wounding and plant hormones, can enhance the nutraceutical content of fresh produce. Wounding increased the phenolic content (TP) of carrots, lettuce, jicama, red onions, white onions and celery with a corresponding increase in antioxidant capacity (AOX). Similarly, phenylalanine ammonia lyase (PAL) activity increased with the corresponding increase in total phenolics. The diversity of antioxidant phenolic compounds present in each fresh produce provided specific antioxidant capacities which could be related to important biological activities. The combination of wounding and ethylene (ET) showed a higher increase in TP and AOX for these same tissues. The reason of the synergistic effect is not clear. However, we propose that both stresses may be sharing in part a common signaling molecule that amplifies the response. Studies with carrot tissue identified the individual phenolics responsible for the AOX of wounded and hormone wounded stressed carrots. ET treated carrot tissue showed accumulation of mainly chlorogenic acid (CHA) and dicaffeoylquinic acid (diCQA), but also a slight increase in the synthesis of the bitter compound isocoumarin. On the other hand, the use of methyl jasmonate (MJ) did not increase the levels of isocoumarins but did increase CHA and diCQA. Studies were performed to understand the mechanism by which wounding and hormone stress stimulated the phenylpropanoid metabolism. The blockers phenidone (PHE), diphenyleneiodonium (DPI), and 1-methylcyclopropene (1MCP) provided information relevant to signaling molecules including reactive oxygen species (ROS), MJ and ET, and their effect on the synthesis of TP. Results with DPI blocker suggest that ROS are

greatly responsible for the accumulation of phenolics in wounded- and wounded-hormone stressed carrot tissue.

DEDICATION

This work is proudly dedicated to my family who I love more than anything else in this life. To my loved wife Yésika Sachiko, without her support, intelligence, motivation and encouragement, this journey would have been more difficult. To my charming and lovely kids, my daughter Paola Abigail, my son Diego Iván and my son Andrés Basilio, for feeding our souls and minds with so much positive energy to keep us moving in the right direction.

Also, because of the unconditional support and the endless love they have always shown to us, I would like to extend this dedication to my dear parents Olivia, Conchita and Alessio. To conclude, I want to honor the memory of my father *Juan*$^{+}$. I know that wherever he is, this work would make him feel more proud of what I am.

I will always have all of you in my heart. May God bless you forever.

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I shall always be in debt with God. Without his blessings this work would not be possible. Thank you so much my Lord.

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Thanks to Grimmway Farms for providing fresh and prompt research plant materials, and to AgroFresh Inc., for supplying the 1MCP tablets and instructions of use.

I would like to recognize the endless support and encouragement of my relatives, particularly my dear brothers Juan, Nicolas, Mauricio, Oscar and Jorge. Similarly, I appreciate the caring and friendship of all my sisters-in-law, especially Karem, Karina, Nairobi and their children. I couldn't forget extending my greetings to my dear grandparents Julia and *Rafael*$^{+}$, and my uncles and aunts Victor, Leopoldo, Ernestina, Lourdes and Francisco.

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CHAPTER I

INTRODUCTION

The need for more convenient, healthy and nutritious foods, including plant-derived products, has constantly increased through the last years (Garret 2002). Fruits and vegetables in the daily diet have been strongly associated with reduced risk for some forms of cancer, heart disease, stroke, and other chronic diseases (Prior and Cao 2000; Amiot and others 1997; Southon 2000; Wargovich 2000; Shahidi and Ho 2003). Some of their components have shown important roles as antimutagenic, antioxidant and anticarcinogenic activities (Otawa 1992; Weisburger 1992).

Plant secondary metabolites are unique sources of pharmaceuticals, food additives and flavors, and food antioxidants (Zhao and others 2005). Also known as phytochemicals, these metabolites have clearly become the nutrition buzzword of recent years, and many food companies are looking for ways to enhance the levels of these phytonutrients in traditional products (Block and others 1992; Gillman 1996: Hasler 2000; Finley 2000). Within each fruit species there is a range of genotypic variation in composition, quality and postharvest life potential (Childs and Witwer 2000; Wu and others 2004). There are many opportunities using plant breeding and biotechnology methods to develop improved cultivars (Kader 2002). However, many preharvest and postharvest factors influence the composition and quality of fruits (Goldman and others 1999; Buescher and others 1999).

The accumulation of antioxidants often occurs naturally in plants subjected to stresses, including various elicitors or signal molecules (Zhao and others 2005). During the process of developing new plant-derived food products, like fresh-cut produce, food scientists can get involved in several challenges associated with the physiology and metabolism of plant tissues. Wounding of fresh fruits and vegetables has been mainly

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related to detrimental changes in fresh-cut produce, and phenolic compounds have been found to be partially responsible for some of those effects (Saltveit 2000; Kang and Saltveit 2003). Recently, a new research trend is showing that, besides the undesired given properties of some wound-stressed phenolics, phenolics are also involved in the increase of antioxidant capacity (Heredia and Cisneros-Zevallos 2002; Cisneros-Zevallos 2003).

Phenolic compounds are secondary metabolites found in plants, which posses an ideal structural chemistry for free radical scavenging activity of primarily reactive oxygen species (ROS) (Ho 1992; Weisshaar and Jenkins 1998; Blokhina and others 2003). Traditionally, ROS were considered to be toxic by-products of aerobic metabolism. However, it has become apparent that plants actively produce ROS as signaling molecules to control processes such as programmed cell death, abiotic stress responses, pathogen defense and systemic signaling (Schreck and Baeuerle 1991; Huang and Ferraro 1992; Mittler 2002; Hodges 2003).

The synthesis of phenolics can be manipulated with the appropriate use of postharvest abiotic stresses in fresh produce (Heredia and others 2001; Cisneros-Zevallos 2003); potatoes (Tudela and others 2002) and fresh-cut lettuce (Kang and Saltveit 2002). Some studies have shown that wounding stress (Saltveit 2000) and plant hormones like ethylene (Sarkar and Phan 1979) and methyl jasmonate (Saniewski and others 1998) can promote the synthesis of phenolic compounds in plant tissues. However, no studies indicating the effect of combining different stresses on the biosynthesis of phenolic antioxidants were found in the literature. Therefore, the objective of this study was to determine the effect of postharvest abiotic stresses and their combinations in the enhancement of the level of bioactive phenolics in whole and wounded produce, as well as understand the responsible signal molecule which elicits the response.

The general hypothesis of this work was that individual abiotic stresses such as wounding, ethylene and methyl jasmonate and their combinations will enhance the accumulation of health-promoting phenolic antioxidants in fresh produce. The role of

reactive oxygen species (superoxide and hydrogen peroxide radicals) as signaling molecules in the phenylpropanoid metabolism in relation to wounding, methyl jasmonate and ethylene stresses will be determined.

This dissertation is composed of 5 chapters. Chapter II evaluated the effect of exogenous ethylene and methyl jasmonate on the accumulation of phenolic compounds of different whole and wounded fresh produce. This part of the work studied the combinations of wounding and hormone applications to enhance the synthesis of health promoting bioactive phenolic compounds on diverse fruits and vegetables.

In Chapter III, cut carrots were chosen to study the effect of intensity of wounding in combination with hormone stresses. This approach was designed to trigger the accumulation of bioactive phenolic compounds more rapidly, and determine if the response of the phenylpropanoid metabolism to the combination of stresses is synergistic, additive, antagonistic or hierarchical.

The experiments in Chapter IV were done to evaluate the role of primary reactive oxygen species (superoxide and hydrogen peroxide) on PAL and LOX activity, and the accumulation of phenolic compounds in wounded and hormone-stressed carrot tissue. This information will contribute toward identifying the involved signaling pathways as part of the plant wound response.

CHAPTER II

EFFECT OF EXOGENOUS ETHYLENE AND METHYL JASMONATE ON THE ACCUMULATION OF PHENOLIC COMPOUNDS OF DIFFERENT WHOLE AND WOUNDED FRESH PRODUCE

Synopsis

Selected whole and wounded fruits and vegetables were used to determine if the phenylpropanoid metabolism was activated in different types of tissues, exposed to wounding and in combination with methyl jasmonate or ethylene. Lettuce, cilantro, cabbage, green beans, apples, plums, peaches, table grapes, strawberries, bell peppers, asparagus, celery, carrots, radishes, potatoes, and jicama were used in this study. These commodities were evaluated on their total phenolics, antioxidant capacity (AOX), PAL enzyme activity and HPLC phenolics profile. The effect of the studied phytohormone abiotic stresses was greater when plant tissues were first exposed to physical damage (wounding) as compared to whole tissues. Several reasons could be involved in the phenylpropanoid metabolism response to the phytohormone stress, including the plant genetic machinery, the specificity of their defense systems, the pre-harvest conditions and cultural practices, and also the possible effect of wounding intensity. The synthesized phenolic compounds increased the overall antioxidant capacity (µg Trolox/g fresh weight) of the tissue. Furthermore, the specific AOX (µg Trolox/mg phenolic) of the synthesized phenolic compounds was influenced by type of tissue and phytohormone used.

Introduction

Epidemiological and clinical investigations have revealed that diets rich in fruits and vegetables are associated with a reduced risk of cardiovascular and neurological diseases and various forms of cancer (Doll 1990; Temple 2000). Phenolic substances can be classified into simple phenols, phenolic acids, hydroxycinnamic acid derivatives and

flavonoids. These phytonutrients are antioxidants which may provide health benefits (Prior and Cao 2000; Wu and others 2004). Cao and others (1996) found that a diverse group of vegetables, which contained a good source of polyphenols, showed high antioxidant capacity. Other studies have shown that fruits also have an excellent quantity and quality of phenol antioxidants (Vinson and others 2001; Sun and others 2002). There is indeed an enormous diversity of phenolic antioxidants found in fruits and vegetables, and their presence and roles can be affected or modified by several pre- and post- harvest cultural practices and/or food processing technologies (Goldman and others 1999; Kaur and Kapoor 2001; Tudela and others 2002). These phenolics are mainly synthesized in plants through the malonic and/or shikimic acid pathways, including a very important role of the key enzyme phenylalanine ammonia lyase (PAL) within the latter pathway (Dangl and others 2000) (figure 2.1).

The fundamental principle underlying quality of wounded fruits and vegetables is that they are living tissues and, as a consequence, show physiological response to diverse processing and handling procedures as well as the package environment in which they are enclosed (Toivonen and DeEll 2002). The effect of wounding on produce varies with the variety, temperature, water content and degree of maturity (Lamikanra 2005). Another important characteristic is related to different mechanical parameters of fruits and vegetables in relation to their specific cell dimension and cell wall structure. Wounding seems to induce the expression of genes encoding defense-related proteins involved in wound healing (Bowles 1991; Saltveit 2000). Therefore, plants can react to diverse stresses like mechanical injury or exposure to plant hormones, like ethylene and methyl jasmonate, by activating a set of responses that include transcriptional activation of wound responsive genes (Lamikanra 2005).

Ethylene (ET) can influence a diverse array of plant growth and development processes including germination, senescence, cell elongation, fruit ripening, and plant defense mechanisms (Kahl and Laties 1989; Kieber 1997). Slight ET applications can increase the anthocyanin accumulation in several crops (Awad and de Jager 2002; Ju and others 1995; Murphey and Dilley 1988). Other studies have shown a positive correlation

between an increase in respiration rate and the phenolic content of plant tissues exposed to ET (Nichols and Laties 1985; Lafuente and others 1996; Sarkar and Phan 1979; Fan and others 2000; Chalutz and others 1969; Yokotani and others 2004).

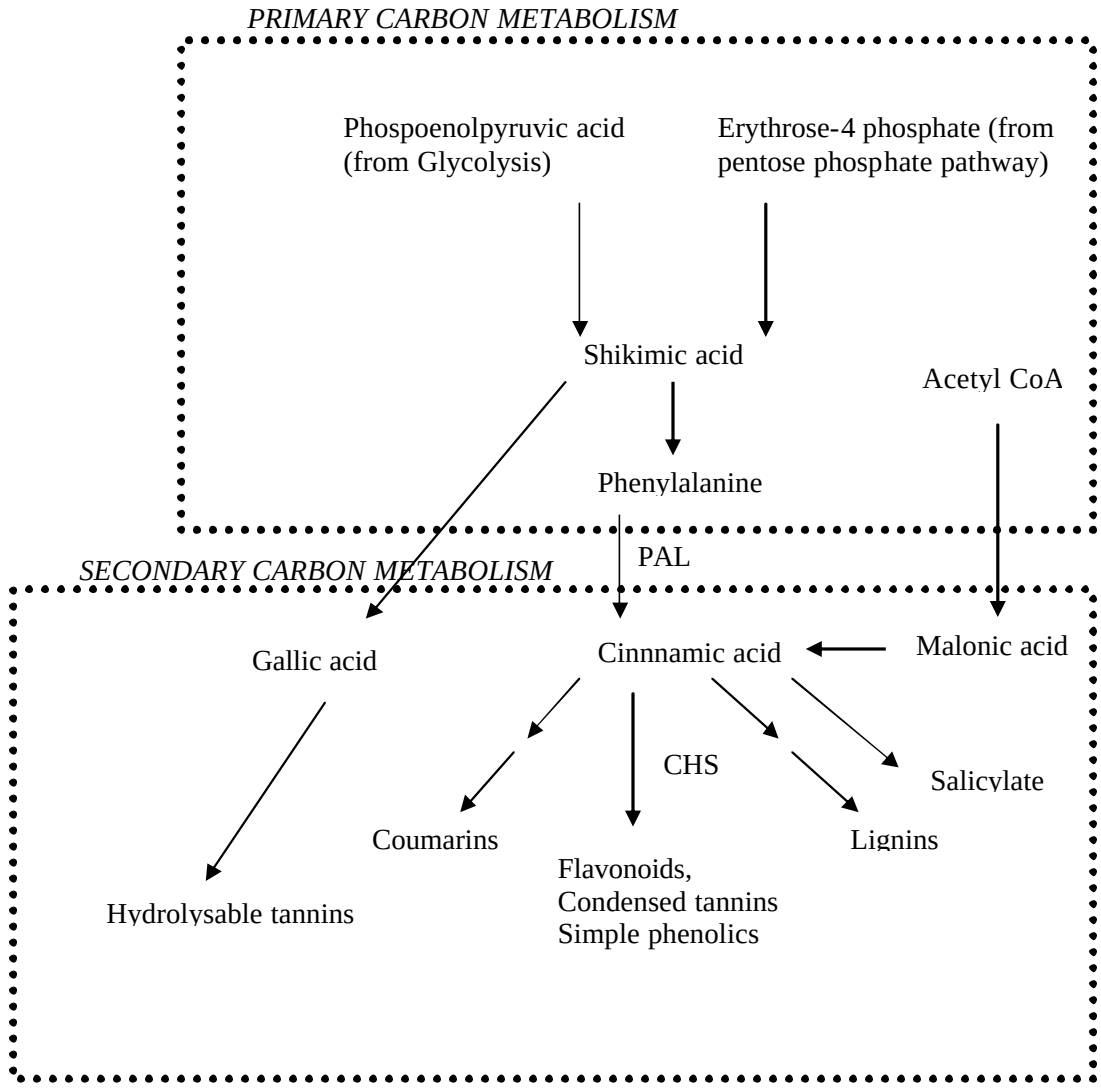

Figure 2.1 Main pathways involved in the synthesis of phenolic compounds. They originate within the primary carbon metabolism and continue until reaching the final products from the secondary carbon metabolism (PAL, phenylalanine ammonia lyase; CHS, chalcone synthase) (Dangl and others 2000).

Methyl jasmonate (MJ) can interact with other phytohormones in eliciting biological activity, and play a prominent role in signaling plant defenses (Sticher and others 1997; Fan and others 1998). Leja and others (1997a; 1997b) and O'Donnell and others (1996) showed that MJ and ET influence each other's concentration in plant tissues and act together to regulate wound-induced gene expression. Other important effects of MJ applications are related to the promotion of color in tomato fruits (Saniewski and others 1987) and in tulip bulbs (Saniewski and others 1998).

Since exogenous ethylene and methyl jasmonate can elicit the synthesis of secondary metabolites, the ability of combinations of wounding and MJ and ET applications to enhance the synthesis of health promoting bioactive phenolic compounds was investigated. In our approach we used a selected group of fruits and vegetables to determine if the phenylpropanoid metabolism was activated in different types of tissues when exposed to these stresses.

Materials and Methods

Plant materials and reagents

Lettuce (*Lactuca sativa*), cilantro (*Coriander sativum*), cabbage (*Brassica oleracea*), green beans (*Phaseolus vulgaris*) , apples (*Malus x domestica*), plums (*Prunus salicina*), peaches and nectarines (*Prunus persica*), pears (*Pyrus communis*), strawberries (*Fragaria x ananassa*), table grapes (*Vitis vinifera*), tomatoes (*Solanum lycopersicum*), bell peppers (*Capsicum annum*), asparagus (*Asparagus officinalis*), onions (*Allium cepa*), celery (*Apium graveolens*), carrots (*Daucus carota*), radishes (*Raphanus sativus*), potatoes (*Solanum tuberosum*), and jicama (*Pachyrrhizus erosus*) were obtained from a local supermarket. All produce was washed with chlorinated water (250 ppm). For the wounded studies, tissues were cut as described in table 2.1. Whole and ~150 g wounded samples were placed inside 1-gallon glass jars, where MJ and ET treatments were continuously applied during the 4 d of storage at 20°C. While ET was directly injected into the jars to obtain 1000 ppm, MJ was applied by wetting a filter paper over a Petri dish to obtain 250 ppm in a headspace-saturated atmosphere.

Table 2.1. Description of fresh cuts for the wounding studies. All samples were manually processed using disinfected stainless steel knifes and plastic cutting boards.

Produce	Type of fresh cut
Green celery	Cut stick in half (long way), then made pieces 4-5 mm thick
White onions	Peel and cut bulb in slices 4-5 mm thick, then made pieces 5x10 mm
Iceberg lettuce	Cut leaves to make pieces 10x10 mm
Orange carrots	Cut roots to make slices 3-4 mm thick
Jicama	Peel and cut in slices 10-12 mm thick, then made 8 triangular pieces
Bell peppers	Cut slices-rings 4-5 mm thick, then made pieces 5x10 mm
Red onions	Peel and cut bulbs in slices 4-5 mm thick, then made pieces 5x10 mm
Asparagus	Cut in slices 4-5 mm thick
Green cabbage	Cut leaves to make small pieces 10x10 mm
Red apples	Cut in slices 10-12 mm thick, then made 8 triangular pieces
Tomatoes	Cut in slices 10-12 mm thick, then made 4 triangular pieces
Nectarines	Cut in slices 10-12 mm thick, then made 8 triangular pieces
Radish	Cut in slices 2-3 mm thick, then made 4 triangular pieces
Red cabbage	Cut leaves to make small pieces 10x10 mm
White potatoes	Cut in slices 10-12 mm thick, then made 8 triangular pieces
Green pears	Cut in slices 10-12 mm thick, then made 8 triangular pieces

Jars were kept in dark conditions with periodic ventilation every 12 h to avoid CO_2 accumulation (>0.5%). Air was used for control samples. Sampling and analysis were done at the initial and the final day of the experiments.

Methyl jasmonate (95%), Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), chlorogenic acid, vanillic acid, ferulic acid, Trolox and 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium phosphate, polyvinylpolypyrrolidone (PVPP), boric acid, sodium hydroxide (NaOH), 2-mercaptoethanol, L-phenylalanine and sulfuric acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). The 1-aminocyclopropene-1-carboxylic acid (1ACC) was purchased from MP Biomedicals (Aurora, OH, USA). Ethylene (CP grade, 99.5%) and sample preparation supplies were obtained from Fisher Scientific (Houston, TX, USA) including filters, membranes and syringes. The solvents, methanol, ethanol, hexane, acetonitrile and water, were quality HPLC grade.

Analysis of total soluble phenolics

Phenolic content was evaluated following the procedure of Swain and Hillis (1959). Samples of 5 g were homogenized with 20 mL methanol using an Ultra-Turrax homogenizer (IKA Works, Inc., Wilmington, NC, U.S.A.) until uniform consistency and then incubated overnight at 4°C. Homogenates were centrifuged (rotor JA-17; centrifuge J2-21; Beckman Coulter, Inc., Fullerton, CA, U.S.A.) at 29,000 x g for 15 min at 4°C. Sample aliquots of 150 µL were taken from the clear supernatant and then diluted with 2400 µL nanopure water, followed by 150µL 0.25N Folin-Ciocalteu and incubated for 3 min at room temperature. The reaction was stopped by adding 300 µL 1N Na_2CO_3 and the mixture was incubated 10 min. Samples were again centrifuged at 29,000 x g for 15 min at 4°C. Absorbance readings at 725 nm of clear supernatant samples were taken using the spectrophotometer with photo diode array detector (model 8452A, Hewlett Packard Co, Waldbronn, Germany). A blank prepared with methanol was used as control. The level of total phenolics for each sample was determined by using a standard curve developed with chlorogenic acid (CHA).

Analysis of antioxidant capacity

The antioxidant or antiradical activity of phenolic compounds was evaluated following the procedure of Brand-Williams and others (1995). Samples were homogenized with methanol until uniform consistency and then refrigerated overnight. Homogenates were centrifuged at 29,000 x g for 15 min at 4°C. Sample aliquots of 150 µL were taken from the clear supernatants and then diluted with a 2,850 µL DPPH solution, previously prepared with methanol until reaching 1.1 units of absorbance at 515 ηm. A blank was prepared with methanol to be used as a control and also to zero the spectrophotometer for further readings of all samples at 515 ηm. The mixture reaction was allowed to react in a shaker until no significant decrease in absorbance was obtained compared to the methanol-based control for antioxidant activity. The decrease in absorbance was obtained and the antioxidant activity was calculated as µg Trolox equivalents using a standard curve.

HPLC phenolics profile

Extraction and analysis

Fresh samples of 5 g were extracted with 20 mL methanol, using the Ultra-turrax homogenizer. Homogenates were centrifuged at 29,000 x g for 15 min at 4°C. Supernatant was passed through nylon membranes (0.2 µm), prior to injection into the chromatography system. Except for the SpectraPhysics SP8792 column heater (San Jose, CA, USA), all the HPLC system was composed of Waters Co instruments (Milford, MA, USA). These are two 515 binary pumps, one 717-*plus* auto-sampler, and one 996 photodiode array detector. The column used to separate the phenolic compounds was a 4.6 x 150 mm, 5 µm, C-18 reverse-phase column (Waters Atlantis, Milford, MA, USA), which was maintained at 40^{o}C. The injection volume was 10 µL. Samples were analyzed under gradient conditions with two mobile phases consisting of acidified water HCl pH 2.3 (solvent A) and acetonitrile (solvent B). The gradient system was 0/85, 5/85, 30/0, 35/0 (min/% solvent A). Data was processed by using a Waters Millennium software v3.2 (Hale 2003). The level of individual phenolics of carrots, including the most

predominantly found chlorogenic (CHA), vanillic (VA) and ferulic acids (FA) (Zhang and Hamauzu 2004), was determined by using standard curves developed with high-purity HPLC-grade commercial phenolic compounds. In the case of isocoumarin (ISO) and dicaffeoylquinic acid (diCQA) compounds, due to unavailability of commercial standards these were synthesized, HPLC-separated, isolated, collected and identified by mass spectrometry.

Synthesis and collection of diCQA and ISO

The synthesis, isolation and collection of ISO and diCQA were done based on previous methodologies (Sondheimer 1957; Talcott and Howard 1999). The process started using 10 kg of fresh carrots that were sprayed with 100 ppm 1ACC and exposed to 1000 ppm ethylene during 4 d of storage at 20°C. Ventilation was allowed every 12 h to prevent CO_2 accumulation above 0.5%. Afterwards, 600 g of peels were used to obtain a crude extract through hexane-ethanol solvent partition. The 600 g peels were immersed in 3 L of hexane for 24 h at 20°C, before partitioning with 1 L ethanol (95%). The recovered ethanol phase contained the suspected ISO and diCQA fractions for the HPLC separation process. A total of 30 injections (125µL) were run with the HPLC-PDA system, and samples of ISO and diCQA were isolated and collected in fractions of 3 mL each using an integrated Waters FC-II fraction-collector (Milford, MA, USA). Based on the λ_{max} of the PDA spectra, in each injection we collected the 2 fractions from 14-17 and 22-25 min, for diCQA and ISO respectively. All collected fractions were evaporated using a Speed-Vac SC-100 concentrator (Savant, NY, USA) and then freeze dried with a FTS PD-6-54A system (New York, NY, USA), under a low pressure of ~500µm Hg.

Confirmation test of diCQA

The diCQA concentrate was re-suspended in water/methanol (50:50 v/v) acidified with 1% acetic acid, and injected into a time of flight (TOF) mass spectrophotometer equipped with electron spray ionization (ESI) in negative ion mode

$(M-H)^-$ (PE Sciex API QStar Pulsar, Concord, Ontario, Canada). The capillary voltage was 4.5 kV. The TOF-MS procedure was done in collaboration with the Department of Chemistry at Texas A&M University (College Station, TX).

Confirmation test of ISO

The ISO concentrate was confirmed using two methods: spectrophotometry and a combined liquid chromatography-mass spectrometry-time of flight-electron spray ionization (LC-MS-TOF-ESI). For the first one, the concentrate was re-suspended in 1 mL of ethanol 95% and absorbance readings were obtained at 267 and 302 nm, in order to confirm and verify the presence of ISO. According to Sondheimer (1957) a pure ISO ethanolic solution should give a value of 2.47 for the Abs 237nm/302nm ratio. The studies showed very similar ratio values of ~2.25-2.29. The confirmation through mass spec (LC-MS-TOF-ESI) was done using the remaining ISO concentrate, which was dissolved in water/methanol acidified with 1% acetic acid. This sample was injected into a QStar Pulsar TOF-MS (Concord, Ontario, Canada) equipped with ESI in positive ion mode $(M+H)^+$ combined with $(M+Li)^+$ for further identification and conformation. The capillary voltage was 4.5 kV. The TOF-MS procedure was done in collaboration with the Department of Chemistry at Texas A&M University. Once confirmed, the quantification of the collected isocoumarins was done by spectrophotometry using a molar extinction coefficient of ε = 14,800/M cm (Sondheimer 1957). The obtained concentration was 1.1031 mg of isocoumarin per 1 mL of ethanolic sample. Several dilutions of this ethanolic sample were injected back into the HPLC to create a standard curve. The standard curve was used to quantify the concentration of ISO in all analyzed carrot samples.

Phenylalanine ammonia lyase enzyme activity

PAL was extracted from 1 g of fresh produce in 25 mL borate buffer (pH 8.5) according to Ke and Saltveit (1986). Samples were placed inside ice and homogenized using an Ultra-Turrax homogenizer, under low-light conditions and at low speed to prevent protein denaturalization. Homogenates were filtered through cheesecloth and

then taken to centrifuge 29,000 x g for 15 min at 4°C. The enzyme activity of PAL was assayed by following the accumulation of cinnamic acid at 290 nm, using 100 mM of L-phenylalanine as PAL substrate and water for control samples. Units were reported as µmol of t-cinnamic acid/h g fresh weight.

Statistical analysis

The experiment followed a completely randomized design. Analyses were done using 9 replicates, unless otherwise indicated. Means, standard deviations, graphs and linear regressions were obtained using Microsoft Excel 2000. For means comparison at the 5% significance level, ANOVA and LSD multiple-range tests were performed using SAS (Raleigh, NC, USA).

Results and Discussion

Effect of MJ and ET on the phenolic content of whole tissues

Results indicated there were non significant differences in the total phenolic content between initial day control vs. air control samples after 4 d of storage at 20°C. The only exception was cilantro which showed a slight decrease in total phenolic content. The postharvest exposure of asparagus, potatoes, apples, peaches, strawberries and table grapes to MJ and ET plant hormones did not induce an increase in the phenolic content in each crop when compared to initial day evaluations or air control after 4 d (table 2.2). Only, carrots with ET, plums with MJ and lettuce and green beans with MJ and ET showed significant increases in the phenolic content of ~10-15%. Although there is limited comparative evidence of the role of both phytohormones as postharvest abiotic stresses, the concentration of phenolics found in these commodities coincides with those reported in the literature (Ho 1992; Wu and others 2004). Furthermore, the response of phenolic compounds on the ET-stressed whole carrots and lettuce studies, showed similarities with previous reports (Sarkar and Phan 1979; Lafuente and others 1996; Campos-Vargas and Saltveit 2002).

Table 2.2. Total phenolic content of selected whole fresh produce. Samples were evaluated on the initial day before treatment (control) and after 4 d of storage at 20°C (AC, 250 ppm MJ and 1000 ppm ET). Results show means of 9 replicates and their standard deviation. Letters in parenthesis show mean comparisons among treatments of each produce (LSD P>0.05).

Produce	Total phenolics [mg CHA/100g FW]			
	Initial day control	Air control	Methyl jasmonate	Ethylene
Iceberg lettuce	18±2(b)	19±4(b)	26±5(a)	23±4(a)
Cilantro	392±21(a)	346±28(b)	355±20(b)	392±13(a)
Green beans	48±10(b)	52±6(b)	59±11(a)	62±10(a)
Asparagus	175±12(a)	163±19(a)	169±23(a)	169±20(a)
Orange carrots	69±12(b)	72±10(b)	74±6(ab)	79±11(a)
White potatoes	84±6(a)	85±6(a)	90±17(a)	88±9(a)
Red apples	358±18(a)	348±19(a)	365±23(a)	364±23(a)
Plums	248±27(b)	250±36(b)	284±16(a)	256±37(ab)
Peaches	86±10(a)	90±7(a)	86±10(a)	84±10(a)
Red table grapes	150±11(ab)	146±15(b)	146±10(b)	157±5(a)
Strawberries	383±40(ab)	376±31(b)	409±42(ab)	416±37(a)

Effect of MJ and ET on the antioxidant capacity of whole tissues

The antioxidant capacity showed no significant differences between initial day and air control evaluations with exception of slight decrease (~15%) for cilantro and asparagus (table 2.3). Whole lettuce and plums exposed to MJ, and strawberries exposed to ET showed increases of ~50, 25 and 20% respectively, after 4 d of storage and compared to initial day or air control samples. The rest of the crops did not show an increase in AOX after exposure to MJ or ET. Lettuce and carrots showed positive correlations (R^2=0.91 and R^2=0.90, respectively) between the newly synthesized phenolics and the antioxidant capacity, which coincides with previous reports (Campos-Vargas and Saltveit 2002; Heredia and Cisneros-Zevallos 2002). In the case of the red-colored tissues, like plums and strawberries, previous reports also show high values of antioxidant capacity, perhaps due to the high anthocyanin content (Vinson and others 2001; Cevallos 2001; Wu and others 2004).

To determine the AOX of the phenolic compounds present in each tissue the term specific AOX was used and defined as the ratio between total AOX and total phenolics. When plotting the calculated specific AOX values, the results showed that these commodities have different specific antioxidant capacities due very likely to different phenolic profiles (*e.g.*, fruits rich in red pigments, which showed the highest ratios) (figure 2.2). Whole produce can be arranged according to the specific AOX in the following decreasing order red table grapes> strawberries= apples> asparagus= plums= peaches> cilantro> carrots> potatoes= green beans> lettuce. Based on this rank, phenolics from table grapes have a higher antiradical scavenging capacity than phenolics from the other studied crops.

Effect of MJ and ET on the phenolic content of wounded tissues

Celery, white and red onions, lettuce, carrots and jicama were the only samples to show an increase in phenolic content due to wounding stress (~15-80%) (table 2.4). Other crops were not affected by wounding. Wounded celery, lettuce and carrots showed a larger increase in phenolic content with applications of MJ and ET (~72-130%), when

Table 2.3. Antioxidant capacity of selected whole fresh produce. Samples were evaluated on the initial day before treatment (control) and after 4 d of storage at 20°C (AC, 250 ppm MJ and 1000 ppm ET). Results show means of 9 replicates and their standard deviation. Letters in parenthesis show mean comparisons among treatments of each produce (LSD P>0.05).

Produce	Antioxidant Capacity [µg Trolox Eq/g FW]			
	Initial day control	Air control	Methyl jasmonate	Ethylene
Iceberg lettuce	58±16(c)	76±18(bc)	101±35(a)	77±22(bc)
Cilantro	2865±266(ab)	2417±342(c)	2623±240(bc)	3016±461(a)
Green beans	228±74(a)	211±43(a)	205±66(a)	216±49(a)
Asparagus	1527±185(a)	1319±244(b)	1327±205(ab)	1298±227(b)
Orange carrots	436±110(ab)	523±157(ab)	406±66(b)	534±159(a)
White potatoes	434±83(a)	439±40(a)	452±70(a)	428±31(a)
Red apples	3319±347(a)	3321±338(a)	3223±383(a)	3191±192(a)
Plums	2139±236(b)	2278±371(b)	2597±95(a)	2363±374(ab)
Peaches	740±119(a)	780±98(a)	739±106(a)	699±127(a)
Red table grapes	1835±458(a)	1710±334(a)	1851±257(a)	1991±318(a)
Strawberries	3668±617(b)	3453±357(b)	3992±719(ab)	4343±566(a)

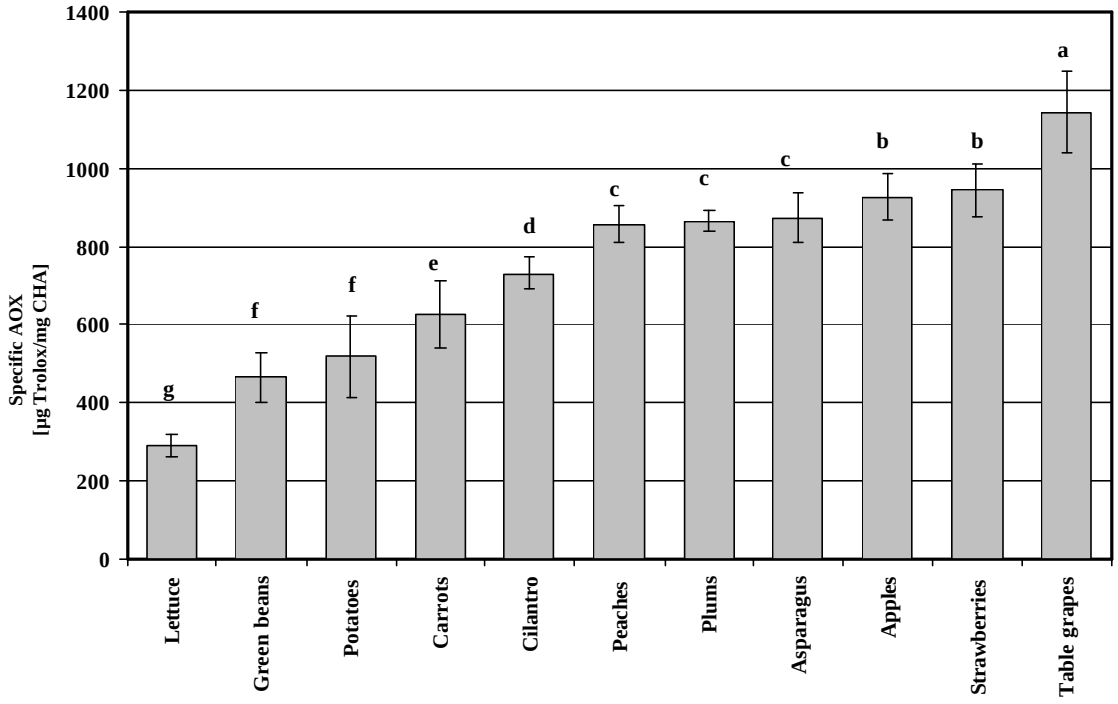

Figure 2.2. Specific antioxidant capacity shown as the ratio between total AOX and total phenolics present in each crop. Total phenolics and AOX analysis were done on the initial day. Results show means of 9 replicates, and bars show standard deviation. Letters over bars show mean comparison (LSD P>0.05).

Table 2.4. Total phenolics content of selected wounded fresh produce. Samples were evaluated on the initial day before treatment (control) and after 4 d of storage at 20°C (AC, 250 ppm MJ and 1000 ppm ET). Results show means of 9 replicates and their standard deviation. Letters in parenthesis show mean comparisons among treatments of each produce (LSD P>0.05).

Produce	Total Phenolics [mg CHA/ 100g FW]			
	Initial day control	Air control	Methyl jasmonate	Ethylene
Celery	18±2 (c)	25±3(b)	32±2(a)	33±4(a)
White onions	105±5(c)	126±8(ab)	120±9(b)	128±5(a)
Lettuce	22±3(d)	34±4(c)	38±4(b)	48±3(a)
Carrots	68±9(d)	138±7(c)	146±7(b)	157±4(a)
Jicama	60±5(c)	71±4(b)	69±6(b)	80±3(a)
Bell peppers	214±17(b)	218±23(b)	225±16(ab)	237±22(a)
Red onions	104±9(c)	123±5(b)	127±6(ab)	132±5(a)
Asparagus	170±17(a)	162±8(ab)	152±9(b)	163±16(ab)
Cabbage	101±4(ab)	97±3(bc)	94±5(c)	104±6(a)
Red apples	308±26(a)	278±23(b)	271±9(b)	271±22(b)
Tomatoes	94±8(a)	95±6(a)	96±9(a)	91±7(a)
Nectarines	85±5(ab)	80±5(b)	86±7(a)	86±4(a)
Radish	115±9(a)	113±5(a)	107±5(b)	117±5(a)
Red cabbage	341±40(a)	341±28(a)	347±28(a)	336±24(a)
White potatoes	115±6(a)	116±10(a)	116±12(a)	116±6(a)
Pears	88±11(a)	76±7(b)	75±8(b)	75±9(b)

compared to wounded tissue alone. Wounded jicama, bell peppers and red onions only increased with ET (~10-30%) but not with MJ.

Wounding is signaling the phenylpropanoid metabolism as observed by the increase in phenolic content of some tissues. Furthermore, the increase in total phenolic content of wounded lettuce, celery, red onions, carrots, jicama, and bell peppers indicates that plant hormone stresses have a greater effect in the presence of wounding stress (table 2.4) compared to whole tissues (table 2.2). The reasons of the observed synergistic effect between wounding and hormones are not clear. However, two possible explanations would be related to the fact that both stresses may be sharing a common signaling molecule which when added may reach a certain threshold that amplifies the response. For example, signaling molecules generated such as superoxide radicals may trigger the production of further superoxide radicals by a feedback mechanism through an increase in activity of NADPH oxidase (Mittler and others 2004; Brandes 2005; Afanas'ev 2006). Another scenario is related to an increased respiration rate which may produce more ROS through the electromagnetic process of moving electrons across the cell membrane (Mittler 2002; Rakwal and Agrawal 2003; Blokhina and others 2003; Apel and Hirt 2004; Murphy and DeCoursey 2006).

Previous studies have shown that wounding elicits an increase in phenylalanine ammonia lyase activity (PAL) with a corresponding increase in phenolic content in tissues like lettuce and carrots (Christoffersen and Laties 1982; Barry-Ryan and O'Beirne 2000; Campos-Vargas and Saltveit 2002).

In the present study asparagus, cabbage, apples, tomatoes, nectarines, radishes, potatoes, and pears did not apparently respond to both stresses and even some tissues showed significant decreases in the phenolic content. This type of response could be due to the presence of reduction-oxidation reactions involving the polymerization and degradation of phenolic compounds (browning) through the activation of diverse antioxidant enzymes (*i.e.*, peroxidases), thus reducing the overall phenolic content of the tissue. In general, tissues that did not respond or showed a reduction in phenolic content would imply that the stress do not affect these tissues at the gene level. Alternatively, it

may indicate that the stresses do affect the tissue. For the apparently non responsive produce, the lack of increase in phenolic content it may be due to a similar kinetics of phenolic synthesis and degradation, while for tissues with a reduction in phenolic content, it would be related to a larger phenolic degradation kinetic compared to phenolic synthesis.

Effect of MJ and ET on the antioxidant capacity of wounded tissues

Similar trends to those of phenolics were obtained for the antioxidant capacity of wounded produce, including celery, white and red onions, lettuce, carrots and jicama. When MJ and ET were applied to the above wounded tissues, a higher increase in AOX was observed with exception white onion and jicama, which showed a lack of response (table 2.5). Among the tissues, lettuce and carrots showed the largest increase in AOX with combinations of wounding and plant hormones (~300-400% over the control). Previously Zang and Hamuzu (2004) and Kang and Saltveit (2002) reported similar responses for carrot and lettuce, respectively.

Among the commodities that responded to wounding or combination of wounding and hormones, wounded carrot and lettuce tissues showed marked increases in the specific AOX values (figure 2.3). For wounded lettuce tissues, applications of hormones further increased the specific AOX. However, for carrot tissues, hormones did not increase the specific AOX compared to wounding *per se*. This suggests that wounding stress or combination of wounding and exogenous hormone applications may influence the phenolic profiles associated to the different specific AOX of lettuce and carrot tissues. On the other hand, jicama did not show an increase in specific AOX after combining wounding or combination of wounding and hormones, indicating that the phenolic profiles was not altered. If a combination of stresses (*e.g.*, wounding and hormones) alters the phenolic profiles, this would imply that individual stresses are triggering the synthesis of specific enzymes of different branches in the phenylpropanoid metabolism.

Table 2.5. Antioxidant capacity of selected wounded fresh produce. Samples were evaluated on the initial day before treatment (control) and after 4 d of storage at 20°C (AC, 250 ppm MJ and 1000 ppm ET). Results show means of 9 replicates and their standard deviation. Letters in parenthesis show mean comparisons among treatments of each produce (LSD P>0.05).

Produce	Antioxidant Capacity [µg Trolox Eq/g FW]			
	Initial day control	Air control	Methyl jasmonate	Ethylene
Celery	70±12(c)	110±12(b)	136±9(a)	114±28 (b)
White onions	291±41(c)	423±59(ab)	378±52(b)	440±42(a)
Lettuce	29±10(d)	129±28(c)	162±28(b)	279±16(a)
Carrots	459±99(c)	2182±230(b)	2320±218(b)	2556±266(a)
Jicama	182±18(c)	216±20(ab)	203±28(bc)	227±27(a)
Bell peppers	1479±222(a)	1462±161(a)	1513±132(a)	1591±170(a)
Red onions	349±52(c)	390±31(b)	397±48(b)	450±35(a)
Asparagus	1445±148(ab)	1503±142(a)	1348±107(b)	1555±109(a)
Green cabbage	657±30(a)	586±31(b)	573±67(b)	684±48(a)
Red apples	2629±207(a)	2523±200(a)	2559±87(a)	2589±169(a)
Tomatoes	559±63(b)	551±69(b)	636±79(a)	558±59(b)
Nectarines	581±89(a)	410±48(b)	385±36(b)	395±48(b)
Radish	630±68(a)	546±45(b)	485±32(c)	569±15(b)
Red cabbage	2117±380(a)	2036±288(a)	2128±285(a)	2113±256(a)
White potatoes	609±99(ab)	678±85(a)	651±78(a)	544±29(b)
Pears	381±68(ab)	371±38(b)	397±49(ab)	430±51(a)

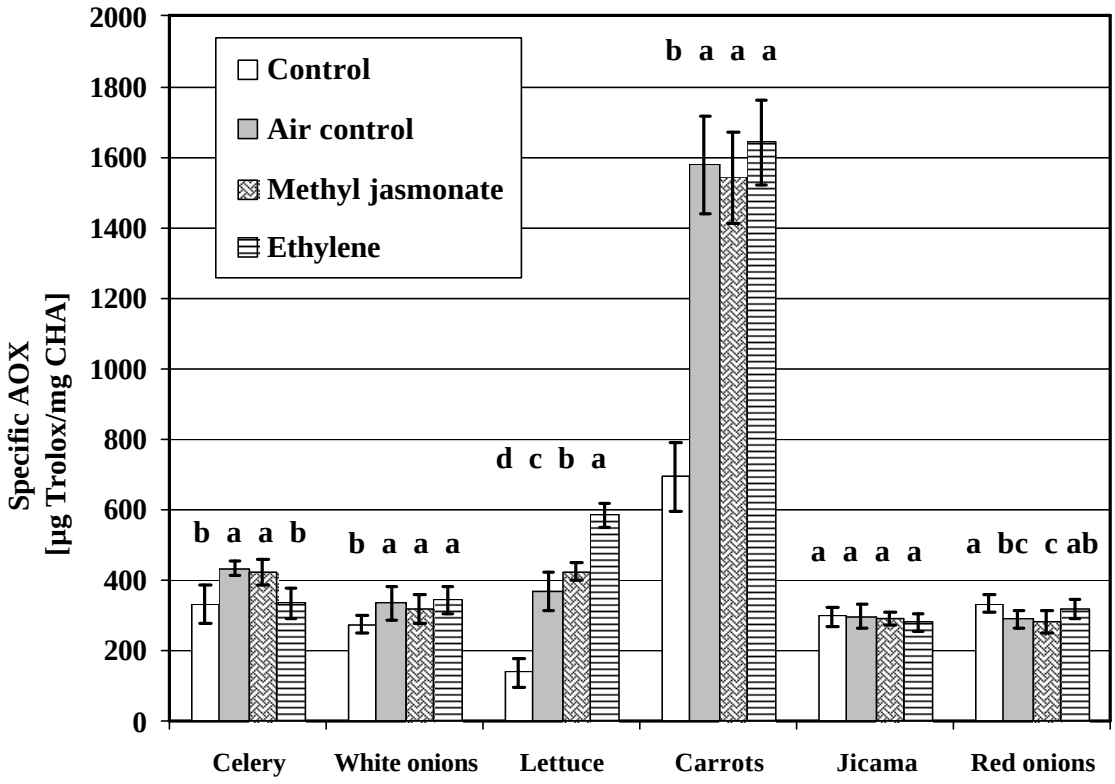

Figure 2.3. Specific antioxidant capacity for wounded produce exposed to MJ (250 ppm) and ET (1000 ppm) for 4 d of storage at 20°C. Results show means of 9 replicates, and bars show standard deviation. Letters show mean comparisons within each produce (LSD P>0.05).

Effect of MJ and ET on PAL activity of wounded tissues

The increase in total phenolic content of carrots, lettuce and jicama corresponds to an increase in PAL activity (figure 2.4). The wounding stress caused a synthesis of the main enzyme of the phenylpropanoid pathway and was further enhanced by both hormones in carrot and jicama but not in lettuce tissues. PAL activity has been previously shown to increase in lettuce after applying MJ (Campos-Vargas and Saltveit 2002) and ethylene (Lopez-Galvez and others 1996). Lettuce response in the present study differs from previous reports and could be related to differences in lettuce cultivars and wounding intensities used among other factors. In general, each tissue studied showed high linear correlations between total phenolics and PAL activity (R^2>0.65) (figure 2.5); however each tissue had different slopes indicating that a certain increase in the amount PAL activity will produce different increases in the amount of synthesized phenolics.

Effect of MJ and ET on the HPLC phenolic content of wounded carrot tissues

The HPLC provided chromatograms with the major peaks found for all treatments (figure 2.6). In order of retention time, the phenolic compounds and their derivatives were chlorogenic acid (CHA, 5.6 min), vanillic acid (VA, 9.8 min), ferulic acid (FA, 14.8 min), dicaffeoylquinic acid (diCQA, 17.4 min) and isocoumarin (23.9 min). The phenolic profile differed for each stressed carrot compared to the control at initial day (figure 2.7). Results indicated that wounded stressed carrot samples accumulated mainly CHA (~80% increase) and its derivative diCQA (~17% increase). These values were similar in MJ treated samples, while in ET treated samples there was a larger increase in CHA (~85%) and a smaller increase in diCQA (~6%). ET-treated samples showed an additional increase in the synthesis of the bitter compound isocoumarin (~450%) compared to the control. On the other hand, the use of MJ did not significantly increase the levels of isocoumarins, suggesting that MJ and ET trigger specific enzymes in the phenylpropanoid pathway other than PAL.

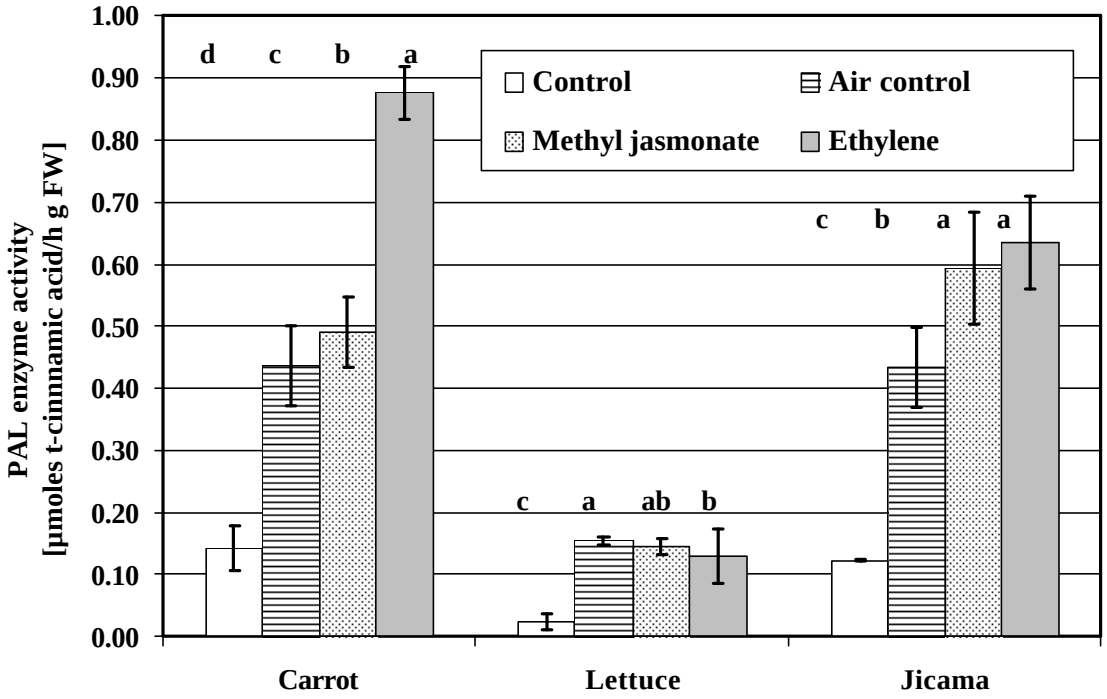

Figure 2.4. PAL enzyme activity from selected wounded commodities. Samples were evaluated on the initial day before treatment (control) and after 4 d of storage at 20°C (AC, 250 ppm MJ and 1000 ppm ET). Results show means of 9 replicates, and bars show standard deviation. Letters show mean comparisons within each produce (LSD P>0.05).

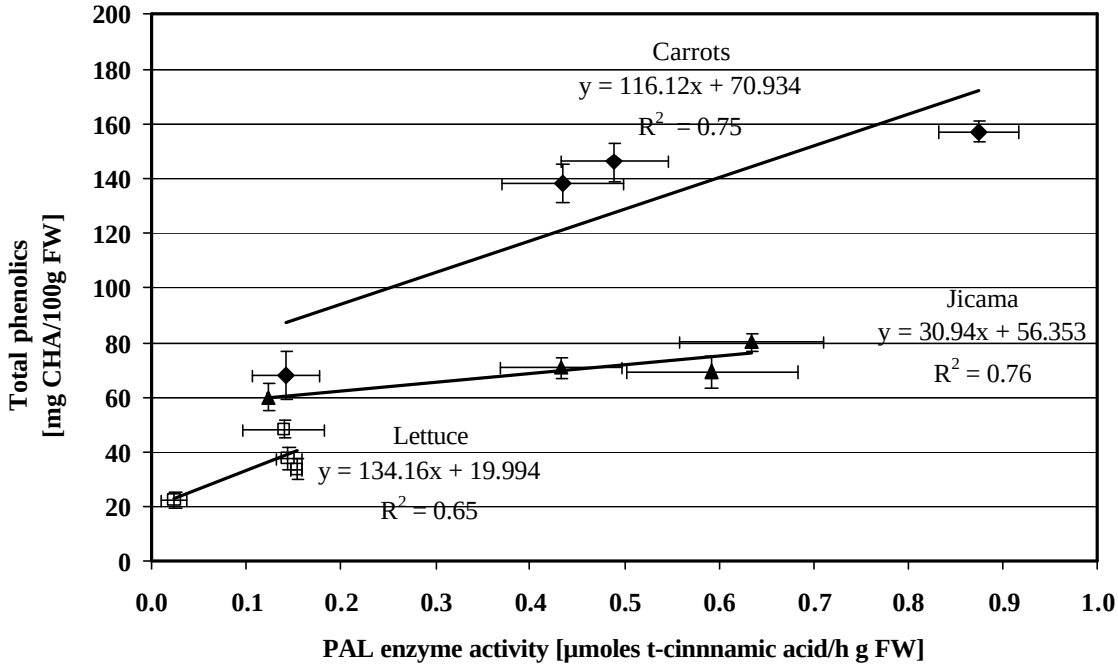

Figure 2.5. Correlation between total phenolics and PAL enzyme activity from selected wounded commodities. Samples were evaluated on the initial day before treatment (control) and after 4 d of storage at 20°C (AC, 250 ppm MJ and 1000 ppm ET). Each data point was obtained from 9 replicates, and bars show standard deviation. R^2 values refer to the coefficient of determination of the fit.

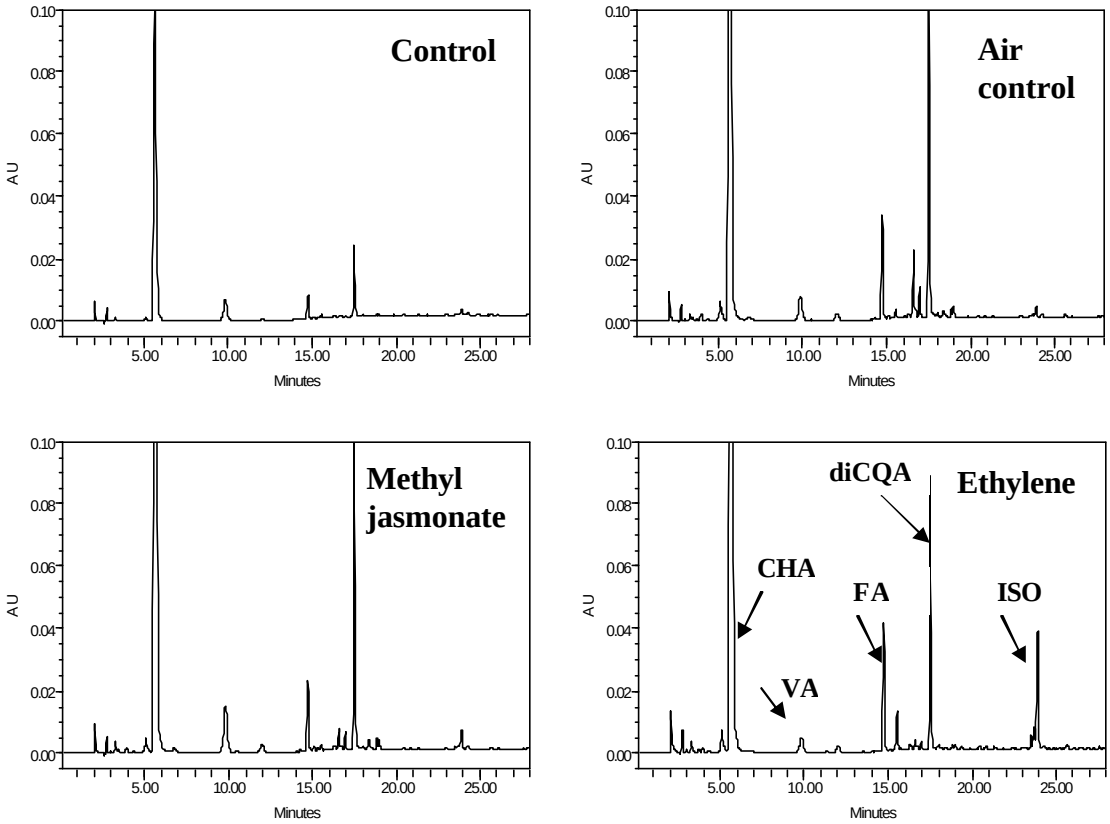

Figure 2.6. Typical HPLC phenolic profile (shown at 320 nm) from methanolic extracts of hormone-stressed wounded carrots. Control represents analysis at initial day, while AC, MJ (250 ppm) and ET (1000 ppm) were done after 4 d of storage at 20°C. The identified peaks are chlorogenic acid (CHA, 5.6 min), vanillic acid (VA, 9.8 min), ferulic acid (FA, 14.8 min), dicaffeoylquinic acid (diCQA, 17.4 min) and isocoumarins (ISO, 23.9 min).

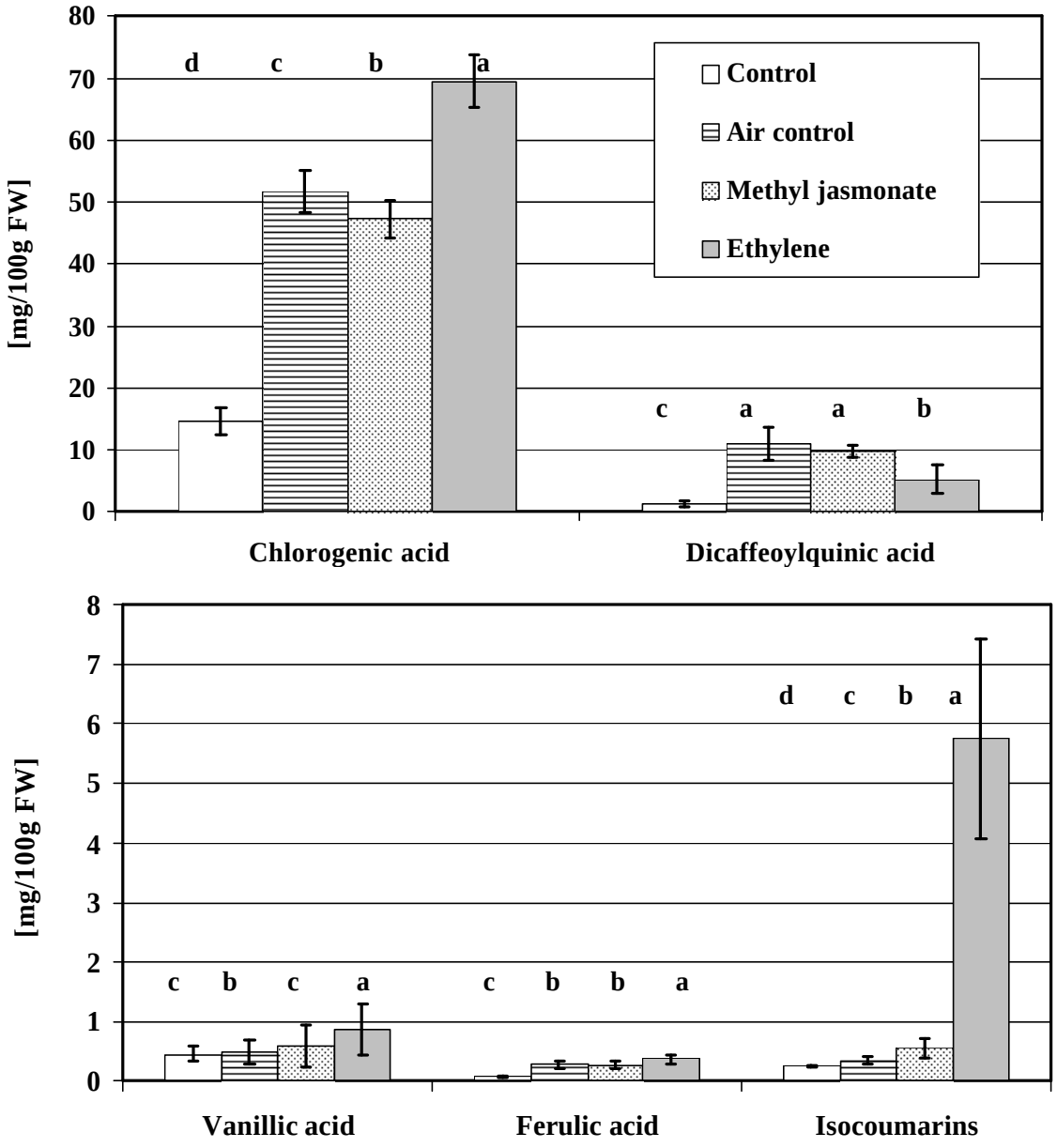

Figure 2.7. Main phenolic acids from methanolic extracts of wounded and wounded+hormone stressed carrots. Phenolics were obtained reading Abs at 320 nm, while Isocoumarins were read at Abs 267nm. Samples were evaluated at initial day (control) and after 4 d of storage at 20°C (AC, 250 ppm MJ and 1000 ppm ET). Results show means of 9 replicates, and bars show standard deviation. Letters show mean comparisons (LSD P>0.05).

Conclusions

The selected whole produce studied did not show an increase in total phenolic content neither in antioxidant capacity after 4 d of storage at 20°C. However, the use of methyl jasmonate slightly enhanced the phenolic content of lettuce, green beans and plums, while ethylene caused an increase in lettuce, green beans and carrots. Whole cilantro, asparagus, potatoes, apples, peaches and table grapes were not affected by exogenous hormones.

Wounding stress enhanced the TP of carrots, lettuce, jicama, red onions, white onions and celery with a corresponding increase in AOX, with exception of wounded bell peppers. Other tissues including asparagus, cabbage, apples, tomatoes, nectarines, radishes, potatoes and pears did not show an increase in TP due to wounding stress.

The effect of plant hormones on lettuce, celery, red onions, carrots, and jicama is larger when plant tissues are first exposed to wounding stress as compared to the corresponding hormone stress. The reason of the synergistic effect is not clear. However, we propose that both stresses may be sharing in part a common signaling molecule which when added may reach a certain threshold that amplifies the response. This synergistic effect may be affected by hormone dose and wounding intensity.

CHAPTER III

EFFECT OF EXOGENOUS ETHYLENE AND METHYL JASMONATE ON THE ACCUMULATION OF PHENOLIC COMPOUNDS AND PAL ACTIVITY ON CARROTS (*DAUCUS CAROTA*) UNDER DIFFERENT WOUNDING INTENSITIES

Synopsis

It is proposed that the intensity of wounding in combination with hormone stresses may accelerate the accumulation of bioactive phenolic compounds. Cut carrots were used as a model system to study the stress-responses. Carrots were cut in slices, pie-cuts and shreds, while whole carrots were used as a control. Carrots under different wounding intensities were exposed to 250 ppm MJ and 1000 ppm ET. Results indicated that synthesis and accumulation of stress-induced phenolic compounds in carrot tissues are dependent upon wounding intensity. Combination of wounding with exogenous applications of MJ and ET further enhanced the accumulation of phenolic compounds. The level of total phenolics was correlated to the antioxidant capacity. The major compounds responsible for the increase of AOX were to be chlorogenic acid and its derivatives. The proportion of chlorogenic acid, dicaffeoylquinic acid and ferulic acid and the interaction among them determined the specific AOX of the phenolic profiles of the effectiveness of the phenolic compounds to neutralize free radicals.

Introduction

Plants have evolved a highly sensitive and efficient system for monitoring changes in their environment (Lamikanra 2005). They can respond to physical damage by an increase in their general metabolism, including respiration rate, and this response appears to be in proportion to the severity of the damage (Barry-Ryan and O'Beirne 2000). Wounding is one of the many abiotic stresses that produce signals that migrate through cells into uninjured tissue and induces a number of physiological responses

(Saltveit 2000). In fact, when plants undergo the onslaught of wound-causing agents they activate mechanisms directed to healing and further defense (Ryan 2000; Rakwal and Agrawal 2003). Most of the induced responses can occur in a time frame of a few minutes to several hours after wounding, and include the generation/release, perception and transduction of specific signals for the subsequent activation of wound-related defense genes (Cantos and others 2001; Leon and others 2001; Orozco-Cardenas and others 2001; Taiz and Zeiger 1998; Zhao and others 2005) (figure 3.1).

The accumulation of phenolic compounds represents a major facet in the inducible defense mechanisms of plants through the phenylpropanoid pathway (Matern and Grimmig 1994; Dangl and others 2000) (figure 2.1). The induction of the phenylpropanoid metabolism could be also achieved experimentally by treatments with elicitors or exposure to specific stress conditions (Saltveit 2000; Cisneros-Zevallos 2003). However, wound signaling in plants is a complex process involving a whole array of molecules with regulatory activity, where the linear signaling pathways can form networks allowing overlapping and interlinking of signals or cross-talk (Rakwal and Agrawal 2003).

For instance, the ET produced by mechanical wounding in higher plants is referred to as ‘stress ethylene’ and previous work support a role for ethylene as a mediator of the wound signal, inducing expression of defense genes (Watanabe and Sakai 1998). Similar effects have been observed with exogenous applications of ET to diverse fresh produce (Christoffersen and Laties 1982; Saltveit 1999). MJ is another phytohormone responsible for the perception and transduction of wound signals, but through the octadecanoid pathway (Rakwal and Agrawal 2003). Studies have shown a positive correlation between exogenous applications of MJ and the synthesis of phenolic compounds, presumably as part of plant defense response (Saniewski and others 1998; Campos-Vargas and Saltveit 2002).

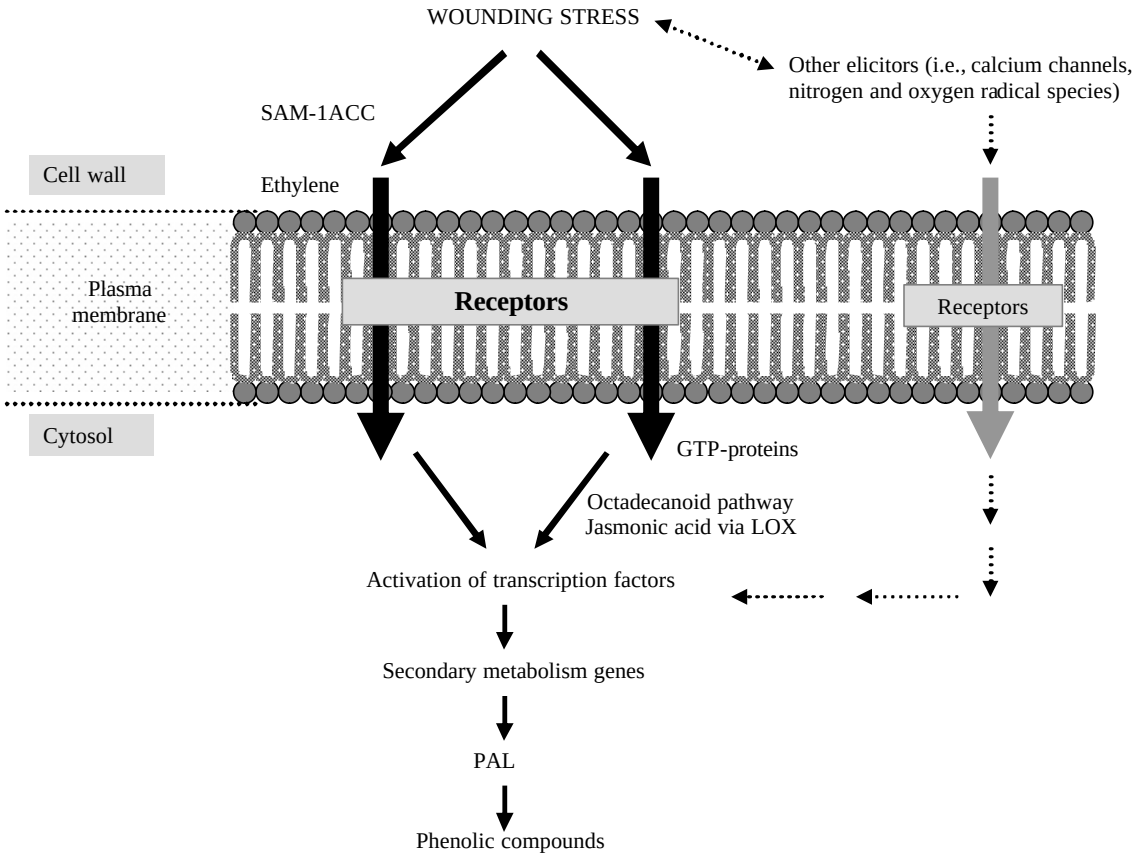

Figure 3.1 Model postulating the relationship between wounding, ethylene, methyl jasmonate and the signal transduction pathways that induce the production of secondary defense compounds (*i.e.*, phenolics). (SAM, s-adenosylmethionine; 1ACC, 1-aminocyclopropane-1-carboxylic acid; LOX, lipoxygenase; PAL, phenylalanine ammonia lyase) (Taiz and Zeiger 1998; Cantos and others 2001; Leon and others 2001; Orozco-Cardenas and others 2001; Zhao and others 2005).

In this part of the study it is proposed that the intensity of wounding in combination with hormone stresses may enhance the accumulation of bioactive phenolic compounds. Carrots were used as a model system. The specific objectives were to determine the effect of wounding intensity in combination with MJ and ET on phenolic content, antioxidant activity and specific phenolic profiles during storage.

Materials and Methods

Plant materials and reagents

Carrots (*Daucus carota,* Cv Choctaw) were provided by Grimmway Farms (Bakersfield, CA). Carrots were sorted, washed and disinfected with chlorinated water (250 ppm). Carrot cuts were produced in the form of slices (3-4 mm thickness), pie-cuts (¼ sections from a slice of 3-4 mm thickness), shreds (2x3x40-60 mm, using a food processor), and whole non-wounded carrots. For each one of the three carrot cuts, a wounding intensity number was calculated by dividing the resulting wounding area [cm^2] over the weight [g] of the carrot cuts. Wounding intensity (A/W) values were 0, 4.2, 6 and 23.5 cm^2/g for whole, slice, pie-cut and shred carrot tissues, respectively. Whole and wounded samples (~150 g) were placed inside 1-gallon glass jars, where MJ and ET treatments were continuously applied during the 12 d of storage at 15°C. While ET was directly injected into the jars to obtain 1000 ppm, MJ was applied by wetting a filter paper over a Petri dish to obtain 250 ppm in a headspace-saturated atmosphere.

The chemicals used were methyl jasmonate (95%), Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), Trolox and 2,2-diphenyl-1-picrylhydrazyl (DPPH), polyvinylpolypyrrolidone (PVPP), sodium hydroxide (NaOH), boric acid, 2-mercaptoethanol, sodium phosphate, L-phenylalanine and sulfuric acid (Sigma-Aldrich, St. Louis, MO, USA). Ethylene (CP grade, 99.5%) and sample preparation supplies were obtained from Fisher Scientific (Houston, TX, USA) including filters, membranes and syringes. The solvents, methanol, ethanol, hexane, acetonitrile and water, were quality HPLC grade.

Analysis of total soluble phenolics

Phenolic content was evaluated following the procedure of Swain and Hillis (1959). Samples of 5 g were homogenized with 20 mL methanol using an Ultra-Turrax homogenizer (IKA Works, Inc., Wilmington, NC, U.S.A.), to uniform consistency and incubated overnight at 4°C. Homogenates were centrifuged (rotor JA-17; centrifuge J2-21; Beckman Coulter, Inc., Fullerton, CA, U.S.A.) at 29,000 x g for 15 min at 4°C. Sample aliquots of 150 µL were taken from the clear supernatant and then diluted with 2400 µL nanopure water, followed by 150µL 0.25N Folin-Ciocalteu and incubated for 3 min at room temperature. The reaction was stopped by adding 300 µL 1N Na_2CO_3 and the mixture was incubated 10 min. Samples were again centrifuged at 29,000 x g for 15 min at 4°C. Absorbance readings of the supernatant samples were taken at 725 nm using the spectrophotometer with photo diode array detector (model 8452A, Hewlett Packard Co, Waldbronn, Germany). A blank prepared with methanol was used as control. The level of total phenolics for each sample was determined by using a standard curve developed with chlorogenic acid (CHA).

HPLC phenolics profile

Fresh samples of 5 g were extracted with 20 mL methanol, using the Ultra-turrax homogenizer. Homogenates were centrifuged at 29,000 x g for 15 min at 4°C. The supernatant was passed through nylon membranes (0.2 µm) prior to injection into the chromatography system. Except for the SpectraPhysics SP8792 column heater (San Jose, CA, USA), the HPLC system was composed of Waters Co instruments (Milford, MA, USA). These were two 515 binary pumps, a 717-*plus* autosampler, and a 996 photodiode array detector. The column used to separate the phenolic compounds was a 4.6 x 150 mm, 5 µm, C-18 reverse-phase Atlantis column (Waters, Milford, MA, USA), which was maintained at 40°C. The injection volume was 10 µL. Samples were analyzed under gradient conditions with two mobile phases consisting of HCl acidified water at pH 2.3 (solvent A) and acetonitrile (solvent B). The gradient system was 0/85, 5/85, 30/0, 35/0 (min/% solvent A). Data was processed by using Waters Millennium software v3.2 (Hale

2003). The level of individual phenolics was determined by using standard curves developed with high-purity HPLC-grade commercial phenolic compounds. Information from Chapter II (see methodology) was used to process isocoumarin (ISO) and dicaffeoylquinic acid (diCQA) results.

Analysis of antioxidant capacity

The antioxidant or antiradical capacity (AOX) of phenolic compounds was evaluated following the procedure of Brand-Williams and others (1995). Samples were homogenized with methanol to uniform consistency and refrigerated overnight. Homogenates were centrifuged at 29,000 x g for 15 min at 4°C. Sample aliquots of 150 µL were taken from the clear supernatants and then diluted with a 2,850 µL DPPH solution, which was previously prepared with methanol until reaching 1.1 units of absorbance at 515 ηm. A blank was prepared with methanol and used as a control, as well as to zero the spectrophotometer for further readings of all samples at 515 ηm. The mixture was allowed to react in a shaker until no significant decrease in absorbance was obtained. The antioxidant capacity was calculated as µg Trolox equivalents using a standard curve. In addition, the specific antioxidant capacity was used in this study and defined as the ratio of total antioxidant capacity over total soluble phenolics and expressed as µg Trolox/mg chlorogenic acid. The specific antioxidant capacity provides information of the effectiveness of phenolic compounds to neutralize free radicals. A higher specific antioxidant capacity means phenolic compounds have a higher capacity to stabilize free radicals.

Phenylalanine ammonia lyase enzyme activity

PAL was extracted from 1 g of fresh produce in 25 mL borate buffer (pH 8.5) according to Ke and Saltveit (1986). Samples were placed inside ice and homogenized using an Ultra-Turrax homogenizer, under low-light conditions and at low speed to prevent protein denaturalization. Homogenates were filtered through cheesecloth and then taken to centrifuge 29,000 x g for 15 min at 4°C. The enzyme activity of PAL was

assayed by following the accumulation of cinnamic acid at 290 nm, using 100 mM of L-phenylalanine as PAL substrate and water for control samples. Units were reported as µmol of t-cinnamic acid/h g fresh weight.

Statistical analysis

The experiment followed a completely randomized design. Analyses were done using 5 replicates, unless otherwise indicated. Means, standard deviations, graphs and linear regressions were obtained using Microsoft Excel 2000. For means comparison at the 5% significance level, ANOVA and LSD multiple-range tests were performed using SAS (Raleigh, NC, USA).

Results and Discussion

Effects of wounding intensity and combination of wounding and hormones on the total phenolics content and PAL activity of fresh carrots through time

The effect of wounding intensity on the synthesis of phenolic compounds during storage for 12 d at 15°C is shown in figure 3.2. Results indicated that wounding intensity affected the phenolic content of air control samples through time. The highest response was for shreds, while the response decreased with less wounding intensity. For whole tissue, phenolic content was the same as the initial day after 12 d of storage. When MJ was applied to wounded tissue, a slightly higher accumulation of phenolics was observed mainly of shred cuts. ET treatments on wounded tissues enhanced even more the accumulation of phenolic compounds through time, and this effect was higher with increased wounding intensity. For ET stressed wounded tissue (*e.g.*, pie-cuts and shreds) the rate of phenolic synthesis during the first 6 d of storage was higher compared to the rate from day 6 to 12.

The synthesis and accumulation of stress-induced phenolic compounds in carrot tissues is dependent upon the intensity of wounding (figure 3.3). With initial values of ~70 mg chlorogenic acid/100 g fresh weight, the phenolic content of air control samples

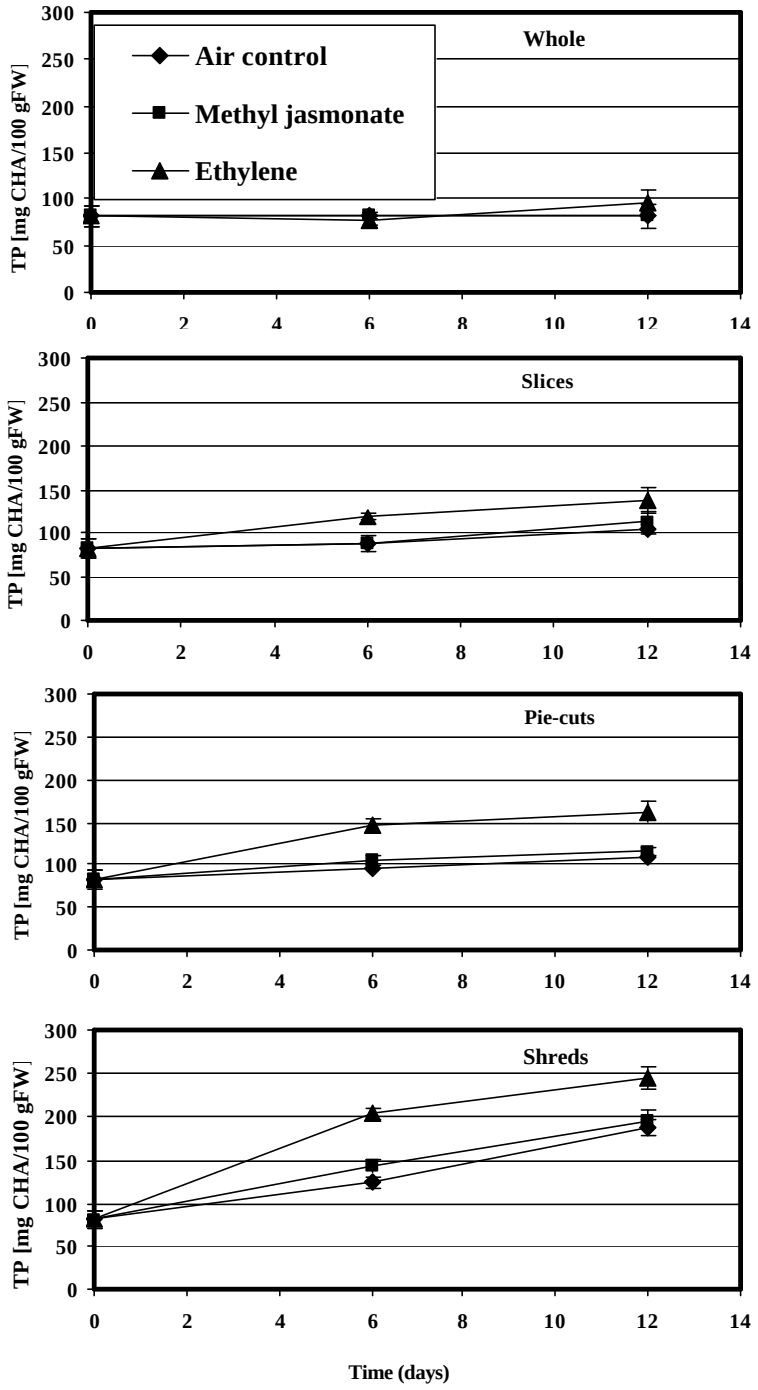

Figure 3.2. Effects of wounding intensity, MJ+wounding and ET+wounding stresses on the total phenolics of fresh carrots through time. The concentration of MJ was 250 ppm, and 1000 ppm for ET. Samples were evaluated after 6 and 12 days of storage at 15°C. Results show means of 5 replicates and their standard deviation.

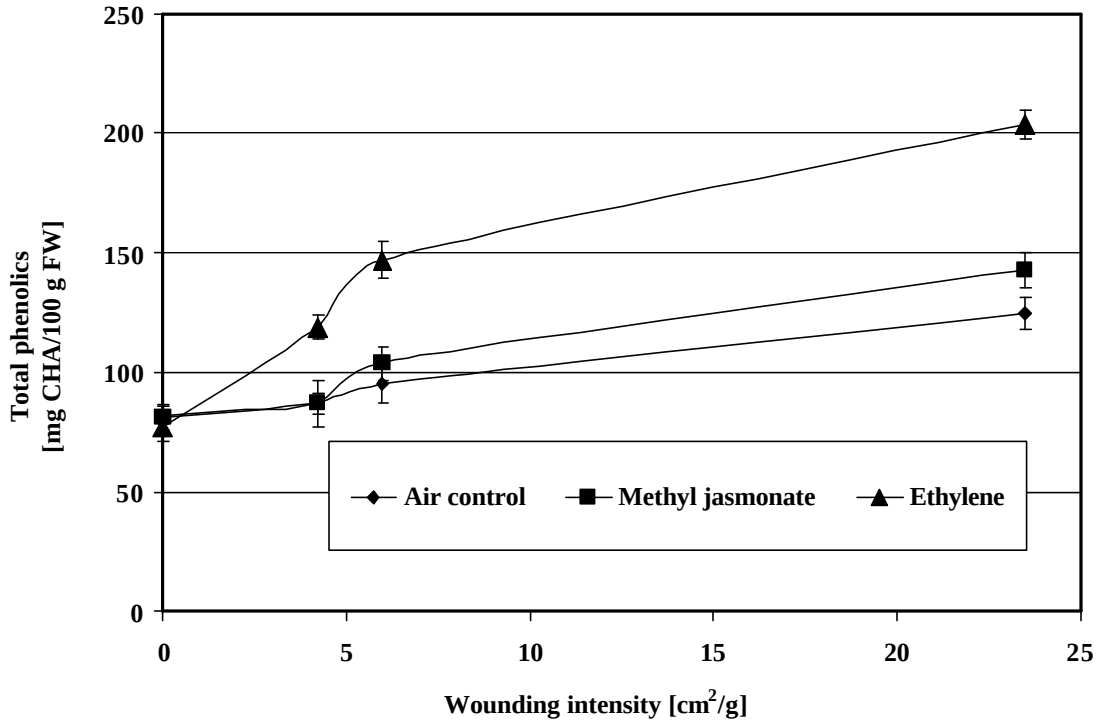

Figure 3.3. Wounding intensity effect on the total phenolic content of hormone-treated carrots. Samples were stored 6 days at 15°C. Results show means of 5 replicates and their standard deviation. A/W values are equal to whole (0 cm^2/g); slices (4.6 cm^2/g); pie-cuts (6 cm^2/g); and shreds (23.5 cm^2/g).

increased ~10, ~20 and ~75% for slices, pie-cuts and shreds, respectively after 6 d at 15°C. When different carrot cuts were exposed to MJ and ET, these hormone stresses enhanced further more the synthesis of phenolic compounds. The largest phenolic content increases were ~150% and ~200% for MJ- and ET-treated shredded carrots. On the other hand, phenolic compounds did not or slightly accumulate in whole carrots when exposed to exogenous hormones, confirming our previous observation that wounding and hormone stresses when combined, they induce a synergistic response in phenolic accumulation.

PAL is the first committed step in the phenylpropanoid metabolism and is a key enzyme which produces many phenolic defense compounds (Dixon and Paiva 1995). The activity of PAL increased with wounding intensity. For an A/W ~4.2 cm^2/g (slices), PAL increased by ~15%, while for an A/W ~23.5 cm^2/g (shreds), PAL increased by ~170% (figure 3.4). PAL activity was enhanced when combining wounding and the phytohormones MJ or ET. This effect was mostly seen for A/W = 6 cm^2/g (pie-cuts). The increase in PAL activities ranged from ~200 to ~800% compared to the initial day evaluations, for wounded and hormone stressed carrots. There was a strong correlation (R^2=0.98) between wound- and wound-hormone- induced PAL activity and phenolic accumulation measured at day 6 of storage at 15°C. Similar results have been reported for lettuce, potatoes and carrots (Lopez-Galvez and others 1996; Leja and others 1997b; Reyes and Cisneros-Zevallos 2003; Choi and others 2005).

The mechanism by which wounding and hormones show a synergistic effect is not clear. However it is possible that both stresses share a common signaling molecule which may amplify the response. For example, reactive oxygen species (ROS) are considered signaling molecules for wounding stress (Hancock and others 2001; Razem and Bernards 2003), and wounding is known to increase respiration (Surjadinata and Cisneros-Zevallos 2003) thus, this increased respiration may increase the presence of ROS (Mittler 2002; Blokhina and others 2003; Apel and Hirt 2004; Murphy and DeCoursey 2006; Brookes 2005). When hormones such as ET are applied there would a further increase in respiration (Kahl and Laties 1989; Saltveit 1999) and possibly a

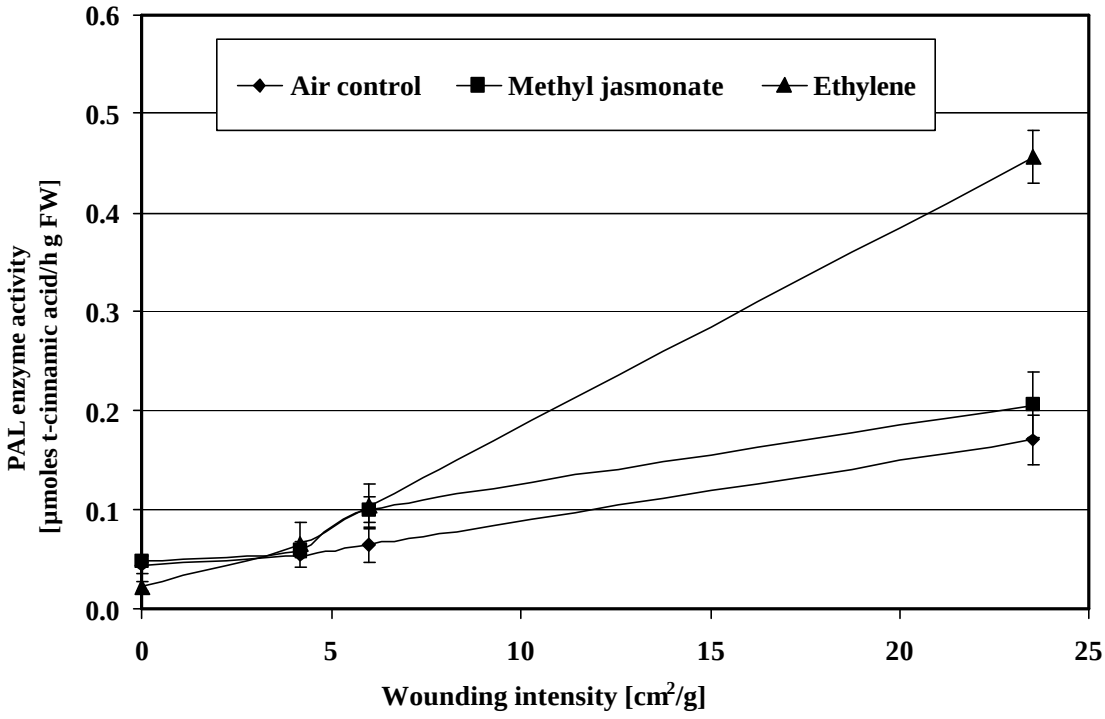

Figure 3.4. Wounding intensity effect on the PAL enzyme activity of hormone-treated carrots. Samples were stored 6 days at 15°C. Results show means of 5 replicates and their standard deviation. A/W values are equal to whole (0 cm^2/g); slices (4.6 cm^2/g); pie-cuts (6 cm^2/g); and shreds (23.5 cm^2/g).

higher increase in ROS amplifying the response. Alternatively, amplification of the response could be achieved through a positive feedback loop of ROS by increasing furthermore the activity of NADPH oxidase and producing more ROS (Mittler and others 2004; Brandes 2005; Afanas'ev 2006). Either these or other mechanism happening, what is known is that crosstalking does take place among different stresses when applied to plants (Rakwal and Agrawal 2003; Zhao and others 2005) thus, synergism would be a result of this interaction through signaling molecules.

Effects of wounding intensity and combination of wounding and hormones on the antioxidant capacity of fresh carrots through time

Wounding enhanced the antioxidant capacity of carrot tissues during a storage period of 12 days at 15°C (figure 3.5). Results showed that increased wounding intensity enhanced the antioxidant capacity of air control samples through time in similar trend as in phenolic compounds. For whole tissue there was no increase in AOX, however for shred cuts AOX increased up to ~300% after 12 d. Compared to air control, MJ application to wounded tissue slightly increased the AOX through time. When ET was applied on wounded tissue, AOX increased even further and the effect was higher with increased wounding intensity. For ET stress wounded tissue (pie-cuts and shreds), the rate of AOX increase during the first 6 d of storage was higher compared to the rate of AOX increase from day 6 to 12. The highest AOX increase of ~3000 µg Trolox/g fresh weight corresponded to shred cuts after 12 d of storage.

In general, the antioxidant capacity (AOX) followed a similar trend to that of phenolic content for tissues exposed to different wounding intensities and hormones (figures 3.2 and 3.3). Wounding stress (shred cuts) increased the AOX by ~120% after 6 d of storage at 15°C from an initial value of ~500 µg Trolox equivalents/g fresh weight corresponding to whole tissue (figure 3.6). When MJ and ET was applied to wounded tissue (shred cuts), the AOX increased by ~150% and ~400%, respectively. The enhanced AOX of ET-treated wounded carrots (~2500 µg Trolox equivalents/g fresh

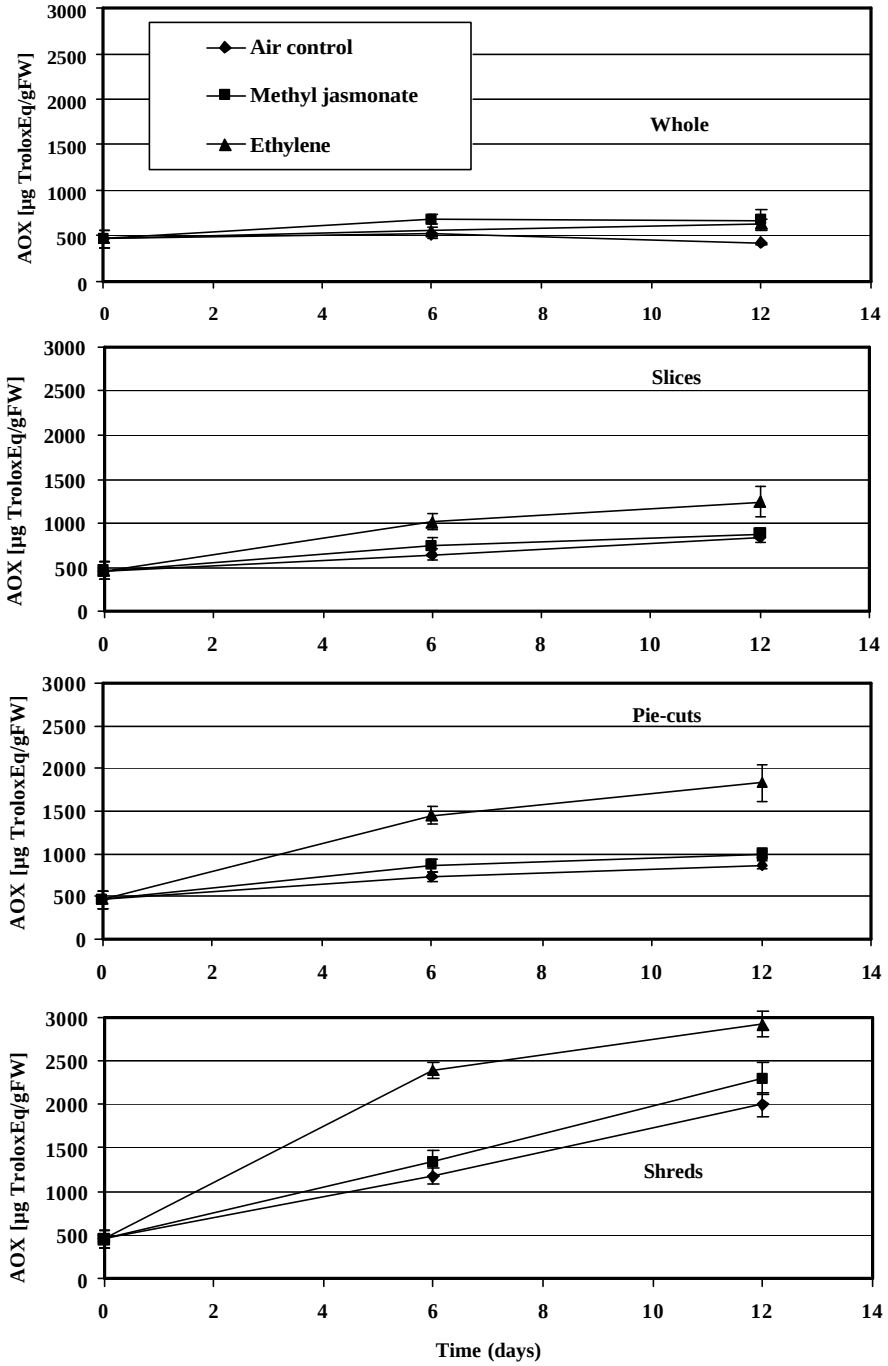

Figure 3.5. Effects of wounding intensity, MJ+wounding and ET+wounding stresses on the antioxidant capacity of fresh carrots over time. The concentration of MJ was 250 ppm, and 1000 ppm for ET. Samples were evaluated after 6 and 12 days storage at 15°C. Results show means of 5 replicates and their standard deviation.

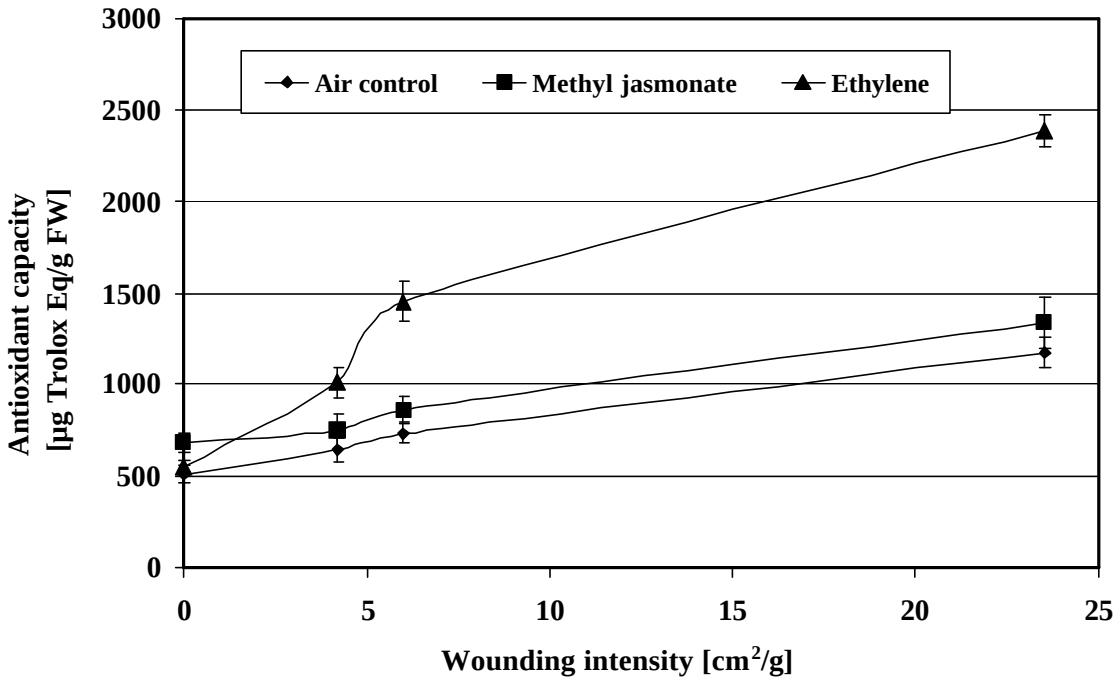

Figure 3.6. Wounding intensity effect on the antioxidant capacity of hormone-treated carrots. Samples were stored 6 days at 15°C. Results show means of 5 replicates and their standard deviation. A/W values are equal to whole (0 cm^2/g); slices (4.6 cm^2/g); pie-cuts (6 cm^2/g); and shreds (23.5 cm^2/g).

weight) could be of great importance, considering that other crops with higher AOX such as blueberries (~5500 µg trolox equivalents/g fresh weight) are promoted as a good source of bioactive antioxidants (Cevallos-Casals and Cisneros-Zevallos 2003). Furthermore, the consumption *per capita* of blueberries is only ~0.4 lb/year (ERS-USDA 2003) compared to that of carrots with ~8 lb/year (ERS-USDA 2004), making carrots a non expensive source of phenolic antioxidants.

The relationship between TP and AOX for each wounding intensity with or without exogenous MJ and ET showed high linear correlation (R^2 =0.86 to 0.98, data not shown) suggesting that the synthesized phenolic compounds are responsible for the increased AOX. In addition, the specific antioxidant capacity (antioxidant capacity on phenolics basis) was determined for each wounded and hormone stressed carrot samples (figure 3.7). Results indicated that the specific AOX increased with wounding intensity from ~600 to 900 µg Trolox/mg phenolics. For MJ stressed wounded tissue, the specific AOX increased and ranged from ~800 to 900 µg Trolox/mg phenolics, while for ET stressed wounded tissue the specific AOX ranged from ~700 to 1150 µg Trolox/mg phenolics. The specific AOX depends on the phenolic profile present in the tissue since it is and indicator of the effectiveness to neutralize free radicals by the phenolic compounds present. Thus, the newly synthesized phenolics due to wounding or wounding combined with hormone stresses are providing phenolic profiles with higher antioxidant capacity to stabilize free radicals compared to phenolic profiles present in whole tissue. For example, the specific AOX of synthesized phenolic compounds from ET treated shred cut carrots are 2-fold higher compared to phenolic compounds from whole carrots (figure 3.7).

Phenolic profiles of whole, wounded and hormone stressed carrots

The main phenolics found in whole carrot tissues were chlorogenic acid (~82%), ferulic acid (~1.2%), dicaffeoylquinic acid (~17%) and in less amount isocoumarins (~0.5%)(table 3.1). Whole carrots non-treated with hormones showed a 1.1 to 1.6-fold

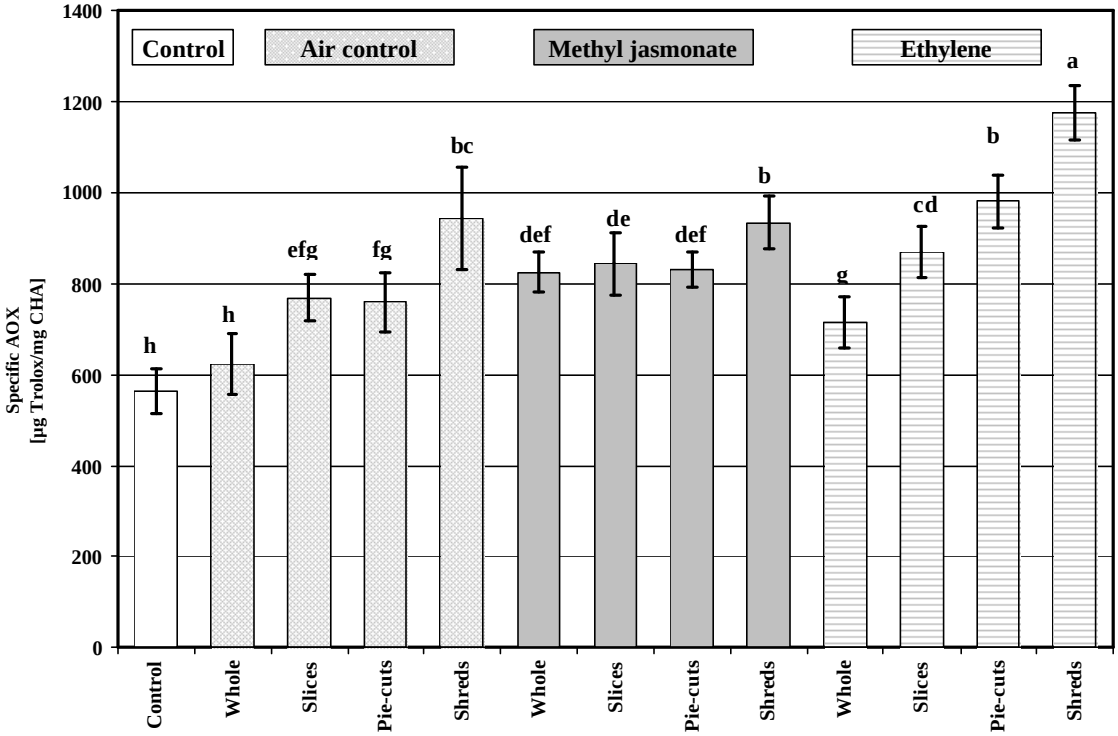

Figure 3.7. Effect of wounding intensity and wounding with hormones on the specific antioxidant capacity of fresh carrots. Samples were stored 6 days at 15°C. Bars show the data for each one of the carrot cuts (wounding intensity). Results show means of 5 replicates, and bars show standard deviation. Letters show mean comparisons among treatments (LSD P>0.05).

Table 3.1. The effect of wounding intensity (A/W) on the individual phenolic content (Abs 320 nm) and isocoumarins (Abs 267 nm) from methanolic extracts of wounded and hormone stressed carrots. Samples were evaluated at initial day (Control) and after 6 days of storage at 15°C (AC, air control; MJ, methyl jasmonate 250 ppm; ET, ethylene 1000 ppm). Results show means of 5 replicates, and the symbol ± shows the standard deviation. Letters show mean comparisons per column (LSD P>0.05).

Treatment	A/W	Phenolic acids [mg/100g FW]			Isocoumarins
		Chlorogenic acid	Ferulic acid	Dicaffeoyl-quinic acid	
Control	**Whole**	14.8±2.2(gh)	0.22±0.05(f)	3.2±0.6(i)	0.09±0.00(e)
Air control	**Whole**	12.5±2.4(h)	0.27±0.06(f)	8.4±2.7(fg)	0.12±0.02(e)
	Slices	19.2±1.8(efg)	0.48±0.03(cde)	8.6±0.6(fg)	0.13±0.00(e)
	Pie-cuts	22.8±5.2(ef)	0.64±0.10(ab)	11.1±1.6(ef)	0.19±0.03(de)
	Shreds	62.8±2.1(b)	0.72±0.03(a)	23.6±1.1(b)	0.47±0.06(d)
Methyl jasmonate	**Whole**	13.1±2.9(h)	0.25±0.05(f)	5.0±1.3(hi)	0.08±0.01(e)
	Slices	17.6±2.6(fgh)	0.51±0.10(cd)	12.6±4.3(de)	0.11±0.02(e)
	Pie-cuts	23.7±3.7(e)	0.65±0.10(ab)	18.0±5.3(c)	0.15±0.01(e)
	Shreds	46.1±5.5(c)	0.40±0.09(e)	11.7±2.3(ef)	0.20±0.05(de)
Ethylene	**Whole**	15.5±3.4(gh)	0.42±0.06(e)	7.0±2.7(gh)	1.27±0.13(c)
	Slices	32.1±4.3(d)	0.45±0.08(de)	9.5±2.1(efg)	1.34±0.17(c)
	Pie-cuts	44.3±5.4(c)	0.63±0.04(b)	15.3±1.5(cd)	3.74±0.45(b)
	Shreds	92.6±11.4(a)	0.54±0.06(c)	28.1±3.0(a)	9.67±0.75(a)

($p<0.05$) increase in dicaffeoylquinic acid (diCQA), while chlorogenic acid (CHA) and ferulic acid (FA) were not mainly affected ($p>0.05$) after 6 d of storage at 15°C. Only isocoumarin showed a ~13.1-fold and FA a ~0.9-fold increase for ET treated whole carrots.

Wounding stress caused an increase in all 4 phenolic compounds compared to whole carrots, including a ~4, 1.6, 1.8 and 1.7-fold increase in CHA, FA, diCQA and isocoumarins, respectively ($p<0.05$) for shred carrots. MJ treated wounded carrots showed a ~2.5, 0.6, 1.3-fold increase in CHA, FA and diCQA, respectively ($p<0.05$) for shred cuts compared to MJ treated whole carrots; while ET treated wounded carrots presented a ~4.9, 0.28, 0.3 and 6.6-fold increase in CHA, FA, diCQA and isocoumarins, respectively ($p<0.05$) for shred cuts compared to ET treated whole carrots.

According to these results, the overall increase in AOX, previously observed in wounded tissue as well as in hormone stress wounded carrots (figure 3.6), would be related to the increases observed in CHA and diCQA. The CHA values obtained were linearly correlated to total phenolics (Folin assay) and with the AOX (DPPH assay) with $R^2 = 0.91$ and $R^2 = 0.90$, respectively (figure 3.8).

The percentage contribution of CHA, FA and diCQA (the 3 main phenolic compounds) on the phenolic profiles of whole and wounded and hormone stressed carrots after 6 d of storage at 15°C are present in table 3.2. CHA ranged from ~56 to 82%, FA from ~0.45 to 1.9% and diCQA from ~16.9 to 41.8%. As the percentage of CHA increased the percentage of diCQA decreased in wounded and hormone stressed carrots. The percentage of FA also decreased as the percentage of CHA increased in the phenolic profiles. According to this, the observed increase in the specific AOX values (figure 3.7) in the stressed tissues is due very likely to an increase in the percentage of CHA and the decrease percentage of diCQA and FA for each phenolic profile. The exact specific AOX values will depend on the proportions of CHA, diCQA and FA, and interactions among them, including additive, inhibiting or synergistic effects.

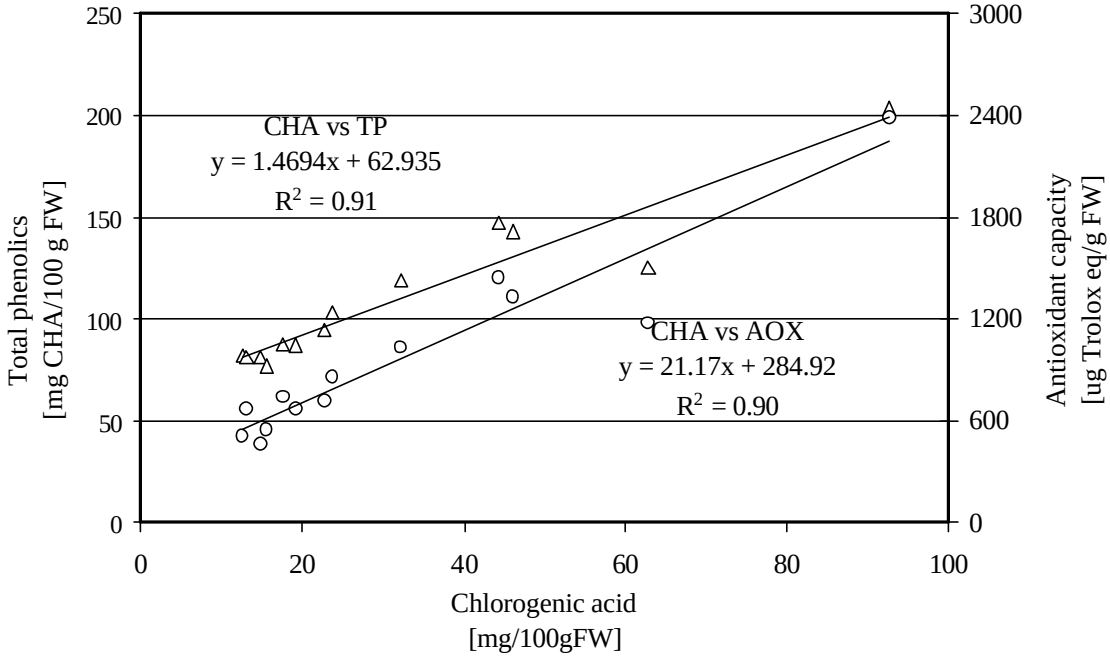

Figure 3.8. Correlation between CHA and total phenolics (left Y axis) and CHA and AOX (right Y axis) from wounded and hormone stressed carrots. Samples were evaluated on the initial day before treatment and after 6 d of storage at 15°C. R^2 values refer to the coefficient of determination of the fit.

Table 3.2. Percentage contribution on the phenolic profile (HPLC) of carrot pie-cuts, exposed to different wounding intensities and exogenous applications of methyl jasmonate (MJ, 250 ppm) and ethylene (ET, 1000 ppm) and air as control (AC). Samples were evaluated at initial day (Control) and after 6 days of storage at 15°C for all AC, MJ and ET. Results show means of 5 replicates, and the symbol ± shows the standard deviation. Letters show mean comparisons per column (LSD P>0.05).

Treatment	Wounding intensity (A/W)	Phenolic acids (%)			Specific AOX [µgTrolox/gCHA]
		Chlorogenic acid	Ferulic acid	DiCaffeoyl-quinic acids	
Control	**Whole**	81.9±2.1(a)	1.2±0.10(de)	16.9±1.98(i)	564±49(h)
Air control	**Whole**	62.1±1.9(ij)	1.3±0.05(d)	36.6±1.85(b)	623±67(h)
	Slices	67.7±1.3(gh)	1.7±0.07(b)	30.6±1.27(c)	768±52(efg)
	Pie-cuts	65.7±4.3(hi)	1.9±0.50(a)	32.4±3.88(cd)	759±67(fg)
	Shreds	72.1±0.5(def)	0.8±0.02(f)	27.1±0.49(def)	942±112(bc)
Methyl jasmonate	**Whole**	58.1±5.1(jk)	1.7±0.17(b)	40.3±5.15(ab)	826±43(def)
	Slices	56.6±4.6(k)	1.6±0.11(bc)	41.8±4.65(a)	843±67(de)
	Pie-cuts	71.4±1.8(efg)	1.4±0.09(cd)	27.3±1.83(de)	831±37(def)
	Shreds	79.3±1.2(ab)	0.7±0.05(f)	20.1±1.12(hi)	933±58(b)
Ethylene	**Whole**	68.7±6.4(fgh)	1.7±0.11(ab)	29.9±6.08(cd)	715±57(g)
	Slices	73.5±1.3(cde)	1.1±0.09(e)	25.4±1.19(efg)	868±57(cd)
	Pie-cuts	76.3±3.9(bcd)	1.1±0.12(e)	22.7±3.83(gh)	980±57(b)
	Shreds	76.3±1.1(bc)	0.5±0.03(g)	23.2±1.09(fgh)	1174±59(a)

The antiradical activity of phenolic compounds depends on their molecular structure (availability of phenolic hydrogens), and on the possibility for stabilization of the resulting phenoxyl radicals (Silva and others 2000). Generally, antioxidant capacity increases with an increase in the hydroxyl groups and a decrease in glycosylation (Kweon and others 2001). Wounding and hormone stresses both increased the production of the less polar forms of hydroxycinnamic acids like the dicaffeoylquinic acids. It is not clear the actual role of this synthesized compound in the cell. However, the strong radical scavenging activity it has (due to the higher availability of hydroxyl groups which provide more H-donor capability) may suggest a role in scavenging reactive oxygen species generated within the stressed carrot cells. Further work is needed to verify this point.

In relation to quality, Lafuente and others (1996) showed that 20 mg of isocoumarins per 100 g fresh weight can provide bitterness in carrots. Previously it was shown that ethylene can affect the isocoumarin levels of carrots (Lafuente and others 1996; Leja and others 1997b). In the present study, the synthesis of isocoumarins in ET treated wounded carrot tissues reached 9.67 mg/100 g fresh weight, which is below the limits of bitterness detection. For MJ treated samples, isocoumarin levels accumulated were minimal.

Conclusions

Wounding intensity plays a major role in activating the synthesis and accumulation of phenolic compounds. The combination of wounding with exogenous applications of MJ and ET further enhanced the accumulation of phenolic compounds by triggering the phenylpropanoid metabolism through an increase in PAL activity. This effect was higher in ET compared to MJ.

The phenolic compounds synthesized by these stresses showed high antioxidant capacity. This is based on the strong correlation among the total phenolics and the antioxidant capacity. From the phenolic compounds found in carrots, chlorogenic acid was the main compound and was well correlated with both the increase in total phenolics (Folin assay) and the antioxidant capacity (DPPH assay). For this reason, chlorogenic acid and its derivatives are responsible for enhancing the antioxidant capacity of stressed carrot tissues. Wounding and hormone stresses also affected the specific antioxidant capacity which is the AOX expressed on phenolics basis and is dependent on the phenolics profile. The specific proportions of CHA, diCQA and FA and the interactions among them, determined the specific AOX.

Despite of the increased isocoumarin content for ET stressed wounded carrots compared to MJ and controls, the obtained levels remained below the 20 mg/100 g fresh weight considered the limit for bitterness detection in carrots.

In general, these results indicate that wounding and hormone stressed wounded tissue can be used to enhance and change the phenolic profiles of carrots. This approach can be introduced in carrot processing operations to tailor specific phenolic profiles with higher AOX or other biological properties.

The enhancement in biological properties of these plant derived products using abiotic stresses could provide more antioxidants to regular diets, at low cost and be an alternative to genetic modifications and breeding activities.

CHAPTER IV

ROLE OF THE REACTIVE OXYGEN SPECIES (SUPEROXIDE AND HYDROGEN PEROXIDE), ON PAL ACTIVITY AND THE ACCUMULATION OF PHENOLIC COMPOUNDS IN WOUNDED AND HORMONE-STRESSED CARROT TISSUE

Synopsis

Plants generate and accumulate reactive oxygen species (ROS) in response to a wide variety of biotic and abiotic stresses including ultraviolet irradiation, wounding, hormones, and pathogens. ROS are in part toxic to the cell because they damage cellular constituents such as proteins, lipids, and nucleic acids, however, cells have evolved ways to cope with the oxidative damages. While high levels of ROS promote programmed cell death, low levels of ROS may trigger signaling responses to stresses. In the present study the role of MJ, ET and ROS as signaling molecules were evaluated in carrot tissue under wounding stress and hormone stresses. Blockers for NADPH oxidase activity, lipoxygenase activity (LOX) and ethylene action were used for determining the role of the targeted signaling molecules in relation to the synthesis of phenolics and PAL activity in stressed carrots. Dynamic studies of total phenolics, PAL activity, LOX activity and ROS were done during days of storage at 15°C. Results indicated that exogenous MJ and ET accelerated the synthesis of phenolics in relation to air control samples. The studies using blockers PHE, DPI and 1MCP and their combinations indicated that MJ, ROS and ET act as signaling molecules. However, ROS plays a key role in the accumulation of phenolics in wounded and hormone stressed wounded tissues. An integral mechanistic diagram is proposed to explain the complex relationship among signaling molecules which trigger the phenylpropanoid metabolism.

Introduction

Plants are frequently exposed to stresses that can promote the synthesis of reactive oxygen species (ROS) (Bray and others 2000). The primary ROS involved in oxidative stress are superoxide and hydrogen peroxide, while all other oxygen radicals are produced via secondary reactions of these initially formed metabolites (Eberhardt 2001; Agarwal and others 2005). Rapid generation of superoxide and accumulation of hydrogen peroxide is a characteristic early feature of the hypersensitive response of stressed plants (Toivonen 2003; Apel and Hirt 2004). Emerging data indicate that the oxidative burst reflects the activation of the enzyme NADPH oxidase, which is a central component of a highly amplified and integrated signal system (Lamb and Dixon 1997). In plants, ROS and jasmonic acid can act as mediators in early responses to wounding or attack by pathogens (Low and Merida 1996). In response to wounding, potato tubers generate ROS in association with suberization (Razem and Bernards 2003). The production of ROS metabolites can be immediate and transient as their levels decrease after wounding or in combinations with other stressors (Dempsey and Klessig 1994). Therefore, identifying which responses promote or maintain plant growth and development during stress is important for understanding the stress response processes.

Hydrogen peroxide can function as a signaling molecule in plants and a wide range of biotic and abiotic stresses results in its generation (Neill and others 2002). However, plants possess a battery of antioxidant mechanisms, both enzymatic and non-enzymatic, by which hydrogen peroxide is removed from the cells (Mittler 2002; Kang and Saltveit 2001). Excessive accumulation of reactive oxidants is toxic, and their intracellular level is, therefore, tightly regulated by several antioxidants (Goldkorn and others 2003). That toxicity can lead to damage of phospholipids and proteins (Kader and Ben-Yehoshua 2000).

Wound signaling is known to activate the octadecanoid pathway which interacts with other defense/stress related signaling pathways (e.g., ethylene) to orchestrate plant responses to many abiotic stresses (Rakwal and Agrawal 2003) (figure 4.1). Jasmonates are some of the molecular components responsible for the perception and transduction of

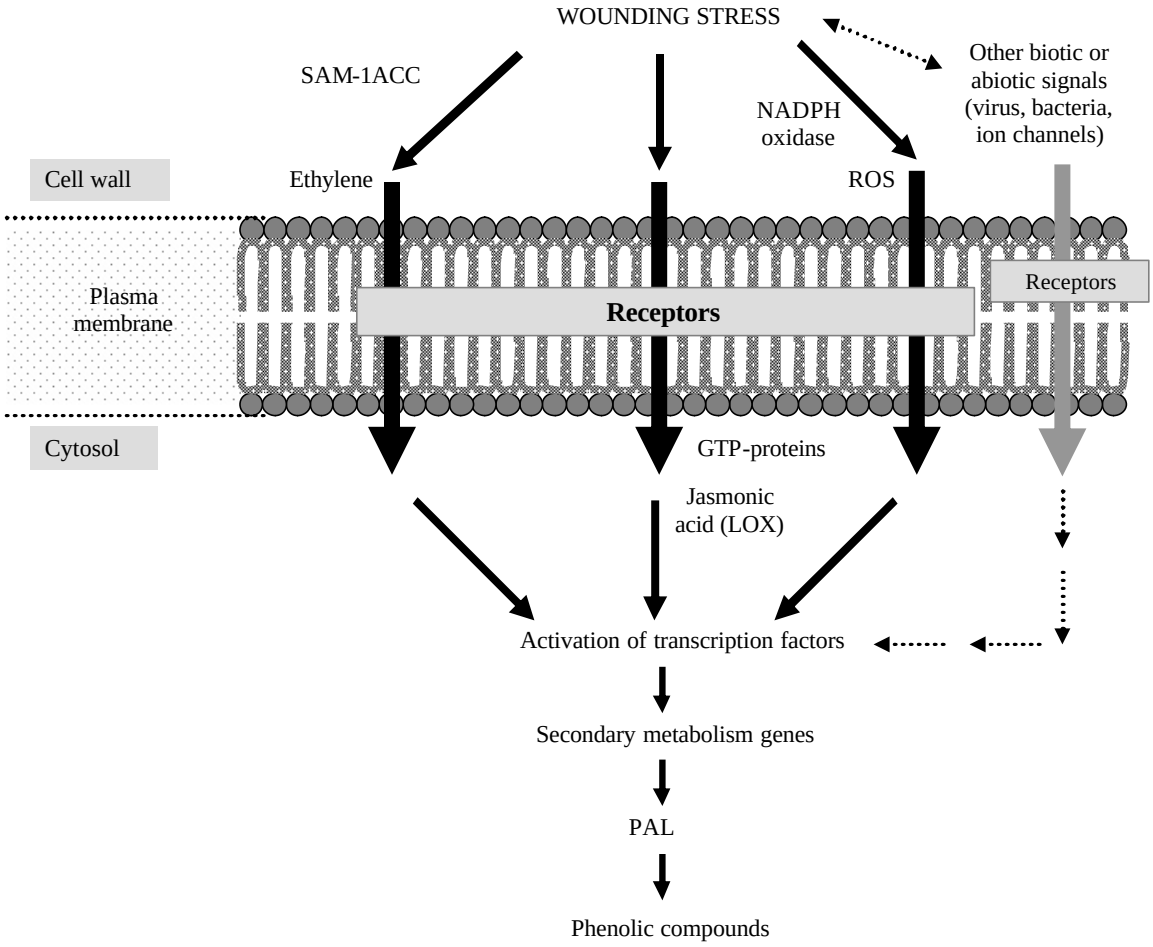

Figure 4.1 Model postulating the relationship between wounding, ROS, ethylene, and methyl jasmonate and the signal transduction pathways that induce the production of secondary defense compounds (*i.e.*, phenolics). (ROS, reactive oxygen species; SAM, s-adenosylmethionine; 1ACC, 1-aminocyclopropane-1-carboxylic acid; LOX, lipoxygenase; PAL, phenylalanine ammonia lyase).

wound signals (Bowles 1991). Lipoxygenase (LOX) mediates an essential step in the biosynthesis of jasmonic acid. In fact, when plants are treated with LOX inhibitors they have reduced ability to synthesize jasmonic acid (Creelman and Mullet 1997). LOX activities have been positively correlated with ROS production in bean cotyledons (Lynch and Thompson 1984) and potato cells (Blokhina and others (2003), respectively. Despite the abundant research involving the role of ROS in plants, there is insufficient scientific data to correlate ROS with the synthesis of compounds known to enhance health benefits, e.g., bioactive phenolic compounds. For that reason, the main objective of this work was to evaluate ROS and understand their role in the synthesis of total phenolics and phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX) enzyme activity from carrot tissues exposed to methyl jasmonate (MJ), ethylene (ET) and wounding stresses.

The approach used in this part of the study includes:

1) Dose response studies. Study the dose response effect of exogenous MJ and ET applications and their blockers, as well as optimize the dose-response of ROS blockers in relation to the phenolic accumulation and PAL activity (table 4.1).

2) Dynamic studies of total phenolics, PAL and LOX activity and ROS. To evaluate the dynamic synthesis of total phenolics, the activity of PAL and LOX enzymes and their correlation with the levels of the harmful radicals hydrogen peroxide (H_2O_2) and superoxide in MJ- and ET-stressed pie-cut carrots .

3) Inhibition studies. To study the effects of blocker applications to challenged MJ- and ET-stressed pie-cut carrots in relation to phenolic accumulation (tables 4.2 and 4.3).

Table 4.1. Concentration of hormones and ROS blockers used in the study of the accumulation of total phenolics in pie-cut carrots.

Concentration of hormones and blockers	**Concentration [units]**
1) Applying hormones to wounded and non-wounded tissues	
Methyl jasmonate (MJ)	2.5, 25, 250 ppm
Ethylene (ET)	10, 100, 1000 ppm
2) Blocking hormones in challenged wounded tissues with MJ and C_2H_4	
Phenidone (PHE) to block lipoxygenase activity, precursor for MJ	20, 200, 2000 µM
1-Methylcyclopropene (1MCP) to block ET action	100, 500, 2000 ppb
3) Blocking ROS in challenged tissues with MJ and C_2H_4	
Diphenyleneiodonium (DPI) to inhibit NADPH oxidase activity	2.12, 21.2, 212 µM

Table 4.2. Treatments in combination with MJ (250 ppm) used for the determination of the contribution of the elicitors in the accumulation of total phenolics in pie-cut carrots. The values used come from the optimized dose-response studies.

Block signals of MJ, ET and ROS and then apply MJ [250 ppm]	Concentration [units]
Control (no blocker, no MJ)	
Control (only MJ)	250 ppm
Block MJ with PHE	2000 µM
Block ET with 1MCP	2000 ppb
Block ROS with DPI	212 µM
Block MJ and ET with PHE and 1MCP	2000 µM, 2000 ppb
Block MJ and ROS with PHE and DPI	2000 µM, 212 µM
Block ET and ROS with 1MCP and DPI	2000 ppb, 212 µM
Block MJ, ET and ROS with PHE, 1MCP and DPI	2000 µM, 2000 ppb, 212 µM

Table 4.3. Treatments in combination with ET (1000 ppm) used for the determination of the contribution of the elicitors in the accumulation of total phenolics in pie-cut carrots. The values used come from the optimized dose-response studies.

Block signals of MJ, ET and ROS then apply ET [1000 ppm]	Concentration [units]
Control (no blocker, no ET)	
Control (only ET)	1000 ppm
Block MJ with PHE	2000 µM
Block ET with 1MCP	2000 ppb
Block ROS with DPI	212 µM
Block MJ and ET with PHE and 1MCP	2000 µM, 2000 ppb
Block MJ and ROS with PHE and DPI	2000 µM, 212 µM
Block ET and ROS with 1MCP and DPI	2000 ppb, 212 µM
Block MJ, ET and ROS with PHE, 1MCP and DPI	2000 µM, 2000 ppb, 212 µM

Materials and Methods

Plant materials and reagents

Carrots (*Daucus carota,* Cv Choctaw) were provided by Grimmway Farms (Bakersfield, CA). Carrots were sorted, washed and disinfested with chlorinated water (250 ppm). Carrots were cut into pie forms (as used in Chapter III, ¼ sections from a 3-4 mm thickness slice). Carrot pie-cut were used in all the experiments involved within this Chapter IV. Glass jars (1-pint) were used for storage of samples (up to 6 d at 15°C) after they were exposed to the hormones, blockers and/or enzyme inhibitors of the targeted signal molecules. All experiments were carried out treating ~100 g of pie-cut carrots per jar with 5 replicates.

The chemicals methyl jasmonate (95%), Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), iron sulfate ($FeSO_4$), Trolox and 2,2-diphenyl-1-picrylhydrazyl (DPPH), polyvinylpolypyrrolidone (PVPP), sodium hydroxide (NaOH), boric acid, 2-mercaptoethanol, xylenol orange, sorbitol, epinephrine, sodium phosphate, sodium linoleic acid, Tris-HCl, L-phenylalanine, diphenyleneiodonium chloride (DPI) and sulfuric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylene (CP grade, 99.5%), ammonium sulfate (NH_4SO_4), dimethyl sulfoxide (DMSO), phenidone, hydrogen peroxide (H_2O_2) and sample preparation supplies were obtained from Fisher Scientific (Houston, TX, USA) including filters, membranes and syringes. 1-Methylcyclopropene (1MCP) was provided by AgroFresh (Spring House, PA, USA). The solvents, methanol, ethanol, hexane, acetonitrile and water, were quality HPLC grade.

Dynamic studies of total phenolics, PAL activity, LOX and ROS metabolites in hormone stressed pie-cut carrots

In this experiment, carrot pie-cuts were exposed to 250 ppm MJ solution, while 1000 ppm ET was continuously applied as headspace vapors. MJ was applied by immersing pie-cut carrots into aqueous MJ solutions containing 1% tween-20, for 15 min at 20°C. ET treatments were continuously applied through storage by directly

injecting into the 1-pint glass jars the desired concentrations. The evaluations performed in this experiment were total phenolics, PAL activity, LOX activity, and the ROS metabolites superoxide and hydrogen peroxide. All these assays were performed at 0, 0.25, 0.5, 0.75, 1, 2, 4, 12, 24, 48, 96 and 144 h after storage at 15°C.

Extraction and assay of superoxide radical

The production of the superoxide radical was estimated by the oxidation of epinephrine to adenochrome (Misra and Fridovich 1972). A sample of 2 g of carrot pie-cuts was shaken with 40 mL of 1 mM epinephrine (pH 7), for 15 min at 20°C and 120 rpm. The absorbance was determined spectrophotometrically by measuring at 480 nm, and changes were recorded after the incubation time. A blank was run with another 2 g of carrot pie-cuts, but this time the incubation was done in distilled water for 15 min.

Extraction and assay of hydrogen peroxide radical

The xylenol orange assay was used to quantify this radical (Gay and Gebicki 2000). Carrot pie-cuts were incubated in distilled water for 15 min at 20°C to produce the incubation solution. Two reagents were prepared. Reagent A was composed of 25 mM $FeSO_4$, 25 mM $(NH_4)_2SO_4$ and 25 mM H_2SO_4, and reagent B was produced with 125 uM xylenol orange and 100 mM sorbitol. The working reagent contained 0.1mL of reagent A and 10 mL of reagent B. The reaction mixture had 3 mL of that combined working reagent and 0.6 mL of the incubation solution. After 15 min, absorbance readings were performed at 560 nm. A standardized solution of H_2O_2 was used to construct a calibration curve.

Analysis of total soluble phenolics

Phenolic content was evaluated following the procedure of Swain and Hillis (1959). Samples of 5 g were homogenized with 20 mL methanol using an Ultra-Turrax homogenizer (IKA Works, Inc., Wilmington, NC, U.S.A.), until uniform consistency, then incubated overnight at 4°C. Homogenates were centrifuged (rotor JA-17; centrifuge

J2-21; Beckman Coulter, Inc., Fullerton, CA, U.S.A.) at 29,000 x g for 15 min at 4°C. Sample aliquots of 150 µL were taken from the clear supernatant and diluted with 2400 µL nanopure water, followed by 150µL 0.25N Folin-Ciocalteu and incubated for 3 min at room temperature. The reaction was stopped by adding 300 µL 1N Na_2CO_3 and the mixture was incubated 10 min. Samples were again centrifuged at 29,000 x g for 15 min at 4°C. Absorbance readings at 725 nm of clear supernatant samples were taken using the spectrophotometer with photo diode array detector (model 8452A, Hewlett Packard Co, Waldbronn, Germany). A blank prepared with methanol was used as a control. The level of total phenolics for each sample was determined by using a standard curve developed with chlorogenic acid (CHA).

Phenylalanine ammonia lyase enzyme activity

PAL was extracted from 1 g of fresh produce in 25 mL borate buffer (pH 8.5) according to Ke and Saltveit (1986). Samples were placed in ice and homogenized using an Ultra-Turrax homogenizer, under low-light conditions and at low speed to prevent protein denaturalization. Homogenates were filtered through cheesecloth and centrifuged at 29,000 x g for 15 min at 4°C. The enzyme activity of PAL was assayed by following the accumulation of cinnamic acid at 290 nm, using 100 mM of L-phenylalanine as PAL substrate and water for control samples. Units were reported as µmol of t-cinnamic acid/h g fresh weight.

Lipoxygenase enzyme activity

For LOX, the crude extract was made using 3 g fresh sample, 0.5 g PVPP and 15 mL cold Tris-HCl buffer (100 mM at pH 8). Homogenates were centrifuged at 29,000 x g for 30 min at 4°C. This assay was determined by measuring the formation of conjugated dienes at 234 nm and 30°C (Perez and others 1999). The reaction mixture was prepared with 2.85 mL sodium phosphate buffer (100 mM at pH 6), 50 µL of a 10 mM sodium linoleic acid solution and 0.1 mL enzyme extract. The LOX specific activity

was expressed as U/mg min, where one unit was expressed as 1 µmol of hydroperoxide/min at 30°C.

Dose response studies of MJ, ET and blockers of NADPH oxidase, LOX and ET action

Pie-cut carrots were exposed to three different MJ concentrations (2.5, 25 and 250 ppm) and three different ET concentrations (10, 100 and 1000 ppm). MJ was applied by immersing pie-cut carrots into aqueous MJ solutions containing 1% tween-20, for 15 min at 20°C. ET treatments were continuously applied through storage by directly injecting into the jars the desired concentrations. The second study used three different concentrations of hormone blockers and three concentrations of the ROS blocker. For ET, we used 1-methylcyclopropene (1MCP; 100, 500 and 2000 ppb), which inhibits ethylene's mode of action (Fan and Mattheis 2000). Phenidone (PHE; 20, 200, 2000 µM) is used to block lipoxygenase activity related to the synthesis of MJ through the octadecanoid pathway (Huang and others 2005). Diphenyleneiodonium (DPI; 2.12, 21.2, 212 µM) is a compound that inhibits the activity of NADPH oxidase and therefore, blocks the production of superoxide at the cell membrane level (Li and others 2003).

1MCP was applied exposing whole carrots to headspace vapors for 12 h at 15°C, following instructions provided by AgroFresh (Spring House, PA, USA). 1MCP-treated carrots were immediately processed into pie-cuts for further treatments. PHE and DPI were applied by dipping pie-cut carrots into prepared solutions. PHE solutions were prepared in 1% ethanol, and carrot samples were immersed for 2 min at 20°C. The solutions of DPI were prepared in 1% DMSO, and carrot samples were immersed for 2 min at 20°C. Treated samples were allowed to dry, and then placed inside 1-pint glass jars and store at 15°C for 6 days. All three 1MCP, PHE and DPI were applied before the carrot pie-cuts were exposed to MJ and ET. Sampling and evaluations were done at initial and final day of storage. The total phenolic content and the PAL enzyme activity were analyzed.

To study if possible diffusion effects of the signal molecule during immersion of wounded samples stressed with MJ and ET in the blocker solutions occurred, wet control samples were evaluated by immersing wounded samples for 2 min and 15 min in water, MJ stressed samples for 2 min and ET stressed samples for 2 min. Different effects were determined by final levels of phenolics accumulated after storage for 6 d at 15°C.

Percentage contribution of MJ, ET and ROS on the synthesis of phenolic compounds

The information from the blockers study was used for determining the percentage contribution of MJ, ET and ROS on phenolic synthesis. Blockers under one saturated concentration, 1MCP (2000 ppb), PHE (2000 µM) and DPI (212 µM) were used to block individually or in combined effects on wounded or hormone challenged wounded carrots. The percentage contribution data was calculated comparing the difference in phenolic content between either, the air control (day 6), MJ or ET treatments and the initial day controls, versus the amount of phenolic content reduction observed for a treatment with individual or combined blockers. Results are shown in tables 4.2 and 4.3. Sampling and evaluations were done at initial day and after 6 days of storage at 15°C. The total phenolic content was analyzed as previously described.

$$\% \text{ Contribution} = \frac{(\text{Total phenolics}_{\text{Treatment}} - \text{Total phenolics}_{\text{After blockers}})}{(\text{Total phenolics}_{\text{Treatment}} - \text{Total phenolics}_{\text{Initial day}})} \text{ X } 100$$

Statistical analysis

The experiment followed a completely randomized design. Analyses were done using 5 replicates, unless otherwise indicated. Means, standard deviations, graphs and linear regressions were obtained using Microsoft Excel 2000. For means comparison at the 5% significance level, ANOVA and LSD multiple-range tests were performed using SAS (Raleigh, NC, USA).

Results and Discussion

Dose response effect of exogenous MJ and ET on the phenolic content and PAL activity of wounded carrots

The response of carrot pie-cuts on the accumulation of total phenolics was dependent on the MJ concentrations (figure 4.2). The treatment with 250 ppm MJ showed a significant increase of ~100% over the air control, and a higher increase of ~400% over the initial day control. Similarly, MJ was applied to tulip bulbs and phenolic compounds increased at higher concentrations (Saniewski and others 1998). MJ is present and known to act at the cell level in wounded plants (Rakwal and Agrawal, 2003). In contrast to the MJ response, all the evaluated ET concentrations showed the same increase in phenolic content, ~700% over the initial day control and ~200% over the air control, which means that the ET levels used saturated the response. It is very likely that lower concentration of ET would have shown a dose response effect such as that observed for MJ. Sarkar and Phan (1979) also reported a similar increase in total phenolic content in whole carrots with ET. They found that the higher the concentration of ethylene used the faster the rate of synthesis of phenolic compounds (100, 2000 and 50000 ppm).

PAL activity showed a significant increase in the phenolic content of MJ-stressed carrot pie-cuts only with the dose of 250 ppm (~0.5 µmoles ŧ-cinnamic acid/h g fresh weight) when compared to the wet control (figure 4.3). Both 2.5 and 25 ppm of MJ did not increased PAL activity. On the other hand, the increase in concentration of ET showed a dose dependent increase in PAL activity, in contrast to the response observed for phenolic content for ET treated wounded carrots. The highest response in PAL activity was observed with the 1000 ppm ET dose (~1.5 µmoles ŧ-cinnamic acid/h g fresh weight). These results suggest that an exogenous application of 10 ppm ET can stimulate a minimum PAL activity to produce the same amount of phenolic compounds obtained with larger ET concentrations and further stimulation of PAL enzyme.

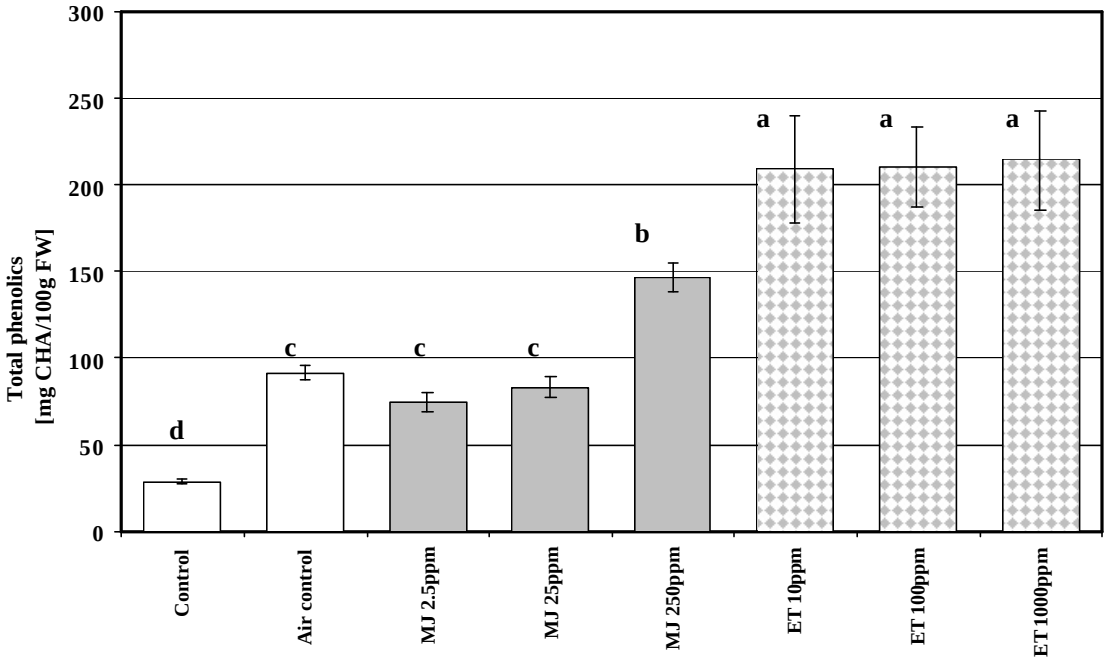

Figure 4.2. Accumulation of total phenolics from carrot pie-cuts exposed to 3 concentrations of MJ and 3 of ET. Samples were stored 6 d at 15°C. Results show means of 5 replicates and their standard deviation. Letters show mean comparisons among treatments (LSD P>0.05).

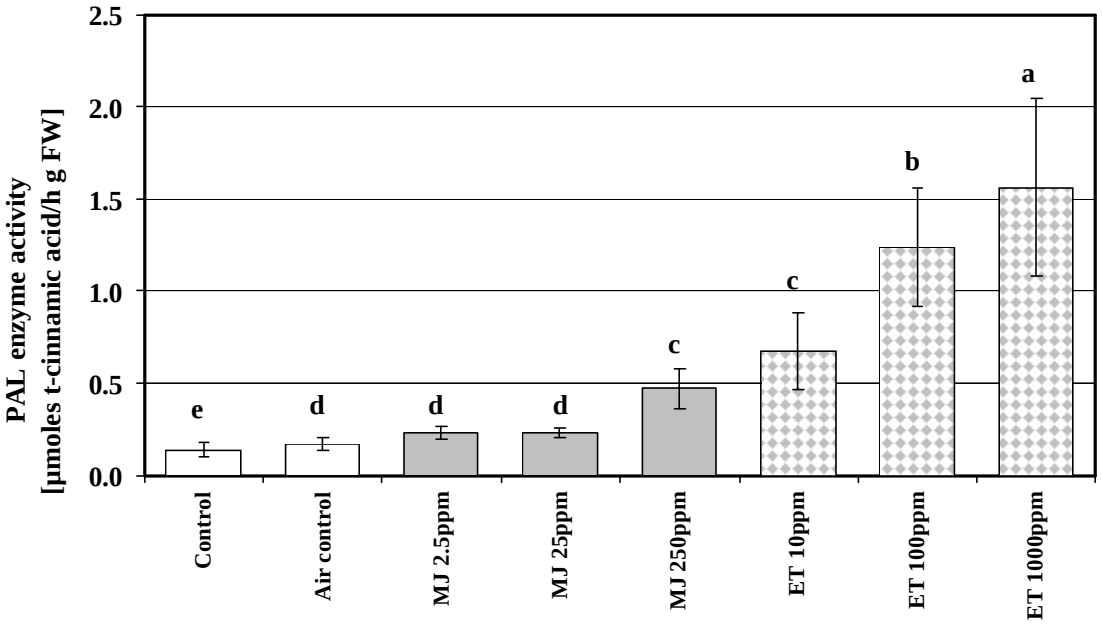

Figure 4.3. Effect of dose-response on PAL enzyme activity from carrot pie-cuts exposed to 3 concentrations of MJ and 3 of ET. Samples were stored 6 d at 15°C. Results show means of 5 replicates and their standard deviation. Letters show mean comparisons among treatments (LSD P>0.05).

Total phenolics, PAL activity and ROS metabolites in hormone stressed wounded carrots during storage

Phenolics accumulated overtime, with the initial increase within the first 12 h in ET-stressed samples compared to air control samples (figure 4.4). ET-treatment reached ~220 mg/100 g fresh weight compared to initial values of ~35 mg/100g fresh weight after 6 d of storage at 15°C. On the other hand, the treatment with MJ showed an increase in the amount of phenolics similar to the wounded air control after 2 days. This implies that ET caused an increase in the rate of phenolic synthesis.

The results for PAL activity coincide with the accumulation of phenolics for the ET effect (figure 4.5). PAL activity for the ET treatment showed a large increase at around 2 h. The continuously exposure of ET over carrot pie-cuts accelerated the phenylpropanoid pathway, responsible for phenolic synthesis, affecting the activity of PAL.

Lipoxygenase activity (LOX) showed a peak in the first 1 h (~0.05 µmol/mg Protein min) for the MJ stressed carrots and the ET treatment showed a peak at 1 d and a larger one after 20 h (~0.04µmol/mg Protein min) (figure 4.6). Interestingly, control wounded carrots showed a higher peak than hormone stressed carrots within the first hour. According to this, exogenous MJ and ET are not affecting the early wounding response through the octadecanoid pathway and the key enzyme, LOX (Creelman and Mullet 1997). After 12 h, the treatments MJ and AC did not show detectable LOX activity levels. Only the ET treated samples showed LOX activity which decreased through time.

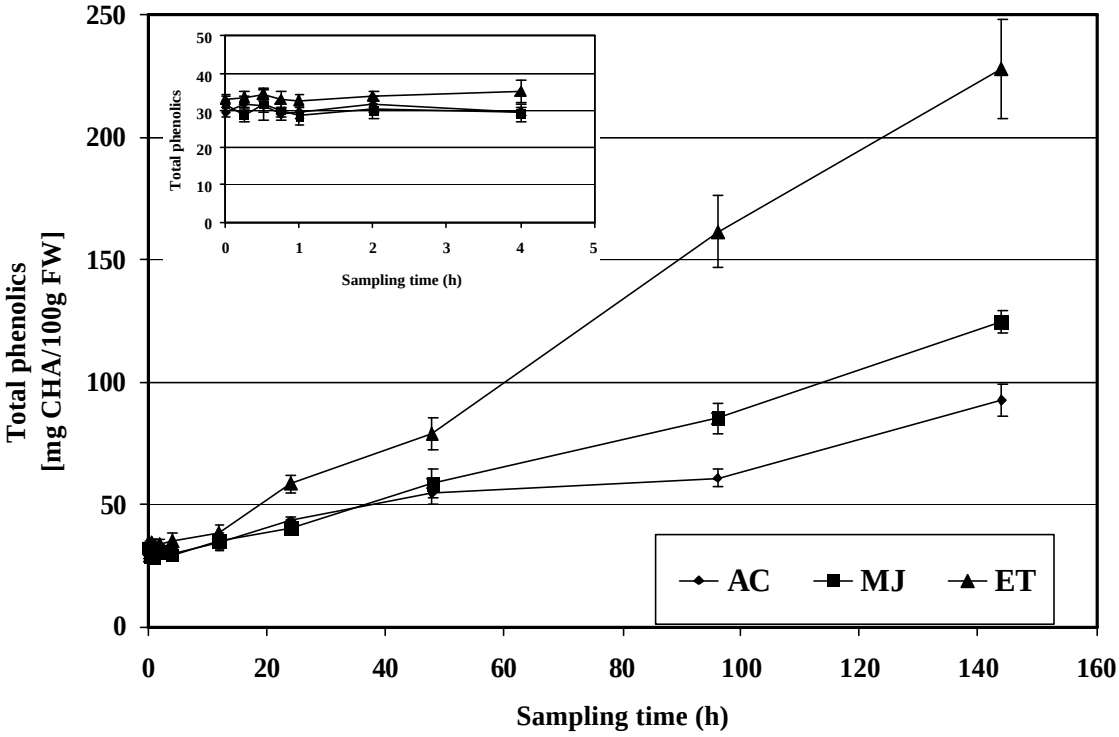

Figure 4.4. Dynamic studies on the accumulation of total phenolics of wounded MJ- and ET-stressed carrot pie-cuts. Samples were stored at 15°C. Results show means of 5 replicates and their standard deviation (AC, air control; MJ, methyl jasmonate; ET, ethylene).

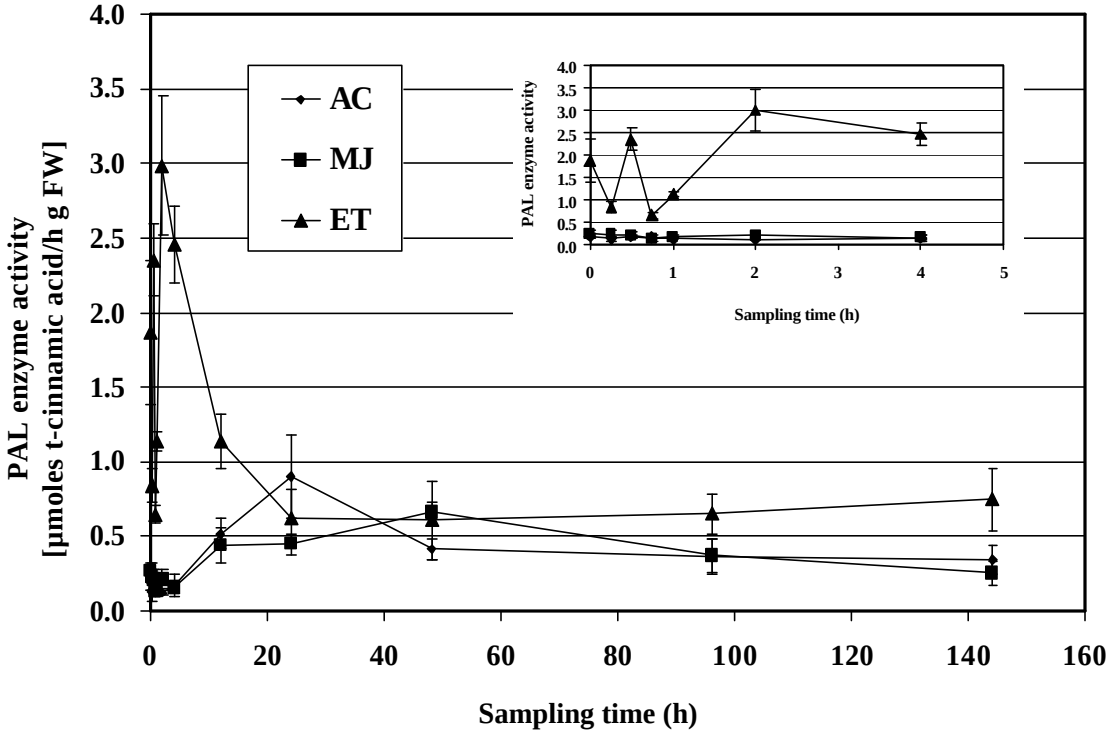

Figure 4.5. Dynamic studies on phenylalanine ammonia lyase (PAL) activity of wounded MJ- and ET-stressed carrot pie-cuts. Samples were stored at 15°C. Results show means of 5 replicates and their standard deviation (AC, air control; MJ, methyl jasmonate; ET, ethylene).

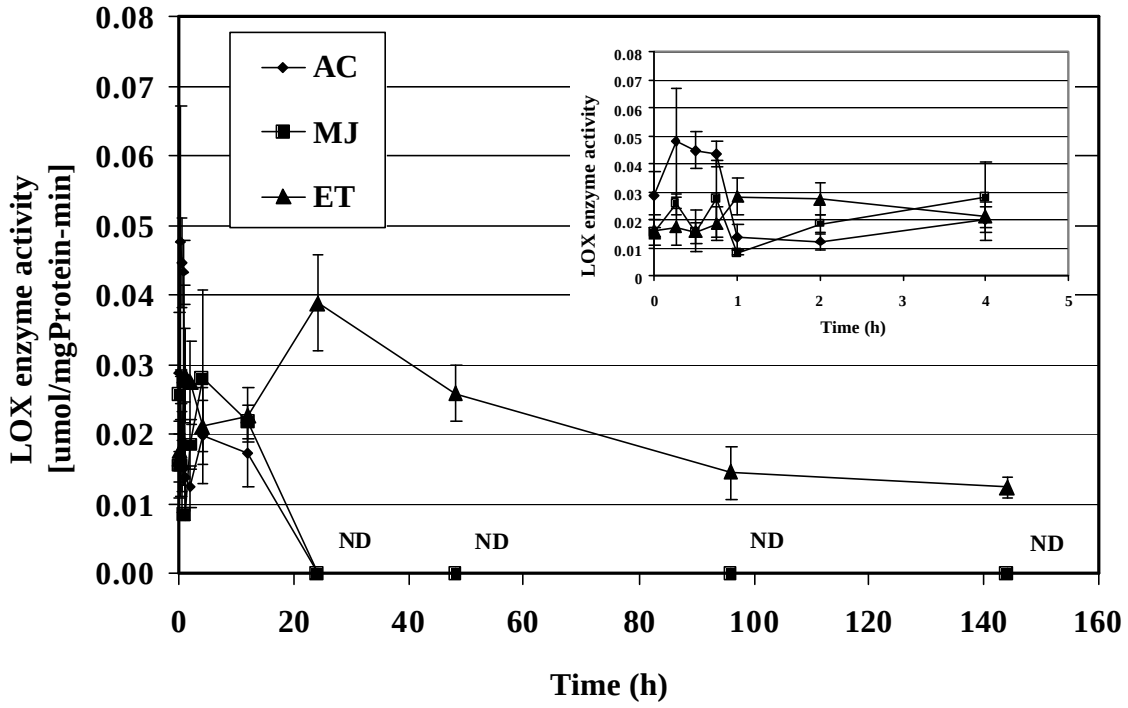

Figure 4.6. Dynamic studies on lipoxygenase (LOX) activity of wounded MJ- and ET-stressed carrot pie-cuts. Samples were stored at 15°C. Results show means of 5 replicates and their standard deviation (AC, air control; MJ, methyl jasmonate; ET, ethylene). AC and MJ treatments showed no LOX activity after 12 h (ND means activity not detected).

The production of reactive oxygen species (ROS) was affected by wounding and hormone stresses. Hydrogen peroxide (H_20_2) showed a significant peak or oxidative burst at 15-30 min in the ET-stressed samples (figure 4.7). For control wounded carrots, there was also a slight increase in H_20_2 within the first 30 min, while MJ did not increase, but rather a decreased through time in the first 12 h. Razem and Bernards (2003) reported the presence of four oxidative bursts of hydrogen peroxide at 30-60 min, and later at 42, 63, 100 h after wounding potato tissue. They also reported that in response to wounding, the generated ROS was associated with the synthesis of phenolic compounds. In the present study treatments showed decreases of H_2O_2 through time, more significant at 45 min for ET, and after 1 h for control and MJ samples. After 1 h, H_2O_2 levels were higher for ET stressed samples through time compared to MJ stressed and controls, while after 20 h, MJ stressed samples showed levels of H_2O_2 higher for controls through time.

The evaluation of the superoxide radical showed a similar oxidative burst in the first 15-30 min after wounding (figure 4.8) for both ET and control samples, and a second peak of superoxide radicals was produced at 144 h only for the ET treated samples. MJ treatments did not show an increase in the superoxide level but rather a decrease through time. After 100 h, there was a slight gradual increase in superoxide radicals for both wounded samples and MJ stressed carrots. Superoxide radicals are converted to H_2O_2 by the enzyme superoxide dismutase (SOD). Since MJ samples showed higher levels of H_2O_2 after 20 h, it is likely that MJ samples may have higher activity of SOD. Based on the results of H_2O_2 and superoxide radicals for wounded tissue, ET treated samples and MJ treated samples, it seems that these stresses are mediating the expression of genes for transcription of PAL through the increase in reactive oxygen species. The dynamics of ROS production are different among the stresses and this could explain in part the differences observed in PAL activity as well in the synthesis rate of phenolic compounds.

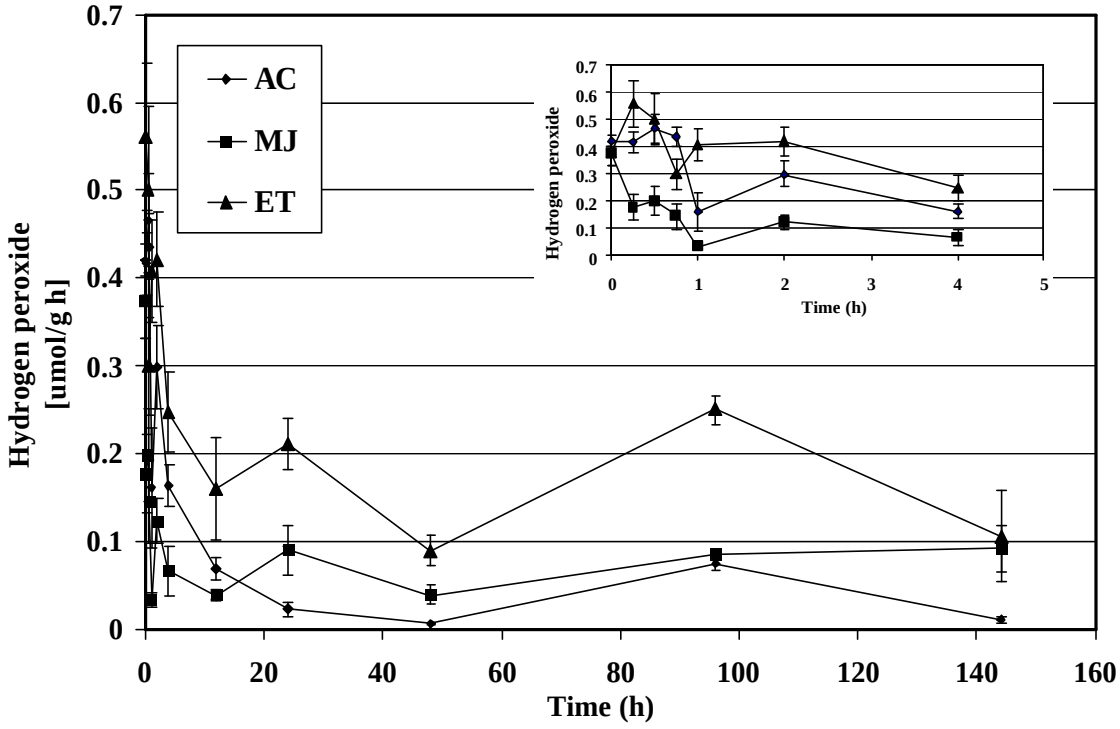

Figure 4.7. Dynamic studies on hydrogen peroxide (H_2O_2) accumulation of wounded MJ- and ET-stressed carrot pie-cuts. Samples were stored at 15°C. Results show means of 5 replicates and their standard deviation (AC, air control; MJ, methyl jasmonate; ET, ethylene).

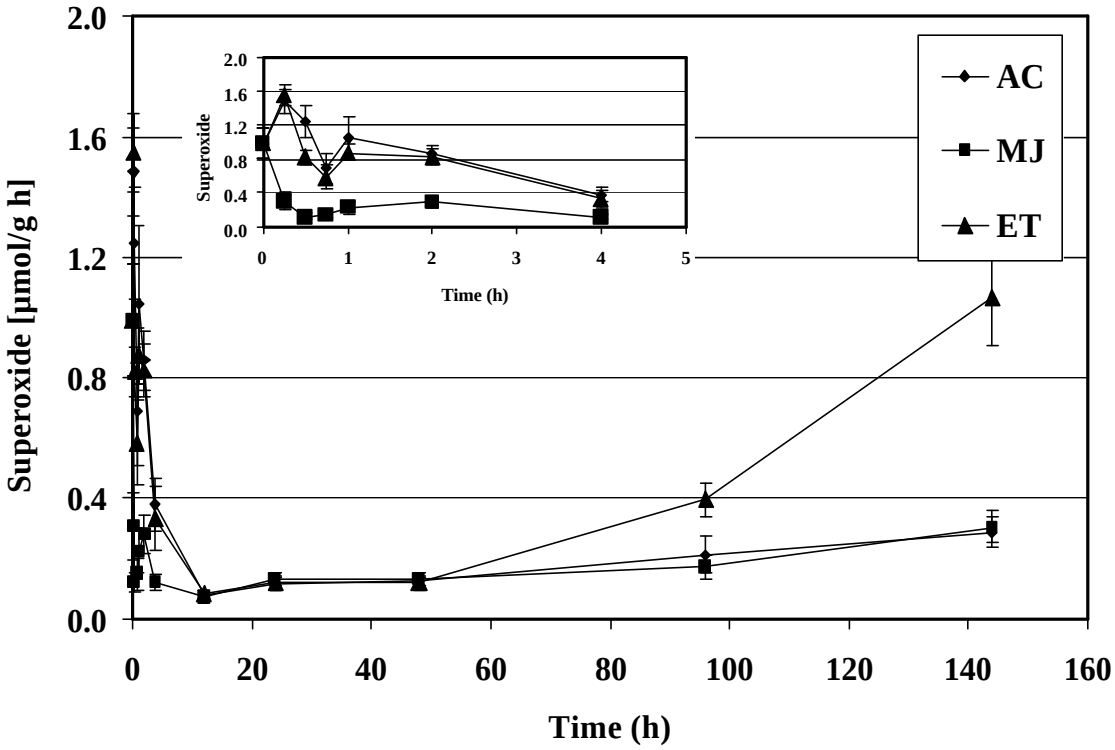

Figure 4.8. Dynamic studies on superoxide accumulation of wounded MJ- and ET-stressed carrot pie-cuts. Samples were stored at 15°C. Results show means of 5 replicates and their standard deviation (AC, air control; MJ, methyl jasmonate; ET, ethylene).

Effect of 1MCP, DPI and PHE blockers on the phenolic content of wounded carrots

The 1MCP blocker did not significantly reduce the phenolic content of carrot pie-cuts, while DPI (~59% reduction) and PHE (~42% reduction) affected the newly synthesized phenolic content, when compared to air control samples and initial day control after 6 d of storage at 15°C (figure 4.9, table 4.4). This implies that wounding stress elicits the production of reactive superoxide radicals through NADPH oxidase and endogenous MJ through the octadecanoid pathway and both, ROS and endogenous MJ play roles as signaling molecules, stimulating the phenylpropanoid pathway and the synthesis of phenolic compounds. Endogenous ET did not have an apparent effect as signaling molecule in wounded carrots under the intensity of wounding used.

Effect of 1MCP, DPI and PHE blockers on the phenolic content of MJ-challenged wounded carrots

Carrot tissue exposed to wounding stress (air control), showed an increase of ~200% in total phenolics over the initial day (control), while samples exposed to 250 ppm of MJ showed ~300% increase over the initial day control after 6 d of storage at 15°C. The use of 1MCP at different concentrations showed a decrease in the accumulation of total phenolics in relation to the MJ control (figure 4.10). The percentage decrease of total phenolics ranged from 29 to 49% compared to MJ treated samples and initial day control (table 4.4). 1MCP is specific in blocking ethylene action as reported previously in carrot tissue (Fan and Mattheis 2000), thus the observed reduction implies that exogenous MJ stimulates phenolic synthesis in part through ET production. The synthesized ET could have been triggered through MJ or through ROS. Wounding is less likely to have triggered ET production as observed in figure 4.9. It also suggests that the phenolics synthesized after using 1MCP could be triggered directly by the exogenous MJ or alternatively through the endogenous MJ and ROS. The use of DPI on challenged MJ stressed carrots showed the largest effect in reduction of phenolic synthesis among the blockers evaluated (figure 4.10). There was a 64% decrease with the 212 µM DPI dose (table 4.4). This result indicates that exogenous MJ stimulates

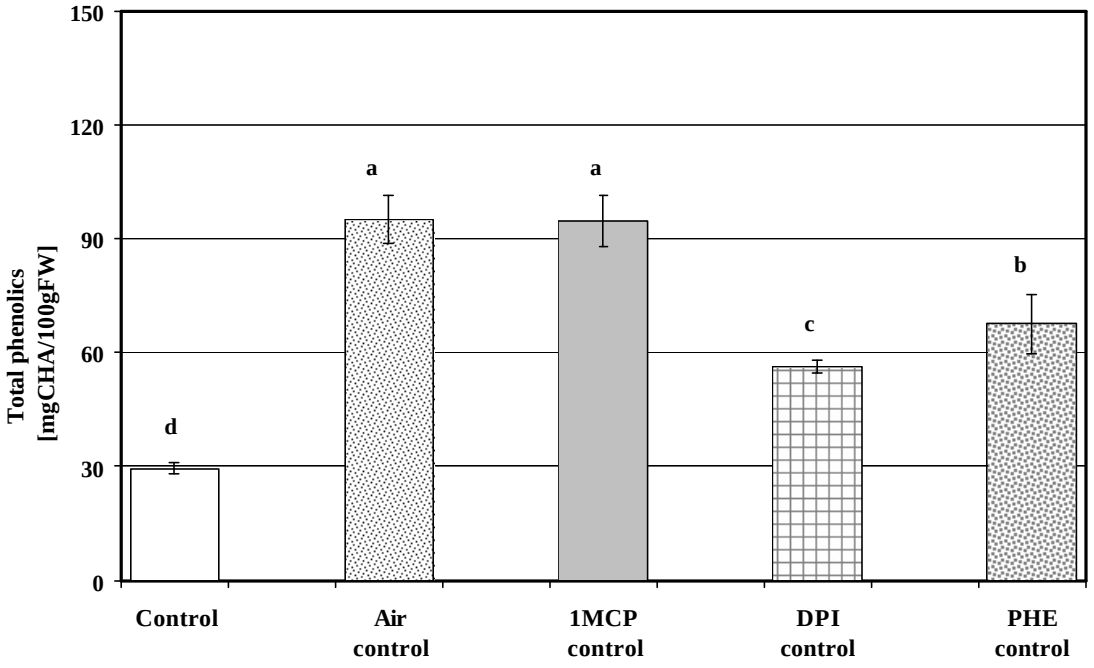

Figure 4.9. Effect of 1MCP, PHE and DPI blockers on the accumulation of total phenolics in wounded stressed carrot pie-cuts. Blocker concentrations were 1MCP, 2000ppb; DPI, 212µM; and PHE, 2000µM. Samples were stored 6 d at 15°C. Results show means of 5 replicates and their standard deviation. Letters show mean comparisons among treatments (LSD P>0.05).

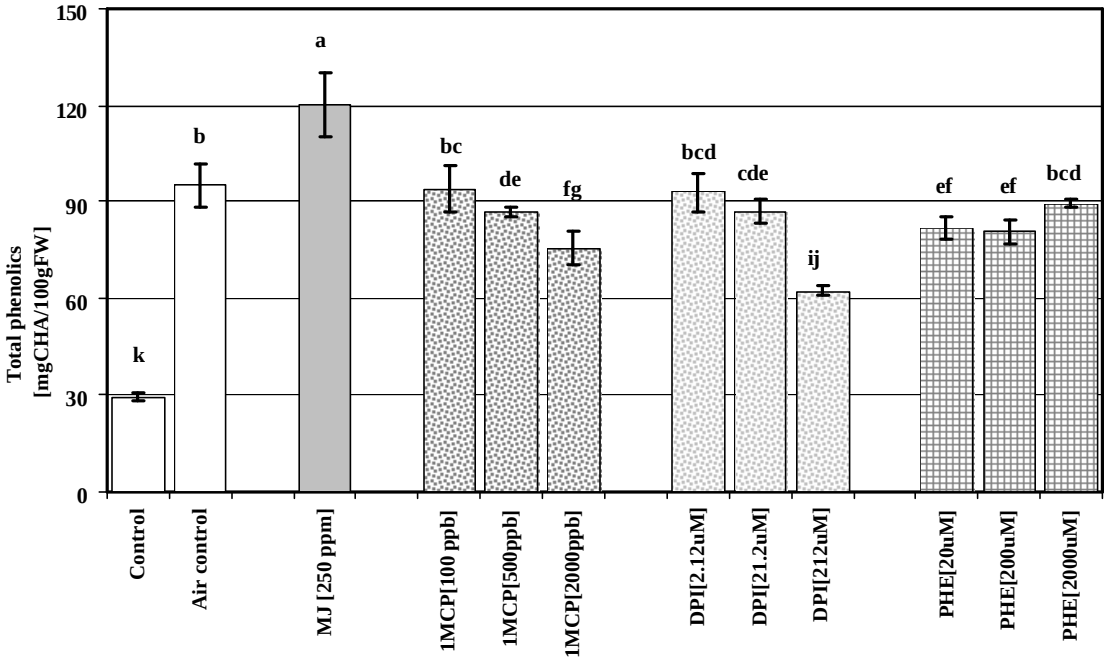

Figure 4.10. Dose-response of 1MCP, PHE and DPI blockers on the accumulation of total phenolics in MJ-wounded stressed carrot pie-cuts. MJ treatments were done with 250 ppm. Blocker concentrations are mentioned in the X axis. Samples were stored 6 d at 15°C. Results show means of 5 replicates and their standard deviation. Letters show mean comparisons among treatments (LSD P>0.05).

Table 4.4. Dose-response effects of blockers and their combinations on the percentage decrease contribution of synthesized phenolics from wounded, hormone stressed carrots, in relation to control MJ stressed and ET stressed carrot pie-cuts. Samples were stored 6 d at 15°C. Results show means of 5 replicates and their standard deviation. Letters show overall mean comparisons (LSD P>0.05). *Data for wounding alone was obtained for the highest concentration of blockers.

Blockers [concentration]	*Wounding [%]	Wounding combined with:	
		Methyl jasmonate [%]	Ethylene [%]
1MCP [100 ppb]		29±8(mn)	37±4(ijkl)
1MCP [500ppb]		37±2(ijkl)	40±7(hijk)
1MCP [2000ppb]	0.26±10(o)	49±6(fg)	58±4(de)
DPI [2.12uM]		30±7(lmn)	33±7(lmn)
DPI [21.2uM]		36±4(ijkl)	54±4(ef)
DPI [212uM]	59±4(de)	64±4(n)	85±2(a)
PHE [20uM]		42±4(ghij)	35±6(klm)
PHE [200uM]		43±4(ghi)	36±3(jklm)
PHE [2000uM]	42±12(hij)	34±2(klmn)	43±7(ghi)
1MCP+PHE		33±5(lmn)	51±5(f)
DPI+PHE		71±8(b)	69±3(bc)
1MCP+DPI		47±6(fgh)	70±4(b)
1MCP+DPI+PHE		63±7(cd)	72±2(b)

phenolic synthesis in part through activation of NADPH oxidase and superoxide production. It also implies that the synthesized phenolics observed after using DPI could be triggered directly by the exogenous MJ or alternatively through the endogenous MJ and ET action.

The use of PHE showed a reduction on the synthesis of phenolics at the concentrations tested (figure 4.10). The reduction obtained with 200 µM PHE, was ~53% (table 4.4). Phenidone blocks LOX activity and endogenous synthesis of MJ thus when exogenous MJ was supplied phenolic synthesis occurs in part through the octadecanoid pathway. Since phenolic synthesis still took place, it is likely that the exogenous MJ could be triggering directly the synthesis of phenolics or alternatively through ET and ROS production.

Further assays using combinations of blockers were performed to obtain additional information of the interactions among the signaling mechanisms involved. Combination treatments of 1MCP, PHE and DPI blockers applied to MJ-challenged carrot pie-cuts are presented in figure 4.11. Combined treatments containing DPI, showed the highest reduction in phenolic synthesis (table 4.4). The combination of DPI+PHE and 1MCP+DPI+PHE showed reductions of 71 and 63%, respectively, and similar to DPI alone. These results imply that ROS plays a major role in the synthesis of phenolics among the other blockers studied. When combinations of blockers 1MCP+DPI+PHE were used the reduction observed in phenolic synthesis was due to the blockers of ET action, and the blocking of ROS and endogenous MJ production. The remaining synthesized phenolics observed were triggered mainly by exogenous MJ.

In similar analysis, when combination of blockers DPI+PHE were used, the reduction observed in phenolic synthesis was due to blocking of ROS and endogenous MJ production. The synthesis of phenolics that still took place would be associated to the triggering effect of the exogenous MJ used and/or the presence of endogenous ET. Since the levels of synthesized phenolics in DPI+PHE and 1MCP+DPI+PHE treated sample are similar, this suggests that exogenous MJ could trigger directly the synthesis of phenolics or in part through the production of endogenous ET.

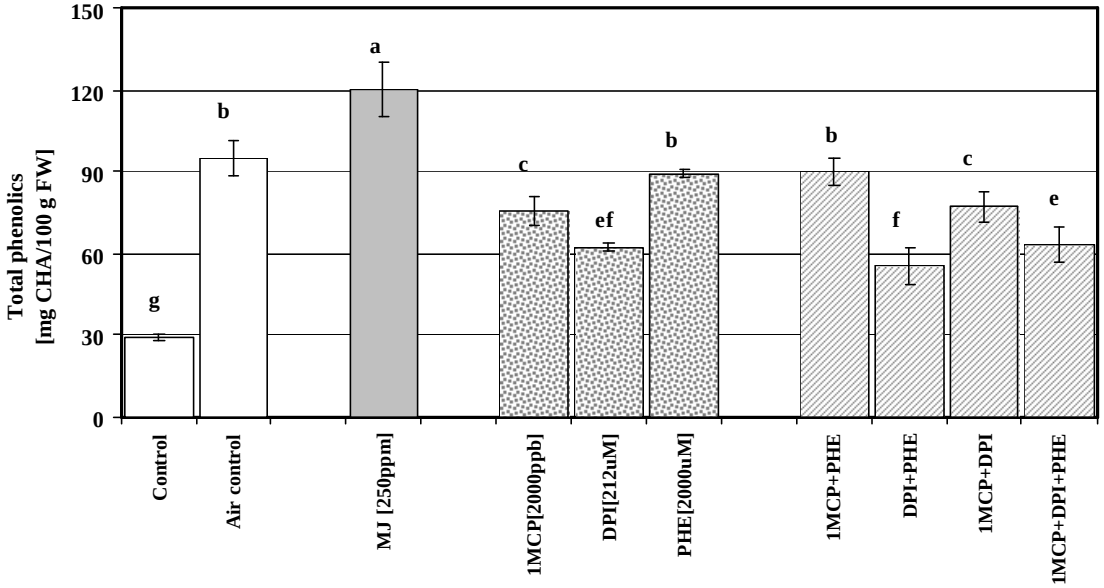

Figure 4.11. Effect of 1MCP, PHE and DPI combined blockers on the accumulation of total phenolics in MJ-wounded stressed carrot pie-cuts. MJ treatments were done with 250 ppm. Blocker concentrations were 1MCP, 2000ppb; DPI, 212µM; and PHE, 2000µM. Samples were stored 6 d at 15°C. Results show means of 5 replicates and their standard deviation. Letters show mean comparisons among treatments (LSD P>0.05).

The model shown in figure 4.12, describes how these blockers may interact within these complex signaling mechanisms in carrot tissue exposed to wounding and MJ. The blockers 1MCP, PHE and DPI show the sites where they can potentially block a signal molecule action or enzyme activities. In this approach, the filled lines are connecting pathways demonstrated and derived from the present study using individual blockers or in combinations. These are known pathways already demonstrated in different plant tissues (Zhao and others 2005) but not in an integral form as presented in this work for carrot tissue. The dotted lines are proposed interactions between endogenous ET and the other signal molecules. According to the present study, synthesis of ET is present and could be triggered by MJ or MJ and ROS. This proposed integral model of exogenous MJ effects on the phenolic synthesis in carrot tissue maybe used in other plant tissues as well (Bowles 1991; Dempsey and Klessig 1994; Dixon and Paiva 1995; Creelman and Mullet 1997; Mittler 2002; Blokhina and others 2003; Rakwal and Agrawal 2003). The model indicates that when wounding takes place, ROS and MJ are generated which in turn trigger the synthesis of PAL and the accumulation of phenolic compounds. MJ as well as ROS also stimulate the production of ethylene, which in turn triggers the phenylpropanoid metabolism. Any application of exogenous MJ may act by stimulating ethylene production or perhaps by acting directly on PAL synthesis.

Effect of 1MCP, DPI and PHE blockers on the phenolic content of ET-challenged wounded carrots

The carrot pie-cuts challenged with 1000 ppm ET showed a large effect in phenolic synthesis (~700% increase) compared to initial day control, while air control carrots only increase by ~300% (figure 4.13). Furthermore, this effect of ET stress on wounded carrots was synergistic, since previously we showed that ET applied to whole carrots only increased phenolic content by 10 to 15% (Chapter II). The use of blockers in ET-challenged carrot pie-cuts caused large reductions in phenolic synthesis (table 4.4). The blocker of ET action, 1MCP, showed up to 58% reduction of phenolic synthesis when ET was exogenously applied to carrot pie-cuts. DPI blocked the synthesis of

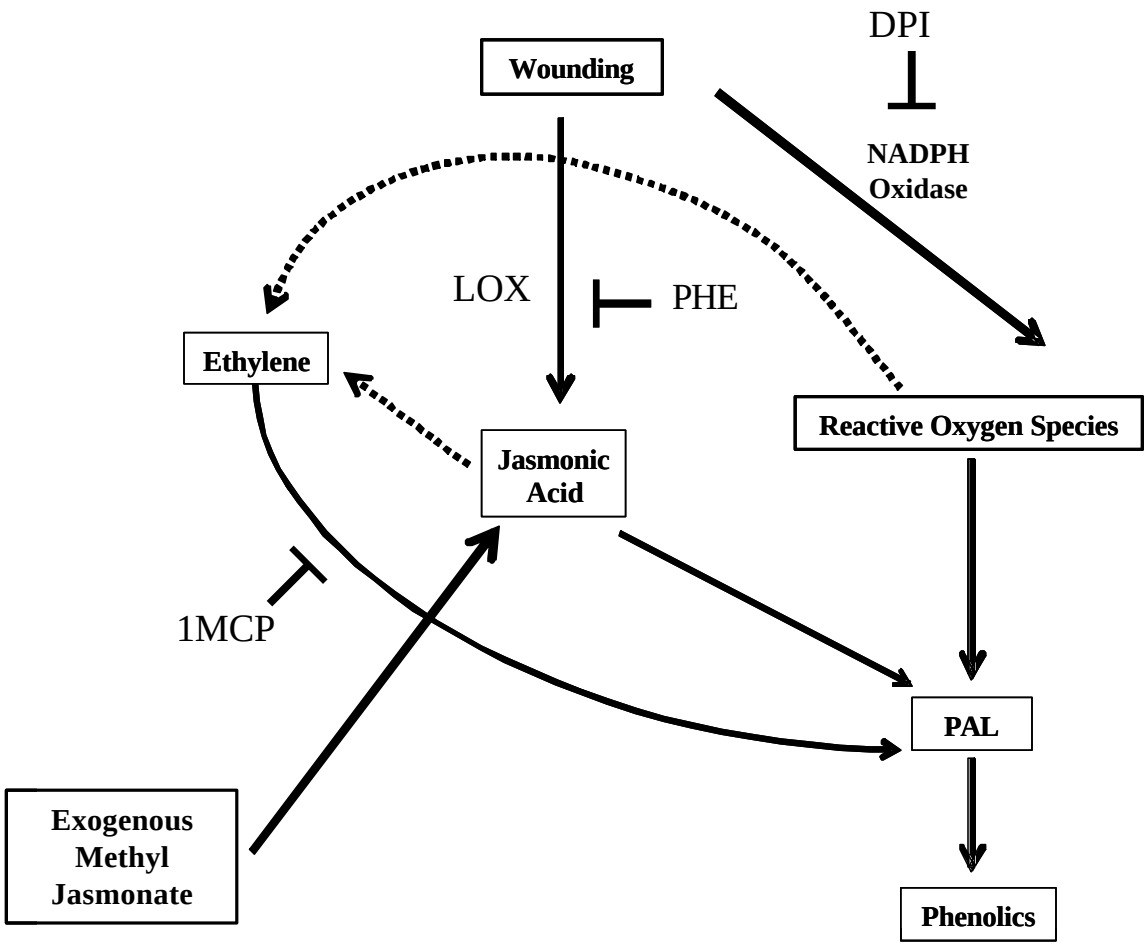

Figure 4.12. Proposed signal pathways involved in the synthesis of phenolic compounds in stressed carrot pie-cuts exposed to exogenous MJ (250 ppm). Filled lines are proposed connecting pathways for carrot tissues shown in this study. Dotted lines are proposed connecting pathways that may take place in wounded carrot tissue. 1MCP, PHE and DPI show the sites where these can potentially block active sites or enzyme activities.

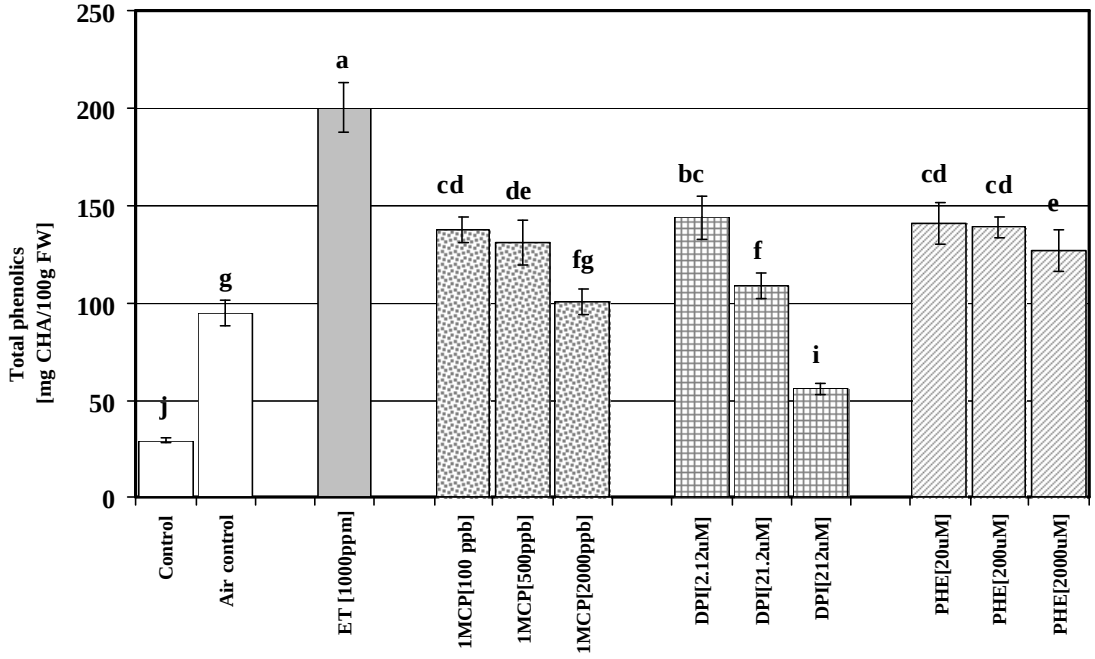

Figure 4.13. Dose-response of 1MCP, PHE and DPI blockers on the accumulation of total phenolics in ET-wounded stressed carrot pie-cuts. ET treatments were done with 1000 ppm. Blocker concentrations are mentioned in the X axis. Samples were stored 6 d at 15°C. Results show means of 5 replicates and their standard deviation. Letters show mean comparisons among treatments (LSD P>0.05).

phenolics by ~85%, showing once more a large effect on phenolic synthesis (table 4.4). The PHE blocker applied to ET challenged carrot pie-cuts (figure 4.13) showed a ~43% decrease in phenolic synthesis (table 4.4). According to this, ET induced more phenolics synthesis compared to MJ in ET challenged wounded carrots.

Studies using combinations of blockers applied to ET-challenged carrot pie-cuts are shown in figure 4.14. The combinations containing DPI showed the largest reduction, with 69 to 72%. The trend is similar to those observed in MJ-challenged tissues and their combinations of blockers, confirming that ROS is an important signaling molecule triggering the synthesis of phenolic compounds. When combination blockers MJ+ROS are used, the reduction in phenolic synthesis is due to the blocking action of LOX and NADPH oxidase. The phenolic synthesis that still took place is due mainly to the action of ET (exogenous and endogenous) (figure 4.14).

When combination blockers ET+ROS are used, the phenolic synthesis that took place is due mainly to MJ. Interestingly, the phenolic content levels synthesized by ET and MJ in this part of the study are similar and differ from the results obtained using individual blockers, where ET showed larger reduction in phenolic synthesis compared to MJ. This result suggests that ET not only triggers PAL directly but ET may induces ROS through either the activation of NADPH oxidase of through and increase in respiration which in turn may increase the levels of ROS (Mittler 2002; Blokhina and others 2003; Apel and Hirt 2004; Murphy and DeCoursey 2006; Brookes 2005), thus triggering more phenolic synthesis.

This could also explain the synergistic effect observed when ET is applied to wounded tissue. According to the model (figure 4.12) ROS may trigger ET production, and since ET can trigger also ROS production (figures 4.7 and 4.8), a loop is formed which can amplify the signal response of both stresses. An alternative explanation to the synergistic effect would be a positive feedback loop of ROS triggering NADPH oxidase to produce more ROS (Mittler and others 2004; Brandes 2005; Afanas'ev 2006). This ROS-producing ROS loop is still under study and not completely clear.

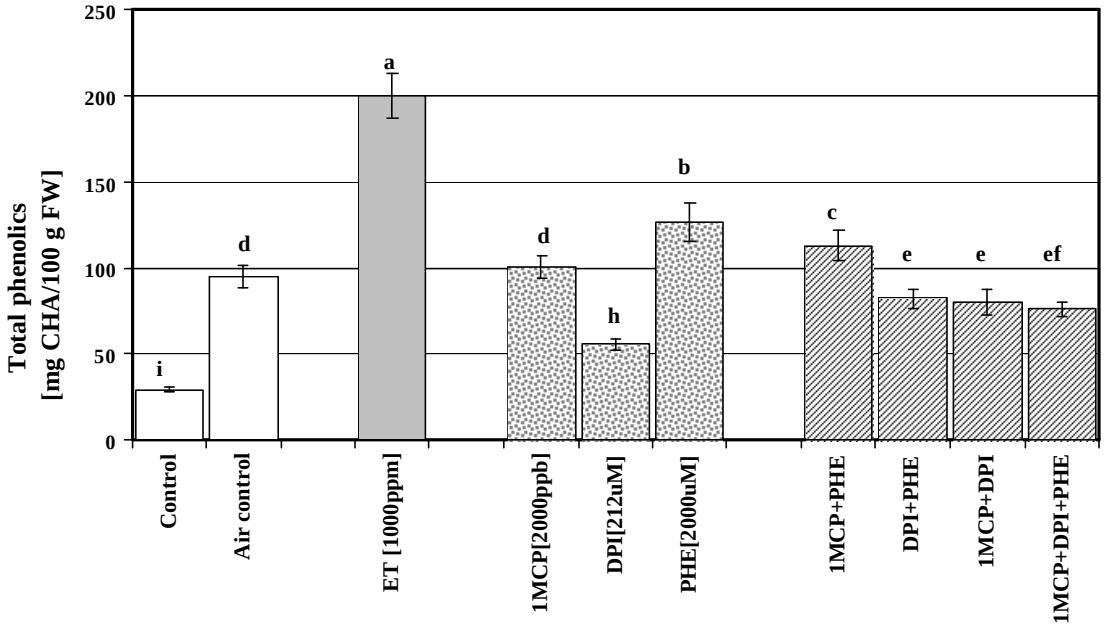

Figure 4.14. Effect of 1MCP, PHE and DPI combined blockers on the accumulation of total phenolics in ET-wounded stressed carrot pie-cuts. ET treatments were done with 1000 ppm. Blocker concentrations were 1MCP, 2000ppb; DPI, 212µM; and PHE, 2000µM. Samples were stored 6 d at 15°C. Results show means of 5 replicates and their standard deviation. Letters show mean comparisons among treatments (LSD P>0.05).

The model that describes the role of ET, MJ and ROS as signal molecules involved in the synthesis of phenolic compounds in carrot pie-cuts exposed to exogenous ET is presented in figure 4.15. In the diagram, the blockers 1MCP, PHE and DPI show the sites where they can potentially block signaling sites or enzyme activities. The filled lines are connecting known pathways obtained from the previous diagram (figure 4.12) and confirmed from this part of the study. This is an integral model present in carrot tissue when exposed to wounding and hormone stresses. The dotted lines represent the proposed pathways which include the action of ET in carrot tissue when exposed to exogenous ethylene. According to this model ET would trigger PAL synthesis indirectly through an increase in respiration which in turn increases ROS or through ROS production by increasing NADPH oxidase activity (Mittler 2002; Rakwal and Agrawal 2003; Blokhina and others 2003). Ethylene will also have a direct effect in triggering PAL activity.

Possible diffusion effects of signal molecules from wounded and hormone stressed wounded tissue.

Immersing carrot pie-cuts in water did not affect the accumulation of total phenolics of wounded and MJ stressed wounded carrots after 6 d of storage at 15°C (figure 4.16). Wounded air control samples showed slight but not significant decreases ($p>0.05$) in the total phenolic content after immersion of wounded sample for 2 min and 15 min. Immersion in water of previously MJ treated wounded sample for 2 min did not affect the accumulation of phenolics after storage ($p>0.05$). However immersion in water for 2 min of ET treated samples showed a slight decrease in phenolic content (~20%) after 6 d of storage at 15°C. According to these results diffusion of signaling molecules did not take place in wounded and MJ treated samples. In ET treated samples slight diffusion took place, however, as seen on blockers studies of ET treated samples (figure 4.13 and 4.14), reduction by blockers treatments were higher (33 to 85%) thus diffusion effects did not alter the overall results.

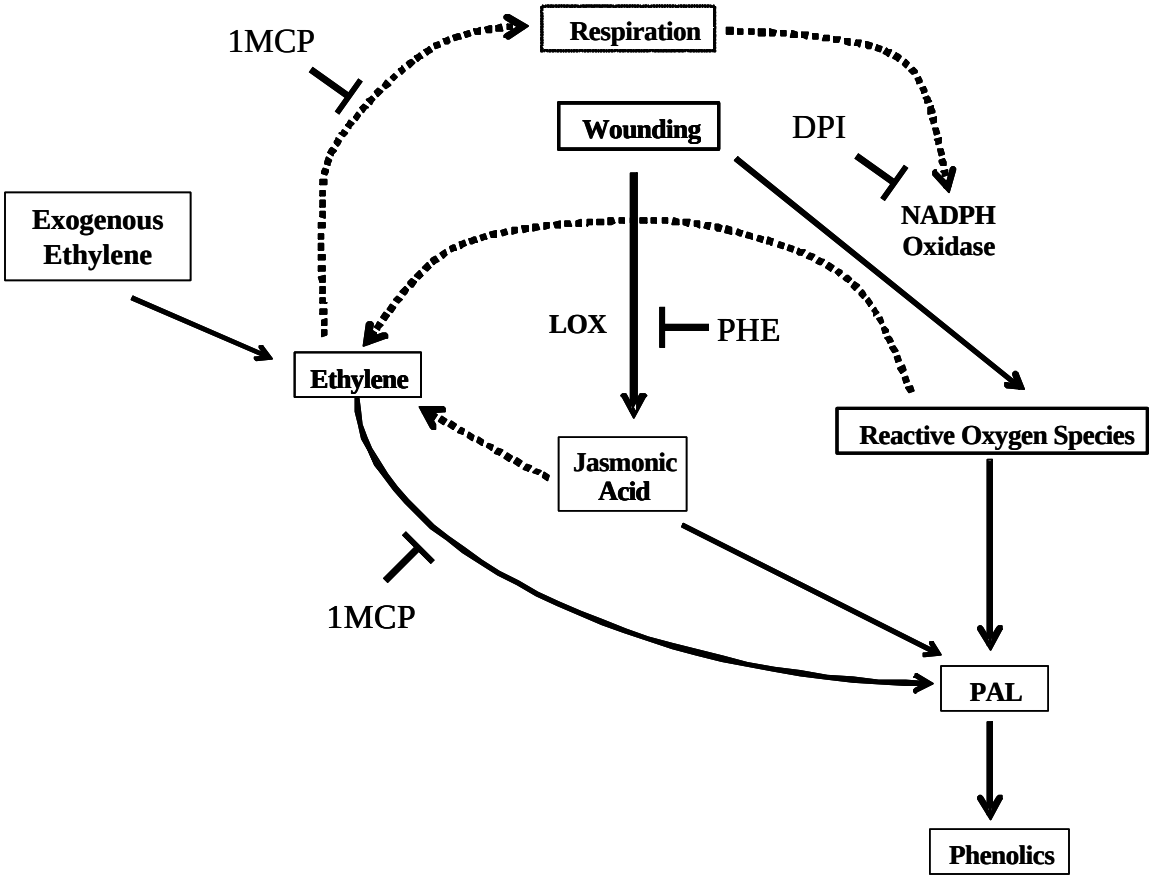

Figure 4.15. Proposed signal pathways involved in the synthesis of phenolic compounds in stressed carrot pie-cuts exposed to exogenous ET (1000 ppm). Filled lines are proposed known connecting pathways for carrot tissues. Dotted lines are proposed effects of exogenous ET applied to wounded carrot tissue. 1MCP, PHE and DPI show the sites where these can potentially block active sites or enzyme activities. NADPH oxidase activity is needed for both wounding and respiration pathways to produce ROS.

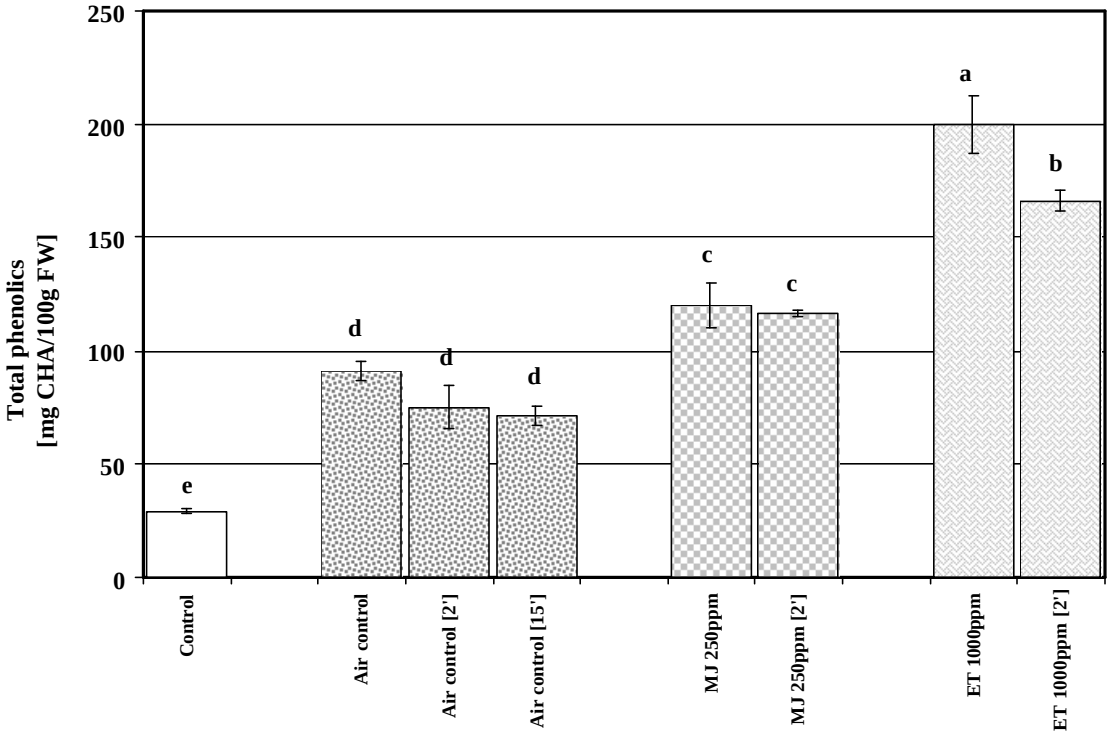

Figure 4.16. Possible diffusion effects of signaling molecules wounded and hormone-stressed wounded carrots during immersion in water. All treatments were evaluated for phenolic content. Values between brackets correspond to the number of minutes of water immersion for air control (wounded samples), wounded carrots immersed previously 15 min in 250 ppm MJ, and wounded carrots treated with 1000 ppm ET continuously during storage. Samples were stored 6 d at 15°C. Results show means of 5 replicates and their standard deviation. Letters show mean comparisons among treatments (LSD P>0.05).

Conclusions

Results indicated that exogenous MJ and ET trigger the accumulation of phenolic compounds over time, with the latter hormone showing a faster rate of synthesis and larger increase in phenolic content.

PAL activity increased with ET and MJ concentration. Phenolic accumulation showed saturation levels at 10 ppm ET, while for MJ a saturation level was not reached for concentrations = 250 ppm MJ.

The blockers PHE, DPI and 1MCP provided information of the role of signaling molecules jasmonic acid (MJ), ROS (NADPH oxidase) and ethylene (ET) in the synthesis of phenolic compounds in wounded carrot tissue alone or exposed to exogenous MJ and ET. Results with DPI blocker suggest that ROS is greatly responsible for the accumulation of phenolics in wounded- and wounded-hormone stressed carrot tissue. MJ also plays an important role as well as ET. The links between these signaling pathways are proposed in a mechanistic diagram. The diagram also explains the synergistic effect between wounding stress and ET hormone in the phenolic synthesis.

The role of exogenous MJ and ET over the ROS signaling molecules needs to be further explored through the evaluation of antioxidant-related enzymes, including superoxide dismutase (SOD), catalase (CAT), xanthine oxidase (XO), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR), which are involved mainly in the regulation of ROS levels under stress conditions.

CHAPTER V

GENERAL CONCLUSIONS AND RECOMMENDATIONS

The enhancement of antioxidant capacity of some fresh produce can be achieved when plant tissues are exposed to combined treatments of wounding stress, followed by ethylene or methyl jasmonate treatments. The synthesis of phenolic compounds is highly correlated with the increase in antioxidant capacity. Not all the commodities responded to these stresses with further synthesis of bioactive phenolics. Nevertheless, the diversity of antioxidant compounds in fruits and vegetables and their distinct responses to abiotic stresses make them an attractive research subject for further exploration of their secondary metabolism.

The intensity of wounding stress and the use of ethylene played major roles in the synthesis of phenolic compounds in selected commodities. In the case of stressed carrots, the main phenolic compounds synthesized were cinnamic and benzoic acid derivatives (*i.e.*, chlorogenic acid, dicaffeolyquinic acid, ferulic and vanillic acid). Chlorogenic acid showed the highest correlation with both the total phenolic content using (Folin assay) and the antioxidant capacity (DPPH assay). The levels of isocoumarins remained below the detectable levels for bitterness perception in all treatments.

The studies using blockers of signaling molecules that trigger selected plant defense response pathways provided information on their contribution in the phenylpropanoid pathway. The signaling molecules jasmonic acid, ROS, and ethylene were involved in the synthesis of stressed phenolics, as determined by the use of the corresponding blockers PHE, DPI and 1MCP, respectively. DPI caused a greater effect in reducing phenolic synthesis and therefore implies a larger role of ROS in the phenylpropanoid metabolism of stressed carrots.

Elicitors of signaling molecules should be investigated targeting the interaction of several secondary metabolites, which could provide more information about the complex metabolic fluxes.

Besides the role of exogenous MJ and ET on the synthesis of phenolics, further research should involve the effect of exogenous H_2O_2 on the signaling molecules JA, ET and ROS. It is also suggested to evaluate other antioxidant-related enzymes (*i.e.*, SOD, CAT, XO, APX, GPX, GR, POD and PPO) which could be related to the synthesis and degradation of ROS, and their role as ROS modulators.

The effect of the abiotic stresses on the quality and safety parameters of the stressed produce that may reach either the fresh-cut, processed and pharmaceutical markets should also be considered when designing these postharvest technologies.

The manipulation of plant stresses in order to produce phenolic compounds that can enhance the antioxidant capacity of plant extracts, could provide foods or derived supplements that can enrich diets and prevent or even repress important degenerative and age-related diseases in humans.

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VITA

In December 1991, Jose Basilio Heredia received his Bachelor of Science degree in food engineering from Instituto Tecnológico de Culiacán, México. During the senior year, Mr. Heredia started an internship program at a local vegetable processing plant. He was supervising Quality Control activities during the manufacturing of canned foods for Del Monte Co. Within the first 6 months of the internship, Mr. Heredia was hired as a full time employee and continued working at that company for another 6 months. The next job opportunity was in a Plant Nutrition Laboratory where Mr. Heredia worked for ~12 months.

By August 1993, he started graduate studies at the Centro de Investigación en Alimentación y Desarrollo, Sonora, México. In 1996, Mr. Heredia obtained his MS degree majoring in nutrition and foods, primarily focusing his research on metabolic changes associated with sugar accumulation in seedless table grapes. Once graduated, Mr. Heredia was offered a Research Associate position at CIAD-Culiacán, where he worked until August 2000. All those years he was involved in diverse plant-derived research projects, assisting 22 students with their theses.

The interaction of researching-teaching motivated Mr. Heredia to pursue a Ph.D. in Food Science and Technology at Texas A&M University. His research was related to manipulation of plant abiotic stresses to enhance the antioxidant capacity of fruits and vegetables. As a result of these studies, Mr. Heredia has presented 4 research papers at meetings of the Institute of Food Technologists, which will be published in related journals. Part of this investigation has been used by the Fresh-Cut Industry of California.

After the spring 2006 Ph.D. graduation, Mr. Heredia may be reached at the Department of Horticultural Sciences (http://aggie-horticulture.tamu.edu), Texas A&M University (http://www.tamu.edu), 2133 TAMU, College Station, TX 77843. His email address is jbheredia@tamu.edu.