MODULATION OF THE BIOSYNTHESIS OF PHENYLPROPANOIDS AND HYDROLYZABLE TANNIN DERIVATIVES IN FRUITS THROUGH LONG-DISTANCE STRESSES

A Dissertation

by

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

DOCTOR OF PHILOSOPHY

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May 2015

Major Subject: Molecular and Environmental Plant Sciences

ABSTRACT

The importance of secondary metabolites (SM) in plant defense mechanisms against environmental stresses as well as its benefits in human health have led to the study of how preharvest factors enhance their biosynthesis in fruits and vegetables. Pecans and strawberries have high level of bioactive phenolics including ellagitannins, gallotanins and proanthocyanidins. Wounding affects the production of SM as a local response, and as a systemic response in leaves, but this has never been tested in fruits. Organic agriculture claims that under this method of production, plants suffer more biotic stress and accumulate more SM in fruits.

In this dissertation, the preharvest effects of biotic stress due to insect feeding and mechanical wounding were evaluated as modulators of phenolics in fruits. Wounding did not produce any differences in quality and vitamin C in fruits at harvest compared with the control. However, the level of total phenolics and soluble sugars in fruits from treated plants increased significantly 12% and 20% respectively. Moreover, increments in the level of specific phenylpropanoids were observed: epicatechin (160%), quercetin (190%) and rutin (190%), the ellagitannins/gallotanins derivatives ellagic acid (58% and gallic acid (130%). In addition, several genes related to phenolics biosynthesis and sugar metabolism were overexpressed. A hypothetical model is proposed to explain the modulation of phenolic compounds in fruits based on source/sink transport of sugars in favor of fruits from wounded leaves. In the following studies were used a generalist insect chewing in organic strawberry plants, and a specialist aphid feeding on pecan

leaves. In strawberries no significant increments were detected for quality parameters, soluble sugars, phenolics, and related gene expression (except for the cell wall invertase gene). In pecan kernels, no differences were found in proanthocyanidins, gallotanins o ellagitannins derivatives levels due to the insect sucking the leaves.

The results could explain how the wounding factor attributed to insect damages is connected to higher levels of phytochemicals in organic fruits. Furthermore, controlled mechanical wounding applied in leaves during preharvest could be used to increase phytochemicals in fruits.

ACKNOWLEDGMENTS

I sincerely want to thank my committee co-chairs, Dr. Lombardini and Dr. Cisneros, for their ongoing support and leadership. I am especially thankful to Dr. Cisneros for letting me work in his lab; working under his direction was indeed a great opportunity for me. Thanks to my committee members, Dr. Medina for his encouragement, motivation, and words of wisdom, and Dr. Finlayson for opening me the doors of his lab and for his guidance in analytical methods.

My gratitude to my colleagues from Plant Bioactive and Bioprocessing Research Laboratory, I have really enjoyed working with you over these four years. Indeed, it feels amazing working in such a positive work environment. Special thanks to Dr. Bang for his unconditional help and knowledge.

Finally, I would like to thank to the *Instituto Nacional de Investigación Agropecuaria* (INIA) of Uruguay and the Fulbright Foreign Student Program of the US Department of State for their financial support.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

In the last decade, the organic food market has grown between 17% and 21%, compared to the 2-4% growth for conventional food products market (Bezawada and Pauwels 2013). European countries and the United States lead the global market as producers and consumers, and several other countries, including Australia, China, Argentina, Brazil and Uruguay are important producers for export markets (Sahota 2008). For the US Department of Agriculture, organic products should certify that during all the processes implied, only approved substances are used, the natural resources are preserved, the biodiversity is respected, and uses only approved substances (USDA 2013). Certification and labeling of organic products have a positive effect on consumers selection and support higher prices in an ever-growing market (Bauer et al. 2013). Some studies showed that organic products it may also have higher dry matter, higher levels of healthy fatty acids in dairy products, less nitrates and higher levels of antioxidants, mainly polyphenols and vitamin C, in fruits and vegetables (Lairon and Huber 2014; Lairon 2010). Regarding the higher levels of phytochemicals, several studies supported that organic fruit and vegetables contain higher levels of secondary metabolites related to the plant defenses (Brandt and Mølgaard 2001; Zuchowski et al. 2011; You et al. 2011; Lima and Vianello 2011; Brandt et al. 2011; Petkovsek et al. 2010; Faller and Fialho 2010; Crinnion 2010; Lima et al. 2008; Lombardi-Boccia et al. 2003). A meta-analysis, contrasting unique results from 84 studies, showed that

secondary metabolites content in organic products is 12% higher than in conventional ones (Brandt et al. 2011). Products derived from organic agriculture contained greater amounts of flavonoids, anthocyanins and carotenoids (Crinnion 2010). Several authors speculate that higher levels of phytochemicals, particularly phenolic compounds, are related to higher levels of biotic stress when plants are grown in organic conditions (Young et al. 2005; Zhao et al. 2009; Cohen and Kennedy 2010). Phytochemicals are especially relevant for human health since they may play a role in treatment and prevention of chronic cardiovascular and inflammatory diseases or cancer (Sarkar and Shetty 2014; Krishnaiah et al. 2011; Rajendran et al. 2014; Wang et al. 2011).

Research on secondary metabolites production induced by biotic stresses has shown an induction of synthesis of phenolic compounds and phytoalexins as a plant defensive response. These studies were conducted in the same tissues where damage was caused by either piercing-sucking insects (Cabrera et al. 1995; Goggin 2007; Morkunas et al. 2011; Pickett et al. 1992; Smith and Boyko 2007; Chen et al. 2009; Eleftherianos et al. 2006) or leaf-chewing insects (Arimura et al. 2005; Bricchi et al. 2010; Howe and Schaller 2008; Maffei et al. 2007; Rodriguez-Saona et al. 2010; Valladares et al. 2002). Systemic induction of secondary metabolites has been demonstrated for the same kind of plant organ, like leaf-leaf model in tobacco, tomato and poplar (Keinänen et al. 2001; Schmidt et al. 2005; Schwachtje and Baldwin 2008; Voelckel et al. 2004; Woldemariam et al. 2011). However, there is no reported scientific information regarding the production of phenolic secondary metabolites (phytochemicals) in fruits and how it is

affected by stresses (biotic and mechanical) produced in the leaves during fruit development.

Plant secondary metabolites are regulated by signal transduction pathways that can be triggered and regulated by several abiotic and biotic stress factors (Rhodes et al. 2006; Maffei et al. 2007). Stresses such as wounding and herbivores induce changes in plant secondary metabolism (War et al. 2012; Salminen and Karonen 2011; Howe and Schaller 2008; Chen 2008; Arimura et al. 2005). Wounded tissues affect the production of phenylpropanoid secondary metabolites as a local response and also as a systemic response in the same organ tissue (e.g., leaves) (Silva et al. 2012; Maffei et al. 2007; Chen et al. 2006; Housti et al. 2002; Campos-Vargas and Saltveit 2002; Dixon and Paiva 1995; Bennett and Wallsgrove 1994; Woodhead 1981). An early study showed that wounded potato and tomato leaves enhanced the production of proteinase inhibitors (Green and Ryan 1972). Since then, several studies have shown how chemical signals produced at the wounded tissue travel through the plant and activate response in undamaged leaves (Howe and Schaller 2008; de Bruxelles and Roberts 2001; Chen 2008; van Verk et al. 2009; Shah and Zeier 2013). After the recognition of the wounding event, plants under insect attack respond with direct and indirect defense mechanisms. There are two kinds of direct defenses in plants, the ones already formed such as secondary metabolites and physical barriers (e.g., waxes or spines), and those inducible by wounding or insect damages (Howe and Schaller 2008; Chen 2008). The indirect defense mechanisms of plants include a third trophic level like predators and parasitoids (Alba et al. 2012; Arimura et al. 2005). Affected plants develop an efficient defense

system that include a crosstalk between signal molecules that include phytohormones, such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), indolacetic acid (IAA) and gibberellic acid (GA), plus several reactive oxygen species (ROSs) (Morkunas et al. 2011; Wasternack 2014; Wang and Wu 2013; De Geyter et al. 2012). ROSs have critical roles in signaling related to plant defenses and several other functions (Fluhr 2009; Suzuki and Mittler 2012; Smékalová et al. 2014; Mori et al. 2009). The specific elicitors released by insect damage activate several signaling pathways that interact each other (crosstalk) producing a metabolic rearrangement expressing defense-related genes and sometimes directly releasing directly volatile organic compounds (Kessler and Baldwin 2002). JA, methyl jasmonate (MeJA) and its precursor, 12-oxo-phytodienoic acid (OPDA), are inducers of proteinase inhibitors as a main defense against herbivore feeding (Korth and Thompson 2006; Heil et al. 2012). JA and ET play important roles as positive regulators of plant defense against attack from insect and some pathogens, whereas SA has been associated with resistance against most pathogens (Monaghan et al. 2009). Apparently, the crosstalk allows the plant to optimize the responses against herbivores and pathogens, a strategy that produces a very complex defensive system (Morkunas et al. 2011; Smékalová et al. 2014; Robert-Seilaniantz et al. 2011; Mori et al. 2009; Bari and Jones 2009; Wasternack 2007; Vandenbussche et al. 2007; Rakwal and Agrawal 2003; Thaler et al. 2002; Ding et al. 2002).

The importance of plant defensive compounds (phytochemicals) for human health has led to the study of pre- and post-harvest factors that influence the production

of bioactive phenylpropanoids (Cisneros-Zevallos 2003; Young et al. 2005; Jin et al. 2011; Wang et al. 2012; Wang et al. 2008; Wang and Frei 2011; Cohen and Kennedy 2010). Phenylpropanoids and ellagitannins received attention for their biological activity associated with health benefits, such as antioxidant, anti-allergy, anti-hypertensive, and antitumor effects *in vitro* and *in vivo* (Krishnaiah et al. 2011; Leopoldini et al. 2011; Rajendran et al. 2014; Saeidnia and Abdollahi 2013; Wang et al. 2011). Strawberry (*Fragaria* x *ananassa*) and pecan (*Carya illinoinensis*) have been reported as foods with very high content of bioactive ellagitannins (Arapitsas 2012; Landete 2011; Ascacio-Valdes et al. 2011; Pinto Mda et al. 2010; Larrosa et al. 2010; Tomás-Barberán et al. 2009; Nohynek et al. 2006).

Strawberry is one of the most important small fruits produced worldwide; in 2012, the United States was the main producer (1.366,850 tons), followed by Mexico (360,426 tons) (FAOSTAT 2012). In South America, the main producers are Argentina, Brazil, and Chile (Antunes and Peres 2012; Gambardella and Pertuzé 2006; Kirschbaum and Hancock 2000). Uruguay has a small production area (120 ha) with high yields (37 tons/ha) which supply the local market with national cultivars (Antunes and Peres 2012; Vicente et al. 2014). Despite the fact that the main biotic stresses affecting strawberry culture are caused by bacteria and fungi (Maas 2004; Louws 2009), some arthropods, such as the aphid *Chaetosiphon fragaefolii* (Bernardi et al. 2013) and the spider mite *Tetranychus urticae* (Monteiro et al. 2014), can negatively affect the commercial production. Several studies showed that the level of phytochemicals was higher in strawberries grown organically compared to those grown with conventional methods. A

comparison between both production approaches for two strawberry cultivars found that fruit from organic production accumulate more total phenolics and that the differences are maintained at different storage temperatures (Jin et al. 2011). Ellagic acid is a phenolic compound found in high concentrations in strawberry, ranging from 39.6 to 52.2 mg/100 g fresh weight in nine cultivars analyzed (Häkkinen and Törrönen 2000). A high variation was reported in 13 clones, showing values between 43 to 464 mg/100g dry weight (Maas et al. 1991). Comparing the organic vs. conventional techniques of production, the cultivar "Jonsok" had higher level of total phenolics and showed a significant increment in kaempferol content (0.9 vs. 0.5 mg/100g fresh weight) and ellagic acid (58.6 vs. 52.2 mg/100 g fresh weight) for the organic system (Häkkinen and Törrönen 2000). The authors speculated that the higher incidence of pathogen attack on this cultivar could increase the levels of these phenolics (Häkkinen and Törrönen 2000). In another study comparing organic (using compost and cow horn manure as a soil supplement) and conventional production (D'Evoli et al. 2010), the results showed higher antioxidant activity and levels of kaempferol (1.99 vs. 1.26 mg/100g fresh weight) and ellagic acid (53.3 vs. 37.9 mg/100g fresh weight) for organically grown strawberry. This higher level in phenolics was correlated with better antiproliferative activity in Caco-2 cell lines, derived from a kind of human colon adenocarcinoma (D'Evoli et al. 2010). The levels of ellagic acid glucoside in "Allstar" and "Earliglow" strawberry cultivars increased from 14.7 to 18.6 µg/g fresh weight and 12.2 to 18.5 µg/g fresh weight respectively for the organic growth conditions (Jin et al. 2011). The addition of organic and conventional soil nutrients did not affect strawberry yield and

quality parameters related to phenolics such as antioxidant capacity (Hargreaves et al. 2008).

Pecan is an important horticultural crop in the southern United States. The value of US pecan production grew from 430 million of dollars in 2009 to 507 million dollars in 2014 (NASS 2015). Furthermore, it is the most significant indigenous nut crop in the US, mainly in the states of Texas, Oklahoma, Louisiana, Arkansas, Mississippi, Kansas, Missouri, Tennessee and Kentucky, with cultivation extended to other southern states (Thompson and Conner 2012). Several pests are associated with pecan orchards. The most important ones are pecan nut casebearer (*Acrobasis nuxvorella*), the black margined aphid (*Monellia caryella*) and the yellow pecan aphid (*Monelliopsis pecanis*) (Ree 1999; Smith 1995). One study reported increments on total terpenes, condensed tannins, hydrolysable tannins and lignin in tissues damaged by fruit tree borer insect (*Euplatypus segnis*) and associated fungi (*Fusarium solani*, *Fusarium oxysporum*, *Alternaria alternata* and *Botryodiplodia theobromae*) on pecan (Alvidrez-Villarreal et al. 2011). Another study, reported the effects of black pecan aphid (*Melanocallis caryaefoliae* Davis) on the activity of oxidative enzymes (peroxidase, catalase, lipoxygenase) and esterase in field conditions (Chen et al. 2009). Recently, over 100 volatile terpenic derivatives from dormant buds were reported for two pecan cultivars (Western Schley and Wichita) grown in Mexico (Corella-Madueño et al. 2011).

Our group has studied the kernel phytochemistry of several pecan varieties and reported different kinds of phenylpropanoids and ellagic acid derivatives (Ortiz-Quezada et al. 2011; Villarreal-Lozoya et al. 2007, 2009). A study carried out to detect

differences in phenolic compounds and ellagic acid for conventional and organic pecan orchards, showed that the levels of ellagic acid and catechin in organically grown 'Desirable' were between two and four times higher than in a conventionally grown orchard (Malik et al. 2009).

The objective of this dissertation is to test the hypothesis that the production of phenolic secondary metabolites (such as phenylpropanoids and ellagic acid derivatives) in fruits is increased by stresses (biotic and mechanical) produced in the leaves during fruit development. In the study described in Chapter II, the hypothesis was tested by identification and quantification of the effects of mechanical wounding on leaves in the biosynthesis of phenolic secondary metabolites in fruits, using strawberry as a plant model. The experiment was then extended to a commercial strawberry field (organic production conditions), using a generalist insect (*Spodoptera exigua*) feeding on leaves as a biotic stressor (Chapter III). The hypothesis was finally tested in a contrasting model, using the specific relationship between pecan trees and Black Pecan Aphid (*Melanocallis caryaefoliae*). In this experiment the production of phenolic secondary metabolites in fruits in response to aphid feeding on leaves was also tested (Chapter IV). In Chapter V the results are summarized and analyzed taking into consideration the similarities and differences between the two plant models, the two stresses in the same plant and the insect feeding behavior related to the plant responses. Conclusions and future research suggestions are also presented in this chapter. This dissertation contributes knowledge about how the phytochemical levels in strawberry fruits and pecan nuts are affected by stresses (biotic and mechanical) produced in leaves of the

plant. The knowledge generated in this work could contribute to explain at chemical and molecular levels the higher phytochemical content found in organic fruits and vegetables. In addition, as a technological tool, controlled stress applied during preharvest in leaves could be used to increase the phytochemical content in fruits.

CHAPTER II

PHENOLIC COMPOUNDS IN STRAWBERRY FRUITS ARE INDUCED BY MECHANICAL WOUNDING ON LEAVES

Introduction

It has been supported by several studies that organic fruit and vegetables contain higher levels of secondary metabolites related to plant defenses (Brandt and Mølgaard 2001; Zuchowski et al. 2011; You et al. 2011; Lima and Vianello 2011; Brandt et al. 2011; Petkovsek et al. 2010; Faller and Fialho 2010; Crinnion 2010; Lima et al. 2008; Lombardi-Boccia et al. 2003). Meta-analysis showed that the content of secondary metabolites in organic products was 12% higher than in those grown with conventional practices (Brandt et al. 2011). In general, vegetables and fruits from organic production contain greater amounts of flavonoids, anthocyanins, and carotenoids (Crinnion 2010). Higher levels of phytochemicals, particularly phenolic compounds, could be related to higher levels of biotic stress, such as insect damage, when plants are grown in organic conditions (Young et al. 2005; Zhao et al. 2009; Cohen and Kennedy 2010). Stresses like wounding and those induced by herbivores (biotic stress) cause changes in plant secondary metabolism (War et al. 2012; Salminen and Karonen 2011; Howe and Schaller 2008; Chen 2008; Arimura et al. 2005). Wounded tissues alter the production of phenylpropanoid secondary metabolites as a local response, and also as a systemic response in the same organ type (e.g. leaves) (Silva et al. 2012; Maffei et al. 2007; Chen et al. 2006; Housti et al. 2002; Campos-Vargas and Saltveit 2002; Dixon and Paiva

1995; Bennett and Wallsgrove 1994; Woodhead 1981). Plants under attack from herbivores develop an efficient defense system that includes a crosstalk between signaling molecules that include phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), indolacetic acid (IAA), gibberellic acid (GA), and several reactive oxygen species (ROSs) (Morkunas et al. 2011; Wasternack 2014; Wang and Wu 2013; De Geyter et al. 2012). The ROSs have critical roles in signaling related to plant defenses, among several other functions (Fluhr 2009; Suzuki and Mittler 2012; Smékalová et al. 2014; Mori et al. 2009). Specific elicitors released by the insect activate several signaling pathways that interact with each other (crosstalk) producing a metabolic rearrangement, expressing defense related genes and sometimes directly releasing directly volatile organic compounds (Kessler and Baldwin 2002). Jasmonic acid, methyl jasmonate (MeJA) and its precursor 12-oxo-phytodienoic acid (OPDA) are inducers of proteinase inhibitors as a main defense against herbivore feeding (Korth and Thompson 2006; Heil et al. 2012). Jasmonic acid and ET play an important role as positive regulators of plant defense against insect attack and some pathogens, whereas SA has been associated with resistance against pathogens (Monaghan et al. 2009). The crosstalk allows the plant to optimize responses against herbivores and pathogens, and this strategy produces a very complex defensive system (Morkunas et al. 2011; Smékalová et al. 2014; Robert-Seilaniantz et al. 2011; Mori et al. 2009; Bari and Jones 2009; Wasternack 2007; Vandenbussche et al. 2007; Rakwal and Agrawal 2003; Thaler et al. 2002; Ding et al. 2002).

The importance of plant defensive compounds (phytochemicals) for human health has led to the study of pre- and post-harvest factors that influence the production of bioactive phenylpropanoids (Cisneros-Zevallos 2003; Young et al. 2005; Jin et al. 2011; Wang et al. 2012; Wang et al. 2008; Wang and Frei 2011; Cohen and Kennedy 2010). Phenylpropanoids and ellagitannins received attention for their biological activity associated with human health benefits like antioxidant, anti-allergy, anti-hypertensive, antitumor effects *in vitro* and *in vivo* (Krishnaiah et al. 2011; Leopoldini et al. 2011; Rajendran et al. 2014; Saeidnia and Abdollahi 2013; Wang et al. 2011). Studies evaluating secondary metabolite responses to biotic stress have shown an induction of phenolic compounds production and other phytoalexins as a local and systemic plant defensive response; however, these studies were conducted in the same tissue where the damage had been caused by piercing-sucking insects (Cabrera et al. 1995; Goggin 2007; Morkunas et al. 2011; Pickett et al. 1992; Smith and Boyko 2007; Chen et al. 2009; Eleftherianos et al. 2006) or leaf chewing insects (Arimura et al. 2005; Bricchi et al. 2010; Howe and Schaller 2008; Maffei et al. 2007; Rodriguez-Saona et al. 2010; Valladares et al. 2002). The objective of this study was to evaluate the systemic induction of secondary metabolites in fruits when the stress is applied in a different organ of the plant (leaves).

Materials and methods

Field experiment. The experiment was conducted on a strawberry (*Fragaria* × *ananassa*) field at the end of the harvesting season, from December 2013 to January

2014, at INIA-Las Brujas Research Station, Uruguay (lat. 34.66 S, long. 56.34 W). An evaluation plot of the advanced selection LBM 10.3 (cv. Albion X SGG 31.1) was used for the experiment (Fig. 2-1A). The plot in a brunisol soil, was divided in three subplots, and randomly assigned to each treatment. Inside each subplot, 20 plants were assigned to each treatment as follow:

1) Control with no perforations applied to the plants;

2) Low mechanical wounding (W50) consisted in small perforation of 3-5 perforations per leaf (Fig. 2-1B), for a total of 50 perforations and a 10-cm^2 loss of foliar area per plant;

3) High mechanical wounding (W100) with 100 perforation on each plant, and 20-cm^2 loss of foliar area per plant.

Fertilization consisted in the application of 30 kg of N and 50 kg of P per hectare in April 2013 before the planting season. The use of insecticides or fungicides was not necessary because there was neither incidence of *Botrytis* sp. nor noticeable presence of insects. The wounding was applied to plants with fully developed fruits (25% red color). Each plant was harvested one and two weeks after the treatment was applied, selecting the full ripen fruits (over 80% of full color).

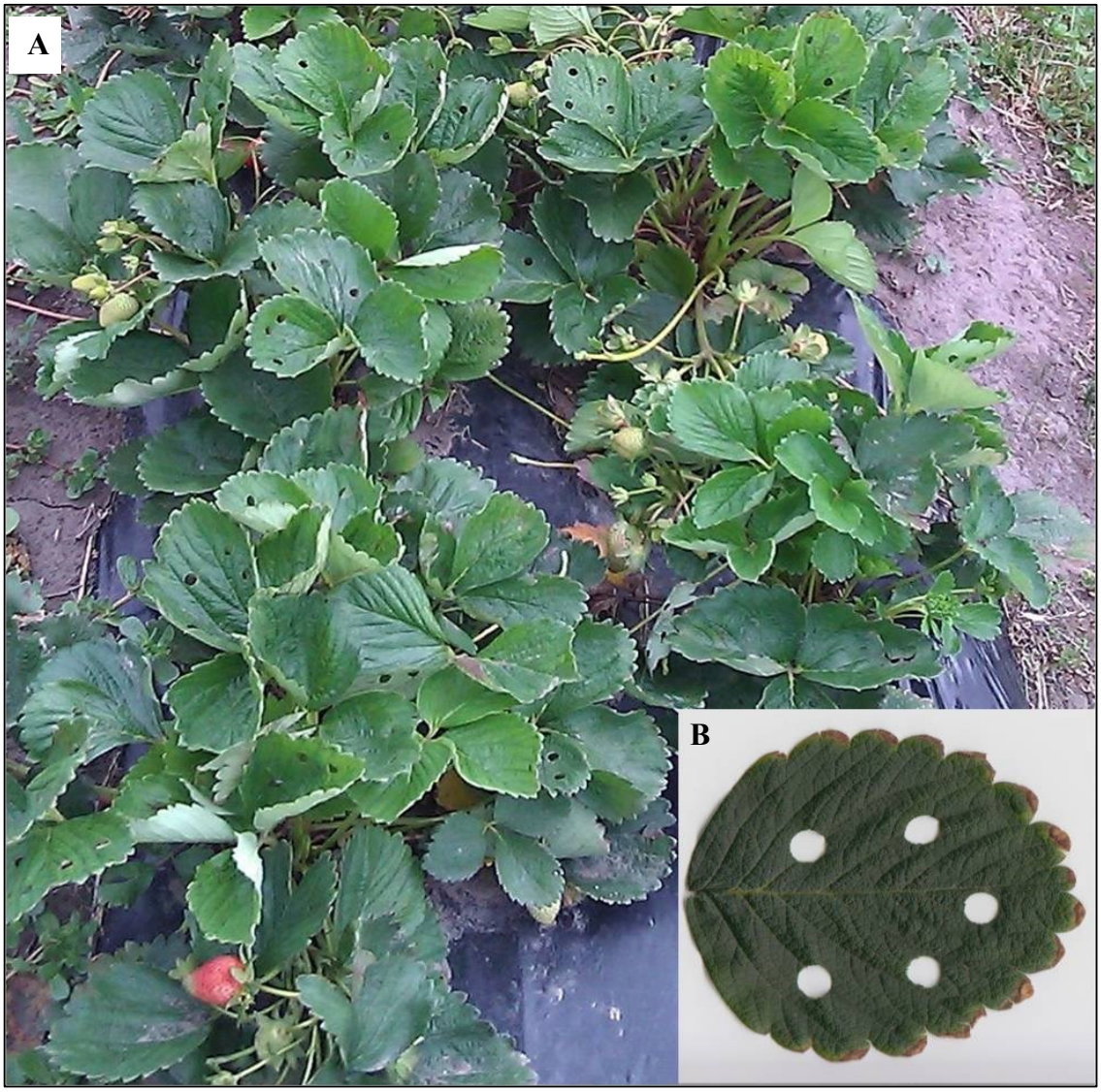

Fig. 2-1. Picture of strawberry wounding experiment (W50). **A**, Field experiment. **B**, sample leaf with mechanical wounding.

Harvest evaluation. Immediately after harvest, fruits were evaluated for harvest quality by measuring physicochemical parameters. For each fruit, the weight was registered and color was determined by two measures using a digital colorimeter (CR-200, D65 illuminant; Minolta, Tokyo, Japan), recording the L*a*b* coordinates values. Fruit firmness was determined using a TA.XTPlus Texture Analyzer (Stable Mycro System, Surrey, United Kingdom). The soluble solids concentration (SS) was determined in the juice by an Atago RX-1000 digital refractometer (Atago Co. Ltd, Tokyo, Japan). After these determinations, samples were frozen in liquid nitrogen and stored at -80 ºC for subsequent freeze drying and further analyses. All samples were freeze-dried in a FreeZone benchtop freeze dry system (Labconco, Kansas City, MO) until they were completely dried.

Total phenolics and vitamin C analysis. Total phenolic compounds and total vitamin C were determined in the same analysis according to the method reported by Sanchez-Rangel et al. (2013). Briefly, 50 mg of freeze dried strawberry powder was extracted with 1.00 mL of MeOH:H_2O (80:20 v/v) in a centrifuge tube using an ultrasonic bath for 30 min. Samples were then centrifuged at 14.000 rpm. In a 96 plate was added 15 µl of extract and 240 µl of distilled water followed by 15 µl of Folin-Ciocalteau reagent. The mixture was incubated for 3 min and the absorbance was read at 725 nm for estimation of vitamin C. After that a Na_2CO_3 solution (30 µl, 1N) was added and incubated at room temperature in dark conditions for 2 h, then the absorbance was measured again at 725 nm. In parallel, standards of ascorbic and chlorogenic acid were

run in addition of blanks. Absorbance was recorded using a Synergy-HT Microplate Reader and analyzed using KC4 software (Bio-Tek Inc., Winooski, VT).

HPLC analysis of phenolic compounds. Chromatographic separation was implemented on a LCQ Deca XP Max LC-MS/MS system (Thermo Finnigan, CA) equipped with an autosampler, a quaternary pump and a UV 2000 PDA detector, using a 150 × 2.00 mm Synergi 4µ Hydro RP 80A column (Phenomenex, Torrance, CA) and a guard column of the same chemistry. The elution mobile phase was executed with solvent solution A [0.5% formic acid -water] and solvent mixture B [0.5% formic acid in acetonitrile]. A linear gradient was set up with A and B: 0 min 98% A, 10 min 75% A, 20 min 75% A, 30 min 25% A, 35 min 0% A, 38 min 98% A. The flow rate was 200µl/min. The injection volume was 10 µl. Retention time and spectral profile were used for identification detected by a photodiode array detector (PDA) scanning between 190-600 nm, the quantification was done by comparison with external standards obtained from Sigma-Aldrich (St. Luis, MO).

Gene expression. Total RNA extraction from strawberry fruits was carried out by combining the method previously described by Christou et al. (2014), with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Briefly, 0.1 g of freeze-dried strawberry fruits was mixed with 1 ml of the extraction buffer (0.5 M Tris–HCl pH 8.8 and 1% sodium dodecyl sulfate [SDS]). Subsequently, 1 ml of phenol:chloroform:isoamyl alcohol (PCI) (25:24:1 [v/v]) was added to the mixture, which was gently agitated and then centrifuged at 14,000 rpm for 5 min at 4 °C for phase separation. The upper aqueous phase (~800 µl) was further subjected to the PCI extractions (three times). After

the third PCI extraction, the upper aqueous phase (~400 µl), whose phenol traces were removed completely, was collected into a fresh chilled tube, where 0.1 volume of 3 M NaOAC (pH 5.6) and 1 volume of 100% ethanol were mixed, incubated at −80 °C for 20 min and then centrifuged at 12,000 rpm for 8 min at 4 °C for RNA precipitation. After drying at room temperature, RNA pellets were finally dissolved in RNase-free water and further purified by RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Willmington, DE). Aliquots of 0.7 µg RNA, treated with DNase I to avoid DNA contamination, were reverse-transcribed into cDNA using the SuperScript III first-strand synthesis supermix (Invitrogen, Carlsbad, CA) following the manufacturers protocol. Finally, the cDNAs were used for real-time qRT-PCR analyses, which were performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. cDNA amplification was carried out using a 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The primer sets used in this study were provided by Integrated DNA Technologies (IDT, Coralville, IA), and their sequence information is shown on Table 2-1. The relative expression of each gene was normalized by the *FaGAPDH* and was calculated following the comparative Ct method (ΔΔCt), known as the $2^{-\Delta\Delta Ct}$ method. Strawberry gene were selected based on implication in the shikimate pathway, phenolic compounds biosynthesis, and sugar transport and metabolism. All of these genes have been reported for strawberry fruits, and primer

sequences are available (Landi et al. 2014; Amil-Ruiz et al. 2013; Amil-Ruiz et al. 2011).

Foliar area. Estimation of losses of foliar area for each strawberry plant were done using the software ImageJ (Schneider et al. 2012). Two average leaflets were picked, submitted to wounding and a digital picture was taken. Twelve leaves was considered the average amount of leaves for this advanced selection.

Statistical analysis. Analysis of Variance (ANOVA) was applied to the data and the statistical differences between treatment means were determined using the Duncan´s Test ($p \leq 0.05$ and $p \leq 0.1$). Those tests were conducted using the software InfoStat (National University of Cordoba, Argentina).

Table 2-1. Sequence of primers from *F. x ananassa* used in qRT-PCR analyses. The sequences belong to genes of enzymes involved in phenolics biosynthesis and other related enzymes.

Primer	Sequence
FaPAL-F	5'-CACCTGCTCTCAGTCGTGGACC-3'
FaPAL-R	5'-GCA TGTTCTACTAGCTCTGCCCTCAG-3'
FaCHS-F	5'-GTTGGGCTCACATTTCACCTCCTCA-3'
FaCHS-R	5'-AATTGCTGGGCCACCTGGGTG-3'
FaEPSPS-F	5'-GGAGACTTGGTCACTGGTCTTA-3'
FaEPSPS-R	5'-GAAGGCCTCCCTTTCCAATTAC-3'
FaDAHPS-F	5'-CGCAACTGGTGGGTATGCGGC-3'
FaDAHPS-R	5'-CCCGGTGAGCAAGTTCCCGG-3'
FaDHQS-F	5'-GCAGCTGGCATGATCATGGCTG-3'
FaDHQS-R	5'-CGGTCACAGACTCAGGAGGGC-3'
FaDHD/SDH 1-F	5'-AGCTCCTGGTCAACCTACTATC-3'
FaDHD/SDH 1-R	5'-GCTGACGGGCTTTCCAATAA-3'
FaDHD/SDH 2-F	5'-CGTTGGGATTCCTCACAAAGA-3'
FaDHD/SDH 2-R	5'-CATCAGTTGGCCTCCTTACAA-3'
FaDHD/SDH 3-F	5'-GAGGAAGGACTTCGAGGATTAG-3'
FaDHD/SDH 3-R	5'-GCTCCCATGACCACAAATAAC-3'
FaSI-F	5'-GGTATGTGGGAGTGCATTGA-3'
FaSI-R	5'-CGTCCAAGCTAGCCTTTAGAA-3'
FaCWI-F	5'-CCAGGCAATTCCAAGGACTAT-3'
FaCWI-R	5'-CTTGACCTCGTTTGTTCTAAGTTT C-3'
FaLOX-F	5'-CCGGGACACGATGAACATAA-3'
FaLOX-R	5'-GGCATATTGAGCTGGGAAGA-3'
FaJMT-F	5'-AATAAGCAGCGGCGAGCGAGTAGC-3'
FaJMT-R	5'-AAGCGATCACTGACGAGCTCTGCG-3'
FaGAPDH-F	5'-TCCATCACTGCCACCCAGAAGACTG-3'
FaGAPDH-R	5'-AGCAGGCAGAACCTTTCCGACAG-3'

Results

The evaluation of several fruit quality parameters was carried out immediately after harvest. Average fruit weight (Fig. 2-2A), firmness (Fig. 2-2C, and color (Fig. 2-2D, E, F), at harvest was the same after 7 and 14 days of applied wounding. For soluble solids, a significant increment was observed in W100 (19.7%), meaning an increase in the levels of soluble sugars and organic acids after two weeks (Fig. 2-2B). Moreover, the amount of total phenolics (TP) in fruits of all treated plants increased significantly, 12.8% for one week and 10.7 % over the control after 2 week of the application of mechanical wounding (Fig. 2-2G). Total ascorbic acid (Fig. 2-2H) was significantly less for the higher level of wounding (W100) after 7 days but the difference was not apparent at 14 days.

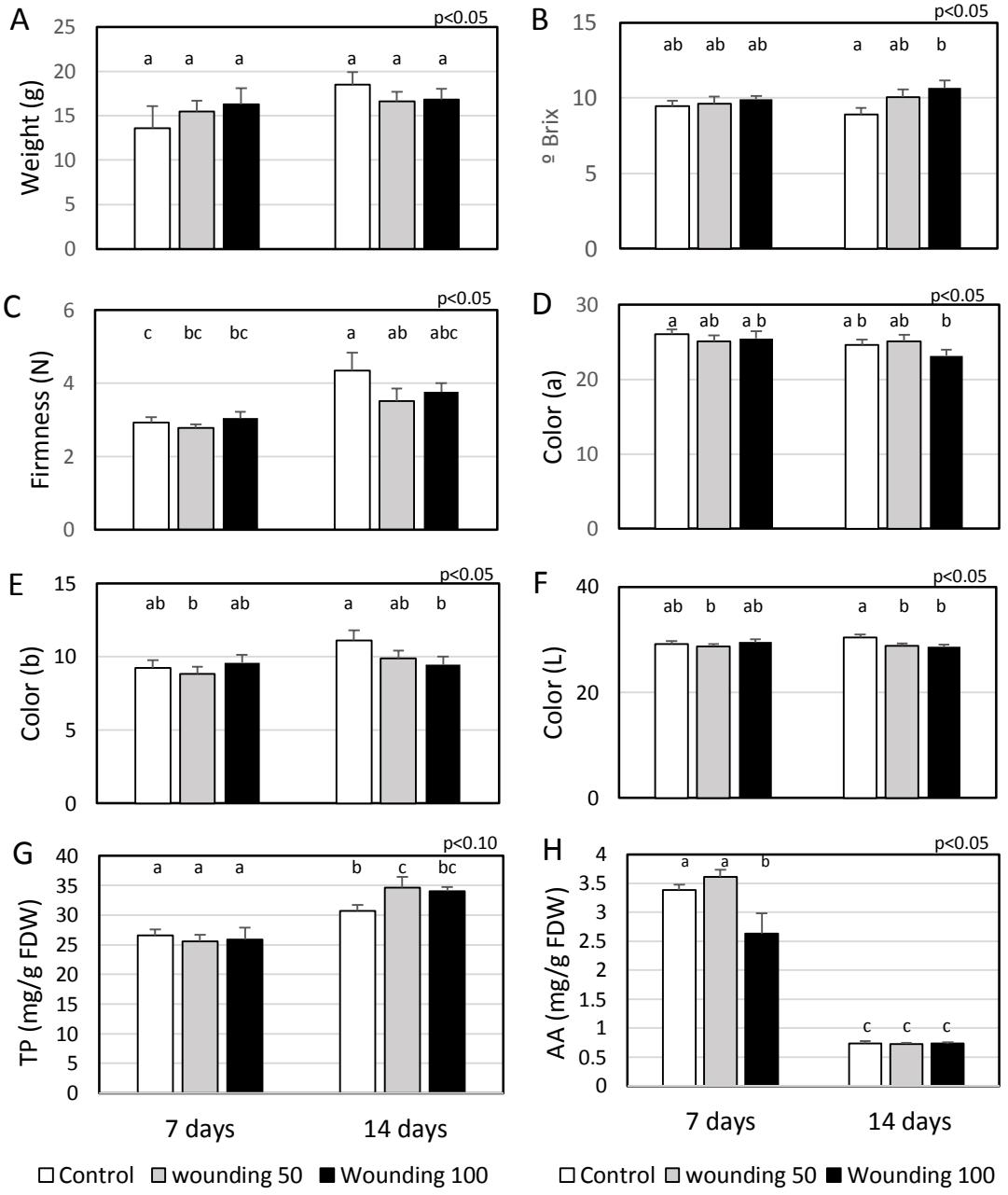

Fig. 2-2. Application of two levels of pre-harvest wounding on leaves and its effects in strawberry fruit at two harvest times (7 and 14 days). Each bar represent the average ± SE of individual fresh fruit evaluated from 15-20 plants; weight (A); soluble sugars (B); firmness (C) color (L a b system, D, E, F, respectively; G, total phenolics (TP); H, ascorbic acid (AA). TP and AA were measured in freeze-dried fruits and expressed per g of freeze-dried weight (FDW). In each group different letters indicate a significant difference in comparison to control (Duncan´s test, $p<0.05$, $p<0.10$).

A significant increase in the level of specific phenylpropanoids and tannins derivatives was observe for W100 after 2 weeks compared with the control; ellagic acid (+58%), epicatechin (+100%), gallic acid (+68%), quercetin (+190), and rutin (+137%) (Table 2-2).

Figure 2-3 shows the effect of wounding on the transcription of specific genes. Phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) are important enzymes involved in polyphenol biosynthesis (Singh et al. 2010; Petersen et al. 2010). PAL is the first and limiting step in the phenylpropanoid pathway and CHS is the first committed enzyme in flavonoid biosynthesis (Vogt 2010). After two weeks, for W100 on leaves, PAL increased 1.85 fold and CHS 1.73 fold (Fig. 2-3A-B). The expression of gene encoding 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase gene (*Fa*DAHPS) and 3-dehydroquinate synthase (*Fa*DHQS) were not affected by the application of wounding after 7 or 14 days (Fig. 2-3 C-D). DAHPS is the first enzyme in the shikimate pathway and catalyze the reaction of phosphoenolpyruvate with D-erythrose 4-phosphate to produce 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) and releasing phosphate. DHQS catalyzes the second step in the shikimate pathway using DAHP as a substrate to produce 3-dehydroquinate and phosphate (Vogt 2010). Fig. 2-3E shows the relative expression of 3-dehydroshikimate Synthase (*Fa*DHD-SDH2) implicated in the synthesis of gallic acid from shikimic acid (Muir et al. 2011). The expression of this gene was 15.2-fold over control for the higher wounding level after 14 days of wounding. *Fa*EPSPS 5-enolpyruvylshikimate-3-phosphate synthase gene expression (Fig. 2-3F) was also greater in fruits from wounded

plant after 14 days (6.25 fold). This enzyme catalyzes the reaction that transforms shikimate-3-phosphate and phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate (EPSP). Lipoxigenase (LOX) is an enzyme involved in the first steps of the biosynthesis of JA, catalyzing the oxidation of alpha-linolenic acid, producing the hydroperoxide (Schaller and Stintzi 2009). Fig. 2-4A shows a 10.7 fold increment in the transcript for the LOX gene in strawberries from wounded plants (W100). The expression of jasmonate methyl transferase (JMT), which catalyzes the conversion of JA to MeJA by adding a methyl group (Schaller et al. 2004), increased 6.2 fold in wounded plants (Fig. 2-4B). The expression of sucrose invertases gene also increased; 2 fold for cell wall invertase (CWI, Fig. 2-4C) and 7.7 fold for soluble invertase (SI, Fig. 2-4D).

Table 2-2. Ellagic acid, epicatechin, gallic acid, quercetin and rutin content in strawberry fruits evaluated after one or two weeks of wounding applied to leaves.

Harvest time	Treatment	Ellagic acid[a,b,d] (µg/g FDM)[c]	Epicatechin[a,b] (µg/g FDM)[c]	Gallic acid[a,b] (µg/g FDM)[c]	Quercetin[a,b] (µg/g FDM)[c]	Rutin[a,b] (µg/g FDM)[c]
7 days	Control	0.21 ± 0.06 a	0.56 ± 0.16 a	2.55 ± 0.60 a	0.29 ± 0.25 a	0.27 ± 0.09 a
	Wounding 50	0.78 ± 0.42 a	1.39 ± 0.93 a	3.26 ± 1.38 a	0.05 ± 0.02 a	0.24 ± 0.01 a
	Wounding 100	0.35 ± 0.30 a	0.31 ± 0.19 a	0.64 ± 0.38 a	0.05 ± 0.01 a	0.26 ± 0.13 a
14 days	Control	178.75 ± 52.21 b	37.28 ± 5.01 b	380.20 ± 48.78 b	4.26 ± 0.63 ab	35.30 ± 6.18 a
	Wounding 50	211.77± 23.90 bc	97.19 ± 6.78 c	905.51 ± 69.67 c	8.84 ± 1.26 ab	102.54 ± 17.67 b
	Wounding 100	283.17 ± 50.73 c	74.37 ± 16.44 c	628.82 ± 62.16 d	12.39 ± 6.10 b	83.80 ± 21.75 b

[a] Data expressed as means ± SE

[b] Means with a common letter in the same column are not significantly different at $p \leq 0.05$ or P ≤0.10 for ellagic acid[d] (Duncan´s Test)

[c](µg/g FDM) : micrograms of compound per g of freeze dried mass of strawberry fruits

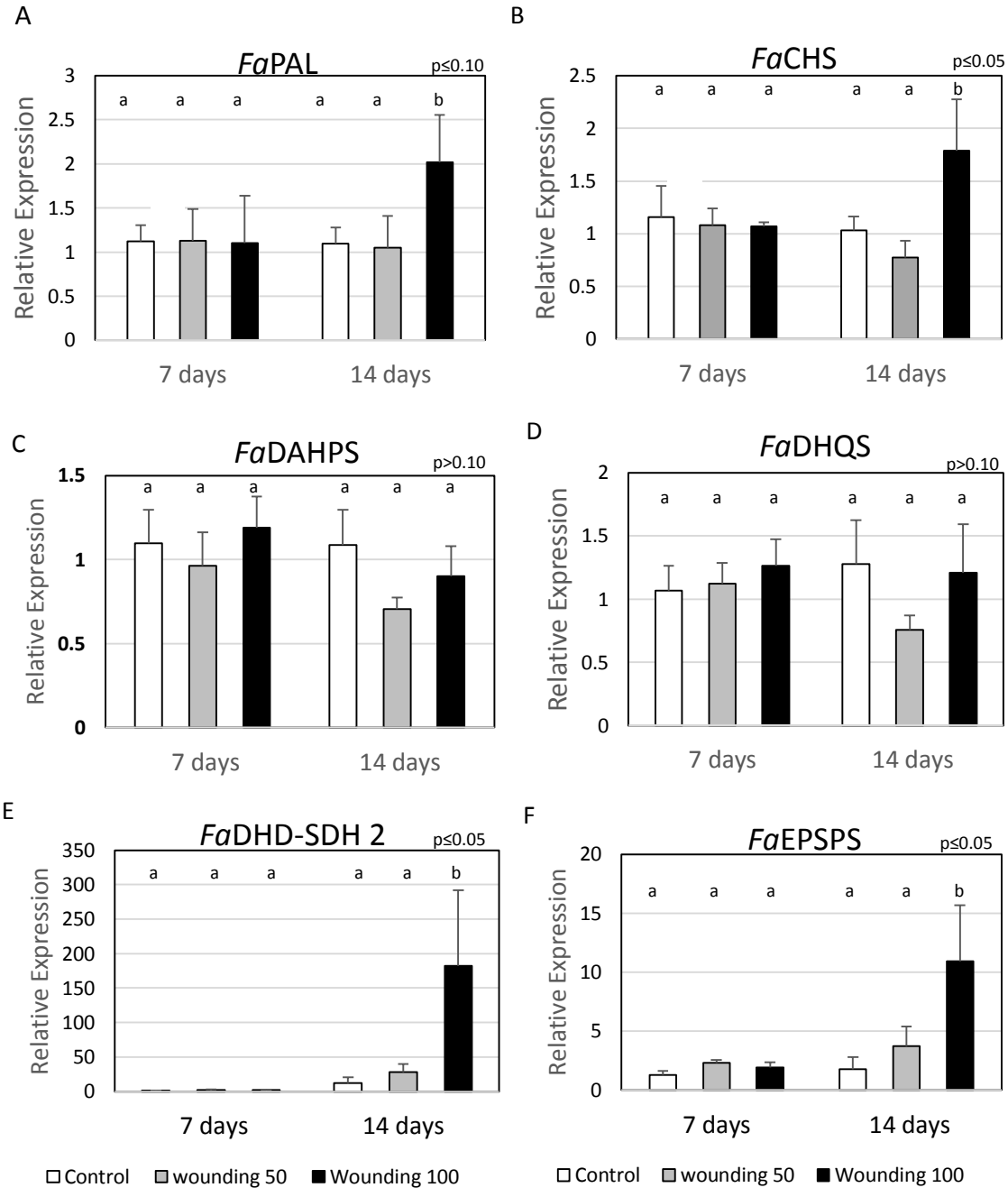

Fig. 2-3. Relative expression of phenylpropanoid intermediates gene. A, Phenylalanine ammonia lyase (*Fa*PAL). B, Chalcone synthase (*Fa*CHS). C, 3-deoxy-D-arabinoheptulosonate 7-phosphate Synthase (*Fa*DAHPS). D, 3-dehydroquinate Synthase (*Fa*DHQS). E, 3-dehydroshikimate Synthase (*Fa*DHD-SDH2). F, 5-enolpyruvylshikimate 3-phosphate Synthase (*Fa*EPSPS). Each bar represent the result of three technical replicates from five experimental samples (n=5) ± SE. In each group, different letter indicate significant differences in comparison with the control (Duncan´s test, the *p*-value is indicated in the upper right corner).

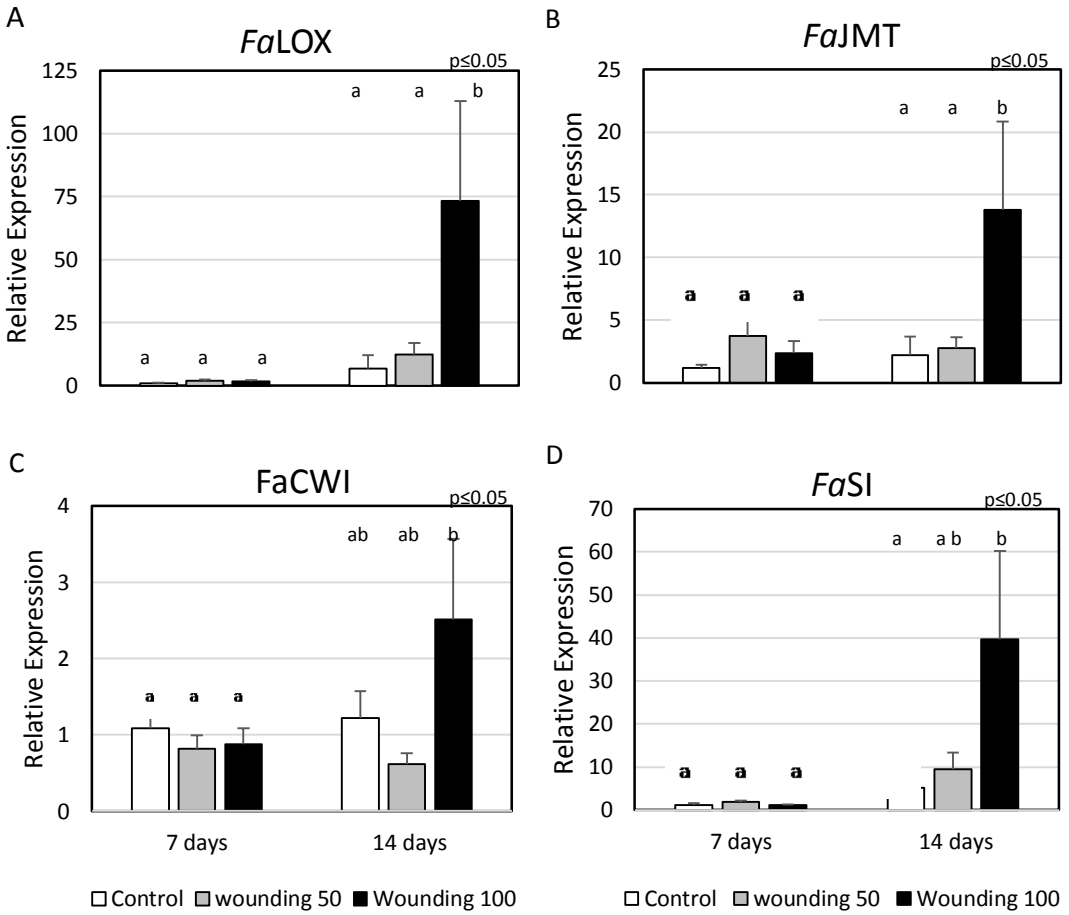

Fig. 2-4. Relative expression of sugar transport involved gene. A, Lipoxigenase (*Fa*LOX). B, Jasmonic acid carboxyl methyltransferase (*Fa*JMT). C, Cell wall invertase (*Fa*CWI). D, Soluble invertase (*Fa*SI). Each bar represent the result of three technical replicates from five experimental samples (n=5) ± SE. In each group, different letter indicate significant differences in comparison with the control (Duncan´s test, the *p*-value is indicated in the upper right corner).

Table 2-3 shows the average losses in the foliar area for the treatments, resulting in approx. 2.43% for W100 and 1.22% for W50.

Table 2-3. Estimated area losses for averages leaflet and strawberry plants.

	Area (mm^2)	Area loss (%)
Leaflet no-wounded	30.81	
Leaflet wounded (5 holes)	29.46	4.38
Total foliar area/ plant	1109.16	-
Wounding (50/plant0	13.50	1.22
Wounding (100/plant)	27.00	2.43

Discussion

Organic agriculture claims that under this kind of management fruits produce more phytochemicals than under the conventional approach (Brandt et al. 2011; Lima and Vianello 2011). This claim is supported by several reports comparing both systems (Amodio et al. 2007; Asami et al. 2003; Carbonaro and Mattera 2001; Carbonaro et al. 2002); alternatively, many studies indicate there are no differences (Dangour et al. 2010; Dimberg et al. 2005; Faller and Fialho 2010; Häkkinen and Törrönen 2000; Hargreaves et al. 2008; Juroszek et al. 2009), setting a controversial matter for several years. There is a speculation that biotic stress due to insect and pathogen attacks triggers the production of defensive secondary compounds, but this hypothesis has never been tested before. The wounding component of the biotic stress is already known as a cause of the

overproduction of secondary metabolites, mainly phenolic compounds, that are accumulate in the damaged leaf and also in other distant leaves as a systemic defensive response (de Bruxelles and Roberts 2001; Engelberth et al. 2012; Koo and Howe 2009; Korth and Thompson 2006). The relation between invertase activity and carbon transport is important for the modulation of plant defense and secondary metabolism as carbon is the source for phenolic compounds and sucrose and glucose play roles as signaling molecules (Proels and Hückelhoven 2014; Lemoine et al. 2013; Ayre 2011; Bolouri-Moghaddam et al. 2010; Schwachtje and Baldwin 2008). There is evidence that raises in translocation of sucrose from source tissues to distant sink tissues increases the production of defensive molecules (Arnold et al. 2004; Arnold and Schultz 2002; Ferrieri et al. 2013). Invertases bounded to plant cell walls facilitate phloem unloading at fruit tissues by hydrolyzing sucrose into glucose and fructose. The cell wall invertase (CWI) is overexpressed by application of wounding and jasmonic acid to leaves, but the systemic induction in other leaves was not detected in pea (Zhang et al. 1996). Systemic induction of CWI by wounding was shown in *Populus* sp. source-sink model (Arnold and Schultz 2002; Babst et al. 2005; Babst et al. 2008).

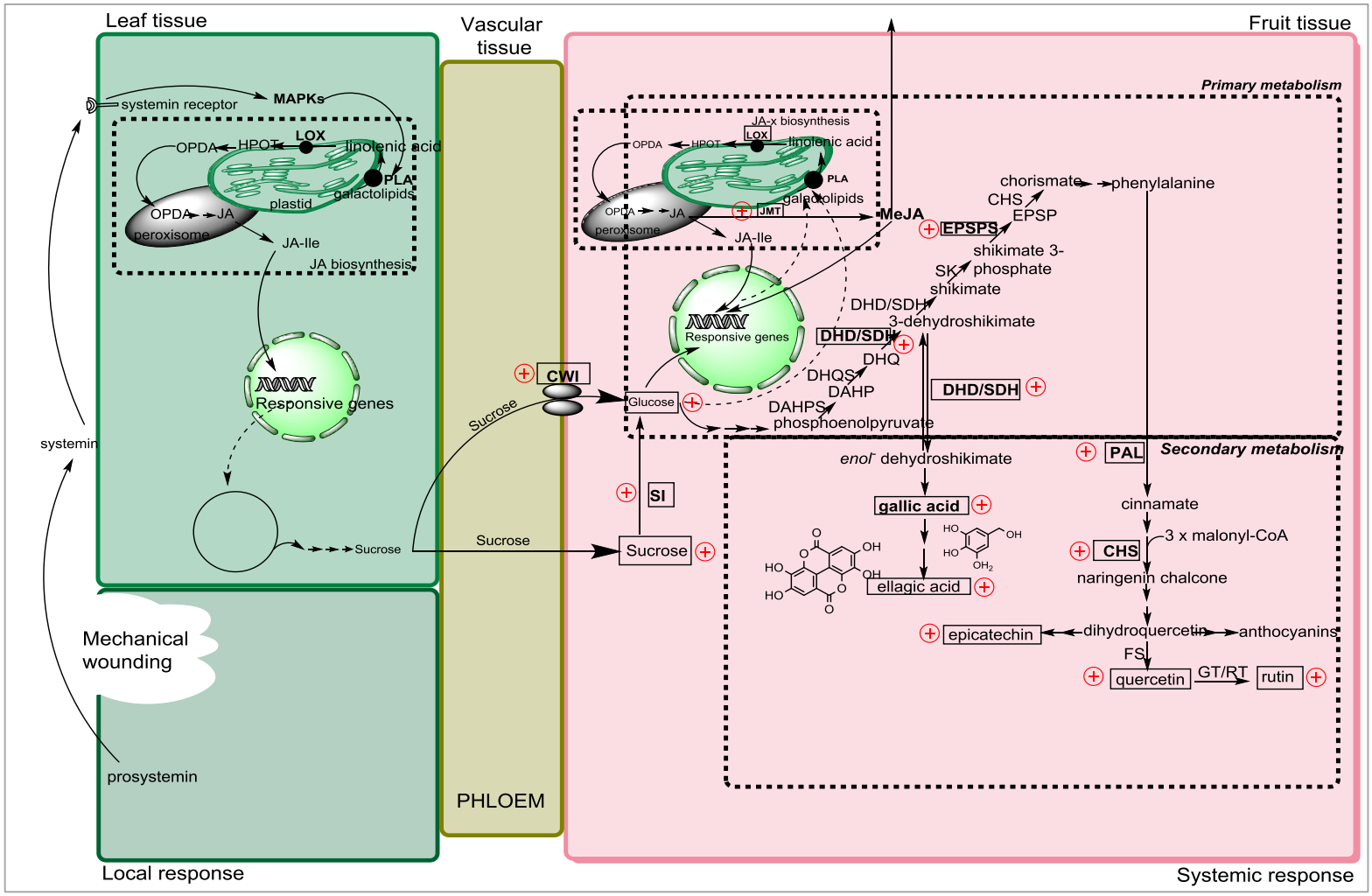

Fig. 2-5. Proposed hypothetical model for phytochemical production in strawberry fruits induced by wounding on leaves. JA, Jasmonic acid; JA-X, Jasmonic acid derivatives; LOX, Lipoxigenase; JMT, Jasmonate methyl transferase; OPDA, 12-oxo-phytodienoic acid; HPOT, 9-/13-hydroperoxy-octadecatrienoic acid. Up regulation of enzyme genes and secondary metabolites increments are represented by ⊕.

Based on the results found in this dissertation and on previous research, I propose a hypothetical model explaining the possible mechanism (Fig. 2-5). The qRT-PCR analyses detected the overexpression of several genes on the phenolics pathway and carbohydrate metabolism on the strawberry fruits as a late response for wounding produced on leaves. In this model, the wounding produced in the leaves triggers the local response that reconfigures the sugar metabolism, producing an upload of sucrose in vascular tissue that is transported to fruit. In fruits, the up-regulation of sucrose invertases genes (CWI and SI), allow the increase of soluble sugar in the cells. The imbalance in sucrose/glucose triggers the octadecanoic pathway increasing the JMT transcripts and defensive genes related to phenolic compounds biosynthesis. The greater accumulation of soluble sugar in fruit cells also increases the availability of carbon for the biosynthesis of secondary metabolites with high C/N ratio, such as the phenylpropanoids (quercetin, rutin and epicatechin) and hydrolysable tannin derivatives (ellagic acid and gallic acid).

Conclusions

Here is reported for the first time the accumulation of phenolic compounds in fruit through long distance wounding applied to leaves, in an experiment conducted in the field (leaf-fruit). The results clarify the role of wounding for the accumulation of defensive compounds in fruit. The results support the idea that higher levels of phytochemicals reported in organic fruits and vegetables could be due to the wounding

component of the biotic stress attributed to herbivore insects feeding on leaves, to which the plant is exposed. The delayed response of fruits in synthetizing phenolics is the result for a late defensive response. This produced an accumulation of soluble sugars in fruits as source of carbon for high rate C/N secondary metabolites production. As a technological application of these results, the controlled mechanical wounding applied during preharvest in leaves could be used to increase phytochemicals in fruits.

CHAPTER III

PHENOLIC COMPOUNDS IN STRAWBERRY FRUITS ARE NOT AFFECTED BY A GENERALIST INSECT CHEWING ON LEAVES

Introduction

Strawberry (*Fragaria* x *ananassa*) is one of the most important small fruits produced worldwide; its production volume is twice the amount of all other berries combined (Liston et al. 2014). In 2012, the United States was the main producer (1,366,850 tons), followed by Mexico (360,426 tons) (FAOSTAT 2012). Strawberry is a good source of nutrients, such as vitamin C, folate, and essential microelements, and is also a source of helpful phytochemicals (Giampieri et al. 2012). The phytochemical profile in strawberry contains anthocyanins, ellagitannins, gallotanins, ellagic acid, and other phenolic compounds that contribute to the antioxidant potential and health benefits (Giampieri et al. 2012; da Silva Pinto et al. 2008a; da Silva Pinto et al. 2008c; Giampieri et al. 2014a; Giampieri et al. 2013). The biological activity of strawberries related to phytochemicals includes antiproliferative effects on human colon carcinoma (D'Evoli et al. 2010), anti-inflammatory effects on macrophages (Liu and Lin 2013), modulation of balance oxidant-antioxidant in blood phagocytes (Bialasiewicz et al. 2014), anti-hyperglycemic potential (da Silva Pinto et al. 2008a), mitochondrial protection (Giampieri et al. 2014b), neuroprotective potential (Heo and Lee 2005), and antimicrobial properties against human pathogens (Nohynek et al. 2006). Ellagic acid is

the phenolic found in highest concentration in strawberry, ranging from 39.6 to 52.2 mg/100 g fresh weight in nine cultivars analyzed (Häkkinen and Törrönen 2000; Maas et al. 1991). A higher variation was reported in 13 clones, showing values between 43 to 464 mg/100g dry weight (Maas et al. 1991). Comparing the organic vs. conventional techniques of production, the cultivar "Jonsok" had higher levels of total phenolics and showed a significant increment in kaempferol content (0.9 vs. 0.5 mg/100g fresh weight) and ellagic acid (52.2 vs. 58.6 mg/100 g fresh weight) for the organic system (Häkkinen and Törrönen 2000). The authors speculated that the higher impact of pathogen attack on this cultivar could increase the levels of these phenolics (Häkkinen and Törrönen 2000). In another study comparing organic and conventional production (D'Evoli et al. 2010), the results showed higher antioxidant activity and levels of kaempferol (1.99 vs. 1.26 mg/100g fresh weight) and ellagic acid (53.3 vs. 37.9 mg/100g fresh weight) for organically grown strawberry. This higher level in phenolics was correlated with better antiproliferative activity in Caco-2 cell lines (D'Evoli et al. 2010). The levels of ellagic acid glucoside in "Allstar" and "Earliglow" strawberry cultivars increased from 14.7 to 18.6 μg/g fresh weight and 12.2 to 18.5 μg/g fresh weight respectively for the organic growth conditions (Jin et al. 2011). The addition of organic and conventional soil nutrients did not affect strawberry yield and quality parameters related to phenolics such as antioxidant capacity (Hargreaves et al. 2008). Despite the fact that the main biotic stress affecting strawberry culture are diseases caused by bacteria and fungi (Maas 2004; Louws 2009), some arthropods, such as the aphid *Chaetosiphon fragaefolii* (Bernardi et

al. 2013) or the spider mite *Tetranychus urticae* (Monteiro et al. 2014), affect negatively the commercial production. Several studies showed that the level of phytochemicals was higher in strawberries grown organically compared to those grown with conventional methods. A comparison between both production approaches for two strawberry cultivars found that fruit from organic production accumulate more total phenolics, and these differences are maintained at different storage temperatures (Jin et al. 2011).

In this experiment, the hypothesis that biotic stress due to generalist insect larva chewing on leaves affects the biosynthesis of phytochemicals in fruits of strawberry growing organically was tested.

Materials and methods

Field experiment on strawberries. For the biotic stress, *Spodoptera exigua* (beet armyworm) was selected due to its commercial availability; larvae are polyphagous; and they are reported as a pest for strawberries (UC-IPM 2010). A field experiment was conducted in a commercial strawberry (cv. Festival) field near the end of the harvesting season, during February to April 2014, in Jollisant Farm, Plantersville, Texas (lat. 30.33 N, long. 95.82 W). In a row, 20 plants were assigned randomly to each treatment as following:

1) Control (C) with no application of larvae.

2) Low insect damage (T1) consisting of the application of one second-instar larva of *S. exigua* per plant;

3) High insect damage (T2), consisting in the application of two second-instar larvae of *S. exigua* per plant;

The larvae were applied to each leaflet inside a mesh bag (Fig. 3-1). Leaves of control plants were treated in the same way but with no insect larvae inside the bags. The treatment was applied to plants with fully developed fruits (~15% red color). Fully ripe fruits (over 80% of full color) were harvested from each plant one and two weeks after the treatment application. The farm followed the guidelines for organic production (not certified) with no application of insecticides and organic soil amendments.

Fig. 3-1. Field experiment with *S. exigua* larvae. Two levels of insect damage were set in each treatment plant (1 or 2 larvae).

Harvest evaluation. Immediately after harvest, fruits were evaluated for harvest quality by measuring physicochemical parameters. For each fruit, the weight was registered and firmness was determined using a TA.XTPlus Texture Analyzer (Stable Mycro System, Surrey, United Kingdom). The soluble solids concentration (SS) was determined in the juice by an Atago RX-1000 digital refractometer (Atago Co. Ltd, Tokyo, Japan). After these determinations, samples were immediately frozen in liquid nitrogen and stored at -80 ºC for freeze-drying and further analyses.

Total phenolics and vitamin C analysis. Determination of total phenolic compounds and total vitamin C were determined in the same analysis according with the method reported by Sanchez-Rangel et al. (2013). Briefly, 50 mg of freeze-dried strawberry powder was extracted with 1.00 ml of MeOH:H_2O (80:20 v/v) in a centrifuge tube using an ultrasonic bath for 30 min. After that, samples were centrifuged at 14,000 rpm. In a 96 plate was added 15 µl of extract and 240 µl of distilled water followed by 15 µl of Folin-Cicalteau reagent. The mixture was incubated for 3 minutes and the absorbance read at 725 nm. After that a Na_2CO_3 solution (30 µl, 1N) was added and incubated at room temperature in dark conditions for 2 hours, then the absorbance was measured again at 725 nm. In parallel standards of ascorbic and chlorogenic acid in addition of blanks were run. Absorbance was recorded using a Synergy-HT Microplate Reader and analyzed using KC4 software (Bio-Tek Inc., Winooski, VT).

HPLC analysis of phenolic compounds. Chromatographic separation was implemented on a LCQ Deca XP Max LC-MS/MS system (Thermo Finnigan, CA) equipped with an autosampler, a quaternary pump and a UV 2000 PDA detector, using a 150 × 2.00 mm Synergi 4μ Hydro RP 80A column (Phenomenex, Torrance, CA) and a guard column of the same chemistry. The elution mobile phase was executed with solvent solution A [0.5% formic acid -water] and solvent mixture B [0.5% formic acid in acetonitrile]. A linear gradient was set up with A and B: 0 min 98% A, 10 min 75% A, 20 min 75% A, 30 min 25% A, 35 min 0% A, 38 min 98% A. The flow rate was 200μl/min. The injection volume was 10 μl. Retention time and spectral profile were used for identification, the quantification was done by comparison with external standards obtained from Sigma-Aldrich (St. Luis, MO).

Gene expression. The total RNA extraction from strawberry fruits was carried out by combining the method previously described by Christou et al. (2014), with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Briefly, 0.1 g of freeze-dried powder was mixed with 1 ml of the extraction buffer (0.5 M Tris–HCl pH 8.8 and 1% sodium dodecyl sulfate [SDS]). Subsequently, 1 ml of phenol:chloroform:isoamyl alcohol (PCI) (25:24:1 [v/v]) was added to the mixture, which was gently agitated and then centrifuged at 14,000 rpm for 5 min at 4 °C for phase separation. The upper aqueous phase (~800 μl) was further subjected to the PCI extractions (three times). After the third PCI extraction, the upper aqueous phase (~400 μl), whose phenol traces were removed completely, was collected into a fresh chilled tube, where 0.1 volume of 3 M NaOAC (pH 5.6) and 1

volume of 100% ethanol were mixed, incubated at −80 °C for 20 min and then centrifuged at 12,000 rpm for 8 min at 4 °C for RNA precipitation. After air dry at room temperature, RNA pellets were finally dissolved in RNase-free water and further purified by RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Willmington, DE). The 0.7 µg RNA, treated with DNase I to avoid DNA contamination, was reverse-transcribed into cDNA using the SuperScript III first-strand synthesis supermix (Invitrogen, Carlsbad, CA) following the manufacturers protocol. Finally, the cDNAs was used for the real-time qRT-PCR analyses, which were performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. cDNA amplification was carried out using a 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The primer sets used in this study were provided by Integrated DNA Technologies (IDT, Coralville, IA), and their sequence information are shown on Table 3-1. The relative expression of each gene was normalized by the *FaGAPDH* and calculated following the comparative Ct method (ΔΔCt), known as the $2^{-\Delta\Delta Ct}$ method. Strawberry gene were selected regarding its implication in shikimate pathway, phenolic compounds biosynthesis, and sugar transport and metabolism. All these gene were reported for strawberry fruits, and the sequence primers are available (Landi et al. 2014; Amil-Ruiz et al. 2013; Amil-Ruiz et al. 2011).

Table 3-1. Sequence of primers from *F. x ananassa* used in qRT-PCR analyses. The sequences belong to the genes of phenolics intermediates biosynthesis and related enzymes.

Primer	Sequence
FaPAL-F	5'-CACCTGCTCTCAGTCGTGGACC-3'
FaPAL-R	5'-GCA TGTTCTACTAGCTCTGCCCTCAG-3'
FaCHS-F	5'-GTTGGGCTCACATTTCACCTCCTCA-3'
FaCHS-R	5'-AATTGCTGGGCCACCTGGGTG-3'
FaEPSPS-F	5'-GGAGACTTGGTCACTGGTCTTA-3'
FaEPSPS-R	5'-GAAGGCCTCCCTTTCCAATTAC-3'
FaDAHPS-F	5'-CGCAACTGGTGGGTATGCGGC-3'
FaDAHPS-R	5'-CCCGGTGAGCAAGTTCCCGG-3'
FaDHQS-F	5'-GCAGCTGGCATGATCATGGCTG-3'
FaDHQS-R	5'-CGGTCACAGACTCAGGAGGGC-3'
FaDHD/SDH 1-F	5'-AGCTCCTGGTCAACCTACTATC-3'
FaDHD/SDH 1-R	5'-GCTGACGGGCTTTCCAATAA-3'
FaDHD/SDH 2-F	5'-CGTTGGGATTCCTCACAAAGA-3'
FaDHD/SDH 2-R	5'-CATCAGTTGGCCTCCTTACAA-3'
FaDHD/SDH 3-F	5'-GAGGAAGGACTTCGAGGATTAG-3'
FaDHD/SDH 3-R	5'-GCTCCCATGACCACAAATAAC-3'
FaSI-F	5'-GGTATGTGGGAGTGCATTGA-3'
FaSI-R	5'-CGTCCAAGCTAGCCTTTAGAA-3'
FaCWI-F	5'-CCAGGCAATTCCAAGGACTAT-3'
FaCWI-R	5'-CTTGACCTCGTTTGTTCTAAGTTT C-3'
FaLOX-F	5'-CCGGGACACGATGAACATAA-3'
FaLOX-R	5'-GGCATATTGAGCTGGGAAGA-3'
FaJMT-F	5'-AATAAGCAGCGGCGAGCGAGTAGC-3'
FaJMT-R	5'-AAGCGATCACTGACGAGCTCTGCG-3'
FaGAPDH-F	5'-TCCATCACTGCCACCCAGAAGACTG-3'
FaGAPDH-R	5'-AGCAGGCAGAACCTTTCCGACAG-3'

Statistical analysis. Analysis of Variance (ANOVA) was applied to the data and the statistical differences between treatment means were determined using the Duncan´s Test ($p \leq 0.05$ and $p \leq 0.1$). Those tests were conducted using the software InfoStat (National University of Cordoba, Argentina).

Results

The weight of the fruits was the same at harvest after one or two weeks of the insect larvae chewing the leaves (Fig. 3-2A). For soluble solids, no significant differences were observed when one or two larvae were applied per plant, indicating that there were no changes on the levels of soluble sugars and organic acids (Fig. 3-2B). Firmness (Fig. 3-2C) was not affected either. Total ascorbic acid (Fig. 3-3A) was not significantly affected by any level of larvae feeding after one or two weeks compared with the control. There was a significant decrease of the level in week 2 (approx. 3-fold) for all the plants, similar results were obtained in the field experiment in Uruguay with the other cultivar. Likewise, the amount of total phenolics (TP) in fruits of treated plants did not increase significantly over the control after one or two weeks of the application of the treatments (Fig. 3-3B).

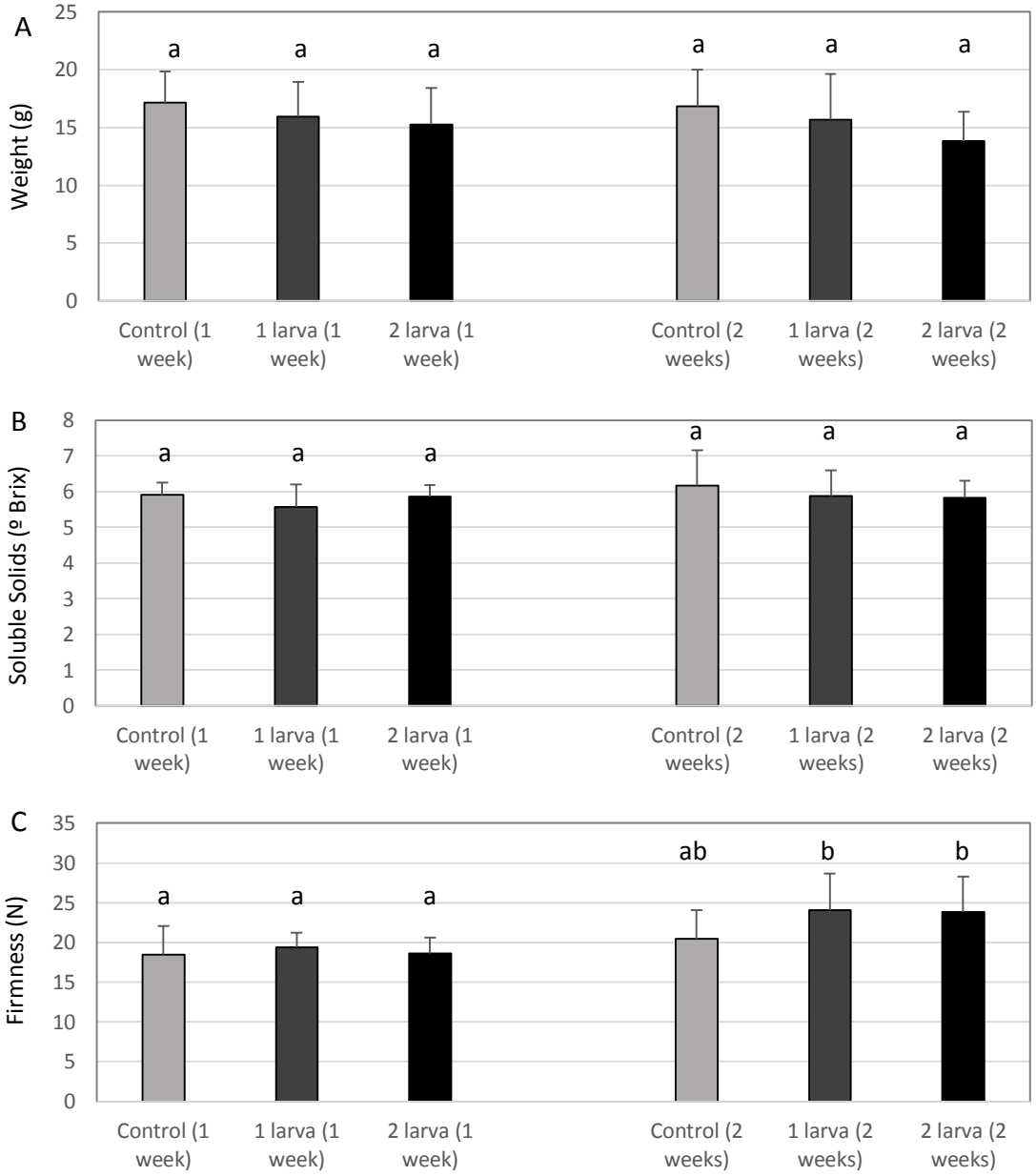

Fig. 3-2. Effects of two levels of *S. exigua* larvae feeding on leaves on strawberry fruit quality at two harvest times (1 and 2 weeks). Each bar represent the mean ± SE of individual fresh fruit evaluated from 5-10 plants; weight (a); firmness (b) soluble sugars (c). Same letter indicate no significant differences between means (Duncan´s test, $p \leq 0.05$).

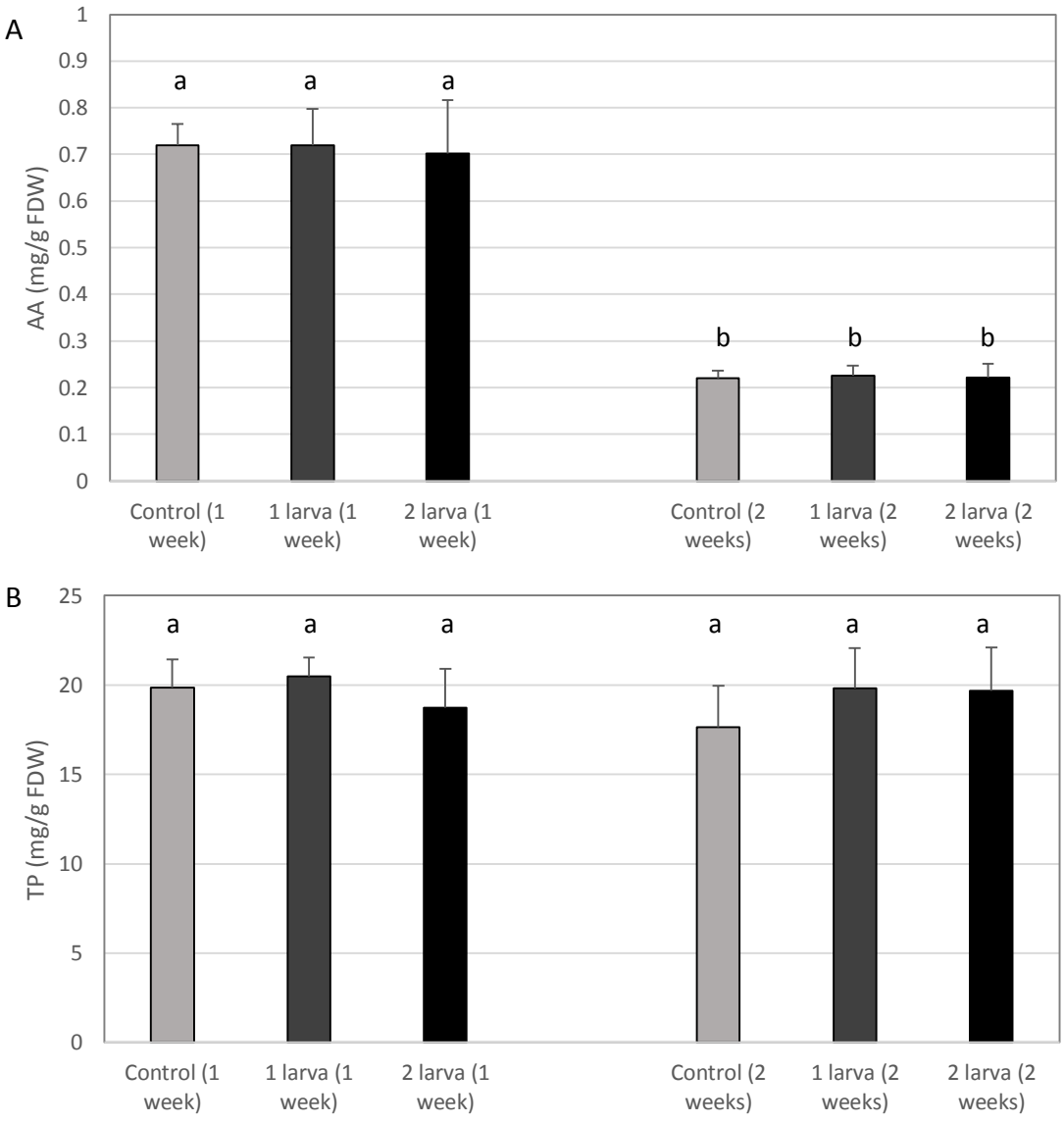

Fig. 3-3. Effects of two levels of *S. exigua* larvae feeding on leaves on strawberry fruit Ascorbic acid (**A**) and Total Phenolics (**B**) content at two harvest times (1 and 2 weeks). Each bar represent the average ± SE from 5-10 plants. Total phenolics (TP) and ascorbic acid (AA) were measured in freeze dried fruits and expressed as g of freeze dried weight (FDW). Same letter indicate no significant differences between means (Duncan´s test, $p \leq 0.05$).

Table 3-2. Changes of ellagic acid, epicatechin, gallic acid and quercetin content in strawberry fruits after one and two weeks of *S. exigua* larvae feeding on leaves[a,b]

arvest time	Treatment	Ellagic acid[c]	Epicatechin[c]	Gallic acid[c]	Quercetin[c]
1 Week	Control	9.85 ± 2.84 a	40.89 ± 17.66 a	57.26 ± 15.55 a	6.46 ± 3.18 a
	1 Larva	12.89 ± 7.03 a	43.77 ± 12.0 a	71.31 ± 18.65 a	10.97 ± 5.33 a
	2 Larvae	14.17 ± 8.06 a	69.68 ± 31.02 a	67.64 ± 17.02 a	4.58 ± 1.50 a
2 Weeks	Control	17.09 ± 4.79 a	43.53 ± 20.55 a	145.24 ± 39.44 b	3.69 ± 1.96 a
	1 Larva	13.54 ± 2.76 a	46.65 ± 13.07 a	100.67 ± 32.02 b	4.50 ± 1.37 a
	2 Larvae	18.57 ± 9.01 a	69.68 ± 31.02 a	135.87 ± 27.81 b	5.29 ± 1.28 a

[a] Data expressed as Means ± SE

[b] Means with a common letter in the same column are not significantly different at $P \leq 0.05$ (Duncan´s test)

[c] Data expressed as micrograms of compound per g of freeze dried weight of strawberry fruits (µg/g FDW)

Phenylalanine ammonia lyase (*Fa*PAL) and chalcone synthase (*Fa*CHS), two important enzymes involved in polyphenol biosynthesis (Singh et al. 2010; Petersen et al. 2010) were not affected by the insect larvae (Fig. 3-4A-B). PAL is the first and limiting step in the phenylpropanoid pathway and CHS is the first committed enzyme in flavonoid biosynthesis (Vogt 2010). The expression of 3-deoxy-D-arabino-heptulosonate 7-phosphate Synthase gene (*Fa*DAHPS) and 3-dehydroquinate Synthase (*Fa*DHQS) was not affected by the application of one or two larvae after 2 weeks (Fig. 3-4C-D). DAHPS is the first enzyme in the shikimate pathway and catalyze the reaction of phosphoenolpyruvate with D-erythrose 4-phosphate to produce DAHP and releasing phosphate. DHQS catalyze the second step in the shikimate pathway using DAHP as a substrate and produce 3-dehydroquinate and phosphate (Vogt 2010). Fig. 3-4E shows the relative expression of 3-dehydroshikimate synthase (*Fa*DHD-SDH2) implied in the synthesis of gallic acid from shikimic acid (Muir et al. 2011). The expression of this gene was slightly lower than control for the two level of larva damage after 2 weeks of feeding, but statistically significant (*t*-Student test, $p \leq 0.05$). On the contrary, *Fa*EPSPS gene expression (Fig. 3-4F) was higher in fruits from plant with two larvae but not significantly. This enzyme catalyzes the reaction that transforms shikimate-3-phosphate and phosphoenolpyruvate to 5-enolpyruvylshikimate-3-phosphate (EPSP).

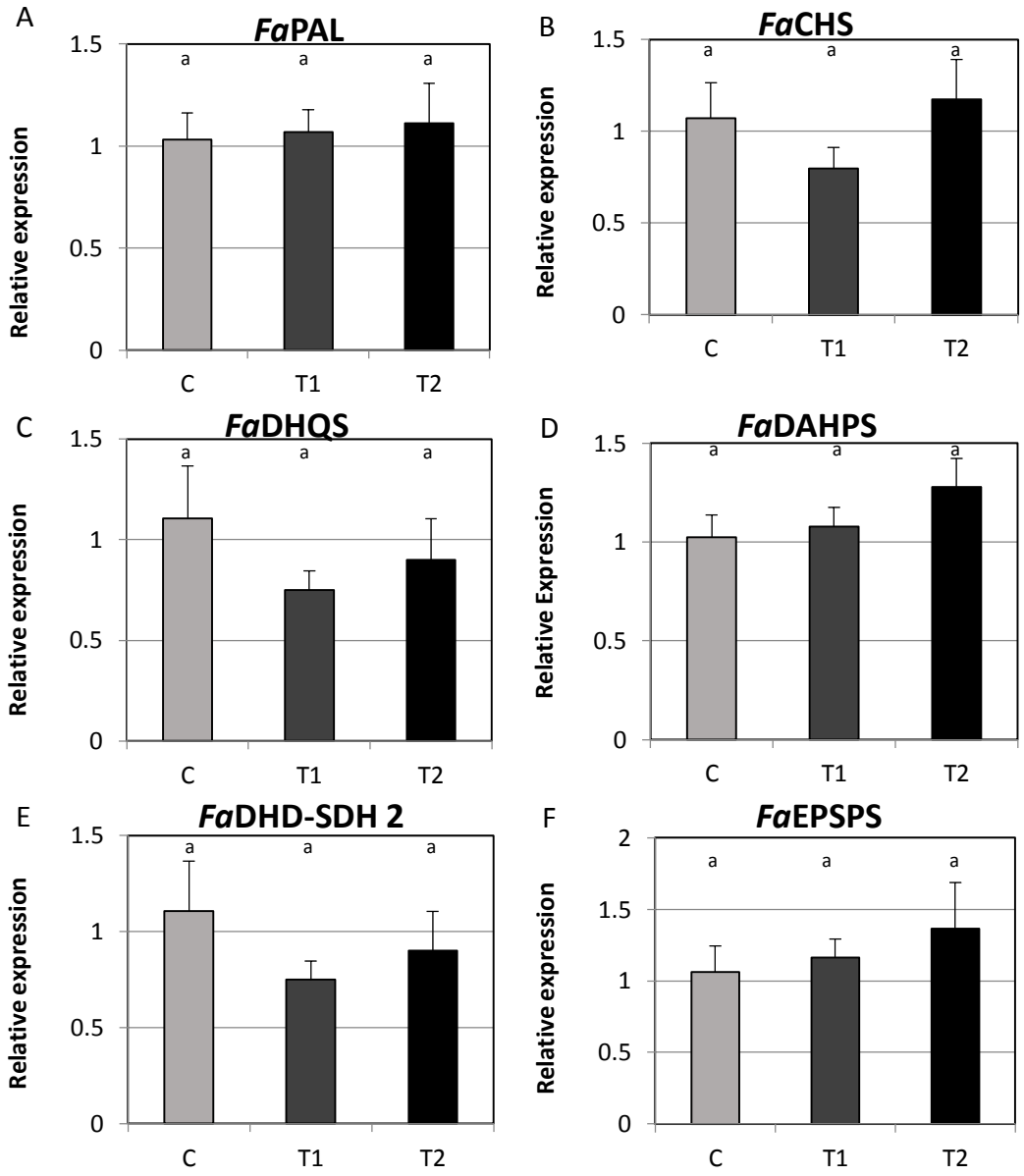

Fig. 3-4. Relative gene expression of shikimate pathway and phenylpropanoid intermediates biosynthesis. **A)**, Phenylalanine ammonia lyase (*Fa*PAL). **B)**, 3-dehydroquinate Synthase (*Fa*DHQS) **C)**, Chalcone synthase (*Fa*CHS). **D)**, 3-deoxy-D-arabinoheptulosonate 7-phosphate Synthase (*Fa*DAHPS). **E)**, 3-dehydroshikimate Synthase (*Fa*DHD-SDH2). **F)**, 5-enolpyruvylshikimate 3-phosphate Synthase (*Fa*EPSPS). C: control, T1: treatment with one *S. exigua* larva per plant; T2: treatment with two larvae of *S. exigua* per plant. Each bar represents the results of three technical replicates from five experimental samples ± SE (n=5). Same letter indicates no significant difference in comparison with the control (Duncan´s test, $p>0.05$).

Lipoxigenase (LOX) is an enzyme implied in the first steps of the biosynthesis of JA, catalyzes the oxidation of alpha-linolenic acid, producing the corresponding hydroperoxide (Schaller and Stintzi 2009). Fig. 3-5A shows a 30% decrease in the transcript for LOX gene in strawberries from one larvae treated plants but was not significant. Jasmonate methyl transferase (JMT), that catalyzes the conversion of JA to MeJA adding a methyl group (Schaller et al. 2004), was not affected its expression in any treatment (Fig. 3-5B). The gene expression of sucrose invertases is shown in Fig. 3-6; increased 2 folds for cell wall invertase (CWI) and kept the same expression level for soluble invertase (SI).

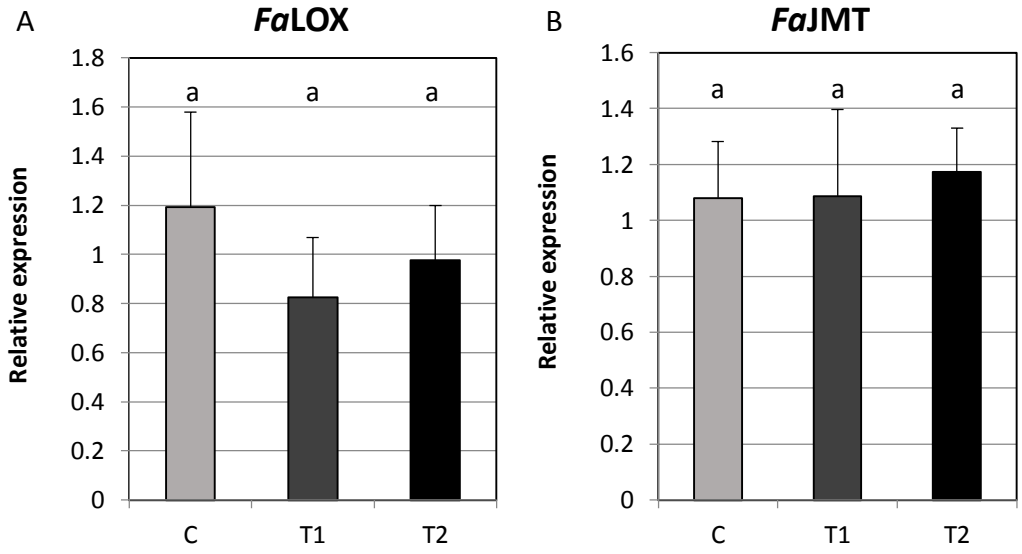

Fig. 3-5. Relative expression of gene implied in jasmonates biosynthesis. **A)**, Lipoxigenase (*Fa*LOX). **B)**, Jasmonic acid carboxyl methyltransferase (*Fa*JMT). Each bar represents the results of three technical replicates from five experimental samples. Same letter indicates no significant difference in comparison with the control (Duncan´s test, $p>0.05$).

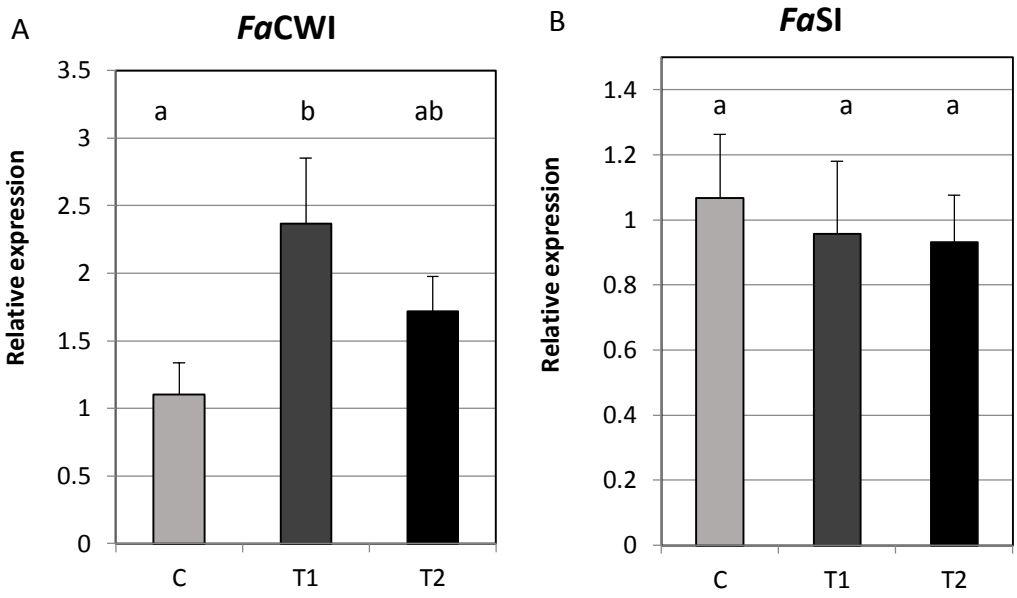

Fig. 3-6. Relative expression of gene involved in sugar transport and metabolism. **A)**, Cell wall invertase (*Fa*CWI). **B)**, Soluble invertase (*Fa*SI). Each bar represents the results of three technical replicates from five experimental samples ± SE (n=5). Same letter indicate no significant differences between means (Duncan´s test, $p \leq 0.05$)

Discussion

All the analyses performed showed that none of the parameters measured in fruit were affected when the *S. exigua* larvae damaged the leaves. The only gene that increased its expression was *Fa*CWI, by 2-fold. This may suggest an increase in sugar transport to the fruits due to unloading in the fruit cell wall; however, no significant differences were detected in the fruit's soluble sugars content, thus indicating that this increase in *Fa*CWI gene was not sufficient.

This experiment showed that the isolation of insect chewing on leaves in preharvest was not enough to release a response that increases the level of

phytochemicals in the fruit at harvest time. This could be possible through two potential mechanisms working alone or in a synergistic way.

Firstly, the plant could depend on indirect defenses, like the release of herbivore-induced plant volatiles. These volatile compounds can be used by predators of *S. exigua* to detect them and protect the plant from the ongoing damage. *S. exigua* produces a fatty acid-L-glutamine conjugate, named volicitin, that triggers the release of volatiles in maize plants attracting the parasitoid *Micriplitis croceipes* (Turlings et al. 2000). In that way, the plant instead of using the high-cost constitutive or inducible defenses, releases volatiles from the surrounding damaged tissue providing a long term benefit if the parasitoid is present in the crop`s nearby area (Hoballah et al. 2004).

Secondly, the wounding damage by the larvae may not be great enough to start the cascade of induced plant defenses. The production of the defensive pathway that involves jasmonic acid derivatives is costly for plants. Redman et al. (2001) demonstrated that the production of jasmonic acid-related defenses by tomato plants under *Manduca sexta* attack affected fruit maturation, delaying fruit setting and decreasing the amount of seeds. It is already known that jasmonic acid is highly correlated with the activity of invertases and phenolic compound accumulation in source/sink relationship in poplars (Appel et al. 2012; Arnold et al. 2012). From the results showed in Chapter II, it could be inferred that the release of jasmonic acid is necessary to force the translocation of sugar from leaves needed for the biosynthesis of more phytochemicals in fruits. In the results showed here, the fruit maturity was not

affected, meaning that damage in leaves by *S. exigua* was not enough to affect the processes.

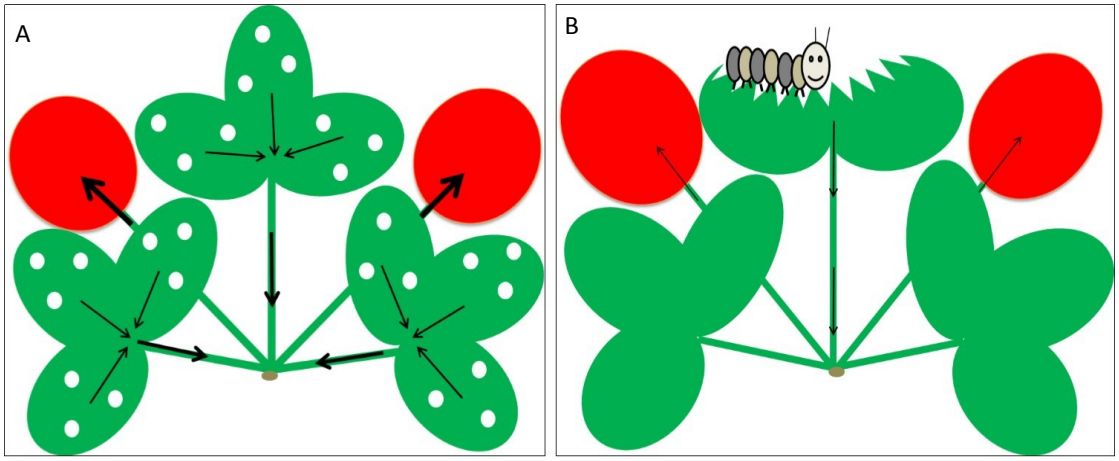

Fig. 3-7. Schematic view of the hypothetical explanation for leaf stress on fruit responses. A), Mechanical wounding distributed in the whole plant produces systemic signals and biochemical changes in fruits. B), Insect with limited feeding to one leaf does not produce enough signal to make changes in fruits. The thickness of the arrow corresponds with signal and source/sink strength from damage leaf to fruit.

In Fig. 3-7, this hypothesis is summarized. Mechanical wounding distributed on the whole plant is enough to release the jasmonic acid cascade and increase the source/sink strength in favor of fruits (Fig. 3-7A). The area chewed by one larva was similar to low level of wounding (W50) in Chapter II and the damaged produced by two larvae were comparable to the foliar area lost in high level of wounding (W100). When the *S. exigua* larvae chewing is limited to one leaf, the source of the signal is limited to one leaf and the damage is not enough to release the responses related to jasmonic acid

and source/sink relationships, so the carbon source necessary in fruit tissue for phenolic compounds biosynthesis is not affected at a significant level.

Conclusions

Differences among treatments were not significant for any parameter measured. After the analysis of all the results, I accepted the null hypothesis (the phytochemicals were not affected). In these experimental conditions, the biotic stress applied on leaves did not produced detectable changes in the phytochemical profile. I hypothesize that damage produced by the larvae was not enough to rearrange the secondary metabolism in fruit because the sugar translocation in favor of fruits was not elicited by larvae feeding only in one leaf. This hypothesis could be tested in future experiments.

CHAPTER IV

PHENOLIC COMPOUNDS IN PECAN KERNELS ARE NOT AFFECTED BY A SPECIALIST APHID FEEDING ON LEAVES

Introduction

Pecans have been promoted as a healthy a food that prevents diseases related to oxidative stress in humans due to its high content of phytochemicals with biological activities such as ellagitannins, gallotanins, and proanthocyanidins (Ortiz-Quezada et al. 2011; Ronald and Pegg 2008; Serrano et al. 2009; de la Rosa et al. 2014). Several pests are associated with native and improved pecan orchards. The most important ones are pecan nut casebearer (*Acrobasis nuxvorella*), the black margined aphid (*Monellia caryella*), and the yellow pecan aphid (*Monelliopsis pecanis*) (Ree 1999; Smith 1995). The pecan leaves are mainly affected by three seasonal aphids; the two above mentioned and a third one, the black pecan aphid (*Melanocallis caryaefoliae*) (Bumroongsook and Harris 1992; Paulsen et al. 2013; Wood and Reilly 1998). Aphids are important arthropod pests that cause damage by piercing-sucking on plant phloem and feeding on assimilated carbon compound from sap, thus weakening the plants and producing economic losses, and also by as acting as vectors of other diseases to the plant (Pickett et al. 1992; Jaouannet et al. 2014). Black pecan aphid is a specialist insect feeding almost exclusively on pecan leaves and its damage corresponds with the final stages of kernel maturity, at the end of harvest season in summer (Wood and Reilly 1998).

There is insufficient knowledge about changes in pecan phytochemistry caused by insect damages. Alvidrez-Villarreal et al. (2011) reported increments on total terpenes, condensed tannins, hydrolysable tannins and lignin in tissues damaged by fruit tree borer insect (*Euplatypus segnis*) and associated fungi (*Fusarium solani*, *Fusarium oxysporum*, *Alternaria alternata* and *Botryodiplodia theobromae*). In another study, Chen et al. (2009) reported the effects of black pecan aphid on the activity of oxidative enzymes (peroxidase, catalase, lipoxygenase) and esterase in pecan leaves under field conditions; however, phytochemical levels were not reported in the study. Despite the lack of information with regard to specific phytochemicals produced by pecan and its effects on insects, it is known that hydrolysable tannins have negative effects on pests (Barbehenn and Constabel 2011; Barbehenn et al. 2009; Moilanen and Salminen 2008). Ellagitannins like geraniin and peduncalagin inhibited the growth of green peach aphid (*Myzus persicae*), and the hydrolysable derivative ellagic acid had a potent activity against barley greengub (*Schizaphis graminum*) (Jones and Klocke 1987). Klocke et al. (1985) proposed that geraniin is a protoxin that releases ellagic acid; this hydrolysis product is detrimental for insects feeding in plants. Barbehenn et al. (2006) suggested that the greater the level of ellagitannins compared to total tannins produced by a plant, the more harmful is the impact on caterpillars. In the study presented here, I wanted to measure the abundances of the most relevant phytochemicals, in particular the ellagitannins derivatives, produced in pecan kernels when the leaves are damaged by a specialist aphid.

Materials and methods

Field experiment. The experiment was conducted on 10-year-old pecan trees (cv. Choctaw) planted in the Texas A&M University Pecan Orchard located near Somerville, TX (lat. 30.52 N, long. 96.42 W). The orchard is managed according to commercial pecan production in a tree spacing of 10x10m (Stein et al. 2012). The soil in the area is a Westwood silt loam and the irrigation of the orchard is through microsprinklers. The experiment was established in early September, when the natural population of pecan black aphid increases. Twelve trees were selected, and then randomly assigned to either the aphid treatment or the control. Three branches of each experimental tree were selected for the treatments with the aphid, taking into account size similarities and position within the canopy (Fig. 4-1). The terminal shoot of one branch was enclosed in a mesh nylon bag (45 x 90 cm; Bioquip, Compton, CA) as shown in Fig. 2 and infested with growth chamber-reared *M. caryaefoliae* by using aphid-infested foliage as described by Chen et al. (2009). The control trees were set with the bag in the same conditions but with no aphids inside. In Fig 4-2 shows the setting of the experiment in a tree:

Treatment 1 (T1): Infested branch.

Treatment 2 (T2): the closest branch to the infested one.

Treatment 3 (T3): The furthest branch to the infested one.

Fig. 4-1. Treatment with black aphid. The branch was covered with a commercial bag designed for insect rearing in trees.

The aphids for the inoculation were obtained from a field collection in the orchard of the USDA-ARS Pecan Breeding & Genetics. A fully expanded leaflet, from greenhouse-grown seedlings with similar aphid infestations (approx. 100 aphids, including adults and all instars) was inserted in the bag to initiate the aphid infestation in

the field experiment. Kernels were collected from the infested branch (**T1**); and non-infested pecan shoots: the closest branch or **T2**, and the furthest branches or **T3**, as shown in Figure 2. The whole production from each branch was collected at harvest in late October (between 3 to 6 kernels), and they were immediately frozen in liquid nitrogen and kept at -80 °C for further analyses.

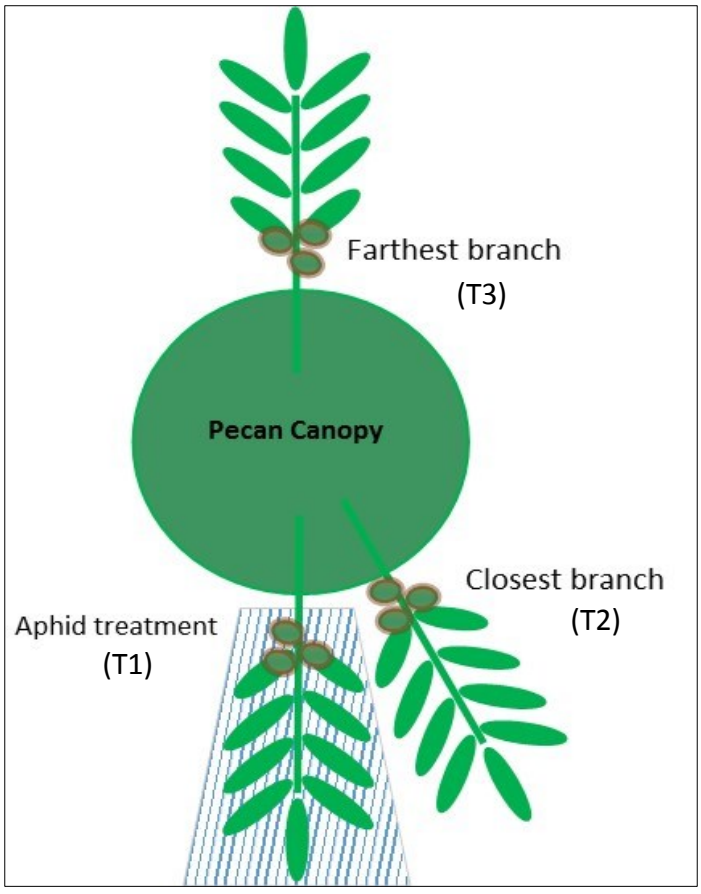

Fig. 4-2. Diagram of the experiment set on pecan trees in the field conditions. Treatment 1: Aphids enclosed in the branch with the mesh bag. Treatment 2: The closest branch to the aphid-treated branch. Treatment 3: The opposite branch in the tree canopy.

Extraction of phenolics. Pecan kernels from each branch were powdered using a food processor. The samples were placed in plastic tubes and extracted with hexane (1:20 w/v) using a high speed homogenizer (Ultraturrax T25, IKA, Wilmington, NC). The homogenate was centrifuged at 5,000 rpm x 5 minutes; the supernatant was collected and the residue was extracted twice with hexane. The remained defatted powder was used for extraction with acetone:water 70:30 (v/v) in proportion 1:20 (w:v), based on a described method for pecans (Villarreal-Lozoya et al. 2009). After 16 h in a shaker at 5 ºC, the extract was centrifuged at 14,000 rpm for 10 min and used for total phenolics and HPLC analyses.

Total phenolics determination. The acetone:water extract was diluted accordingly with nanopure water and the determination was carried out following the microplate method reported by Villarreal-Lozoya et al. (2007) with minor modifications. Briefly, 15 µL of each diluted extract was placed in a 96 microplate well, nanopure water (208 µL) was added with an automatic dispenser, followed by 13 µL of Folin-Ciocalteau reagent. The solutions were allowed to react for 3 min and then 25 µL of 1N Na_2CO_3 was added. The mixture was placed for 2 h in dark conditions and the absorbance was measured at 725 nm using a microplate reader (Sinergy HT, Bio-Tek Instruments, Inc., Winooski, VT). At the same time, blanks were run with nanopure water, and standard calibration curve was made with chlorogenic acid in water. Total phenolic was reported in each sample as mg chlorogenic acid equivalent (CAE) per g of defatted kernel powder.

HPLC analysis of hydrolyzed phenolic compounds. The acetone: water (70:30, v/v) extracts (1.5 ml) were evaporated under vacuum at 35 ºC using a SpeedVac concentrator (Thermo, Marietta, OH), and the residues were dissolved in 300 µL of 8 N NaOH. They were flushed with nitrogen, capped, and allowed to react overnight (approx. 16 h). After this basic hydrolysis, 500 µL of 6N HCl was added to each sample and flushed with nitrogen, capped, and heated for 45 min using a block heater (Fisher Scientific, Houston, Texas) set at 85 ºC. After the acid hydrolysis, the solutions were filtered using a 0.2 µm PTFE filter and put in HPLC vials. The HPLC analysis was performed following the procedure described in Villarreal-Lozoya et al. (2009) with some modifications. The HPLC system consisted in two Waters 515 gradient pumps, a Waters Atlantis C18 column (5 µm particle, 4.5mm × 150mm), coupled with a Waters 717 autosampler, and a Waters 916 photodiode array detector (Waters Corp., Mildford, MA). Photodiode array detector was set to scan absorbance from 190 nm to 600 nm. For determination of compounds each peak spectra was used and the retention times were compared with external pure standards of catechin, gallic acid and ellagic acid (SigmaAldrich, Milwaukee, WI). Nanopure water, acidified to pH 2.3 with 2 M HCl (solvent A), and acetonitrile (solvent B) were used as mobile phases. Solvent gradient was set as follows: from 0 to 5 min 85% of A in isocratic flow mode, from 5 to 20 min a linear gradient of 85% A to 100% B, and from 25 to 30 min isocratic conditions of 100% B. After that, a linear gradient was set to 85% A and kept in isocratic mode for 10 min before next injection. The injection volume for samples and pure compounds was 20 µL.

For quantification of the identified hydrolysable tannin derivatives, standard curves with pure compounds were made.

Statistical analysis. Analysis of Variance (ANOVA) was applied to the data and the statistical differences between treatment means were determined using the Duncan´s Test ($p \leq 0.05$). Those tests were conducted using the software InfoStat (National University of Cordoba, Argentina).

Results

The content of total phenolic and tannin derivatives after hydrolysis was not significantly different for all treatments and respective controls (Fig. 4-3). Total phenolics (Figure 3A) ranged from 22.47 to 31.11 CAE / g defatted pecan kernel (DPK). Gallic acid content was 20.19 to 24.96 mg/g DPK (Figure 3B), catechin between 0.28 and 0.39 DPK (Figure 3C), and ellagic acid ranged from 0.15 to 0.22 mg/g DPK (Figure 3D). Treatment 1 (aphids feeding) and Treatment 2-3 (the closest and the farthest branches respectively) did not produce any significant variation in ellagitannins derivatives in the kernels from the branches.

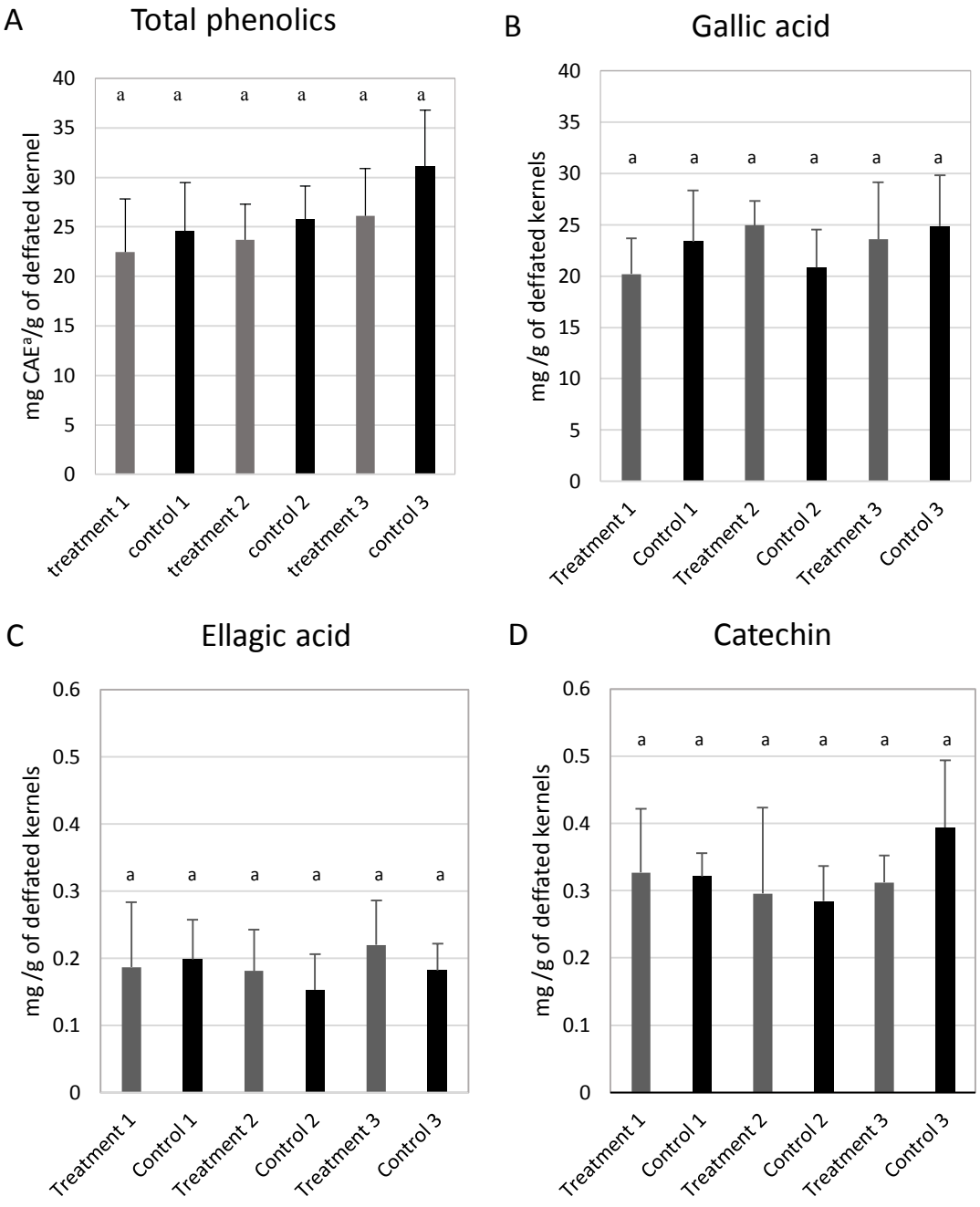

Fig. 4-3. Levels of total phenolics and specific phenolic compounds from treatments 1-3. **A)**, Total phenolic content expressed as [a]CAE (Chlorogenic acid equivalents) per g of defatted pecan kernel (DPK). **B),** Gallic acid content mg/g DPK. **C),** Ellagic acid levels in mg/g DPK. **D)**, Catechin levels expressed as mg/g DPK. All values expressed as mean ± SE of the six field repetitions, compared by Duncan´s test ($p \leq 0.05$).

Phytochemical levels for this cultivar have been not reported before. The values of total phenolic reported here are less than half the range reported for six other varieties (62 to 106 mg CAE/g) (Villarreal-Lozoya et al. 2007), showing the great variability among cultivars. The gallic acid level was higher than the values reported for these six cultivars (0.6-1.3 mg/g DPK), and the content of ellagic acid reported here was about one tenth less than the range in these other varieties (2.5-4.7 mg/g DPK) (Villarreal-Lozoya et al. 2007). The values reported by Daniel et al. (1989) of 0.33 mg/g of ellagic acid in pecans after acid hydrolysis with trifluoroacetic acid are within the range found here, but the authors did not report the cultivar analyzed. The high gallic acid content obtained after the hydrolysis was caused by the strong basic and acid conditions for the hydrolysis. Under these conditions, all the hydrolysable tannins were hydrolyzed to gallic acid, which is the monomeric form for gallotanins and the precursor of ellagitannins found in pecans (Jourdes et al. 2013; Muir et al. 2011; Ascacio-Valdes et al. 2011).

Discussion

The Choctaw cultivar is cultivated in approx. 2500 hectares representing 1.2% of total production in the US (Thompson and Conner 2012). This old variety produces good nut quality and yields. Nevertheless, it has several problems related to challenging soils management, temperatures and pests (Mc Eachern et al. 2012; Knuston et al. 2012); but most importantly, Choctaw shows high susceptibility to black pecan aphid (Wood and Reilly 1998). Wood and Reilly (1998) suggested that varieties susceptible to this aphid

were not able to produce chemical compounds (not mentioned) that affect the aphid preference. In a study comparing three susceptible and three resistant cultivars of pecans to black pecan aphid it was reported that susceptible varieties increased the activity of enzymes related to oxidative stress, and decreased lipoxygenases implicated in resistance against insects. Lipoxygenases are important enzymes in the production of jasmonic acid derivatives that have important roles in the plant systemic responses and production of defensive phenolic compounds (Erb et al. 2012; Kessler and Baldwin 2002; Chauvin et al. 2013). These enzymatic activities were measured in the same leaves where the aphids were applied however non systemic effects were mentioned (Chen et al. 2009). Aphid feeding on plants affects the production of phenolic compounds and its variation is unique for the plant-insect interaction. In an experiment using plant of maize and barley attacked by *Sitobion avenae*, Eleftherianos et al. (2006) found that the levels of phenolics decreased compared to undamaged plants; however, the phenolic levels were unaffected when using the aphid *Rhopalosiphum padi* in the same conditions. Pest damage in pecan is highly dependent on crop management. When plants are under good irrigation, good nitrogen availability and low fruit load, pecan leaves were more able to host pests (Wood and Reilly 2000). Because black aphids are typically a threat during the late growing season, the leaves used in this study had already suffered some kind of biotic stress by the time the treatments were established.

The total phenolics content was lower than that previously reported for pecans kernels at harvest time (Villarreal-Lozoya et al. 2007; Malik et al. 2009). The conditions of the trees used in this study plus the high susceptibility of Choctaw pecans to black

pecan aphid could cause a reduction in the translocation of photosynthates from leaves to fruits for synthesis of phenolic compounds. Results showed here support this hypothesis; since the total phenolic compounds were half and ellagitannins derivatives about one tenth than what reported for other more resistant pecan varieties such as Pawnee, Shawnee or Kiowa (Villarreal-Lozoya et al. 2007). The aphid-host relationship is highly dependent on the chemical interaction between the piercing-sucking part of the insect and the phloem, and several secondary metabolites are implied in the interaction (Smith and Chuang 2014; Pickett et al. 1992). In the case of the interaction between black pecan aphid and Choctaw trees, the aphid may be able to avoid the local defenses from the plant, and the continuing sucking of photosynthates may produce a depletion of sugars that cannot be translocated to the sink tissues (kernels) for production of high C/N compound as the ellagitannins derivatives reported here.

Conclusions

Differences among treatments were not significant for any tannin derivative measured. After the analysis of all the results, I concluded that the levels of the phytochemicals investigated in pecan kernels were not affected by aphids. Under these experimental conditions, the biotic stress applied on leaves did not produce detectable changes in the phytochemical profile. The damage produced by the black pecan aphid was not enough to rearrange the secondary metabolism in fruit, probably because the sugar transport in favor of fruits was depleted by the aphids sucking the leaves. This

hypothesis could be tested in a future experiment under more controlled environmental conditions of the trees.

CHAPTER V

CONCLUSIONS

The importance of phytochemicals for human health has led the study of pre- and post-harvest factors that influence the production of bioactive phenylpropanoids. Organic agriculture claims that under this method of production, plants suffer more biotic stress and accumulate more secondary metabolites (SM) in fruits. Wounded tissues affect the production of SM as a local response, and also as a systemic response in the same organ type (e.g. leaves) but this has not been tested in other kind of organ so far. Pecans and strawberries are known for their high level of bioactive phenolic compound including ellagitannins, gallotanins and proanthocyanidins. In this dissertation, the preharvest effects of biotic stress due to insect feeding and mechanical wounding were evaluated as modulators of tannin derivatives in fruits.

In Chapter II, a preharvest leaf wounding was applied to measure the effects on production of bioactive compounds on fruits. The experiment was conducted in an experimental plot subjected to two levels of mechanical wounding applied on strawberry leaves, 7-12 days before harvest time. No differences in color, fresh weight, firmness and vitamin C were detected at fruit harvest compared with the control ($p>0.05$). However, the level of total phenolics and soluble sugars in fruits of treated plants increased significantly 20% and 12% over the control, respectively. Moreover, significant increments ($p\leq0.05$) in the level of specific phenylpropanoids were observed: epicatechin (185%), and rutin (190%) and the gallotanins derivative gallic acid (130%).

In addition, several genes related to phenolic compounds biosynthesis and sugar metabolism were overexpressed in the fruits from the stressed plants. In this study, it was found that the accumulation of phenolic compounds in fruits can be triggered by the application of mechanical wounding on a different tissue of the plant. A hypothetical model was proposed to explain the modulation of phenolic compounds in fruits based in source/sink transport of sugars in favor of fruits. Furthermore, controlled mechanical wounding applied in leaves during preharvest could be used to increase phytochemicals in fruits through changes in source/sink sugar transportation. Phenolic compounds are very important for example in wine and olive oil industries. This innovative approach could be used for increasing the level and modulating the profile of phenolic compounds in preharvest. Wineries rely on grapes phenolics composition for producing high quality wines (Holt et al. 2008; Pardo-García et al. 2013; Mulero et al. 2010). Several quality parameters in virgin olive oil such as its shelf and storage life, its organoleptic attributes, and its benefits for human health depend on the phenolic profile in olives (Ninfali et al. 2008; Gallardo-Guerrero et al. 2012; Mraicha et al. 2010). These phenolics could be enhanced by using the abovementioned approach.

In chapters III and IV, studies were conducted to evaluate the phenolics in fruits when a generalist insect is chewing organic strawberry plants, and a specialist aphid feeding on pecan leaves. In strawberry fruits evaluated at harvest, there were no significant increments in quality parameters, soluble sugars, phenolic compounds, or the related gene expressions, with the exception of CWI gene after two weeks when two larvae feeding in one leaf. This increment of CWI transcription could indicate that the

level of damage just started to develop the late response in fruits as discussed for Chapter II. In the pecan kernel harvested, there were no detected differences in proanthocyanidins, gallotanins o ellagitannins derivatives levels due to the insects sucking phloem sap from the leaves.

Overall, the results did not support the organic agriculture claim that higher levels of phytochemicals in fruits could be attributed directly to the biotic stress when plants are exposed to organic management. However the wounding component of this biotic stress could be important part of those higher levels. Other parameters should be taking into consideration when phytochemicals are evaluated in organic fruits. There are several factors on phenolic compounds differences between organic and conventional approach, the most studied are: soil and nutrient managements (Mitchell et al. 2007; Lester and Saftner 2011; Caris-Veyrat et al. 2004; Omar et al. 2012); genetic variation (Malik et al. 2009; Faller and Fialho 2010; Kovačević et al. ; Petkovsek et al. 2010); location and cultural practices (Juroszek et al. 2009; Lombardi-Boccia et al. 2003; Mitchell et al. 2007); abiotic stresses (Oliveira et al. 2013; Wang and Frei 2011; Akula and Ravishankar 2011; Hodges and Toivonen 2008; Jaleel et al. 2009; Keutgen and Pawelzik 2008). Most studies have focused on the evaluation of these parameters isolated, probably due to the difficulties to set factorial design experiments and control all the factors. However, phytochemical levels in fruits is a complex function which is derived by the interaction of several agents, such as genetics, soil, nutrients, abiotic stresses, biotic stresses, cultural practices, location, climate).

The results showed in this dissertation stated that the biotic stress isolated in these experimental conditions was not enough to produce changes in phytochemical levels in fruits. In future experiments, more variables such as the distribution of insect damage in the whole plant and the duration of insect feeding on leaves and its effect in fruit phytochemicals should be evaluated. The high variation of phenolics values and mRNA transcripts plant-to-plant was observed in these studies and it could be a common situation in comparison of organic vs. conventional practices (Luthria et al. 2010), another experimental design should be considered in future studies.

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