UNIVERSIDADE ESTADUAL DE PONTA GROSSA
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LETÍCIA REIS

ROLE OF ETHYLENE IN NON-VOLATILE COMPOUNDS DURING STRAWBERRY MATURATION AND CHANGES IN SUGAR METABOLISM DURING MELON MATURATION – New studies about non-climacteric fruit ripening

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Thesis presented to Universidade Estadual de Ponta Grossa to obtaining the Doctor (Ph.D.) title in Agronomy – Concentration: Agriculture; Research area: Physiology, Breeding and Crop management.

Advisor: Prof. Dr. Ricardo Antonio Ayub

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Título da Tese: "**Role of ethylene in volatile and non-volatile compounds during strawberry maturation and changes in sugar metabolism during melon maturation – new studies about non-climateric fruit ripening**".

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To my Family for being my first and eternal teachers,

I dedicate.

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“The task is, not so much to see what no one has seen yet; but to think what nobody has thought yet, about what everybody sees.”

Arthur Schopenhauer

ROLE OF ETHYLENE ON NON-VOLATILE COMPOUNDS DURING STRAWBERRY MATURATION AND CHANGES IN SUGAR METABOLISM DURING MELON MATURATION – New studies about non-climacteric fruit ripening

ABSTRACT

Based on respiration, ethylene production and ethylene response, fleshy fruit are classified as climacteric or non-climacteric. Generically, climacteric fruit are those whose maturation occurs concomitantly to a peak in respiration and increase in ethylene production; are extremely responsive to exogenous ethylene; and are able to complete ripening detached from the mother plant if physiological maturation occurs prior to harvest. On the other hand, non-climacteric fruit are classified as having no respiratory peak nor rise in ethylene production during maturation; do not respond to exogenous ethylene in most cases; and are unable to ripen detached from the mother plant, since physiological ripeness must coincide with the time of harvest. Studies conducted mainly on tomatoes, a model climacteric fruit, generated important information on ethylene production, perception, and signal transduction, as well as their influence on change of color, flavor, texture and aroma, which allowed the development of more efficient production, harvesting and post-harvest technologies. However, in the maturation of non-climacteric fruits, although much research has been conducted, there are still many questions to be elucidated. Among the non-climacteric fruits, strawberry (*Fragaria ananassa* L. Dutch) is the most studied system for understanding the role of ethylene in ripening regulation with several genes related to ripening already characterized. Recently, the melon fruit (*Cucumis melo* L.), is emerging as a new model to study fruit ripening due to the presence of climatic and non-climatic cultivars, allowing the comparison of results among model fruit of the same species. Therefore, in the present study 'Albion' strawberry fruit on the plant at four developmental stages (Green, White, Pink and Red) were immersed in three different treatments (Ethephon – an ethylene-generating compound, 1-MCP - an ethylene perception inhibitor, or Water - containing solvents and diluents), plus one absolute control that received no treatment. At ripeness all fruit were harvested and evaluated to show the effect of ethylene on important physical-chemical attributes and non-volatile quality compounds. Ethephon treatment was observed to affect fruit dimension, firmness, anthocyanins and amino acid content. Treatment with 1-MCP at any developmental stage had no effect on any of the variables measured. A second study was conducted with a non-climacteric melon fruit (*Cucumis melo* cv. 'Yellow') to characterize the expression of important sugar metabolism genes during four fruit development stages (small green, large green, color change and full ripe) to relate sugar metabolism of non-climacteric melon with possible linkage to sugar metabolism and shelf life of these fruits. The target genes were choosen using previous information from a RNAseq sequencing of 'Yellow' melon. Genes encoding enzymes that metabolise sugar for energy were observed to be more expressed early in development at the large green development stage, while genes encoding enzymes that synthesize sugar and/or direct this sugar to storage were observed to be expressed later in development, at the color change and full ripe stages. Both studies provided important information about non-climacteric fruit ripening that can be used to improve management, harvest and post-harvest technologies in the future.

Key-words: Non-climacteric, New insights, Fruit ripening.

PAPEL DO ETILENO NOS COMPOSTOS NÃO-VOLÁTEIS DO MORANGO DURANTE A MATURAÇÃO E MUDANÇAS NO METABOLISM DE AÇÚCARES DURANTE A MATURAÇÃO DO MELÃO – Novos estudos sobre a maturação de frutos não-climatéricos

RESUMO

Em virtude da respiração e, produção e resposta ao etileno, os frutos carnosos foram classificados em climatéricos e não-climatéricos. De maneira genérica, os frutos climatéricos são aqueles cujo amadurecimento ocorre concomitantemente a um pico na respiração e aumento na produção de etileno; são extremamente responsivos ao etileno exógeno; e são conhecidos por serem capazes de completar a maturação mesmo destacados da planta mãe, já que o ponto de maturação fisiológica antecede o ponto de colheita. Já os frutos não-climatéricos, não apresentam pico respiratório e de produção de etileno durante a maturação; não respondem ao etileno exógeno na maioria dos casos; e não são capazes de amadurecer destacados da planta mãe, já que o ponto de maturação fisiológica coincide com o ponto de colheita. Estudos realizados principalmente em tomate, modelo climatério de fruto de polpa, geraram informações importantes sobre a produção, percepção e transdução de sinal do etileno, bem como, sua influência na mudança de cor, sabor, textura e aroma dos frutos, o que possibilitou o desenvolvimento de tecnologias de produção, colheita e pós-colheita mais eficientes. Todavia, no amadurecimento de frutos não-climatéricos, embora muitos trabalhos estejam sendo realizados, ainda existem muitas questões a serem elucidadas. Dentre os frutos não-climatéricos, o morango (*Fragaria ananassa* L Dutch) é o sistema mais estudado para o entendimento do papel do etileno na regulação da maturação, incluindo vários genes relacionados com a maturação já caracterizados. Recentemente, o melão (*Cucumis melo* L.), vem surgindo como uma nova proposta de modelo de estudos devido a presença de variedades climatéricas e não-climatéricas, possibilitando a comparação entre frutos modelo da mesma espécie. Assim, no presente estudo, frutos de morango var 'Albion' ligado à planta mãe em quatro estágios de desenvolvimento (verde, branco, rosa e vermelho) foram imersos em três diferentes tratamentos (Ethephon - composto gerador de etileno, 1-MCP - inibidor de percepção de etileno, ou água - contendo solventes e diluentes), mais um controle absoluto que não recebeu tratamento. Na maturação, todos os frutos foram colhidos e avaliados para mostrar o efeito do etileno em importantes atributos físico-químicos e compostos de qualidade não voláteis. Observou-se que o tratamento com ethefon afetou a dimensão dos frutos, a firmeza, o teor de antocianinas e o teor de alguns aminoácidos. O tratamento com 1-MCP em qualquer fase de desenvolvimento não teve efeito em nenhuma das variáveis medidas. Um segundo estudo foi conduzido com melão não-climatérico (*Cucumis melo* var. Amarelo) para caracterizar a expressão de importantes genes do metabolismo do açúcar durante quatro estágios de desenvolvimento do fruto (verde pequeno, verde grande, mudança de cor e totalmente maduro), pois pouco se sabe sobre o metabolismo do açúcar do melão não-climatérico e também devido à suposta ligação entre o metabolismo do açúcar e a vida de prateleira desses frutos. Os genes-alvo foram escolhidos usando informações prévias de um sequenciamento RNAseq do melão Amarelo. Os genes que codificam enzimas que consomem açúcar para fornecimento de energia foram mais expressos no início do desenvolvimento, principalmente no estágio de desenvolvimento verde grande, enquanto os genes que codificam enzimas que sintetizam açúcar e / ou direcionam esse açúcar para armazenamento foram mais expressos geralmente no estágio de mudança de cor e mantendo alta expressão em frutos maduros. Ambos estudos fornecem informações importantes sobre o amadurecimento de frutos não climatéricos que podem ser usados para melhorar as tecnologias de manejo, colheita e pós-colheita no futuro.

Palavras-chave: Não-climatérico, Novas ideias, Maturação de frutos.

FIGURE LIST

TABLES LIST

ACRONYMS AND TERM LIST

1MCP - 1-Methylcyclopropene;

AAG - Acid Alpha galactosidase

AIN - Acid invertase

C3g - Cyanidin-3-glucoside

CIN - Cell wall invertase

D.A.P. - Days after pollination

DEGs - Differentially expressed genes

EST - Expressed sequence tag

FaACO1 – Fragaria's 1 aminocyclopropane-1-carboxylic acid oxidase 1

FaACO2 – Fragaria's aminocyclopropane-1-carboxylic acid oxidase 2

FaERS1 – Fragaria's Ethylene Response Sensor 1

FaETR1 – Fragaria's Ethylene Receptor 1

FaETR2 – Fragaria's Ethylene Receptor 2

FaSAMS1 – Fragaria's S-adenosylmethyonine

FK - Fructokinase

GAE - Gallic acid equivalents

GK - Galactokinase

HXK - Hexokinase

ID - Identification number

INH - Invertase inhibitor

mRNA - Messenger RNA

NAG - Neutral Alfa galactosidase

NGE - Next generation sequencing

NIN - Neutral invertase

P3g - Pelargonidin-3-glucoside

PAL - Phenylalanine ammonia lyase

PCA - Principal Component Analysis

PCR - Polymerase Chain Reaction

RFOs - Raffinose family oligosaccharides

RNA - Ribonucleic acid

RPKM - Reads Per Kilobase Million

RT-qPCR - Relative quantitative real-time polymerase chain reaction

SPP - Sucrose-phosphate phosphatase

SPS - Sucrose-phosphate synthase

SUS - Sucrose synthase

UDP-glc - UDP-glucose

uHPLC - ultra-high-performance liquid chromatography

SUMMARY

PRESENTATION

This thesis was written according to "Manual de normatização bibliográfica para trabalhos científicos, UEPG (2012)" and is composed of the following chapters:

- Role of ethylene in non-volatile quality attributes of 'Albion' strawberry fruit during ripening on the plant.

- Sugar metabolism in non-climacteric 'Yellow' melon (*Cucumis melo* L.) fruit during maturation.

All the chapters will be submitted for publication in scientific journals and despite interconnections, each chapter is independent of the others (the reader of any chapter should presume the acronyms, equations, figures, tables, or references refers only to the current chapter).

Preceding the first chapter there is a general introduction and the last section is composed of general conclusions.

GENERAL INTRODUCTION

Fruit development and maturation have received considerable attention because of its importance both to the biology of plants and as a significant component of the human diet (ADAMS-PHILLIPS; BARRY; GIOVANNONI, 2004; BARRY; GIOVANNONI, 2007; GIOVANNONI, 2001).

Overall, ripening is an event coordinated by biochemical and physiological changes triggered by a series of molecular events that initiate signaling and stimulate the transcription of specific genes, involving expressive changes of color, texture, flavor and aroma, which makes the fruit attractive for consumption (GIOVANNONI, 2004; PECH et al., 2012). However, with maturation, the fruit also becomes perishable, making it difficult to harvest and market. Therefore, understanding the processes that determine the maturity of the fruit is important for reducing losses and maintaining fruit quality.

Ripening has been categorized as either ‘climacteric’ or ‘non-climacteric’ according to the fruit’s ability to produce ethylene and an associated increase in respiration rate at the onset of ripening (BARRY; GIOVANNONI, 2007).

The role of ethylene as the ‘‘ripening hormone’’ in climacteric fruits such as tomato, apple, banana and some melons has been extensively studied (ADAMS-PHILLIPS; BARRY; GIOVANNONI, 2004; AYUB et al., 1996; BARRY; GIOVANNONI, 2007; GIOVANNONI, 2001, 2004, 2007; PECH et al., 2012; PECH; BOUZAYEN; LATCHÉ, 2008). In non-climacteric fruit ripening, the role of ethylene is not clear. However, there is increasing experimental evidence that implicates ethylene in the ripening of fruits that have been classically thought of as non-climacteric (BÖTTCHER et al., 2013; JIANG; JOYCE; TERRY, 2001; LOPES et al., 2015; MERCHANTE et al., 2013; SUN et al., 2013; SYMONS et al., 2012; TESNIERE et al., 2004; TRAINOTTI; PAVANELLO; CASADORO, 2005; VILLARREAL et al., 2010), suggesting ethylene-dependent and independent processes differs among species (FREILICH et al., 2015).

Among the non-climacteric fruits, strawberry has emerged as the most widely studied system for understanding the role of ethylene in ripening regulation (GIOVANNONI, 2001), and has been useful in identification and characterization of numerous ripening-related genes that affect cell wall metabolism, color, and aroma (AHARONI, 2002).

Strawberry is considered an easy model to study due to its small size, well-defined development stages, easy propagation, short vegetative stage of growth and rapid fruit development (approximately 30 days from flowering to ripening) (SYMONS et al., 2012).

Although strawberry fruit have low respiration and ethylene production at onset of ripening, an extensive number of researchers reported ethylene had a positive influence on strawberry quality attributes or that its competitive inhibitor 1-MCP inhibited this effect. These effects were reported on softening enzymes (VILLARREAL et al., 2008; VILLARREAL; MARTÍNEZ; CIVELLO, 2009), anthocyanin accumulation and phenylalanine ammonia lyase (PAL) activity (LOPES et al., 2015; MERCHANTE; ALONSO; STEPANOVA, 2013; SUN et al., 2013; VILLARREAL et al., 2010; VILLARREAL; MARTÍNEZ; CIVELLO, 2009), phenolic compound accumulation (LOPES et al., 2015; VILLARREAL et al., 2010), and in sugar and acid content (ELMI et al., 2017; LOPES et al., 2015; VILLARREAL et al., 2010, 2016), suggesting that even in non-climacteric fruit, there are ethylene-dependent events during fruit development and ripening.

In a similar way, melon fruit (*Cucumis melo* L.) have been suggested to be an ideal alternative model fruit for studies on ethylene perception and sensitivity, because members of the *Cucumis* genus exhibit both climacteric and non-climacteric cultivars, making it possible to use similar fruit to study both ripening patterns (EZURA; OWINO, 2008; SALADIÉ et al., 2015).

Melon is an important horticultural crop that is cultivated worldwide. Its development has distinct stages and the availability of genetic and genomic resources including EST collections such as the Cucurbit Genomics Database (CuGenDB - http://www.icugi.org) and the MELONOMICS melon genome (GARCIA-MAS et al., 2012) are available.

In melon fruit, differences in shelf-life of climacteric and non-climacteric cultivars have been suggested to be associated with different patterns of expression of key-genes in sugar accumulation metabolism during fruit development, and sugar degradation metabolism post-harvest (SALADIÉ et al., 2015). This provides important information about fruit ripening and potential management stratagies to prolong fruit quality during storage.

Therefore, the present study was conducted in two parts to provide important information about non-climacteric fruit ripening that can improve management, harvest and post-harvest technologies in the future. The aim of the first part of this study was to observe the effect of ethylene on important physical-chemical attributes and non-volatile quality compounds of ripe ‘Albion’ strawberry fruit following three pre-harvest treatments (Ethephon – an ethylene-generating compound, 1-MCP - an ethylene perception inhibitor, or water - containing solvents and diluents), plus one absolute control that received no treatment. Fruit on the plant were treated at four developmental stages (Green, White, Pink and Red). The aim

of the second part of this study was to characterize the expression of important sugar metabolism genes during four fruit development stages (small green, large green, color change and full ripe) of 'Yellow' melon fruit, to increase the knowledge about the sugar metabolism of non-climacteric melons and try to link this information with shelf life metabolism. Both studies aim to contribute to the knowledge on the maturation of non-climacteric fruit.

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Chapter - ROLE OF ETHYLENE IN NON-VOLATILE QUALITY ATTRIBUTES OF 'ALBION' STRAWBERRY FRUIT DURING RIPENING ON THE PLANT

Abstract: In contrast to the ripening of climacteric fruit, knowledge of non-climacteric fruit ripening needs to be better understood. Among the non-climacteric fruits, strawberry is the most studied system for understanding the role of ethylene in the regulation of ripening. However, all previous studies that report ethylene effects on strawberry quality were performed with detached fruit, using only specific developmental stages and did not consider the plant-fruit connection during development and ripening. Thus, the aim of this work was to show the effect of ethylene and an ethylene-action inhibitor on important physical-chemical attributes and non-volatile quality compounds in ripe 'Albion' strawberry fruit treated during different developmental stages. Fruit attached to the plant at four developmental stages (Green, White, Pink and Red) were dipped in one of three treatment solutions (Ethephon – an ethylene-generating compound, 1-MCP - an ethylene perception inhibitor, and water - containing solvents and diluents), plus one absolute control that received no dip. Following treatment, the strawberry fruit were grown on the plant until ripe. Strawberry fruit were then assessed for physicochemical properties that included weight, length, diameter, firmness, color, titratable acidity, soluble solids, pH, anthocyanin content, total phenolics content, and sugar, organic acid and amino acid composition. The days required for fruit to ripen following treatment was also recorded, but no treatment effect was observed for this measurement. Some treatment effects on weight, diameter, length and firmness were observed. Ethylene was observed to affect fruit weight, length, diameter and firmness. Ethylene treatment at the white stage increased anthocyanin content in ripe fruit. The amino acid alanine was also increased by the ethylene treatment applied to green and white fruit, while phenylalanine increased in fruit treated with ethylene at all development stages and tryptophan content increased in fruit treated with ethylene at the green, white and pink developmental stages. Treatment with 1-MCP at any developmental stage had no effect on any of the variables measured, suggesting that the effect on strawberry ethylene receptors was not enough to inhibit ethylene signal transduction, the effect was not persistent until ripening or different kinds of ethylene receptors are present in strawberry fruit.

Key Words: Non-climacteric, Maturation, Fruit quality, Ethrel®, Harvista™

List of acronyms: C3g, Cyanidin-3-glucoside; *FaERS1,* Ethylene Response Sensor 1; *FaETR1,* Fragaria's Ethylene Receptor 1; *FaETR2,* Fragaria's Ethylene Receptor 2; *FaACO1,* Fragaria's 1 aminocyclopropane-1-carboxylic acid oxidase 1, *FaACO2,* Fragaria's aminocyclopropane-1-carboxylic acid oxidase 2, *FaSAMS1,* Fragaria's S-adenosylmethyonine GAE, Gallic acid equivalents; 1MCP, 1-Methylcyclopropene; P3g, pelargonidine-3-glucoside; PAL, phenylalanine ammonia lyase; PCA, Principal Component Analysis; SAM, S-adenosilmetionina; uHPLC, ultra-high-performance liquid chromatography.

1.1 INTRODUCTION

The strawberry plant (*Fragaria x ananassa* Duch.) is a crop of high commercial value and world production was ~9.11 million tons in 2016 (http://faostat.fao.org/site/567/default.aspx). The strawberry fruit is composed of dry achenes (true fruit that evolves from ovaries) and the enlarged flower receptacle (flesh part) that shows high metabolic synchrony during fruit development and ripening (FAIT et al., 2008).

During ripening of the receptacle, ethylene is involved in a series of biochemical and physiological changes that affect texture, color, aroma, and flavor (BAPAT et al., 2010; BARRY; GIOVANNONI, 2007; SYMONS et al., 2012). Due to low rates of ethylene production and respiration during ripening, strawberry fruit are classified as non-climacteric (BAPAT et al., 2010; BARRY; GIOVANNONI, 2007). However, recent studies suggest hormonal involvement during strawberry fruit development, including indole-3-acetic acid, gibberellic acid and castasterone (SYMONS et al., 2012) as well as a possible involvement of ethylene (BAPAT et al., 2010; LOPES et al., 2015; MERCHANTE et al., 2013; SUN et al., 2013; TRAINOTTI; PAVANELLO; CASADORO, 2005; VILLARREAL et al., 2010) and its interaction with other plant growth regulators such as abscisic acid (AYUB et al., 2016; JIA et al., 2011, 2013, 2016; SYMONS et al., 2012) and brassinosteroid (AYUB et al., 2018a, 2018b).

In strawberry, a seven-fold increase in ethylene production has been detected during fruit expansion as fruit progress from the green to the white ripening stage, and a 30-fold increase as fruit turn red (IANNETTA et al., 2006; SUN et al., 2013). In red ripe fruit this increase in ethylene production is accompanied by a three-fold increase in respiration rate, similar to that seen in climacteric fruit at the onset of ripening (IANNETTA et al., 2006). In addition, peak expression of the ethylene receptors occurs during anthesis for the *FaERS1* receptor, during the white to red ripening stage for the *FaETR1* receptor and in both stages for the *FaETR2* receptor (TRAINOTTI; PAVANELLO; CASADORO, 2005). The peak expression of the ethylene pathway genes, including *FaACO1* and *FaACO2,* occurs during anthesis, and the ethylene biosynthesis-related gene *FaSAMS1* shows high expression in small green fruit followed by an abrupt decrease in the following developmental stages, with no increase during ripening (IANNETTA et al., 2006; SUN et al., 2013; TRAINOTTI; PAVANELLO; CASADORO, 2005). This suggests an involvement of ethylene in strawberry fruit development and ripening.

During strawberry development and ripening, numerous biochemical changes in primary and secondary metabolism contribute to fruit flavor (AHARONI, 2002; FAIT et al., 2008; SCHWIETERMAN et al., 2014; ZHANG et al., 2011). Previous studies that used ethylene

elicitors or inhibitors have suggested that exogenous ethylene effects some important quality attributes in strawberry fruit (ELMI et al., 2017; JIANG; JOYCE; TERRY, 2001; LOPES et al., 2015; TRAINOTTI; PAVANELLO; CASADORO, 2005; VILLARREAL et al., 2008, 2010, 2016; VILLARREAL; MARTÍNEZ; CIVELLO, 2009). Despite ethylene being present in low concentrations, ethylene may trigger the ripening process. However, all of these studies have been conducted with detached fruit, demonstrating transient effects that may not persist through full ripening on the plant. The reason for the absence of literature for evaluating if the ethylene effect is maintained until ripening is due to unavailability of products that can be applied directly to the plant or fruit, since some products used in previous work, mainly ethylene inhibitors, are gaseous. In this way, using the liquid ethylene formulation - Ethrel® (an ethylene releasing agent) and a new liquid 1-Methylcyclopropene (1-MCP) formulation - Harvista™ (an ethylene perception inhibitor), the aim of this work was to understand the role of ethylene in strawberry ripening by measuring the effects of these compounds on important quality attributes during fruit maturation on the plant.

1.2 MATERIAL AND METHODS

1.2.1 Plant material and growth conditions

In 2017, two hundred eight (208) day neutral strawberry plants (*Fragaria* × *ananassa* cv. Albion) were cultivated in a semi-hydroponic system in a greenhouse at the Kentville Research and Development Centre, Agriculture and Agri-Food Canada (Kentville, Nova Scotia, Canada). Twelve primary or secondary fruit in each of the green, white, pink, and red development stages were characterized and selected daily for treatment. The green stage was characterized as small green fruit with developed, separated green achenes and white receptacle color close to the sepals, but still green on the end of the fruit (Figure 1.1-A). The white stage was defined as fruit with a white receptacle and light green/yellow achenes that are separated from each other at the end of the fruit (Figure 1.1-B). The pink stage was defined as fruit showing the initial change of color being pink in most of the fruit, but with a white color close to the sepals (Figure 1.1-C). The red stage was defined as the first-day fruit were observed to have a uniform red color (ripe fruit) (Figure 1.1-D).

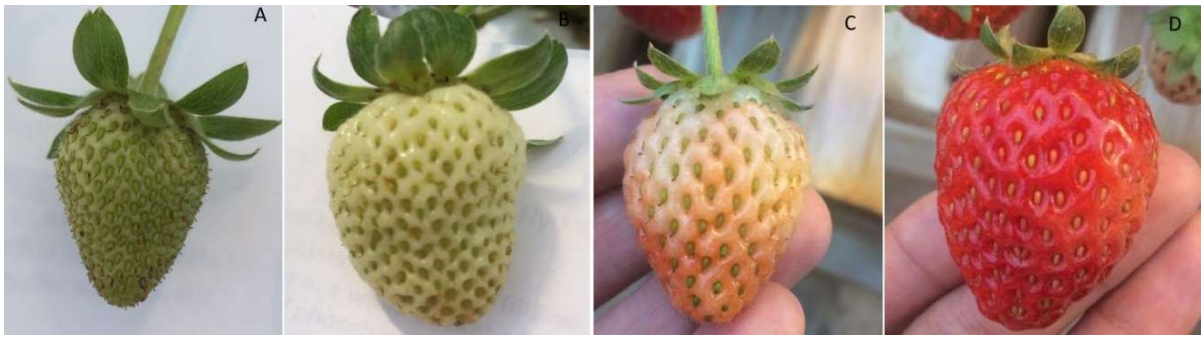

Figure 0.1 Four development stages of Albion strawberry. A- green, B- white, C- pink and D- red.

1.2.2 Treatments

Based on the developmental stages described above, 'Albion' strawberry fruit attached to the plant were labeled and dipped in one of 3 treatments or received no-dip (absolute control). For the ethylene treatment, whole fruit were dipped for 5 min in a solution of 7 mmol L^{-1} ethephon (Ethrel®, 240 $g.L^{-1}$ a.i., 2-chloroethylphosphonic acid metabolized by the plant into ethylene), 0.2 mL L^{-1} Tween 20® and 10 mL L^{-1} ethanol (both used as surfactants) that was prepared immediately before use (SUN et al., 2013; VILLARREAL et al., 2010). For the ethylene inhibitor treatment, whole fruit were submerged for 1 min in a water solution with 1.0 µg L^{-1} aqueous 1-methylcyclopropene (1-MCP, Harvista™ AF-701 formulation, 1.3% a.i, AgroFresh, Inc.). As a control treatment, the whole fruit were submerged for 5 min in a solution of water, 0.2 mL L^{-1} Tween20 and 10 mL L^{-1} ethanol. In addition, fruit were not subjected to a dip to comprise the absolute control. All treated, and control fruit were harvested at the red ripe stage. The strawberry fruit treated at the red ripe stage were harvest 24h after treatment.

1.2.3 Rate of ripening

For all fruit treated at the green, white and pink stages, the number of days from treatment to harvest were recorded to determine the effect of treatment on the rate of ripening.

1.2.4 Physicochemical fruit analysis

All fresh fruit were measured for (1) weight, using an analytical balance and expressed in grams (g); (2) Diameter and length using a digital caliper and expressed in millimeters (mm); (3) epidermis surface color using a Minolta CR 400 colorimeter and expressed as a* (green / red coordinate: -a indicates green tones and + indicates red color), and hue angle (true color value of the fruit, expressed in degrees), and (4) firmness using a penetrometer with a 6 mm

diameter notched tip, expressed in newtons (N). After these measurements, all 12 fruit for each treatment were sliced, frozen in liquid nitrogen and stored as a composite sample in a -80C ultra low freezer and later analyzed for (5) soluble solids (SS, ◦Brix), (6) pH and (7) titratable acidity (%TA, expressed as citric acid equivalents).

1.2.5 Anthocyanins and phenolic compounds

About 6 g of frozen fruit from each composite sample were ground with a mortar and pestle using liquid nitrogen. Approximately 0.5 g of the resultant powder was mixed with 0.7 mL of extraction solvent (methanol/water/trifluoroacetic acid – 70:29:1) in a labeled 2 mL microcentrifuge tube, vortexed for 10 s and sonicated in water for 20 min. The slurry was centrifuged at 10,000×g at 4 ºC for 10 min and the supernatant was transferred to a second labelled tube and set aside. Second and third 0.7 mL extractions were conducted on the tissue pellet and the supernatant of the 3 extractions were pooled. Each treatment was extracted in triplicate.

For anthocyanin measurement, 25 uL of each extracted sample described above were added in triplicate to 275 uL of pH 1.0 buffer (25 mL of 0.2 N KCl and 75 mL of 0.2 N HCl - pH adjusted to 1.0) in a 96 well microplate and as well as to a pH 4.5 buffer (80 mL of 1M $C_2H_3NaO_2$, 50 mL of 1 N HCl and 70 mL of water – pH adjusted to 4.5) in an additional 96 well microplate, according to the pH differential method of Lee et al. (2005). After 5 min, the optical density (OD) at 520 and 700 nm was measured for each plate and the optical density readings for each plate were subtracted to determine anthocyanin concentration. The anthocyanin content was expressed as mg of pelargonidin-3-glucoside (P3g)/100 g fresh weight and as mg of Cyanidin-3-glucoside (C3g) /100 g fresh weight using the formula: Concentration = (Absorbance x MW x Dilution Factor x 1000) / (ϵ x path length x Sample weight (0.5g)). Where, Absorbance is (A520 nm pH 1.0 - A700 nm pH 1.0) - (A520 nm pH 4.5 - A700 nm pH 4.5); Dilution factor is the total volume (300 uL)/extract volume used (25 uL); The microplate path length (cm) is (4 x Vol)/(π x $d2$), where Vol is in cm^3 (mL) and d is the mean diameter of the well in cm (as listed by the manufacturer). When following this protocol, 300 µL gives a path length of 0.8515 cm. To calculate P3g, an extinction coefficient (ϵ) of 26900 and molecular weight (MW) of 433.389 g/mol was used and to calculate C3g an ϵ of 15600 and MW of 449.2 g/mol was used.

For phenolic compound measurement, 25 uL of extract from each sample was added in triplicate to 250 uL milli-Q water and 50 uL prepared Folin-Ciocalteu reagent: Water (1:2 (v:v))

in a 96 well microplate, according to the Folin-Ciocalteu method (SINGLETON; ROSSI JR.; ROSSI J A JR., 1965). A gallic acid (0, 50, 75, 100, 150, 200 and 250 mg/L) standard curve was done in triplicate for each microplate used to quantify total phenolics. After 5 min of agitation, 12 uL of a saturated Na-carbonate solution was added to each microplate well, followed by additional agitation. After a one-hour rest in the dark at room temperature, the optical density (OD) at 750 nm was measured. Total phenolic compounds were calculated as gallic acid equivalents/100 g sample using the absorbance and the regression equation for the standards, where concentration = {[((absorbance - intercept) / slope) /1000] /dilution factor} x 100.

1.2.6 Sugar, organic acid and amino acid analyzes

About 20 g of frozen fruit tissue from each treatment were thawed, crushed with a mortar and pestle using liquid nitrogen and centrifuged in 50 mL tubes for 20 min. The supernatant strawberry juice was filtered through a polyethylene membrane of 0.45 µm pore size (Millipore Corp., Bedford, MA, USA) and collected directly in 3 different vials (2 x 2 mL) for sugar, organic acid and amino acid analysis.

The juice was analyzed for sugar composition using an Agilent uHPLC 1290 Infinity II System equipped with a 1260 Infinity II Refractive Index Detector G7162A held at 40 ºC. Using a 1290 Infinity II Multisampler G7116B, a 20 µL sample of juice was injected onto a BioRad, Aminex HPX-87P column with guard held at 85 ºC with a flow rate of 0.6 mL/min of 100% Nanopure water, that had been vacuum filtered with a 0.22 µ GV Durapore membrane filter and sonicated for about ~10 min. The sugars sucrose, glucose and fructose were identified by retention times, which were 10.11; 11.12 and 18.8 min respectively. All sugars were identified and quantified using external standards. Quantification of these compounds was carried out by calibration curves constructed with three independent sets of dilutions of standard compounds.

For organic acid analysis, the juice was analyzed using an Agilent uHPLC 1290 Infinity II System equipped with a diode array detector. A 20µL sample of juice was injected onto a Phenomenex, Kinetex 2.6 u 100A, 150x4.6 mm column w/ guard held at 30 ºC with a flow rate of 0.5 mL/min of 0.01M H_2SO_4, pH 2.5 that had been vacuum filtered with a 0.22 µ RC filter and sonicated for about 10 min. Malic, shikimic, citric and succinic acids were identified by retention times which were 4.00, 5.93, 6.79 and 20.4 min respectively. All organic acids were identified and quantified using external standards.

For amino acid analysis, the juice was analyzed using an Agilent uHPLC 1290 Infinity II System equipped with a fluorescence detector. A 40 µL sample of juice was injected onto an Agilent, Infinity Lab Poroshell HPH-C18 column w/ guard held at 40 ºC with a flow rate of 0.42 mL/min. The mobile phase consisted of a linear gradient of 98% phase A decreasing to 43% after 13.4 min, then decreasing to 0% and returning to 98% after 15.8 min, where phase A consisted of 10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, pH 8.2 that was vacuum filtered with a 0.22µ RC filter and sonicated for about 10 min and phase B consisted of acetonitrile: methanol: nanopure water 45:45:10 (v:v:v). Detection was carried out using fluorescence, with excitation at 340 nm and emission at 450 nm for the first 10.3 min followed by excitation at 260 nm and emission at 315 nm. The amino acids alanine, arginine, asparagine, aspartate, cystine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine were identified and quantified using external standards.

1.2.7 Experimental design and statistical analysis

The experiment was carried out in a randomized block design in a 3x4 factorial with 1 absolute control. The treatments were composed of 3 dip solutions (1MCP, Ethephon, and water) applied at four development stages (green, white, pink and red), plus an absolute control that received no dip. Each treatment was performed in 4 repetitions (blocks) with each replication comprised of 4 plants. From these 4 plants, 12 strawberry fruit were treated and harvested and assessed when red ripe. These 12 fruit were combined to form composite samples for chemical analysis. The data collected after harvest of ripe fruit were analyzed using Analysis of Variance (ANOVA), and principal component analysis through Genstat 16 (VSN International, 2013) software.

1.3 RESULTS

1.3.1 Ethylene effects on rate of ripening, size and, firmness

The rate of strawberry fruit ripening was not affected by any treatment regardless of the development stage applied (data not shown). For fruit treated at the green stage, the time from treatment until fruit was red ripe averaged 13 days, for the white stage it was 6 days, and for the pink stage it was 4 days.

Ripe ‘Albion’ strawberry fruit were observed to have an average weight of 12.7 ± 0.5 g, an average diameter of 287 ± 4 mm, an average length of 335 ± 7 mm, and an average firmness of 4.5 ± 0.2 N across all development stages and treatments (Table 1.1). For each of these parameters No Dip control was not significantly different than the average of the factorial treatments, but significant interactions between development stages and treatments were observed (Attached Table1).

Table 0.1 - Biometrical characteristics weight (g), diameter (mm), length (mm) and firmness (N) of ripe ‘Albion’ strawberry fruit treated with 1MCP, Ethephon or water plus an absolute control (No Dip) at four development stages (Green, White, Pink and Red). The differences between treatments within a development stage greater than twice the standard error of the mean (SEM) indicate significant difference.

Treatment	Weight (g)	Diameter (mm)	Length (mm)	Firmness (N)
NoDip	13.2	289	347	4.19
Green 1MCP	11.9	279	332	4.32
Green Ethephon	14.1	295	341	4.49
Green Water	12.2	283	327	4.81
White 1MCP	12.9	291	336	4.50
White Ethephon	13.3	297	331	4.35
White Water	13.9	296	342	4.41
Pink 1MCP	13.1	294	343	4.46
Pink Ethephon	10.7	270	308	5.65
Pink Water	12.4	287	327	4.20
Red 1MCP	12.2	286	333	4.36
Red Ethephon	12.4	283	332	4.48
Red Water	12.6	283	351	4.04
Grand Mean	12.7	287	335	4.48
SEM	0.49	4.0	7.0	0.23
F pr				
Stage x Dips	0.002	0.005	0.023	0.008
G,W vs P,R x 1MCP vs Ethep	0.002	<0.001	0.047	0.054
G vs W x 1MCP vs Ethep	0.064	NS	NS	NS
P vs R x 1MCP vs Ethep	0.010	0.023	0.020	0.024

NS- No significant difference.

Based on the difference between treatments greater than twice the standard error of the mean (SEM), fruit treated with Ethephon at the green development stage had 16% and 13.5% greater mass and 5.4% and 4.0% larger diameter when ripe than 1MCP or water treated fruit, respectively. In contrast, pink fruit treated with Ethephon resulted in ripe fruit having 22.5%, 16.2% and 23.7% less mass, and 9%, 6.3% and 7.0% smaller diameter than fruit treated with

1MCP, water or NoDip, respectively. No differences in mass or diameter were observed for any ripe fruit that were treated at the white or red development stage (Table 1.1).

For length, the Ethephon treatment applied at the pink development stage resulted in ripe fruit having 11.4%, 6.2% and 12.7% shorter length than fruit treated with 1MCP, water or No Dip, respectively. Similarly, pink fruit treated with Ethephon were 21.0%, 25.7% and 25.8% firmer when ripe than those treated with 1MCP, water or No Dip, respectively. Ethylene or 1MCP treatments did not affect length or firmness of strawberry fruit treated at the green, white or red development stages (Table 1.1).

1.3.2 Ethylene effects on anthocyanins and phenolic compound content and color development

All ripe fruit, regardless of treatment, had higher concentrations of the anthocyanin pelargonidin 3-O-glucoside (P3g) than the anthocyanin cyanidin 3-O-glucoside (C3g) (Table 1.2). Ripe 'Albion' strawberry fruit had an average P3g concentration of 214 ± 15 mg/100 g and C3g concentration of 129 ± 9 mg/100 g across all development stages and treatments (Table 1.2). For each of these parameters, the No Dip control was not significantly different than the average of the factorial treatments, but significant interactions between development stages and treatments were observed (Attached Table 1).

Based on the differences greater than twice the standard error of the mean (SEM), we observed that the concentration of anthocyanins in white ripe fruit was greater in fruit treated with ethylene than in fruit subjected to the other treatments. Concentrations of both C3g and P3g in ripe fruit were 32.7%, 25.0% and 22.8% greater in ethylene treated fruit at the white development stage than in 1MCP, water or the No Dip treated fruit, respectively (Table 1.2). Anthocyanin content of fruit treated at the green, pink and red development stages was not affected by treatments (Table 1.2).

For all ripe fruit that were treated at different ripeness stages, the average phenolic compound content was 32.6±1.46 mg GAE/100 g. (Table 1.2). No treatment effect was observed in the content of phenolic compounds at all development stages (Attached Table 1).

Table 0.2 - Content of cyanidin 3-O-glucoside (C3g) and pelargonidin 3-O-glucoside (P3g) anthocyanins (mg/100 g) and phenolic compound content (mg of GA/100g) in ripe 'Albion' strawberry fruit treated with 1MCP, Ethephon or water plus an absolute control (No Dip) at four development stages (Green, White, Pink and Red). The differences between treatments within a development stage greater than twice the standard error of the mean (SEM) indicate significant difference.

Treatment	Anthocyanins (mg/100 g)		Phenolic compounds (mg of GA/100 g)
	C3g	P3g	
NoDip	122	203	34.6
Green 1MCP	122	202	32.1
Green Ethephon	134	222	30.4
Green Water	123	205	31.1
White 1MCP	106	177	32.5
White Ethephon	158	263	33.1
White Water	119	197	33.3
Pink 1MCP	133	221	33.2
Pink Ethephon	125	208	32.6
Pink Water	127	210	31.5
Red 1MCP	141	234	31.6
Red Ethephon	135	225	34.6
Red Water	132	220	33.1
Grand Mean	129	214	32.6
SEM	8.99	15.0	1.46
F pr			
Stage x Dips	0.044	0.044	NS
G,W vs P,R x 1MCP vs Ethep	0.005	0.005	NS
G vs W x 1MCP vs Ethep	0.034	0.034	NS
P vs R x 1MCP vs Ethep	NS	NS	NS

NS- No significant difference.

For color, strawberry fruit were observed to have an average hue angle of 27.0±0.92 º (0º – red, 90º yellow), and to have an average a* value of 29.7±0.67 on a scale of 0 to 60 (Table 1.3), indicating a bright red color on the surface of 'Albion' strawberry fruit. No significant treatment effects were observed for fruit color (Attached Table 1).

Table 0.3 - Visible color parameters red color (a*) and Hue angle (h^o) in ripe 'Albion' strawberry fruit treated with Ethephon, 1MCP or water plus an absolute control (No Dip) at four development stages (Green, White, Pink and Red). The differences between treatments within a development stage greater than twice the standard error of the mean (SEM) indicate significant difference.

Treatment	a*	Hue angle (degrees)
NoDip	29.5	25.3
Green 1MCP	29.6	26.7
Green Ethephon	29.4	26.3
Green Water	28.9	26.2
White 1MCP	29.9	28.1
White Ethephon	28.1	25.9
White Water	31.0	28.1
Pink 1MCP	29.5	27.4
Pink Ethephon	28.9	25.8
Pink Water	30.9	27.6
Red 1MCP	30.4	27.6
Red Ehtephon	30.0	29.4
Red Water	30.5	27.7
Grand Mean	29.7	27.1
SEM	0.68	0.92
F pr		
Stage x Dips	NS	NS
G,W vs P,R x 1MCP vs Ethep	NS	NS
G vs W x 1MCP vs Ethep	NS	NS
P vs R x 1MCP vs Ethep	NS	0.076

NS- No significant difference.

1.3.3 Ethylene effects on sugar and organic acid content

In 'Albion' strawberry fruit, glucose and fructose were the most abundant sugars in ripe fruit subjected to all treatments, comprising 39.3% and 43.3% of total sugars, respectively (Table 1.4). Sucrose comprised only 17% of the total sugars suggesting high rates of sucrose hydrolysis in the ripe fruit. No treatment effect was observed for all ripe fruit treated at different ripeness stages (Attached Table 2).

Table 0.4 - Concentration of sucrose, glucose, fructose and total sugars (mg/ml) in ripe 'Albion' strawberry fruit treated with Ethephon, 1MCP or water plus an absolute control (No Dip) at four development stages (Green, White, Pink and Red). The differences between treatments within a development stage greater than twice the standard error of the mean (SEM) indicate significant difference.

Treatment	Sucrose (mg/ml)	Glucose (mg/ml)	Fructose (mg/ml)	Total Sugars (mg/ml)
NoDip	2.19	4.52	4.94	11.7
Green 1MCP	1.74	3.98	4.36	10.1
Green Ethephon	1.78	3.62	4.08	9.47
Green Water	1.84	3.84	4.24	9.92
White 1MCP	1.64	4.03	4.42	10.1
White Ethephon	1.64	4.33	4.81	10.8
White Water	2.21	4.54	4.96	11.7
Pink 1MCP	1.54	3.85	4.25	9.64
Pink Ethephon	1.56	4.13	4.57	10.3
Pink Water	1.57	4.18	4.65	10.4
Red 1MCP	1.83	4.67	5.10	12.2
Red Ethephon	1.87	4.15	4.60	10.6
Red Water	2.06	4.36	4.79	11.2
Grand Mean	1.80	4.17	4.60	10.6
SEM	0.234	0.250	0.275	0.756
F pr				
Stage x Dips	NS	NS	NS	NS
G,W vs P,R x 1MCP vs Ethep	NS	NS	NS	NS
G vs W x 1MCP vs Ethep	NS	NS	NS	NS
P vs R x 1MCP vs Ethep	NS	NS	NS	NS

NS- No significant difference.

For organic acids, as expected, citric acid was the predominant acid comprising 69.7% of total acids for all ripe fruit treated at different ripeness stages, which was followed by malic acid responsible for 23.9% of the total acids. Other organic acids measured included succinic and shikimic acids that were observed in small amounts and accounted for 7.9% and 0.5% of the total acids. resectively (Table 1.5). No treatment effect was observed for all ripe fruit treated at different ripeness stages (Attached Table 2).

Table 0.5 - Concentration of citric, malic, shikimic, succinic and total organic acids (ug/ml) in ripe 'Albion' strawberry fruit treated with Ethephon, 1MCP or water plus an absolute control (No Dip) at four development stages (Green, White, Pink and Red). The differences between treatments within a development stage greater than twice the standard error of the mean (SEM) indicate significant difference.

Treatment	Citric acid (ug/mL)	Malic acid (ug/mL)	Succinic acid (ug/mL)	Shikimic acid (ug/mL)	Total (ug/mL)
NoDip	2410	850	360	18.2	3350
Green 1MCP	2220	799	324	17.2	3420
Green Ethephon	2230	834	335	18.6	3400
Green Water	2270	783	331	16.9	3410
White 1MCP	2350	770	270	16.4	3450
White Ethephon	2320	802	307	17.0	3260
White Water	2290	709	236	19.6	3190
Pink 1MCP	2090	772	316	16.9	3520
Pink Ethephon	2360	835	311	14.9	3690
Pink Water	2450	877	347	19.1	3490
Red 1MCP	2360	839	278	19.3	3620
Red Ethephon	2430	834	333	19.0	3710
Red Water	2530	868	288	19.8	3630
Grand Mean	2330	813	311	17.9	3470
SEM	178	51.7	41.3	2.22	204
F pr					
Stage x Dips	NS	NS	NS	NS	NS
G,W vs P,R x 1MCP vs Ethep	NS	NS	NS	NS	NS
G vs W x 1MCP vs Ethep	NS	NS	NS	NS	NS
P vs R x 1MCP vs Ethep	NS	NS	NS	NS	NS

NS- No significant difference.

For traditional fruit quality measurements, ripe 'Albion' strawberry fruit had an average soluble solids content of 6.9±0.30 ºBrix, pH of 3.5±0.01 and titratable acid of 1.05±0.03% citric acid equivalents (Table 1.6). No treatment effect was observed for all ripe fruit treated at different ripeness stages (Attached Table 3).

Table 0.6 - Concentrations of soluble solids (SS – Brixº), pH and titratable acid (% citric acid) in ripe 'Albion' strawberry fruit treated with Ethephon, 1MCP or water plus an absolute control (No Dip) at four development stages (Green, White, Pink and Red). The differences between treatments within a development stage greater than twice the standard error of the mean (SEM) indicate significant difference.

Treatment	Soluble solids (SS – Brixº)	pH	Titratable acid (% citric acid)
NoDip	6.58	3.51	1.03
Green 1MCP	7.53	3.48	1.05
Green Ethephon	6.91	3.47	1.00
Green Water	7.50	3.49	1.13
White 1MCP	6.63	3.49	1.04
White Ethephon	6.50	3.50	1.08
White Water	6.42	3.50	1.05
Pink 1MCP	7.06	3.51	1.03
Pink Ethephon	6.84	3.49	1.07
Pink Water	7.59	3.50	0.953
Red 1MCP	7.01	3.49	1.06
Red Ethephon	6.48	3.52	1.12
Red Water	6.66	3.50	1.06
Grand Mean	6.90	3.50	1.05
SEM	0.304	0.017	0.032
F pr			
Stage x Dips	NS	NS	NS
.. G,W vs P,R x 1MCP vs Ethep	NS	NS	NS
.. G vs W x 1MCP vs Ethep	NS	NS	NS
.. P vs R x 1MCP vs Ethep	NS	NS	NS

NS- No significant difference.

1.3.4 Ethylene effects on amino acid metabolism

In 'Albion' strawberry fruit submitted to all treatments, asparagine, glutamine and alanine were the most abundant amino acids, comprising 40%, 18.7% and 13% of total amino acids, respectively, followed by glutamate, serine and aspartate that comprised 8.3%, 7.2% and 6.3% of total amino acids, respectively (Table 1.7). The other 13 amino acids identified were observed in very low quantity (Data not shown).

Table 0.7 - The six most abundant amino acids (nm/ml) identified in ripe 'Albion' strawberry fruit treated with Ethephon, 1MCP or water plus an absolute control (No Dip) at four development stages (Green, White, Pink and Red). The differences between treatments within a development stage greater than twice the standard error of the mean (SEM) indicate significant difference.

Treatment	Alanine (nm/ml)	Asparagine (nm/ml)	Aspartate (nm/ml)	Glutamate (nm/ml)	Glutamine (nm/ml)	Serine (nm/ml)	Total (nm/ml)
NoDip	452	1320	208	275	665	260	3380
Green 1MCP	350	119	182	246	502	204	2830
Green Ethephon	506	1200	202	249	656	268	3310
Green Water	375	1230	181	235	516	207	2920
White 1MCP	344	1280	180	250	516	190	2930
White Ethephon	479	1310	208	264	617	252	3360
White Water	367	1310	193	266	560	207	3080
Pink 1MCP	421	1290	199	263	608	212	3170
Pink Ethephon	408	1340	213	275	643	230	3340
Pink Water	456	1340	216	276	603	249	3360
Red 1MCP	436	1330	222	305	647	254	3410
Red Ethephon	410	1330	224	311	677	235	3400
Red Water	426	1360	218	281	614	250	3350
Grand Mean	418	1300	203	269	602	232	3220
SEM	31.8	61.1	12.4	16.0	50.2	23.2	179
F pr							
Stage x Dips	0.021	NS	NS	NS	NS	NS	NS
G,W vs P,R x 1MCP vs Ethep	<0.001	NS	NS	NS	NS	0.060	NS
G vs W x 1MCP vs Ethep	NS	NS	NS	NS	NS	NS	NS
P vs R x 1MCP vs Ethep	NS	NS	NS	NS	NS	NS	NS

NS- No significant difference.

The No Dip control was not significantly different than the average of the factorial treatments, but significant interactions between development stages and treatments were observed for alanine, phenylalanine and tryptophan content (Attached Table 4).

Based on the difference between treatments greater than twice the standard error of the mean (SEM), we observed that alanine content in ripe strawberry fruit that were treated with ethylene at the green development stage was 30.7% and 30% greater than in 1MCP or water treated fruit, respectively (Table 1.7). Similarly, ripe fruit that were treated with ethylene at the white development stage had 28% and 23.3% greater alanine content than 1MCP or water treated fruit, respectively. No treatment effect on alanine content was observed for fruit treated at the pink or red development stages.

For phenylalanine content, ripe fruit treated with ethylene at the green development stage had 44.6%, 33.5% and 33.5% greater content than those treated with 1MCP, water or

NoDip control, respectively. Ripe fruit treated with ethylene at the white development stage had 22%, 19.6% and 14.1% greater phenylalanine content compared to that in 1MCP, water or NoDip control treated fruit, respectively. Ripe fruit that were treated with ethephon at the pink development stage had 21.5% and 15.8% greater phenylalanine content than those subjected to the 1MCP or NoDip control treatments, respectively. Ripe fruit harvested one day after ethylene treatment had 25.3%, 27.8% and 25.7% greater phenylalanine content than those treated with 1MCP, water or No Dip control (Table 1.8).

For tryptophan content, ripe fruit that were treated with ethylene at the green development stage had 31.3%, 20% and 15% greater content than those treated with 1MCP, water or No Dip control. Ethylene treatment at the white development stage increased tryptophan content 31.2%, 28.2% and 18.7% in ripe fruit compared to that in 1MCP, water or No Dip control treated fruit respectively. Ethylene treatment at the pink development stage resulted in ripe fruit having 18.6% greater tryptophan content in ripe fruit than those fruit treated with 1MCP (Table 1.8).

Table 0.8 – Concentration of amino acids alanine (nm/ml), phenylalanine (nm/ml) and tryptophan (nm/ml) in ripe 'Albion' strawberry fruit treated with Ethephon, 1MCP or water plus an absolute control (No Dip) at four development stages (Green, White, Pink and Red). The differences between treatments within a development stage greater than twice the standard error of the mean (SEM) indicate significant difference.

Treatment	Alanine (nm/ml)	Phenylalanine (nm/ml)	Tryptophan (nm/ml)
NoDip NoDip	452	15.5	14.1
Green 1MCP	350	12.9	11.4
Green Etephon	506	23.3	16.6
Green Water	375	15.5	13.3
White 1MCP	344	14.1	11.9
White Etephon	479	18.0	17.3
White Water	367	14.5	12.5
Pink 1MCP	421	14.4	12.6
Pink Etephon	408	18.4	15.5
Pink Water	456	16.9	14.7
Red 1MCP	436	15.6	14.3
Red Etephon	410	20.8	14.2
Red Water	426	15.0	14.1
Grand Mean	418	16.5	14.0
SEM	31.8	1.13	1.03
F pr			
Stage x Dips	0.021	0.039	0.098
G,W vs P,R x 1MCP vs Ethep	<0.001	NS	0.012
G vs W x 1MCP vs Ethep	NS	0.007	NS
P vs R x 1MCP vs Ethep	NS	NS	NS

NS- No significant difference.

A principal component analysis (PCA) was also conducted to observe the interactive effects of treatments and development stages on 19 amino acids in ripe 'Albion' fruit. The biplot of the first 2 scores is shown in Figure 1.2. Score 1 accounted for 60% and Score 2 accounted for 13% of the variance of the data. Together they represent more than 70% of the variance.

The Score 1 showed that all amino acids are negatively related to water and 1MCP treatments at green and white development stages. The amino acids tyrosine, phenylalanine, and isoleucine, and to a lesser extent tryptophan, arginine, valine histidine and alanine, are associated with ethephon treatment of green and to a lesser extent white and pink. The amino acids glutamine, asparagine, aspartate and methionine are associated with the red development stage regardless of treatment, including the NoDip absolute control fruit (Figure 1.2).

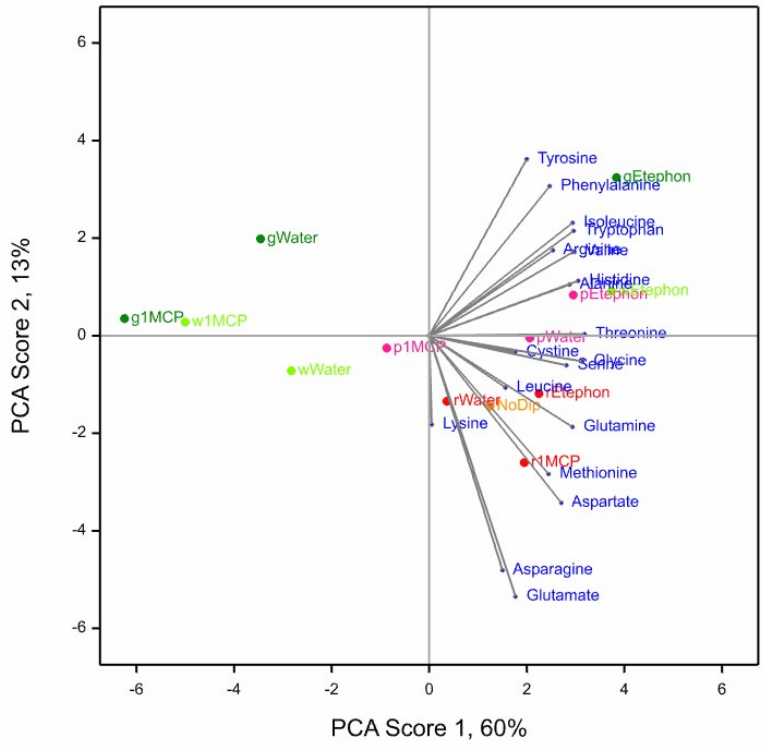

Figure 0.2- Biplot of the first two scores of the PCA for 19 amino acids observed in ripe 'Albion' strawberry fruit treated with Ethephon, 1MCP or water plus an absolute control (No Dip) at four development stages (Green, White, Pink and Red).

1.4. DISCUSSION

1.4.1 Ethylene effects on strawberry ripening rate, size and firmness

The rate of 'Albion' strawberry fruit ripening measured as red color development was not affected by any treatment. Both 1MCP and ethylene treatments did not change the rate of strawberry ripening on the plant, suggesting that an internal regulation of plant hormones is not easily affected by these one-time exogenous treatments. In case of the ethylene treatment, the absence of effects on rate of ripening suggests that, during normal development and maturation (fruit on the plant), ethylene may not play a major role in strawberry fruit ripening in agreement with its non-climacteric behavior. No previous studies have been reported to avaliate the role of ethylene during strawberry ripening on the plant.

In the present study, fruit weight, length, diameter and firmness were similar to those reported for 'Albion' grown in the field under a plastic greenhouse in Mexico, which were reported to be 17.6 ±1.6 g weight, 38.8 ± 0.9 mm length, 29.5 ± 1.3 mm diameter, and 3.8 ± 0.2 N firmness (ORNELAS-PAZ et al., 2013).

For our results, treatment of green fruit with ethylene resulted in greater mass and larger diameter than 1MCP or water treated ripe fruit, while ethylene treatment at the pink stage resulted in smaller and harder strawberry fruit.

Although there are no similar studies that have reported ethylene effects on attached strawberry fruit development and ripening, in the present study ethylene treatment of green fruit increased tyrosine content, which could increase auxin production and might contribute to the increase of mass and diameter observed in those fruit at ripening (Figure 1.2). On the other hand, the ethylene effect on ripe strawberry fruit treated at the pink development stage (smaller and harder fruit) might be related to visual color production and early immature harvest, since the harvesting point was made visually.

In this study, ethylene treatment did not induce fruit softening nor did 1MCP delay softening at ripening. No post-harvest assessments were conduced in the current study to observe a possible effect in firmness during storage, but it would be interesting to assess post-harvest effects in order to observe possible treatment effects on strawberry shelf life.

Previous studies using detached strawberry fruit, without any hormonal influence of the plant, have reported that 1-MCP delays post-harvest changes in ripe strawberry fruit firmness (JIANG; JOYCE; TERRY, 2001), while ethylene treatment increased respiration rate and accelerated cell wall degradation (ELMI et al., 2017). In a similar way, in white detached strawberry fruit, 1MCP treatment reduced the activity of the softening enzymes complex (*PGal* and *β-Gal*), while ethylene treatment showed an increase in its activity, suggesting that ethylene treatment can accelerate cell wall degradation (VILLARREAL; MARTÍNEZ; CIVELLO, 2009). As well, the content of galacturonic acid and pectin was reduced in ethylene-treated fruit, while opposite effects were observed in fruit treated with 1-MCP (VILLARREAL et al., 2016).

1.4.2 Ethylene effects on color formation

Fruit color is one of the most important quality characteristics for strawberries with respect to human consumption. In ripe strawberry fruit, anthocyanins are a group of flavonoids that provide red pigmentation and act as strong antioxidants (AABY et al., 2012). In this study, we observed the anthocyanin P3g to be the predominate anthocyanin followed by C3g for all ripe fruit treated at different ripeness stages. The anthocyanin pelargonidin (mainly 3-O-glucoside, P3g) has been reported to be the predominant pigment in other strawberries and has been associated with bright red color, while cyanidin (mainly 3-O-glucoside, C3g), usually found in less quantity, is associated with dark red color (CEREZO et al., 2010; MUÑOZ et al., 2011). The anthocyanin composition found in ripe ‘Albion’ strawberry in this study is therefore

consistent with its bright red color. In previous studies with 27 cultivars of strawberry, pelargonidin 3-O-glucoside comprised 60–95% to the total anthocyanin content (AABY et al., 2012).

In the present study we observed a positive effect of ethylene on anthocyanin content in ripe 'Albion' strawberry fruit treated at the white stage, showing that even in fruit that are developing and ripening on the plant, a one-time ethylene treatment can cause a positive effect that is persistent until harvest. In agreement, previous studies have shown that most changes associated with ripening start at the white stage, when the fruit has reached almost its final size and the accumulation of anthocyanins has begun (AHARONI, 2002; PERKINS-VEAZIE, 1995). Trainotti et al., (2015) reported that strawberry fruit were more responsive to treatment with exogenous ethylene at the white stage than at later stages. Similarly, anthocyanin content increased and chlorophyll levels decrease in detached strawberry fruit treated with ethephon at the white stage, followed by storage at 22 ºC for 48 h, while the opposite was observed in fruit treated with 1-MCP (VILLARREAL et al., 2010; VILLARREAL; MARTÍNEZ; CIVELLO, 2009). In the same way, transient downregulation of the ethylene biosynthesis-related gene *FaSAMS1* and the signaling gene *FaCTR1* at white stage can inhibit fruit pigment formation, that interestingly can be partially recovered by ethephon treatment (SUN et al., 2013).

It is important to remember that in the present study, while ethylene treatment at the white development stage increased anthocyanin content in ripe strawberry fruit, the 1MCP treatment did not inhibit this response. This suggests that there was no inhibitory effect on strawberry ethylene receptors and that a different ethylene signal transduction in attached strawberry fruit may occur. For example, anthocyanin synthesis may be dependent on plant signals and not only coordinated by fruit ethylene receptors, since an alternative pathway for ethylene signal transduction has been suggested by Zhang et al., (2014).

In the present study, ripe fruit from all treatments had low hue angle (27º) and an a* value of 29.7 indicating a light red color that agrees with high P3g content (Table 1.3). In strawberries, typical hue angle values range from ~26 to 49 (HERNÁNDEZ-MUÑOZ et al., 2008). In agreement, Ornelas-Paz et al., (2013) observed lower hue angle in 'Albion' strawberry fruit (h° = 18.2), suggesting high concentrations of bright red pigments.

Although anthocyanin content was significantly increased by ethylene treatment in white fruit, the treatment effect on anthocyanin content was not reflected in differences in visible color for all ripe fruit treated at different ripeness stages. In agreement, previous studies reported

that alterations of anthocyanin pigmentation may not be reflected in changes in the visible color of strawberry fruit (CORDENUNSI; NASCIMENTO; LAJOLO, 2003).

According to our results from strawberry fruit grown inthe greenhouse in Canada, all ripe fruit treated at different ripeness stages had very low phenolic compound content, reaching only 32.6 mg GAE/100 g, while in 'Albion' ripe strawberry fruit grown in the field under a plastic greenhouse in Mexico, phenolic compounds were observed to be 195.6 mg GAE/100 g (ORNELAS-PAZ et al., 2013), suggesting that phenolic content of strawberries is variable due to growing conditions.

Previous studies have reported that ethylene treatment in detached green and white strawberry fruit increased phenolic compounds content (AYUB et al., 2018b; LOPES et al., 2015), while 1MCP treatment in detached white strawberry fruit significantly reduced phenolic compounds content (VILLARREAL et al., 2010). However, in the present study, no Ethylene or 1MCP treatment effect on phenolic compound content was observed for all ripe fruit treated at different ripeness stages. This suggests that during fruit development and ripening, the content of phenolic compounds in attached fruit is highly dependent on mother plant metabolism and is not easily changed by a one-time treatment of ethylene or 1MCP.

1.4.3 Ethylene effects on sugar and organic acid metabolism

The content of sugars and organic acids plays a significant role in the overall flavor of ripe fleshy fruit. Previous studies have reported fructose, glucose and sucrose as the major soluble sugars (FAIT et al., 2008; MERCHANTE et al., 2013; MOING et al., 2001; ZHANG et al., 2011) and citric and malic acids as the main organic acids in ripe strawberry fruit (CORDENUNSI; NASCIMENTO; LAJOLO, 2003; MERCHANTE et al., 2013; MOING et al., 2001; ORNELAS-PAZ et al., 2013).

In the present study, higher content of glucose and fructose than sucrose for sugars, and higher content of citric acid followed by malic acid for organic acids were observed for all ripe 'Albion' strawberry fruit treated at all development stages. We observed lower soluble solids content (6.9 ºBrix) and pH (3.5) and higher titratable acids (1.05 % of citric acid) compared with previous studies that reported soluble solids around 9.0, pH of 3.8 and titratable acids of 0.7% (ORNELAS-PAZ et al., 2013).

Although some studies have reported that exogenous 1MCP and ethylene treatment can affect sugar and organic acid accumulation in specific developmental stages of strawberry, in the present study, effects were not observed any treatment.

In transgenic strawberry plants that are partially insensitive to ethylene, citric and malic acids were very low in white fruit, but similar to red ripe control fruit (MERCHANTE et al., 2013). In ripe strawberry fruit subjected to continuous ethylene supplementation (50 μL L^{-1}) during 6 days of cold storage,, respiration rate and sucrose hydrolysis (glucose and fructose increased) were enhanced and organic acids declined (ELMI et al., 2017). In a similar way, ethylene treatment was reported to increase total sugar and decrease titratable acidity in green and white detached strawberry fruit (LOPES et al., 2015), but no effect was observed in total sugars when ripe strawberries were treated with ethylene (LOPES et al., 2015; VILLARREAL et al., 2010, 2016).

Previous studies have reported that in detached immature white strawberry fruit, total sugars (VILLARREAL et al., 2010) and neutral sugars (VILLARREAL et al., 2016) increased in ethylene-treated fruit and decreased in 1-MCP-treated fruit, but no effect of ethylene or 1-MCP treatment was reported on strawberry titratable acidity and pH (VILLARREAL et al., 2010). According to the authors, the effect on sugars might be related to degradation of cell wall components, since this is the only possible source of simple sugars in detached strawberry, sincesucrose is no longer translocated into the fruit from photosynthetic tissues after harvest. In the same way, no effect on the accumulation of sugars and organic acids should be expected to be observed in the present study, since the treatments were carried out on fruit attached to the mother plant. These fruit, received simple sugars (sucrose) directly from photosynthesis and converted them into organic acids. Therefore, these fruit were dependenton the plant metabolism and the one-time treatment did not induce change. In agreement, no change in strawberry ripening rate was observed in this experiment as discussed before. Since we can not expect a great change in the hormonal control of an entire plant from a one-time stimulus to a few fruit that are connected to the mother plant for a short time until ripening, this may justifywhy no sugaror acid alteration was observed.

1.4.4 Ethylene effects on amino acid metabolism

In ‘Albion’ strawberry fruit, asparagine, glutamine and alanine were the most abundant amino acids for all ripe fruit treated at different ripeness stages, comprising around 40, 18 and 13% of total amino acids respectively. Previous studies have reported that asparagine, glutamine and alanine are the major free amino acids in strawberry fruit and act as nitrogen reservoirs and sources in the cells (PÉREZ et al., 1992). During the development of strawberry fruit, the total amino acid content decreased gradually as they are utilized in protein synthesis

(FAIT et al., 2008; ZHANG et al., 2011), but can increase in ripe and over-ripe fruit, which might be related to cell degradation (KNEE; SARGENT; OSBORNE, 1977). Amino acids are another soluble constituent of strawberries, and may also directly affect fruit taste (MOING et al., 2001).

In the present study, for all 19 amino acids analyzed in ripe 'Albion' strawberry fruit, only alanine, tryptophan and phenylalanine content were positively affected by ethylene treatment. Ethylene treatment early in development (Green and White stage) increased alanine content suggesting that ethylene might increase pyruvate formation to provide substrate for alanine production. Alanine is reported as an amino acid that changes during strawberry ripening (PÉREZ et al., 1992) and might serve as a precursor of ethyl esters, which are volatile compounds very abundant in strawberry fruit aroma (PEREZ et al., 2002). According to Moing et al., (2001), the most aromatic strawberry cultivar also had the highest alanine concentration during maturation.

In the present study, ethylene treatment applied at all development stages resulted in ripe fruit having greater phenylalanine content, while tryptophan content was positively affected by ethylene treatment at the green, white and pink development stages. Both aromatic amino acids are known to be the precursors for the biosynthesis of anthocyanins and flavonoids through the phenylpropanoid pathway (ZHANG et al., 2011). In this study, higher phenylalanine and tryptophan content in fruit treated with ethylene at the white development stage agrees with higher anthocyanin content observed in ripe 'Albion' strawberry fruit. This suggests an important role of ethylene in strawberry color formation and amino acid production and supports the idea that color and amino acid metabolism can be affected by one-time exogenous ethylene treatment in attached strawberry fruit.

In the PCA analysis, amino acids were negatively related to 1MCP and water treatments early in development (green and white). A positive effect of ethylene treatment at the green, white and pink development stages was observed on the amino acids tyrosine, phenylalanine, isoleucine, tryptophan, arginine, valine, histidine and alanine.

No 1MCP treatment effect was observed for any of the amino acids, suggesting that in attached strawberry fruit, the one-time exogenous 1MCP treatment was not enough to inhibit internal ethylene signaling or that another ethylene signal transduction pathway can be activated in strawberry fruit to supply the ethylene receptors inhibition as discussed before.

1.5 CONCLUSION

Our results showed that one-time exogenous ethylene or 1MCP treatments are not able to change the rate of strawberry ripening when fruit mature and ripen on the plant. Consequently, no changes in sugar and organic acid metabolism were observed. Some treatment effect was observed in biometrical characteristics suggesting that ethylene treatment at the green development stage may improve fruit growth while ethylene treatment at the pink stage may inhibit fruit expansion, resulting in smaller and harder fruit at ripening. As no difference in days between treatment and harvest were observed, we can suggest that pink fruit treated with ethylene developed a good red color, while they were not actually in full ripe stage at harvest. However, no similar reports were found in the to support this hypothesis.

Exogenous ethylene treatment at the white development stage was able to increase anthocyanin content at ripening. Although an increase in red visible color was not observed, the higher content of anthocyanins imparts higher antioxidant activity and gives evidence that the red strawberry coloration is directly ethylene-dependent and may be affected by exogenous treatments. As well, alanine and the aromatic amino acids tryptophan and phenylalanine, which may act as anthocyanin precursors, were positively affected by ethylene treatment at the green and white, and green and red development stages, respectively. Besides that, PCA analysis of all 19 amino acids were grouped close to ethylene treatment for all development stages, showing a good correlation between ethylene and amino acid metabolism.

No 1MCP treatment effect was observed for any of the variables measured at all development stages, suggesting no inhibition of ethylene receptors in strawberries attached to the plant, the inhibition by 1MCP was not persistent to affect changes in metabolism until harvest, or that a different ethylene signal transduction independent of the ethylene receptors could be active in attached strawberry fruit.

1.6 ACKNOWLEDGMENT

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Chapter - SUGAR METABOLISM IN NON-CLIMACTERIC 'YELLOW' MELON (*Cucumis melo* L.) FRUIT DURING MATURATION.

Abstract: The quality of melon fruit is determined by its sugar content. Understanding melon sugar metabolism could contribute to the development of new production management and post-harvest tools to provide consumers with a higher quality product. Next generation sequencing (NGS) or high-throughput techniques and metabolomics technologies are useful tools to reveal the transcriptomic change during development and ripening stages of the melon. In our previous work transcription analysis was performed on small green fruit (10 days after pollination - D.A.P.) and full ripe fruit (40 D.A.P.) of nonclimacteric melons using RNA-seq on the Ion Torrent Proton ™ platform. From fruit sugar metabolism 17 ESTs (expressed sequence tag) were differentially expressed between immature and mature fruit and 10 important genes were described and compared with the literature (CmAAG2, CmNAG2, CmAIN2, CmINH1, putative CmINH-Like3, CmUGE3, CmSUS1, CmSUS2, CmSPS2, and CmSPP1). These genes were analyzed by relative expression of small green (10 D.A.P), large green (20 D.A.P), color change (30 D.A.P) and full ripe (40 D.A.P) fruit. Genes that encode enzymes evolved in sucrose catabolism, for energy supply, were highly expressed at small green (CmSUS2) and large green melon fruit (CmNAG2 and UGE3), while genes evolved in the inhibition of sucrose catabolism were highly expressed at small green fruit (CmINH-Like3) suggesting its importance during all stages of non-climacteric fruit development and ripening. Genes that encode enzymes involved in sucrose synthesis, for sugar accumulation, were more highly expressed in large green melon fruit (CmSPP1) in complementation with those genes at the color change development stage, which were also well expressed in full ripe melon fruit (CmSUS1 and CmSPS2). The CmAAG2 and CmIHN1 genes were weakly expressed and these results were not showed.

Key Words: *Cucumis melo*; Transcriptomic; Ripening, Biotechnology, Bioinformatics.

List of acronyms: AAG - Acid alpha galactosidase; AIN - Acid invertase; CIN - Cell wall invertase; D.A.P. - Days after pollination; DEGs - Differentially expressed genes; EST - Expressed sequence tag; FK - Fructokinase; GK - Galactokinase; HXK – Hexokinase; ID - identification number; INH - Invertase inhibitor; mRNA - messenger RNA; NAG - Neutral alpha galactosidase; NIN - Neutral invertase; NGE - Next generation sequencing; PCR - Polymerase chain reaction; RPKM - Reads per kilobase million; RT-qPCR - Relative quantitative real-time polymerase chain reaction; RFOs - Raffinose family oligosaccharides; SPS - Sucrose-phosphate synthase; SPP - Sucrose-phosphate phosphatase; SUS - Sucrose synthase; UDP-glc - UDP-glucose; RNA - Ribonucleic acid.

2.1 INTRODUCTION

The melon (*Cucumis melo* L.) belongs to the Cucurbitaceae family, which contains numerous species differing greatly in fruit size, shape and color (SCHAFFER; PARIS, 2016). Due to an extensive polymorphism, the melon has been classified into 19 botanical groups (PITRAT, 2015). In Brazil, *Inodorus* and *Cantaloupe* are the most representative botanical groups of melon and production of these melons in Brazil was around 596 thousand tons in 2016 (FAO - http://www.fao.org/faostat/en/#data/QC).

Different from most of the fleshy fruits, melon include both climacteric and non-climacteric cultivars in their botanic groups. Typical climacteric melon genotypes include *Cantaloupe* (such as Charentais and Védrantais melon cultivars) and *Reticulatus* (such as Dulce melon cultivar) groups. Fruit maturity of climacteric melons is characterized by a respiration peak followed by the autocatalytic synthesis of ethylene, and the development of strong aroma, orange pulp, ripening, and stem abscission. These fruit typically have a short shelf life with rapid lost of firmness and flavor. In contrast, non-climacteric melon genotypes that include the *Inodorus* group (such as 'Yellow', 'Piel del Sapo', 'Honey Dew' and 'Hami' melon), have little or no ethylene synthesis, clear or white pulp, low aroma (explained by the ethylene-dependent volatile esters synthesis pathway), no ripening stem abscission and long shelf life (LEIDA et al., 2015; PECH et al., 2012; PECH; BOUZAYEN; LATCHÉ, 2008; SALADIÉ et al., 2015; ZHENG; WOLFF, 2000).

Both climacteric and non-climacteric melon fruit accumulate high levels of sucrose as they ripen (SALADIÉ et al., 2015). Sucrose, glucose, and fructose are the major soluble sugars, and sucrose is the predominant sugar in the melon at maturity (SHIN et al., 2017) being stored in the vacuoles of the pericarp parenchyma cells (DAI et al., 2011). During fruit development, sugar is necessary for energy supply, generates turgor for fruit cell enlargement and accumulates during maturation contributing to fruit taste (OHKAWA et al., 2010).

Sucrose is responsible for fruit sweetness, one of the most important organoleptic traits for the consumer (YAMAGUCHI et al., 1977). The accumulation of sucrose in melon fruit is determined by the metabolism of carbohydrates in the fruit and can be provided from three sources: (1) Melon plants export the raffinose family oligosaccharides (RFOs) such as raffinose and stachyose, as well as sucrose from photosynthetic sources, such as leaves, to sink tissues, such as developing fruit, (2) Imported RFOs are hydrolysed by multiple isoforms of two different families of alpha-galactosidases (either neutral-alkaline NAG or acidic AAG pH optima), keeping the sucrose following the removal of galactose and/or, (3) Sucrose resynthesis,

which is a very important pathway and involves many key enzymes of sugar metabolism. Released galactose is phosphorylated by galactokinase (GK) and the resulting galactose 1-phosphate, can either be respired or used for sucrose synthesis. In sucrose synthesis, galactose 1-phosphate is transformed to glucose-1-P by the actions of UDP-gal/glc pyrophosphorylase (UGGP) and converted to other hexose-phosphates, providing the substrates for synthesis of sucrose by sucrose-phosphate synthase (SPS) and sucrose-phosphate phosphatase (SPP). Sucrose unloaded from the phloem can be hydrolyzed in the apoplast by cell wall invertase (CIN). The resulting hexose sugars (glucose and fructose) are imported into cells by monosaccharide transporters, phosphorylated by hexokinase (HXK) and fructokinase (FK) and used for respiration or sucrose resynthesis. Sucrose can also be unloaded symplastically. Within the cell, sucrose can be synthesized in the cytosol by sucrose synthase (SUS) from fru and UDP-glc, or, in the reverse reaction, sucrose can be cleaved to fru and UDP-glc for energy production. Sucrose also can be hydrolyzed to fructose and glucose for energy production by neutral invertase (NIN), or imported into the vacuole for storage or hydrolyzed by vacuolar acid invertase (AIN). The invertase activity can be post-translationally regulated by invertase inhibitor proteins (INH) (Figure 3.1) (BURGER; SCHAFFER, 2007; CHAYUT et al., 2015; DAI, 2006; DAI et al., 2011; LEIDA et al., 2015; SALADIÉ et al., 2015).

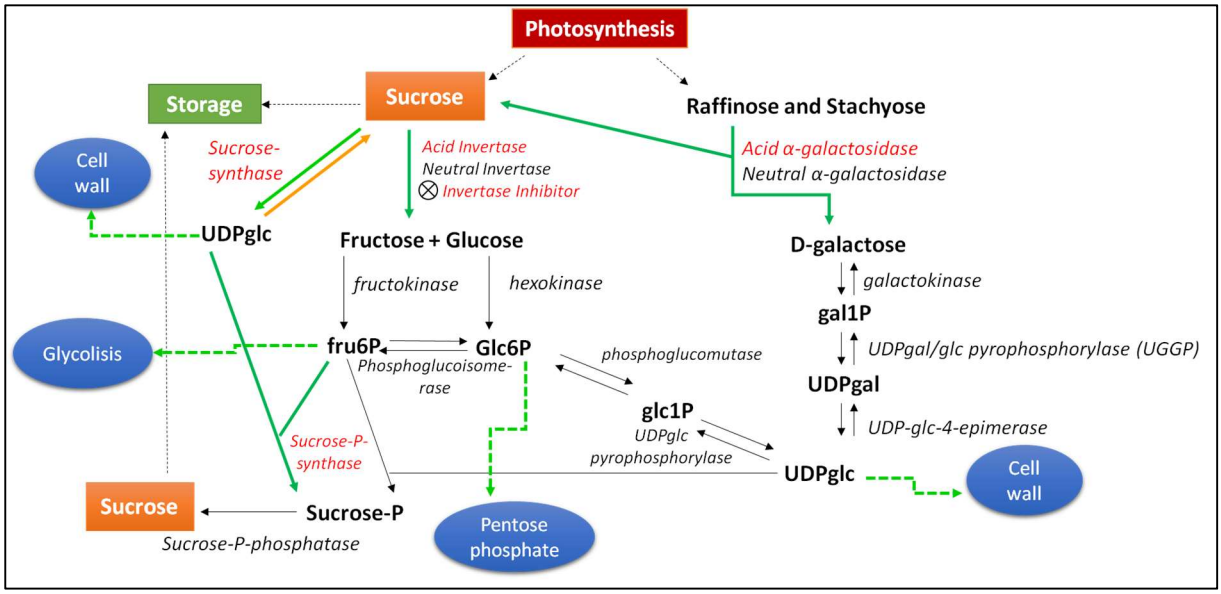

Figure 0.1 - The metabolic pathway of Raffinose Family Oligosaccharides (RFOs) in melon and different routes leading to the accumulation of sucrose. UDPglc - UDP-glucose; Fruc6P - fructose-6-phosphate; Glc6P - glucose-6-phosphate; Glc1P - glucose-1-phosphate; Gal1P - galactose-1-phosphate. Adapted from Chayut et al. (2015) and Dai et al. (2011).

In climacteric melon fruit, after the sugar accumulation period, senescence causes sugar catabolism, taste deterioration and lose of shelf life. The high activity of soluble acid invertase (AIN), which uses sucrose to produce glucose and fructose to be consumed by respiration, might both limit the accumulation of sucrose during climacteric ripening and imparts a stale flavor to the fruit, due to increase in organic acids formation, such as malate, by increase in respiration rate (BURGER; SCHAFFER, 2007; DAI et al., 2011; LEIDA et al., 2015; SALADIÉ et al., 2015). In contrast, previous studies have shown that soluble AIN gene activity is almost 10 times lower in non-climacteric melon fruit and Invertase Inhibitor proteins (INH), are about 30 times higher, preventing sucrose catabolism in non-climacteric melon fruit, which might be an important factor contributing to the long shelf life of this kind of melon (SALADIÉ et al., 2015). In addition, sucrose content can be used as a marker of melon fruit ripeness, as non-climacteric varieties do not show stem abscision or other signs of ripeness (SALADIÉ et al., 2015).

The evidence that shelf life of melon can be related to sugar accumulation metabolism described in the paragraph above and the relevance of sugar content as a ripeness marker in non-climacteric melon, make sugar metabolism studies important to develop new management tools that can improve quality of commercial melons. In this way, comprehensive biomolecular studies that elucidate the metabolic pathways of sugar metabolism are essential tools. Indeed, next-generation sequencing (NGS) or high-throughput techniques and metabolomics technologies allow the generation of massive amounts of information across a range of developmental stages. The NGS sequence identification only is possible because of previous database studies such as the Cucurbit Genomics Database (CuGenDB - http://www.icugi.org) and MELONOMICS melon genome (GARCIA-MAS et al., 2012).

Therefore, the aim of the present work is to elucidate aspects of the metabolism of sugar in 'Yellow' non-climacteric melon (*Cucumis melo* L. - *Inodorus* group), through previous NGE sequence data on the Ion Torrent Proton ™ platform, as well as plot the relative expression profiles of some of those genes during development and ripening using real-time PCR.

2.2 MATERIAL AND METHODS

2.2.1 Previous information

In a previous study (not published data), non-climacteric melon fruit of a 'Yellow' commercial genotype *(Cucumis melo var Inodorus)* was cultivated by Itaueira Agropecuária

SA company at São Paulo. Three biological replicate fruit were harvested at the small green (10 days after pollination - D.A.P.) and at the full ripe development stage (40 D.A.P.). The total RNA was extracted, purified for messenger RNA (mRNA), converted in cDNA and sequenced on the Ion Torrent Proton ™ platform (Thermo Fisher). Quality trimming of the reads samples was performed, and the reads were blasted in the melon reference genome, version 3.5 (GARCIA-MAS et al., 2012) available at MELONOMICS (https://melonomics.net). The Gaussian T-test was carried out, comparing Small Green results (used as testator - control) to Full Ripe results. A total of 2340 DEGs (differentially expressed genes) were observed with a p-value less than 0.05 and with the fold change greater than +2 and less than -2. In addition, the RPKM (Reads Per Kilobase Million) was also analyzed to quantify the expression of the ESTs (expressed sequence tag). Functional notation of the genes was performed using the Blast2GO software. A total of 17 DEGs were related to sugar metabolism (Table 2.1).

Table 0.1 - Differentially expressed sequences during the maturation of 'Yellow' melon (p-value <0.05), their respective short forms, identification number (ID) in the genome MELONOMICS, Fold Change and RPKM. Positive fold change values indicate higher expression in green fruits and negative values, higher expression in mature fruits.

DEG	Short Form	ID MELONOMICS	Fold Change	RPKM (SG)	RPKM (FR)
Acid α-galactosidase 2	CmAAG2	MELO3C011771	3.05	1.72	0.56
Putative acid α-galactosidase	CmAAG-LIKE1	MELO3C010698	3.68	6.28	1.70
Neutral α-galactosidase 2	CmNAG2	MELO3C023110	2.32	88.7	38.1
Putative neutral α-galactosidase	CmNAG-LIKE2	MELO3C009979	4.89	5.08	1.03
Putative neutral α-galactosidase	CmNAG-LIKE3	MELO3C015912	9.17	8.51	0.92
Acid invertase 2	CmAIN2	MELO3C005363	5.15	10.9	2.10
Cell wall invertase 2	CmCIN2	MELO3C024383	4.84	0.34	0.07
Cell wall invertase 4	CmCIN4	MELO3C009488	5.26	2.39	0.45
Invertase inhibitor 1	CmINH1	MELO3C022457	2.08	75.0	36.0
New putative invertase inhibitor	CmINH-LIKE3	MELO3C006266	6.01	208	34.6
Hexoquinase 3	CmHK3	MELO3C007677	31.2	0.25	0.008
New putative hexoquinase	CmHK-LIKE1	MELO3C003755	4.04	0.89	0.22
UDP-glucose epimerase 3	CmUGE3	MELO3C005640	3.33	35.7	10.7
Sucrose synthase 1	CmSUS1	MELO3C015552	-2.95	11.9	35.0
Sucrose synthase 2	CmSUS2	MELO3C025101	18.7	148	7.94
Sucrose phosphate synthase 2	CmSPS2	MELO3C020357	2.68	3.84	1.42
Sucrose phosphate phosphatase 1	CmSPP1	MELO3C009570	2.03	14.7	7.20

The DEGs, CmAAG2, CmNAG2, CmAIN2, CmINH1, CmINH-Like3, CmUGE3, CmSUS1, CmSUS2, Cm SPS2 and CmSPP1 genes were delimited as representatives of genes responsible for transcribing the main enzymes involved in sugar metabolism of melon fruit and

their relative expression was observed in the present study during non-climacteric melon development and ripening.

Genes with low RPKM (> 10) observed in the RNAseq analysis were not analyzed except for the genes CmAAG2 and CmSPS2 that had their relative expression analysed because of their importance to sugar metabolism of melon fruit. The CmINH-Like3 gene was choosing to be analysed due to it very high RPKM, even it is related as a putative invertase inhibitor gene. Genes such as cell wall invertases and hexokinases were not analyzed due to low RPKM and small importance to sugar metabolism of melon fruit.

2.2.2 Plant material

Non-climacteric melon fruit of the 'Yellow' commercial genotype (*Cucumis melo* var *Inodorus*) were cultivated by Itaueira Agropecuária SA company at São Paulo. Fruit were manually pollinated, and three biological replicates were harvested for each of four development stages. Small green fruit were harvested 10 days after pollination (D.A.P.), large green fruit were harvested 20 D.A.P., fruit in color change (visible yellow color) were harvested 30 D.A.P., and fruit at full ripe development stage were harvested 40 D.A.P (Figure 2.2).

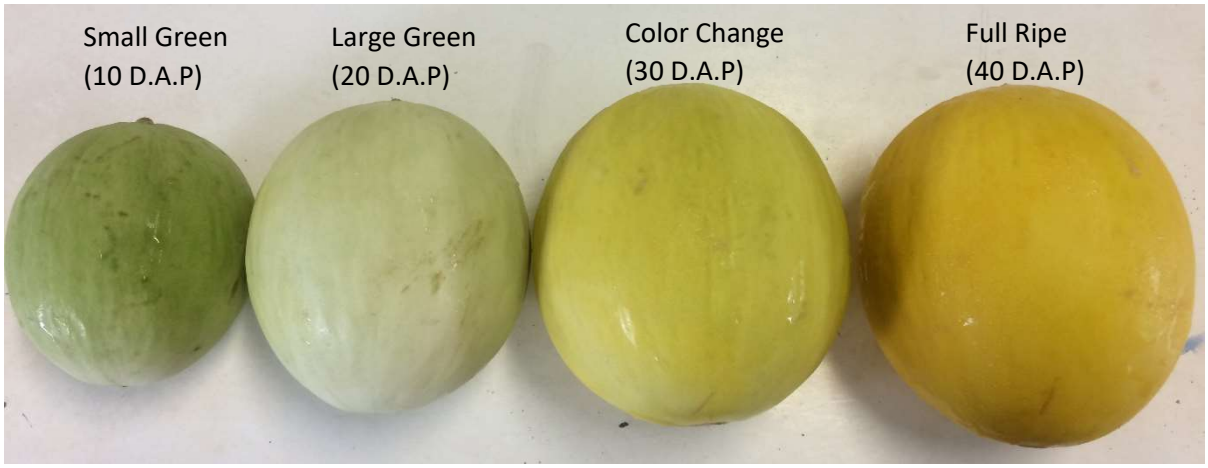

Figure 0.2 – 'Yellow' melon fruit at four development stages. From left to right are small green fruit (10 days after pollination (D.A.P.), large green fruit (20 D.A.P), fruit in Color Change (30 D.A.P.) and full ripe fruit (40 D.A.P).

The fruit were peeled, seeds removed and the mesocarp was immediately cut in small pieces, frozen in liquid nitrogen and stored at -80°C for molecular analysis.

2.2.2 Primer design and specificity test

Specific oligos were designed for each target gene of melon fruit sugar metabolism. The sequences were chosen using the "PrimerBlast" online program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were tested for qPCR using "Beacon designer" online program (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1). The Forward and Reverse sequence were blast against *Cucumis melo* genome in "Blastn" online program(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). To normalize the gene expression, two reference genes (RPS15 and RPL), that are constantly expressed, were chosen according to the reference (KONG et al., 2016) (Table 2.2).

Table 0.2 - Information on the Target genes and Reference genes used in this work.

Gene	Short Form	Primer 5' – 3'	Amp	Tº
Acid alpha-galactosidase2	CmAAG2	5' ATGATTGTTGGGCTGAACTTGTA 3' 5' CCAAGGGAACCAGGCATCTT 3'	178	-
Neutral alpha-galactosidase 2	CmNAG2	F – TTCCTGTGGTATCGATGGCG R – ATGAATGGTGTGGGAGGCAG	258	62ºC
Acid invertase 2	CmAIN2	F – CTCGATCTGTCAAAAAGCAGC R – TTCCCCAAACGCCCCTAAAG	213	62ºC
Invertase inhibitor 1	CmINH1	F - GAGGAGAAAGAAGGGGTGGTT R - CCATGGTGTGGATTGTATTTTGA	205	62ºC
New putative invertase inhibitor	CmINH1-Like3	F' CTCGCATTTTCCTTCGTTGT R' AAAGGTCCGCCGTGTCGCT	164	62ºC
UDPglc epimerase 3	CmUGE3	F – CTGTTGGGGAGAGTGTTGCT R – GCTTCGTCCGACCATAAGGA	203	62ºC
Sucrose synthase 1	CmSUS1	F - AACCGTGTTCATAGCCTCCG R – TCAGGACTTCCCCAAATGCC	195	64ºC
Sucrose synthase 2	CmSUS2	F - TCCAGATGCGAAGGGGACTA R – ATCCGGCTTTGCTTCCATGA	204	62ºC
Sucrose-P-synthase 1	CmSPS2	F - AGTTCGTTTCTTCGTTTGGCT R – TTGGCGCTTCTTTTGTGATGG	198	62ºC
Sucrose-P-phosphatase 1	CmSPP1	F' TATCGTCACTCTTCTCCAC R' CATGAGACGTGCTGAAGCG	182	60ºC
CmRPS15 - *c*ytosolic 40s **Ribosomal protein S15**	RPS15	F - GAAGCTGCGTAAAGCGAAAC R – GGTCTTTCCATTGTAAACTCCAA	132	62ºC
CmRPL -60s ***Ribosomal protein L***	RPL	F - CGACAATACTGGAGCCAAGAA R – CATCACCATATCTCCCACACAA	100	62ºC

The primers specificities were tested in conventional PCR using 50 ng of cDNA in a system containing 2 µL of buffer (10x), 0.6 µL of $MgCl_2$ (50 mM), 0.4 µL of dNTPs (10 mM), 1 µL of forward primer (10 pmol), 1 µL of reverse primer (10 pmol) and 0.2 µL of Taq DNA

polymerase enzyme (5 U\µL) (INVITROGEN). The amplification conditions were 2 min at 94◦C followed by 30 cycles of 45 s at 94°C, 30 s at specific primer temperature, and 30 s at 72°C, and final extension for 10 min at 72ºC. The amplifications were observed in 2.5% agarose gel electrophoresis with TBE buffer and compared with a 100bp BioLabs® molecular weight marker.

2.2.4 Total RNA extraction

The total RNA was extracted in biological triplicate (different fruit) of four development stages using the sodium perchlorate method, described for melon by Campos et al. (2017). The integrity of the total RNA was analyzed by 1% agarose gel electrophoresis with TAE buffer compared with 1 Kb Axygen® molecular weight marker. Those samples that showed sharpness in the bands 18S and 28S without signs of degradation were considered quality sample (Figure 2.3).

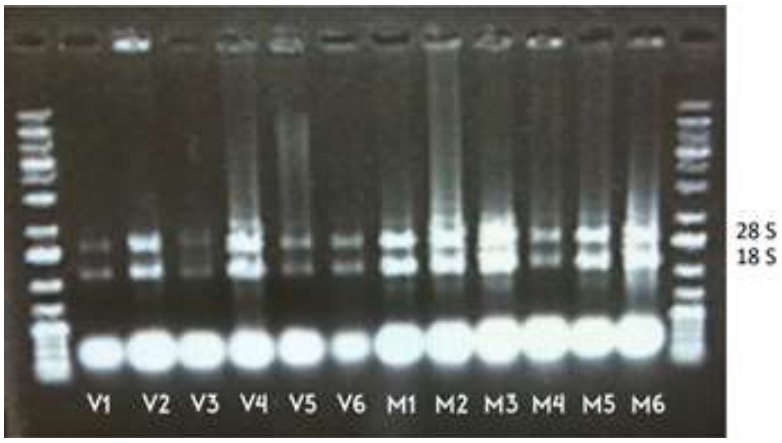

Figure 0.3 - Evaluation of total RNA from small green fruit (V1, V2 and V3), large green fruit (V4, V5 and V6), color change fruit (M1, M2 and M3) and full ripe fruit (M4, M5 and M6) samples by 18S and 28S ribosomal RNA integrity in (1%) gel electrophoresis. M: molecular weight marker of 1 Kb (Axygen, Corning/New York/USA).

RNA quantification was performed using a Nanovue™ spectrophotometer, as well as the A260/A280 and A260/A230 absorbance ratios that characterize extraction purity. Pure samples were considered to have absorbance ratios close to 2.0 and 1.8, respectively (Table 2.3).

Table 0.3 - Quantification analysis and absorbance ratios measurements for RNA quality.

Sample	Replicate (different fruit)	Quantification (ng/ul)	A260/A280	A260/A230
Small green	1	222	2.16	1.70
Small green	2	478	2.07	1.70
Small green	3	340	2.12	1.63
Large green	1	520	1.91	1.87
Large green	2	320	1.96	1.70
Large green	3	215	2.03	1.60
Color change	1	510	2.08	1.76
Color change	2	630	2.08	1.80
Color change	3	1000	1.92	1.60
Full ripe	1	531	2.02	1.79
Full ripe	2	500	1.99	1.53
Full ripe	3	502	1.98	1.74

2.2.5 Synthesis of cDNA

The best quality samples were treated with the TURBO ™ DNase kit (Invitrogen) to remove genomic DNA residues from the extraction and were submitted to cDNA conversion by Maxima H minus First Strand cDNA Synthesis kit (Thermo Scientific) following manufacturer's instructions. The cDNA from each sample was tested in conventional PCR using the RPL Reference gene using the conditions described before in section 2.2.2.

2.2.6 Relative quantitative real-time polymerase chain reaction (RT-qPCR) analysis

RT-qPCR was performed on a LightCycler® Nano platform (Roche Diagnostics GmbH, Mannheim, Germany) using 100 ng cDNA in a system containing 1 µL forward and reverse primer (10 µM), 10 µL FastStart Essential DNA Green Master 2X (Roche), in a final volume of 20 µL. The amplification conditions were 10 min at 94◦C, 45 cycles of 15 s at 95°C, 20 s at specific primer temperature, and 20 s at 72°C, followed by a pre-melting curve for 10 s at 95ºC, initial melting curve for 60 s at 60ºC and final melting curve for 1 s at 97ºC. The relative gene expression analysis was performed in technical triplicate for each sample, plus a negative control without template.

The amplification efficiency for each gene was determined by seven-fold serial dilutions of pooled cDNA at concentrations of 800, 160, 32, 6.4, 1.28, 0.256 and 0.0512 ng μL^{-1}, performed in two technical replicates plus a negative control without template. A value of 2 ± 10% was considered good efficiency. To validate the use of the $2^{-\Delta\Delta Ct}$ method for relative gene expression calculation, the Target and Control Genes' amplification efficiencies were compared in a semi-log regression line. The ΔCT (CT target – CT reference) values of dilution curves were plotted vs. log input amount of cDNA and considered valid if the slope was close to zero (LIVAK; SCHMITTGEN, 2001).

The $2^{-\Delta\Delta Ct}$ method was used for calculation of relative gene expression according to Livak and Schmittgen, (2001). Values were normalized to that of RPS15 and RPL reference genes and the small green fruit was chosen as the calibrator and set to 1. Error bars represent standard errors of the mean of the normalized expression. The numerical values obtained with these calculations were transformed into graphics using Microsoft Excel.

2.3 RESULTS

2.3.1 alpha-Galactosidase gene

In our previous RNA-seq analysis two acid alpha-galactosidases genes (CmAAG1 and putative CmAAG-Like2) were identified and both were expressed more in small green than in full ripe melon fruit. The CmAAG1 gene was 3.05-fold reduced in full ripe (0.56 RPKM) compared to small green (1.72 RPKM) melons, and in a similar way, the putative CmAAG-Like2 gene was 3.68-fold reduced in full ripe melon fruit (1.70 RPKM) compared to small green (6.28 RPKM) melons (Table 2.1). The relative expression of the CmAAG2 gene was not consistent (data not shown), probably because it has a very low expression during `Yellow` melon development.

Three neutral alpha-galactosidase genes were observed in our previous RNA-seq analysis (CmNAG2, CmNAG-Like2 and CmNAG-Like3). All three were expressed more in small green than in full ripe fruit. The putative CmNAG-Like2 and Like3 genes were 4.84-fold and 9.17-fold higher in small green fruit than in full ripe and the RPKM level reduced from 5.08 to 1.03 and from 8.51 to 0.92, respectively (Table 2.1), but no relative expression analysis was performed for these putative genes because of their low RPKM (>10). The CmNAg2 gene was observed to be 2.32-fold higher in small green than in full ripe melon fruit with 88.7 and 38.1 of RPKM, respectively in RNA-seq analysis (Table 2.1). The relative expression analysis

showed that the CmNAG2 gene is actually expressed a little more in full ripe than in small green melon fruit. Using the intermediate development stages, it was possible to observe that the CmNAG2 gene expression pattern increases at the intermediate stages of fruit development and then decreases with ripening. The gene expression increased about 3.5-fold in large green and about 2-fold in color change melons compared to the gene expression level in small green melon fruit (Figure 2.4).

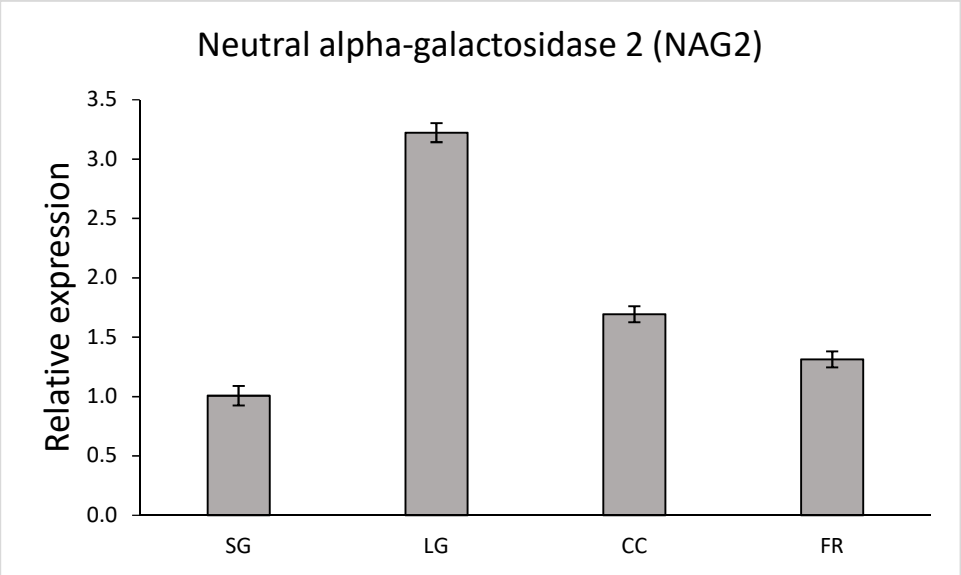

Figure 0.4 - Relative expression profile of neutral alpha-galactosidase 2 gene during fruit development and ripening. Samples are: SG, small green fruit; LG, large green fruit; CC, color change fruit (i.e. visible start of the ripening phase) and FR, full ripe fruit. Values have been normalized to SG, arbitrarily set to 1. Error bars represent standard errors of the mean of the normalized expression. N=3.

2.3.2 Invertases

In our previous RNA-Seq analysis three acid invertase genes were observed. Two of these genes were cell wall acid invertase genes (CmCIN2 and CmCIN4) and one was a soluble acid invertase gene (CmAIN2).

Both cell wall acid invertase genes showed about a 5-fold higher expression in small green than in full ripe melon fruit but the RPKM level of expression was too small and the relative gene expression analysis was not assessed.

The CmAIN2 gene was expressed 5.15-fold more in small green than in full ripe melons with 10.9 and 2.1 RPKM, respectively, in the previous RNA-Seq analysis (Table 2.1). The CmAIN2 gene was assessed in the relative expression analyze and a 4-fold expression increase was observed in the large green compared to the small green melon fruit, followed by about a

0.5-fold reduction in gene expression in color change and full ripe melon fruit compared with the gene expression level in small green melon fruit (Figure 2.5).

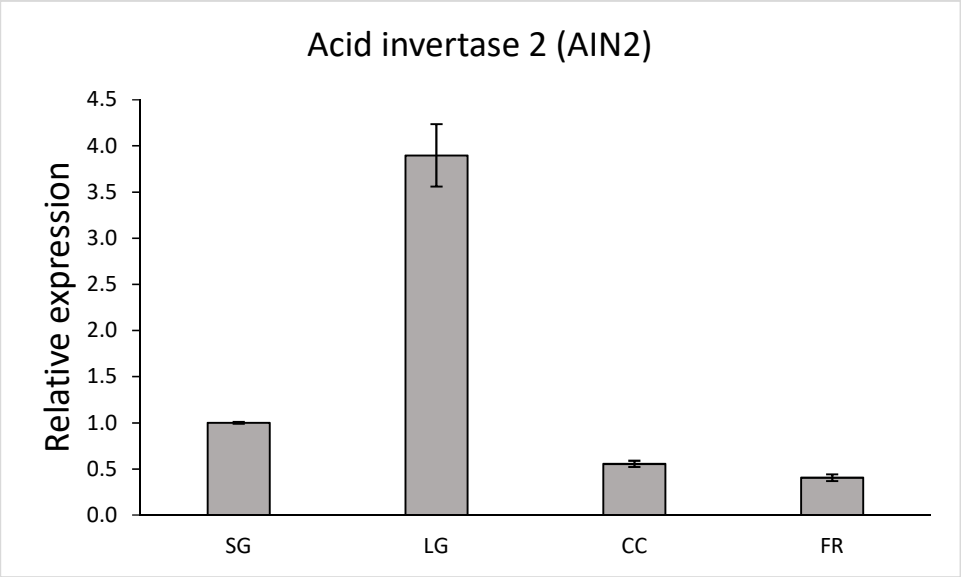

Figure 0.5 - Relative expression profile of acid invertase 2 gene during fruit development and ripening. Samples are: SG, small green fruit; LG, large green fruit; CC, color change fruit (i.e. visible start of the ripening phase) and FR, full ripe fruit. Values have been normalized to SG, arbitrarily set to 1. Error bars represent standard errors of the mean of the normalized expression. N=3.

2.3.3 Invertase inhibitors

In RNA-seq analysis two invertase inhibitor genes (CmINH1 and putative CmINH-Like3) were identified, and both were expressed more in small green than in full ripe melons. The RPKM of CmINH1 was 2.08-fold more expressed in small green (75.0) than in full ripe melon fruit (36.0) and the RPKM of putative CmINH-Like3 was 6.0-fold more expressed in small green fruit (208.0) than in full ripe melon fruit (34.6).

The relative gene expression assessed for CmINH1 showed a very low quantity making it unreliable for discussion (data not shown). However, the CmINH-Like3 gene expression was assessed and showed about 5-fold more expression in small green melon than in large green, color change and full ripe melon fruit (Figure 2.6)

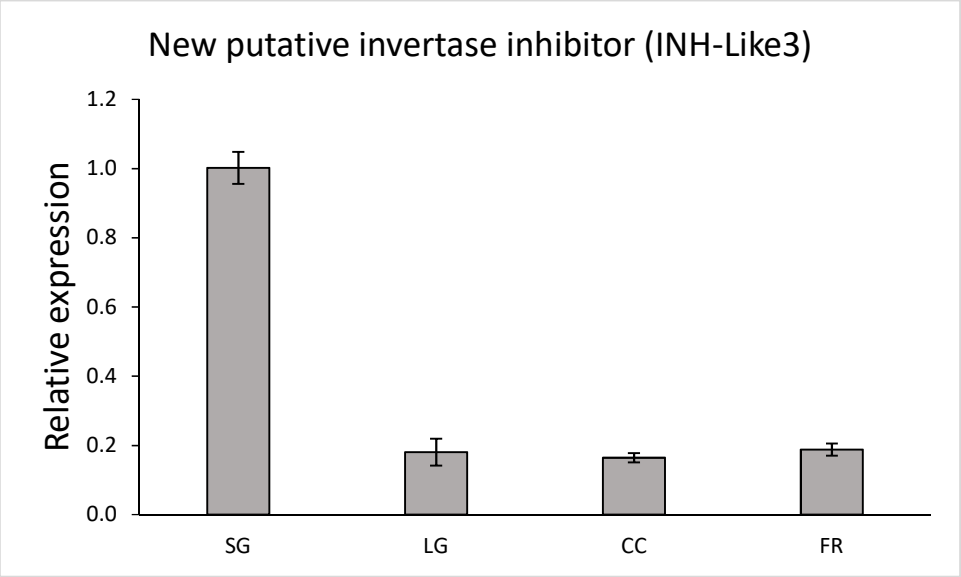

Figure 0.6 - Relative expression profile of new putative invertase inhibitor gene during fruit development and ripening. Samples are: SG, small green fruit; LG, large green fruit; CC, color change fruit (i.e. visible start of the ripening phase) and FR, full ripe fruit. Values have been normalized to SG, arbitrarily set to 1. Error bars represent standard errors of the mean of the normalized expression. N=3.

2.3.4 UDP-glc epimerase

Only a single gene of UDP-glc epimerase (CmUGE3) was observed in our previous RNA-seq analysis. The CmUGE3 gene was 3.33-fold more expressed in small green than in full ripe melon fruit, with a RPKM level of 35.7 and 10.7, respectively (Table 2.1). The relative expression analysis showed that the CmUGE3 gene was about 1.4-fold less expressed in full ripe fruit than in small green melon fruit, showing a similar expression level between immature and mature fruit. Observing the four development stages of melon it was possible to see that CmUGE3 gene expression increased about 2.5-fold from small green to large green fruit, and then, expression decreased in color change and full ripe fruit (Figure 2.7).

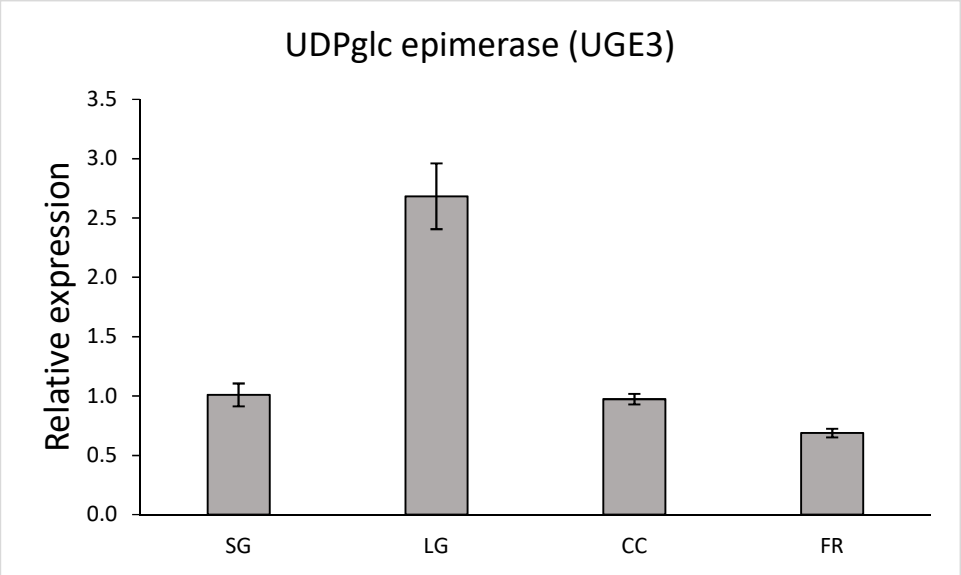

Figure 0.7 - Relative expression profile of UDPglc epimerase 3 gene during fruit development and ripening. Samples are: SG, small green fruit; LG, large green fruit; CC, color change fruit (i.e. visible start of the ripening phase) and FR, full ripe fruit. Values have been normalized to SG, arbitrarily set to 1. Error bars represent standard errors of the mean of the normalized expression. N=3.

2.3.5 Sucrose synthase

In our previous RNA-seq analysis, two sucrose synthase genes (CmSUS1 and CmSUS2) were identified. The CmSUS1 gene was the only one of 17 genes related to melon sucrose metabolism that had higher expression in full ripe than in small green fruit. The CmSUS1 gene expression level increased 2.9-fold from 11.9 RPKM in small green to 35.00 RPKM in full ripe fruit (Table 2.1).

In relative expression analysis we observed that CmSUS1 gene expression increase almost 7-fold from small green to color change fruit and almost 9-fold from small green to full ripe melon fruit (Figure 2.8).

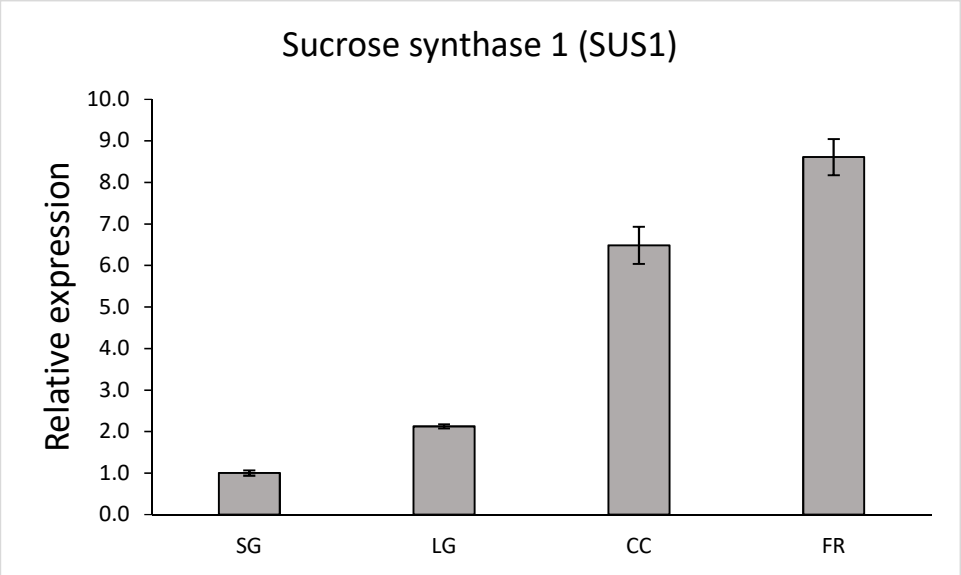

Figure 0.8 - Relative expression profile of sucrose synthase 1 gene during fruit development and ripening. Samples are: SG, small green fruit; LG, large green fruit; CC, color change fruit (i.e. visible start of the ripening phase) and FR, full ripe fruit. Values have been normalized to SG, arbitrarily set to 1. Error bars represent standard errors of the mean of the normalized expression. N=3

In the opposite way, the CmSUS2 gene showed high expression in small green fruit in our previous RNA-seq analysis. The RPKM for small green fruit was 148.6 but was 18.69-fold less in full ripe fruit being only 7.9 RPKM (Table 2.1). The same pattern was observed in the relative expression analysis. The CmSUS2 gene showed a gradual reduction in its expression during maturation, achieving very little expression in full ripe melon fruit (Figure 2.9).

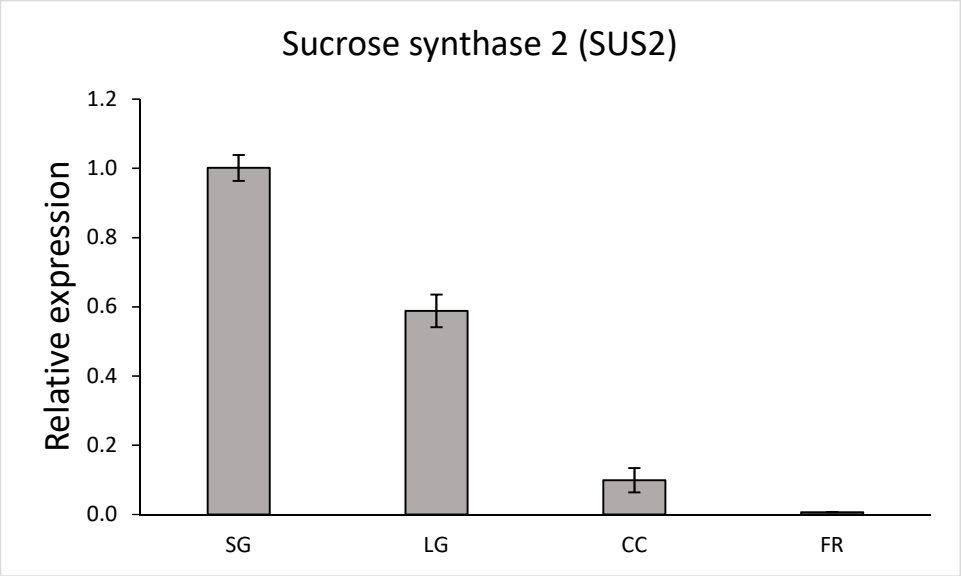

Figure 0.9 - Relative expression profile of sucrose synthase 2 gene during fruit development and ripening. Samples are: SG, small green fruit; LG, large green fruit; CC, color change fruit (i.e. visible start of the ripening phase) and FR, full ripe fruit. Values have been normalized to SG, arbitrarily set to 1. Error bars represent percentage standard errors of the mean of the normalized expression. N=3.

2.3.6 Sucrose-phosphatase synthase

CmSPS1 was the only sucrose-P synthase gene observed in our previous RNA-seq analysis. Expression was 2.68-fold less in full ripe (1.42 RPKM) than in small green (3.84 RPKM) melon fruit (Table 2.1).

Relative expression analysis showed similar expression of the CmSPS1 gene in small green and full ripe fruit. However, during fruit development and ripening, gene expression increased 0.6-fold from small green to large green fruit and increased about 4-fold from small green to color change melon fruit (Figure 2.10)

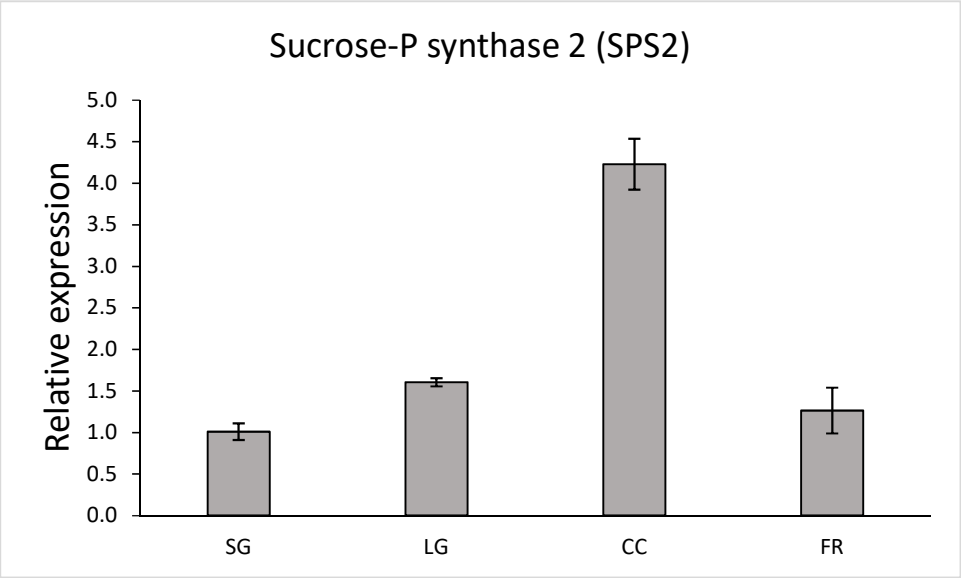

Figure 0.10 - Relative expression profile of sucrose-P synthase 2 gene during fruit development and ripening. Samples are: SG, small green fruit; LG, large green fruit; CC, color change fruit (i.e. visible start of the ripening phase) and FR, full ripe fruit. Values have been normalized to SG, arbitrarily set to 1. Error bars represent standard errors of the mean of the normalized expression. N=3.

2.3.7 Sucrose-phosphate phosphatase

Only a single gene of sucrose-P phosphatase (CmSPP1) was observed in our previous RNA-seq analysis. The CmSPP1 gene expression was 2.03-fold higher in small green than in full ripe melon fruit, with the RPKM level reducing from 14.7 to 7.2 respectively (Table 2.1).

Relative gene expression analysis showed a 2.5-fold decrease from small green to full ripe melon fruit. Using the intermediate stages of fruit development, it was possible to observe that the CmSPP1 gene expression pattern increased about 0.3-fold in large green compared with small green melon fruit, followed by about 0.3-fold decrease of its expression in color change compared with small green melon fruit (Figure 2.11)

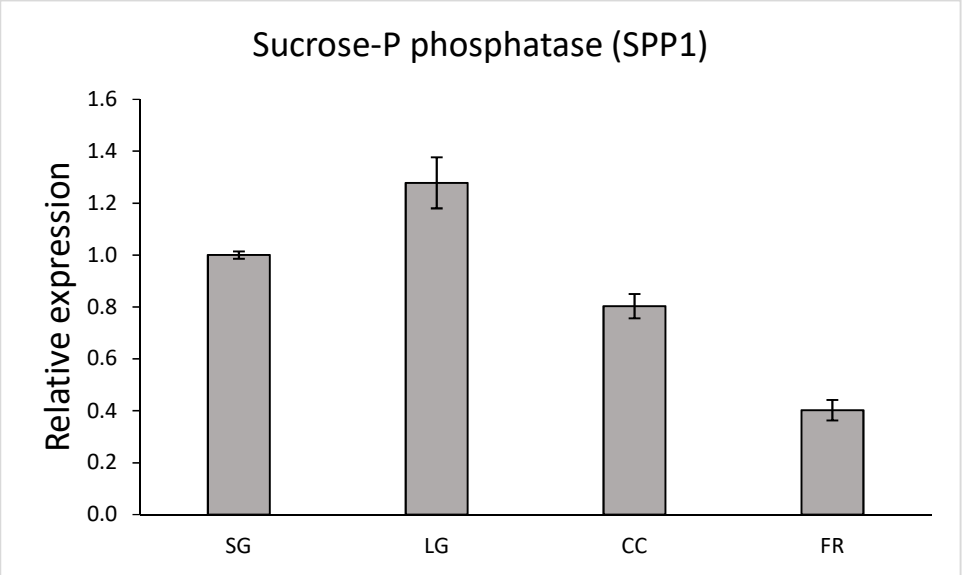

Figure 0.11- Relative expression profile of sucrose-P phosphatase 1 gene during fruit development and ripening. Samples are: SG, small green fruit; LG, large green fruit; CC, color change fruit (i.e. visible start of the ripening phase) and FR, full ripe fruit. Values have been normalized to SG, arbitrarily set to 1. Error bars represent standard errors of the mean of the normalized expression. N=3.

2.4 DISCUSSION

2.4.1 alpha--Galactosidase

In sugar metabolism, alpha-galactosidase enzymes are responsible for hydrolyzing the RFOs translocated from source leaves to the developing melon fruit, producing sucrose and galactose. In melon fruit, alpha-galactosidase includes acid and neutral isozymes and in young fruit both can be used to provide energy for growth metabolism. In mature fruit, sucrose can be stored in the vacuole while galactose can be metabolized to sucrose (DAI et al., 2011; LEIDA et al., 2015; SALADIÉ et al., 2015).

Previous studies with climacteric melon (*C. melo var reticulatus*, cv 'Dulce') have reported three genes encoding for neutral alpha-galactosidase enzymes and two genes encoding for acid alpha-galactosidase enzymes (DAI et al., 2011). However, no neutral alpha-galactosidase (NAG) genes were found in other non-climacteric melons such as 'Piel del Sapo', PI 161375 (SALADIÉ et al., 2015) or 'Hami' melon (ZHANG et al., 2016).

In our previous RNA-seq analysis three genes encoding for neutral enzymes (CmNAG2, CmNAG-LIKE2 and CmNAG-LIKE3) and two genes encoding for acid ones (CmAAG2, CmAAG-LIKE1) were found. All five ESTs were differentially expressed between small green and full ripe melon fruit, suggesting the high importance of these enzymes for sugar metabolism in melon fruit.

In the present study we observed that the CmNAG2 gene was the most expressed gene, among those involved in the metabolism of RFOs (Table 2.1). The CmNAG2 gene expression increases from small green (10 D.A.P) to large green (20 D.A.P) in which its expression was highest. This was followed by a reduction of its gene expression in the color change (30 D.A.P) and full ripe stages (40 D.A.P) (Figure 2.4). The CmAAG2 gene was not consistent (data not shown) because it has very low expression during `Yellow` melon development.

In climacteric melon, CmNAG2 is the most highly expressed gene of sugar metabolism in young fruit (10 and 20 D.A.P), followed by a decrease close to maturity (30 and 40 D.A.P), while other neutral alpha-galactosidase genes were weakly expressed in fruit tissue and acid alpha-galactosidase genes were expressed only in young fruit, suggesting that carbon and energy from alpha-galactosidase sucrose catabolism is needed for growth-related processes only during early fruit development (DAI et al., 2011).

According to Zhang et al. (2016), during melon fruit development, 10 D.A.P ovarian epidermal cells stop proliferating and the fruit is small. Subsequently, following a rapid increase in the volume of the young fruit, the cell diameters of the mesocarp, endocarp and placenta increase quickly resulting in fruit enlargement (20 D.A.P) and the melon fruit enters the expanding stage, with increasing fruit volume, but sugar content is at a very low level.

In climacteric melon fruit at 10 D.A.P the CmNAG2 gene expression is very high (DAI et al., 2011), in contrast to the present study that shows CmNAG2 expression increases only at 20 D.A.P. We suggest that climacteric melon might spend more energy very early in fruit development requiring carbon and energy from sucrose catabolism and high CmNAG2 expression, while in non-climacteric melon fruit the increase in CmNAG2 expression was closer to 20 D.A.P. However, to reach this conclusion it would be necessary to study the energy expenditure during growth of climacteric versus non-climacteric melon. Another hypothesis would be that in non-climacteric fruit, such as ‘Yellow’ melon, the energy required in the first stage of fruit development (10 DAP) can be supplied by the sucrose readily imported from the leaves, which can be better explored by phloem sugar measurement during melon fruit development as previous reported by Chrost and Schmitz, (1997).

2.4.2 Invertases

In melon fruit, sucrose can be catabolized by neutral and acid invertase to fructose and glucose to be used in energy production. While acid invertases have been attributed to vacuole

localization, neutral invertases have generally been attributed to a cytosolic localization, consistent with the neutral pH activity optimum and absence of glycosylation (DAI et al., 2011).

In our previous RNAseq study, only a single gene for soluble (vacuole) acid invertase (CmAIN2) was observed to be differentially expressed in non-climacteric 'Yellow' melon fruit, but no neutral invertase genes were found. In agreement with previous studies with 'Piel del Sapo' and PI 161375 (SALADIÉ et al., 2015) and 'Hami' non-climacteric melon fruit (ZHANG et al., 2016), only one acid invertase (AIN2) and no neutral invertases were reported. In 'Dulce' climacteric melons, four neutral invertase genes (CmNIN1, CmNIN2, CmNIN3 and CmNIN4) were found, as well as one acid invertase (CmAIN2) (DAI et al., 2011). In progenies of the cross between climatic and non-climateric melons, only one CmAIN gene was observed (CHAYUT et al., 2015).

In the present study CmAIN2 gene expression increased 4-fold in large green melon compared to the small green melon fruit, followed by about 0.5-fold reduction of gene expression in color change and full ripe melon fruit compared with the gene expression level in small green melon fruit (Figure 2.5).

All acid and neutral invertase genes are highly expressed in young developing fruit, and subsequently declined substantially at the sucrose accumulation stage (CHAYUT et al., 2015; DAI et al., 2011; SALADIÉ et al., 2015; ZHANG et al., 2016). Previous studies have shown that reduction of soluble acid invertase activity signals the metabolic transition from fruit growth to sucrose accumulation (BURGER; SCHAFFER, 2007; LEIDA et al., 2015). The presence of neutral invertases in climacteric melon fruit suggests cytoplasmatic sugar catabolism might be an additional source of energy, supporting the hypothesis that climacteric melon fruit spend more energy during fruit development, due to respiration, than non-climacteric melon fruit.

Previous studies have reported that the acid invertase gene (CmAIN2) was almost 10-fold higher in 'Védrantais' climacteric melon than in 'Piel del Sapo' non-climacteric melon, which might limit the accumulation of sucrose during ripening, contributing to rapid post-harvest decline in sucrose levels and providing sugars for respiratory pathways, that produce organic acids, such as malate, which may be detrimental to the taste of climacteric melons (SALADIÉ et al., 2015).

Using genotypes with low acid invertase activity or premature reduction of its activity in breeding programs might increase sugar accumulation. Reducing greenhouse nighttime temperatures from 18º to 12ºC during the period beginning with the onset of the sucrose

accumulation phase can also be used as a management strategy after reduction of acid invertase activity, to extend the cultivation time, delaying the harvest and giving more time for sugar accumulation (BURGER; SCHAFFER, 2007).

In the present study, in addition to the vacuolar invertase gene, differential expression of two ESTs corresponding to cell wall invertases (CmCIN2 and CmCIN4) were observed, both with low expression values in green fruit followed by near-silencing in mature fruit (Table 2.1) and because of that, the cell wall invertase gene expression was not assessed.

Previous studies have reported two cell wall invertases (CmCIN2 and CmCIN3) differentially expressed during fruit development in 'Hami' non-climacteric melon. While CmCIN3 gene increased during fruit development, peaking in the premature stage, and declining in ripe fruit, the expression of CmCIN2 was low throughout fruit development (ZHANG et al., 2016).

Comparing both climacteric and non-climacteric melon, Saladié et al. (2015) observed that the cell wall invertase gene was preferentially expressed in non-climacteric melon, and would not have access to intracellular (vacuolar) stores of sucrose, and therefore not affect sugar in the fruit. The contribution of cell wall invertase to the hydrolysis of sucrose in melon is still unclear.

According to Dai et al. (2011), neutral invertase activity may be a metabolic necessity in a tissue that storages sucrose in the vacuole, but still requires limited sucrose hydrolysis for other metabolic processes.

2.4.3 Invertase inhibitors

Invertase inhibitors are responsible for decreasing the activity of soluble acid invertases through post-translational regulation, reducing sugar consumed in respiration and regulating the accumulation of sucrose during fruit development and in ripe melons (BURGER; SCHAFFER, 2007; SALADIÉ et al., 2015).

In our previous RNA-seq study two transcripts encoding invertase inhibitors, CmINH1 and putative CmINH-LIKE3, were found to be differentially expressed between small green and full ripe 'Yellow' non-climacteric melon. The CmINH1 gene has the fourth largest expression and the putative CmINH-LIKE3 gene was superior in expression to all other sugar metabolism genes in small green fruit, declining with development, but maintaining high expression in ripe fruit (Table 2.1).

In the present study, the relative gene expression assessed for CmINH1 showed that it was present in a very low quantity making it unreliable for discussion (data not shown). The putative CmINH-Like3 gene expression showed about 5-fold more expression in small green melon than in large green, color change and full ripe melon fruit (Figure 2.6). The putative CmINH-LIKE3 gene was not found in previous studies with non-climacteric melon fruit nor in new searches for different invertase inhibitor isoform genes.

Previous studies on climacteric 'Dulce' melon reported three genes encoding invertase inhibitors (CmINH1, CmINH2 and CmINH3). CmINH1 and CmINH2 were expressed at high levels at the onset of sucrose accumulation (30 D.A.P) and just prior to sucrose accumulation (20 D.A.P), and both were not expressed at 40 DAA, when CmAIN2 is no longer expressed. This suggests that the inhibitor proteins play a role in reducing invertase activity while invertase expression is still occurring, or while invertase protein is still existent. The remaining gene for an invertase inhibitor, CmINH3, was only weakly expressed (DAI et al., 2011)

Comparing climacteric and non-climacteric melon fruit, Saladié et al. (2015) detected the expression of two genes corresponding to the inhibitory enzymes of invertase (isoforms of CmINH1) that were about 30 times higher in 'Piel del Sapo', a non-climacteric melon, than in 'Védrantais', a climacteric melon, suggesting that a greater expression of invertase inhibitor genes in non-climacteric fruits might maintain high sucrose levels at maturity and also limit post-harvest consumption of sucrose by respiration and consequently production of organic acids that cause stale flavor, contributing to the longer shelf life in non-climacteric melon fruit than in climacteric melon genotypes (BURGER; SCHAFFER, 2007; DAI et al., 2011; LEIDA et al., 2015; SALADIÉ et al., 2015).

In the present study, the putative CmINH-Like3 gene had its highest expression very early in development, before the higher expression of acid invertase 2 gene (CmAIN2), however, the enzime activity can not be confirmed only with gene expression, suggesting more studies to measure the invertase inhibitor enzyme activity during melon fruit development.

2.4.4 UDP-glucose epimerase

In sugar metabolism, UDP- glucose epimerase transforms galactose-1P to glucose-1P, providing the substrates for synthesis of sucrose (DAI, 2006; DAI et al., 2011; LEIDA et al., 2015; SALADIÉ et al., 2015).

In our previous RNA-Seq study, only one UDP-epimerase gene (CmUGE3) was differentially expressed between small green and full ripe 'Yellow' melon fruit. The CmUGE3 had higher expression in small green fruit and decreased with maturation (Table 2.1).

In the present study, we observed an increase in CmUGE3 gene expression in large green fruit, followed by the subsequent decrease until the full ripe stage (Figure 2.7), implying a concomitant gene expression with CmNAG2, which provides the substrate for CmUGE3 activity and suggesting a different pattern for sugar production in young non-climacteric melon fruit.

In climacteric 'Dulce' melon, three epimerase genes were identified and are expressed throughout fruit development (CmUGE1, CmUGE2 and CmUGE3), but CmUGE3 shows the strongest developmentally regulated pattern of the three, increasing significantly during fruit maturation (DAI et al., 2011). No CmUGE gene expression or enzyme activity were found in non-climacteric melon in previous studies (SALADIÉ et al., 2015; ZHANG et al., 2016).

2.4.5 Sucrose synthase

Sucrose synthase (SUS) can both synthetize sucrose or in the reverse reaction cleave sucrose (BURGER; SCHAFFER, 2007; CHAYUT et al., 2015; DAI et al., 2011; LEIDA et al., 2015).

In our previous RNA-Seq study, two genes encoding sucrose synthase (CmSUS1 and CmSUS2) were observed. CmSUS1 was the only gene in sugar metabolism that was expressed more in full ripe than in small green 'Yellow' non-climacteric melon fruit (Table 2.1). In the present study, relative expression of CmSUS1 gradually increased during fruit development reaching its highest expression in full ripe melon fruit (Figure 2.8).

In the opposite way, in our RNA-Seq analysis, the CmSUS2 gene was observed to have high expression in small green fruit followed by low expression at the full ripe stage in 'Yellow' non-climacteric melon (Table 2.1). In the present study, the CmSUS2 gene expression gradually decreased during fruit development and was very low in full ripe fruit (Figure 2.9).

In agreement, 'Hami' (ZHANG et al., 2016) and 'Piel del Sapo' (SALADIÉ et al., 2015) non-climacteric melons were observed to increase CmSUS1 gene expression during fruit ripening, while in contrast, CmSUS2 was highest in young fruit, and consistently decreased during development. No other isoforms were observed.

In contrast, three sucrose synthase genes were identified in 'Dulce' climacteric melon fruit (CmSUS1, CmSUS2 and CmSUS3). CmSUS1 gene was strongly expressed in young

‘Dulce’ climacteric melon fruit, followed by near-silencing in mature fruit. CmSUS2 showed low levels of expression throughout fruit development and CmSUS3 was weakly expressed in the young fruit but expression increased in the maturing fruit. The same work has shown that SUS activity steadily increased during development, suggesting that those activities might be related to the CmSUS3 gene (DAI et al., 2011).

This may suggest that in non-climacteric melons, CmSUS1 is responsible for synthesis of sucrose for storage in the vacuole, contributing to ripe fruit taste, while CmSUS2 acts in an opposite way providing substrate for energy production by sucrose catabolism during early development. It is important to note that similar sucrose synthase isoforms have a different pattern of expression in climacteric and non-climacteric melon fruit, suggesting that new studies are necessary to compare those similarities and differences during melon development and ripening.

2.4.6 Sucrose-P synthase

Sucrose-P synthase is considered the key gene to sucrose accumulation at fruit maturity (BURGER; SCHAFFER, 2007; CHAYUT et al., 2015; DAI et al., 2011).

Our previous RNA-Seq study showed only the CmSPS2 gene encoding for sucrose-P synthase in ‘Yellow’ non-climacteric melon (Table 2.1). The present study showed that CmSPS2 gene expression increased during fruit maturation, reaching the highest level in color change melon fruit (30 D.A.P) (Figure 2.10). This is in agreement with gene expression observed in ‘Hami’ non-climacteric melon (ZHANG et al., 2016).

In climacteric melon fruit, SPS enzyme activity increases during the period of sucrose accumulation (BURGER; SCHAFFER, 2007; DAI et al., 2011). In previous studies, two genes encoding for sucrose-P synthase (CmSPS1 and CmSPS2) were found in climacteric ‘Dulce’ melon. CmSPS1 expression increases dramatically with fruit maturation and is expressed at highest levels at 30 and 40 D.A.P, coinciding with the sucrose accumulation phase in fruit tissue, while CmSPS2 was only weakly expressed during fruit development with no obvious pattern of expression, but is supposedly expressed in vegetative tissue (DAI et al., 2011).

According to Saladié et al., (2015) there were only minor differences in expression of the CmSPS gene between climacteric and non-climacteric genotypes, however which isoforms was not stated. This information suggests that different isoforms of sucrose-P synthase are important in climacteric and non-climacteric melon, but both are required during the sucrose accumulation phase in fruit tissue.

2.4.7 Sucrose-P phosphatase

Sucrose-P phosphatase as well as sucrose-P synthase are responsible for the synthesis of sucrose for accumulation in ripening melons (BURGER; SCHAFFER, 2007; CHAYUT et al., 2015; DAI et al., 2011; LEIDA et al., 2015; ZHANG et al., 2016).

In our previous RNA-seq study only the CmSPP1 gene were observed to encode for sucrose-P phosphatase being more expressed in small green than in full ripe 'Yellow' non-climacteric melon. In non-climacteric 'Hami' melon, genes encoding for sucrose-P phosphatase were not observed (ZHANG et al., 2016)

In the present study the CmSPP1 gene expression pattern increased about 0.3-fold in large green compared with small green melon fruit, followed by about 0.3-fold decrease of its expression in color change and 2.5-fold decrease of its expression in full ripe compared with small green melon fruit (Figure 2.11).

In climacteric 'Dulce' melon fruit two genes (CmSPP1 and CmSPP2) encoding for sucrose-P phosphatase were observed. CmSPP1 expression increased during ripening but was lower than CmSPS1 gene expression, and CmSPP2 was only weakly expressed in melon fruit because it is suggested to be more present in vegetative tissue (DAI et al., 2011).

In the present study, the CmSPP1 gene was most expressed in large green melon fruit while CmSPS2 gene was most expressed at color change and maintained high expression in full ripe melon fruit, as previously discussed, suggesting an alternation between CmSPS and CmSPP genes in non-climacteric melon, in a different way than previously described for the climacteric melon (DAI et al., 2011).

2.5 CONCLUSION

Considering the limited knowledge about non-climacteric melon fruit ripening, next-generation sequencing (NGS) or high-throughput techniques and metabolomics technologies are useful tools to reveal the transcriptomic change during melon development and ripening.

Sugar metabolism was chosen for the present study because of its importance for energy supply during fruit development and sweet taste during fruit ripening, as well it serves as a ripening marker in non-climacteric melons.

We observed that the CmNAG2 gene, which encodes enzymes that produce sucrose and galactose from the RFOs family, CmAIN2 gene, which encodes enzymes that catabolize

sucrose to fructose and glucose to be used in energy production, and CmUGE3 gene, which encodes enzymes that transform galactose-1P to glucose-1P, providing the substrates for resynthesis of sucrose, were expressed more in large green melon fruit suggesting its contribution to energy production during fruit development. In a similar way, SUS2 which encodes enzymes that cleve sucrose for energy supply, was expressed more in small green fruit suggesting sucrose is the main source of energy production in fruit at the beginning of development, both for sucrose imported directly from photosynthesis or sucrose obtained from the degradation of raffinose and stachyose.

The new putative invertase inhibitor CmINH-LIKE3, which encodes enzymes responsible for decreasing invertase activity resulting in reduced sugar consumption, was highly expressed early in development in small green melon fruit, suggesting its importance during all non-climacteric melon fruit development and ripening.

In contrast, CmSUS1 and CmSPS2 genes were highly expressed at color change and maintained high expression in full ripe melon fruit. The enzyme activity of sucrose synthesis in this case might be related to sucrose accumulation rather than sucrose catabolism for energy production.

The CmSPP1 gene was expressed in large green melon fruit and is believed to encode enzymes acting on sucrose accumulation prior to those encoded by CmSUS1 and CmSPS2.

The expression of CmAAG2 and CmINH1 genes were not consistent probably because of its low presence in `Yellow` non-climacteric melon fruit.

This work is pioneering for this variety of melon and provides perspectives for further studies. This information about melon sugar metabolism during development and ripening may contribute to the development of future management practices and molecular tools to improve fruit quality, such as molecular markers for low acid invertase gene expression or higher invertase inhibitor gene expression to be used in breeding programs, as well as external interference in acid invertase and invertase inhibitor enzymes activity like manipulation of environmental factors such as temperature and photoperiod.

2.6 ACKNOWLEDGMENTS

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GENERAL CONCLUSION

Our results showed that one-time exogenous ethylene or 1MCP treatments are not able to change the rate of strawberry ripening when fruit mature and ripen on the plant. Consequently, no changes in sugar and organic acid metabolism were observed. Some treatment effect was observed in biometrical characteristics suggesting that ethylene treatment at the green development stage may improve fruit growth while ethylene treatment at the pink stage may inhibit fruit expansion, resulting in smaller and harder fruit at ripening. As no difference in days between treatment and harvest were observed, we can suggest that pink fruit treated with ethylene developed a good red color, while they were not actually in full ripe stage at harvest. However, no similar literature information was found to support this hypothesis.

Exogenous ethylene treatment at the white development stage was able to increase anthocyanin content at ripening. Although an increase in red visible color was not observed, the higher content of anthocyanins may impart higher antioxidant activity and gives evidence that the red strawberry coloration is directly ethylene-dependent and may be affected by exogenous treatments. As well, the concentration of alanine and the aromatic amino acids tryptophan and phenylalanine was increased in ripe fruit that were treated with ethylene at the green and white, and green and red development stages, respectively. Besides that, PCA analysis of all 19 amino acids were grouped close to ethylene treatment for all development stages, showing an association between ethylene and amino acid metabolism.

No 1MCP effect was observed for any of the variables measured at all development stages, suggesting no inhibition effect on ethylene receptors of attached strawberries, or the inhibition by 1MCP was not persistent to affect fruit composition at harvest, or that a different ethylene signal transduction independent of the ethylene receptors could be in attached strawberry.

Considering the limited knowledge about non-climacteric melon fruit ripening, next-generation sequencing (NGS) or high-throughput techniques and metabolomics technologies are useful tools to reveal the transcriptomic change during melon development and ripening.

Sugar metabolism was chosen for the present study because of its importance for energy supply during fruit development and sweet taste during fruit ripening, as well it serves as a ripening marker in non-climacteric melons.

We observed that the CmNAG2 gene, which encodes for enzymes responsible for the production of sucrose and galactose from the RFOs family, CmAIN2 gene, which encode for enzymes that catabolize sucrose to fructose and glucose to be used in energy production, and CmUGE3 gene, which encode enzymes that transform galactose-1P to glucose-1P, providing

the substrates for resynthesis of sucrose, were expressed more in large green melon fruit suggesting its contribution to energy production during fruit development. In a similar way, SUS2, which encodes enzymes that cleve sucrose for energy supply was expressed more in small green fruit suggesting sucrose is the main source of energy production in fruit at the beginning of development, both for sucrose imported directly from photosynthesis or sucrose obtained from the degradation of raffinose and stachyose.

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ATTACHMENTS

Attached Table 1 - Variance analysis of weight, diameter, length, firmness, anthocyanins (C3g and P3g), phenolic compounds and color measurements a* and hue angle. *P* values < 0.10 are reported. ns = not significant (*P*>0.10).

Source	d.f.	Weight	Diameter	Length	Firmness	C3g	P3g	Phenolic compounds	a*
Block stratum	3	.	.	.	.	.	.	.	.
Block.stPlot.dPlot stratum	*	.	.	.	.	.	.	.	.
Set	1	Ns	ns	0.077	ns	ns	ns	ns	ns
Set.Stage	3	0.018	0.019	ns	0.083	ns	ns	ns	ns
.. Set.G,W vs P,R	1	0.006	0.026	ns	ns	ns	ns	ns	ns
.. Set.G vs W	1	Ns	0.021	ns	ns	ns	ns	ns	ns
.. Set.P vs R	1	Ns	ns	0.028	0.014	ns	ns	ns	ns
Set.Dips	2	Ns	ns	ns	0.048	0.082	0.082	ns	ns
.. Set.Water vs M,E	1	Ns	ns	ns	ns	ns	ns	ns	ns
.. Set.1MCP vs Etep	1	Ns	ns	ns	0.045	0.056	0.056	ns	ns
Set.Stage.Dips	6	0.002	0.005	0.023	0.008	0.044	0.044	ns	ns
.. Set.G,W vs P,R.Water vs M,E	1	Ns	ns	ns	0.006	ns	ns	ns	ns
.. Set.G vs W.Water vs M,E	1	0.070	ns	ns	ns	ns	ns	ns	0.030
.. Set.G,W vs P,R.1MCP vs Etep	1	0.002	<0.001	0.047	0.054	0.005	0.005	ns	ns
.. Set.P vs R.Water vs M,E	1	Ns	ns	ns	ns	ns	ns	ns	ns
.. Set.G vs W.1MCP vs Etep	1	0.064	ns	ns	ns	0.034	0.034	ns	ns
.. Set.P vs R.1MCP vs Etep	1	0.010	0.023	0.020	0.024	ns	ns	ns	ns
Residual	36	.	.	.	.	.	.	.	.
Total	51	.	.	.	.	.	.	.	.

Attached Table 2 - Variance analysis of sucrose, glucose, fructose, total sugars, citric acid, malic acid, shikimic acid, succinic acid and total organic acids P values < 0.10 are reported. ns = not significant (P>0.10).

Source	d.f.	Sucrose	Glucose	Fructose	Total Sugars	Citric Acid	Malic Acid	Shikimic Acid	Succinic Acid	Total Organic Acids
Block stratum	3	.	.	.	.	.	.	.	.	.
Block.stPlot.dPlot stratum	*	.	.	.	.	.	.	.	.	.
Set	1	0.093	ns	ns	ns	ns	ns	ns	ns	ns
Set.Stage	3	Ns	0.033	0.050	0.076	ns	ns	ns	ns	0.014
.. Set.G,W vs P,R	1	ns	ns	ns	ns	ns	0.072	ns	ns	ns
.. Set.G vs W	1	ns	0.023	0.032	ns	ns	ns	ns	0.089	ns
.. Set.P vs R	1	0.066	ns	ns	0.054	ns	ns	ns	ns	0.002
Set.Dips	2	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.Water vs M,E	1	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.1MCP vs Etep	1	ns	ns	ns	ns	ns	ns	ns	ns	ns
Set.Stage.Dips	6	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.G,W vs P,R.Water vs M,E	1	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.G vs W.Water vs M,E	1	ns	ns	ns	ns	ns	ns	ns	ns	0.039
.. Set.G,W vs P,R.1MCP vs Etep	1	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.P vs R.Water vs M,E	1	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.G vs W.1MCP vs Etep	1	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.P vs R.1MCP vs Etep	1	ns	ns	ns	ns	ns	ns	ns	ns	ns
Residual	36	.	.	.	.	.	.	.	.	.
Total	51	.	.	.	.	.	.	.	.	.

Attached Table 3 - Variance analysis of soluble solids, pH and titratable acidity. P values < 0.10 are reported. ns = not significant (P>0.10).

Source	d.f.	Soluble solids	pH	Titratable acidity
Block stratum	3	.	.	.
Block.stPlot.dPlot stratum	*	.	.	.
Set	1	ns	ns	ns
Set.Stage	3	0.009	ns	ns
.. Set.G,W vs P,R	1	ns	ns	ns
.. Set.G vs W	1	0.003	ns	ns
.. Set.P vs R	1	0.079	ns	0.023
Set.Dips	2	ns	ns	ns
.. Set.Water vs M,E	1	ns	ns	ns
.. Set.1MCP vs Etep	1	0.088	ns	ns
Set.Stage.Dips	6	ns	ns	0.029
.. Set.G,W vs P,R.Water vs M,E	1	ns	ns	0.011
.. Set.G vs W.Water vs M,E	1	ns	ns	0.052
.. Set.G,W vs P,R.1MCP vs Etep	1	ns	ns	ns
.. Set.P vs R.Water vs M,E	1	ns	ns	ns
.. Set.G vs W.1MCP vs Etep	1	ns	ns	ns
.. Set.P vs R.1MCP vs Etep	1	ns	ns	ns
Residual	36	.	.	.
Total	51	.	.	.

Attached Table 4 - Variance analysis of alanine, arginine, asparagine, aspartate, cystine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, valine and total amino acids. P values < 0.10 are reported. ns = not significant (P>0.10).

Source	d.f.	Alanine	Arginine	Asparagine	Aspartate	Cystine	Glutamate	Glutamine	Glycine	Histidine	Isoleucine
Block stratum	3	.	.	.	.	.	.	.	.	.	.
Block.stPlot.dPlot stratum	*	.	.	.	.	.	.	.	.	.	.
Set	1	ns	0.057	ns	ns	ns	ns	ns	ns	ns	ns
Set.Stage	3	ns	ns	0.044	0.011	ns	0.001	ns	0.056	0.039	0.063
.. Set.G,W vs P,R	1	ns	ns	0.029	0.002	ns	<0.001	0.020	0.009	0.009	0.062
.. Set.G vs W	1	ns	ns	0.062	ns	0.045	ns	ns	ns	ns	ns
.. Set.P vs R	1	ns	ns	ns	ns	ns	0.041	ns	ns	ns	0.052
Set.Dips	2	0.024	<0.001	ns	ns	ns	ns	0.055	0.002	<0.001	<0.001
.. Set.Water vs M,E	1	ns	0.085	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.1MCP vs Etep	1	0.008	<0.001	ns	0.072	ns	ns	0.031	0.001	<0.001	<0.001
Set.Stage.Dips	6	0.021	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.G,W vs P,R.Water vs M,E	1	0.078	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.G vs W.Water vs M,E	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.G,W vs P,R.1MCP vs Etep	1	<0.001	ns	ns	ns	0.023	ns	ns	0.052	ns	0.031
.. Set.P vs R.Water vs M,E	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.G vs W.1MCP vs Etep	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.P vs R.1MCP vs Etep	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Residual	36	.	.	.	.	.	.	.	.	.	.
Total	51	.	.	.	.	.	.	.	.	.	.

Continue…

Source	d.f.	Leucine	Lysine	Methionine	Phenylalanine	Serine	Threonine	Tryptophan	Tyrosine	Valine	Total AA
Block stratum	3	.	.	.	.	.	.	.	.	.	.
Block.stPlot.dPlot stratum	*	.	.	.	.	.	.	.	.	.	.
Set	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Set.Stage	3	ns	0.038	ns	ns	ns	0.029	ns	ns	0.090	0.074
.. Set.G,W vs P,R	1	ns	ns	ns	ns	ns	0.003	ns	ns	0.057	0.014
.. Set.G vs W	1	ns	0.007	ns	0.076	ns	ns	ns	ns	ns	ns
.. Set.P vs R	1	ns	ns	ns	ns	ns	ns	ns	ns	0.085	ns
Set.Dips	2	ns	ns	ns	<0.001	ns	<0.001	<0.001	0.079	0.004	ns
.. Set.Water vs M,E	1	ns	ns	ns	0.019	ns	ns	ns	ns	ns	ns
.. Set.1MCP vs Etep	1	ns	ns	ns	<0.001	0.069	<0.001	<0.001	0.026	0.001	0.041
Set.Stage.Dips	6	ns	ns	ns	0.039	ns	ns	0.098	ns	ns	ns
.. Set.G,W vs P,R.Water vs M,E	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.G vs W.Water vs M,E	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
.. Set.G,W vs P,R.1MCP vs Etep	1	0.034	ns	0.066	ns	0.060	ns	0.012	ns	0.036	ns
.. Set.P vs R.Water vs M,E	1	ns	ns	ns	0.068	ns	ns	ns	ns	ns	ns
.. Set.G vs W.1MCP vs Etep	1	ns	ns	ns	0.007	ns	ns	ns	ns	ns	ns
.. Set.P vs R.1MCP vs Etep	1	ns	0.080	ns	ns	ns	ns	ns	ns	ns	ns
Residual	36	.	.	.	.	.	.	.	.	.	.
Total	51	.	.	.	.	.	.	.	.	.	.