Article

Identification of Metabolite and Lipid Profile in a Segregating Peach Population Associated with Mealiness in Peach

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Abstract: Peach is the third most important temperate fruit crop considering fruit production and harvested area in the world. Exporting peaches represents a challenge due to the long-distance export markets. This requires fruit to be placed in cold storage for a long time, which can induce a physiological disorder known as chilling injury (CI). The main symptom of CI is mealiness which is perceived as non-juicy fruit by consumers. The purpose of this work was to identify and compare the metabolic and lipid profile between two siblings from a contrasting population for juice content, at harvest and after 30 days at 0°C. A total of 119 metabolites and 189 lipids were identified, which showed significant differences of abundance including mainly in amino acids, sugars and lipids. Our results indicate that some of the top metabolites and lipids could be used as biomarkers associated with mealiness at harvest and after cold storage.

Keywords: chilling injury; mealiness; metabolomics; lipidomics; biomarker

1. Introduction

Peaches and nectarines (*Prunus persica* (L.) Batsch) are among the most important temperate fruit crops, with a world production of 24,453,425 t in 2018 [1]. Chile is among the top ten countries for peach and nectarine production with 319,047 t in 2018 [1]. However, exported fruit quality is compromised due to the long distance to market, since fruits must be maintained at low-temperature storage (0-4ºC), to prolong its shelf life by avoiding its decay development [2]. This prolonged cold storage can negatively affect sensorial characteristics of peaches and nectarines like flavor and texture, due to the development of physiological disorders known as Chilling Injury (CI) [2,3] Chilling Injury corresponds to an internal breakdown, limiting the storage life of peaches and nectarines under refrigeration, and includes dry flesh, hard texture fruit, flesh browning, flesh bleeding and mealiness as main symptoms [2]. Mealiness is a textural disorder that occurs in the mesocarp, and can be observed during shelf life, where the affected ripe fruit display a dry grainy feel when chewed [4]. On a cellular level, cell wall pectin metabolism is altered in mealy peach fruit, a gel is formed when pectic substances in intercellular spaces absorb free water, and intercellular adhesion is reduced [5]. At this level, mealiness is associated with a decline in respiration rate, very low ethylene evolution and reductions in extractable protein of over 50% [5-8]. Cold storage causes a reduction in ethylene-regulated enzymes, which participate in cell wall enzyme activities, including endo-PG, which are required for normal ripening in melting fresh varieties [5]. At a molecular level, mealiness would be

associated with incomplete solubilizing of cell wall macromolecules due to an imbalance in the expression of transcripts and enzymatic activity of cell wall-modifying enzymes such as pectin methylesterases (PMEs) and polygalacturonases (PGs) [5,9]. Besides, a dysfunctional of cell membrane at low temperature is considered a primary molecular event ultimately leading to the development of CI symptoms [10]. Several studies have linked the activity of polygalacturonase with the incidence of mealiness. PGs transcripts cannot accumulate at the normal levels as ripe juicy fruits, also an endopolygalacturose has been identified that co-localizes with an important QTL affecting characters generated by cold damage, such as mealiness and pulp bleeding [4, 11]

Different genomics studies have been performed to elucidate the appearance of mealiness. For example, nine candidate genes were identified for peach mealiness from QTL regions, where LG4 contains a cluster for genetic factor that could regulate mealiness, being a transcription factor one of the most relevant genes in the regulation of this trait [12]. As well, SNP marker for resistance to chilling injury symptoms have been proposed as a potential candidate gene (QTN) [13] The presence of genes related to invertase/pectin methylesterase inhibitor (PMEI) was reported in susceptible fruit for mealiness. This gene produces a de-methyl esterification effect, producing free carboxylic group that alters the charges inside the cells allowing the addition of polyuronides into calcium-linked gel structure which increase the firmness of the cell wall, avoiding the incidence of mealiness in the fruits [14].

Metabolomics and lipidomics provide a tool to understand the physiological processes that occur in response to different types of stress, such as chilling injury, where differences in the abundance of these compounds can be used to characterize phenotypes. For instance, in response to cold and other osmotic stresses, plant organisms can change their metabolism and accumulate metabolites like sterols, glucosides, raffinose, arabinoxylans, and another soluble sugars [15]. In addition, plants also accumulate other solutes such as glutamic acid, amino acids (alanine, glycine, proline and serine), polyamines (putrescine) and betaines. These molecules, which are often degraded once the stress has passed, are referred to as osmolytes, osmoprotectans or compatible solutes [16-18]. Otherwise, lipids are an essential part of biological processes and serve numerous structural and functional roles in these systems inclusive of providing structural molecules for forming cellular membrane bilayers, signaling molecules, energy storage and transport. Advances in lipid detection techniques have been useful for studying the composition of the plasma membrane and how it is remodeled in response to stress such as cold temperature. For this reason, metabolome and lipidome approach could be useful for the study of differential metabolic profiles between phenotypes, helping in the search of biomarkers associated with agronomical traits. Thus, this work aims to identify candidate metabolites and lipids involved in the susceptibility to mealiness in *Prunus persica* by GC-MS and UHPLC MS/MS analysis.

2. Results and discussion

2.1. Phenotypic parameters in response to chilling injury

In order to determine the physiological parameters of the VxV nectarine population, we evaluated phenotypic parameters as firmness [N], background color (I_{ad}), soluble solids (°Brix) and juiciness (%). For firmness parameter, background color, soluble solid content (Figure 1 A, B, C) no significant differences were found between contrasting phenotypes for mealiness (juicy and mealy fruits). In order to evaluate the degree of chilling injury in both phenotypes, we determined the percentage of juice after cold storage (Figure 1D). In this case, juiciness in mealy fruits was significantly lower (21.56% of juice), compared to juicy fruits (52.68% of juice) as revealed by t-test ($p < 0.05$), coinciding with the expected characteristics of this physiological disorder.
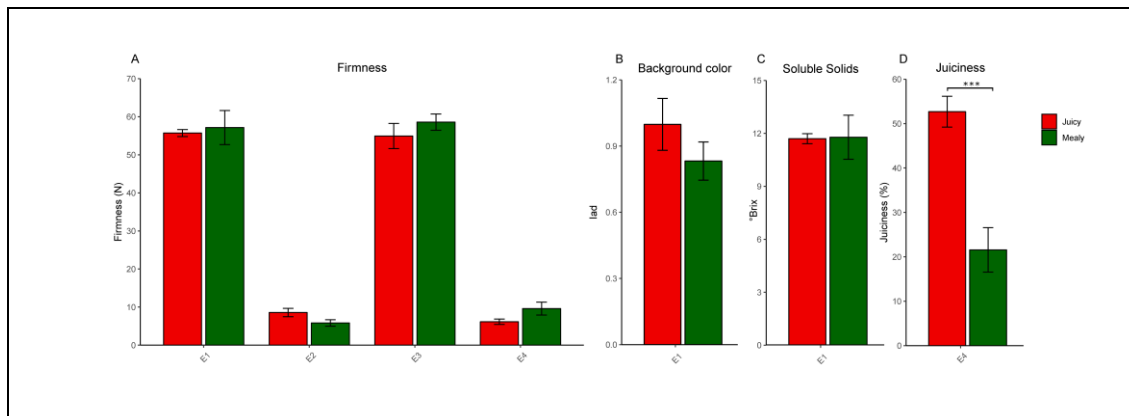
Figure 1. Physiological parameters of nectarine fruits from VxV population. (A) Firmness, (B) Background color, (C) Soluble Solids, (D) Juiciness. These parameters were measured in mealy and juicy fruits during 2016-2017 from contrasting individuals of VxV population for the mealiness (three tolerant siblings to mealiness and three susceptible). Values are the mean of five biological replicates (5 fruits) and bars represent standard deviation. (*) Indicates significant differences between both phenotypes with t-test at 0.05 significant level.

2.2. Comparison of differentially accumulated metabolites and lipids

A total of 308 metabolites and lipids were initially characterized, expanding triglycerides, saturated and unsaturated fatty acids, disaccharides, dicarboxylic acids, amino acids, sugar acids, among others. From these 308 metabolites and lipids, 212 (68.8%) could be mapped to metabolites with unique InChiKeys/Pubchem_ID/SMILES tags, as required by the ChemRICH tool (Figure 2) [19]. Two comparisons were performed: E1 against E3 from juicy phenotype fruits (Figure 2A) and E1 against E3 from mealy phenotype fruits (Figure 2B). When assessing the transition from E1 to E3 in fruit that remained juicy, E1 fruits displayed higher levels of triglycerides, and lower levels of unsaturated phosphatidylcholines, unsaturated fatty acids, and branched chain amino acids compared to E3 (Figure 2A). The same analysis for fruit that would become mealy showed that the decrease in branched chain amino acids was also displayed in the E1 to E3 transition. In addition, saturated lysophosphatidylcholines, diglycerides, sugar acids and hexosephosphates decreased markedly during the E1 to E3 transition in fruit that would become mealy, compared to a null or very slight change in the fruit that remain juicy (Figure 2B).
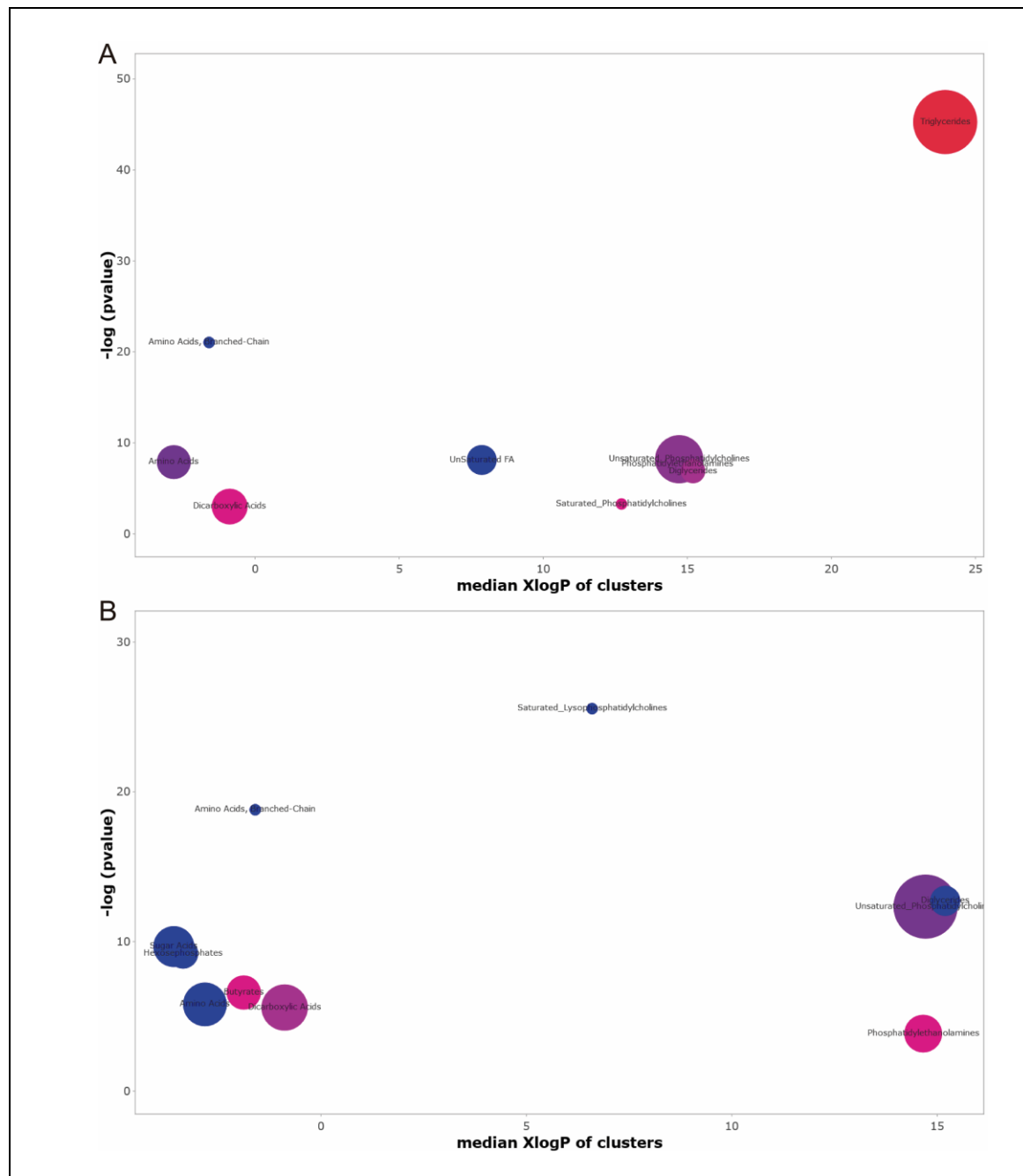
Figure 2. Comparison of E1 vs E3 differentially accumulated metabolites in juicy (A) and mealy (B) fruit. Chemical Similarity Enrichment (ChemRICH) analysis was used to identify metabolites with a significant alteration in their abundance during the transition from firm (E1) to cold stored (E3) fruit. Each circle reflects a significantly altered cluster of metabolites. Circle sizes represent the total number of metabolites in each cluster set. The circle color scale shows the proportion of increased (red) or decreased (blue) compounds. Purple-color circles have both increased and decreased metabolites. Enrichment p-values are given by the Kolmogorov-Smirnov-test.

Changes in lipid profile in response to low temperature revealed that the composition of membrane lipids changes substantially in plants [20]. A significant increase of unsaturated fatty acids has been reported during cold acclimation [21-23] In *Arabidopsis thaliana*, the amounts of lysophospholipid species as LPC and LPE increased in response to cold acclimation at 4°C or exposed to freezing stress [21, 23]. In response to cold, enrichment analysis corresponding to the juicy phenotype showed high levels of TAG (Figure 2A), however, the mealy phenotype showed low

amounts of saturated LPC, amino acids, diglycerides, sugar acids and hexosephosphates (Figure 2B). The exposure of plants to stress conditions like the exposure to low temperatures results in the alteration of their metabolism. It has been reported that this happens adjusting or restoring catalytic properties of enzymes through regulatory mechanisms in response to cold stress [24, 25], and through modifications of the metabolic parameters like the production of some metabolites, osmolytes, and phytohormones [26-33]. These include sugars, amino acids, organic acids, polyamines and lipids which eventually assist in cellular protection from cold-induced damage by various mechanism [26-33]. These results suggest that the conditions of susceptibility or tolerance of the fruit are determined at harvest, and probably, fruits of the juicy phenotype achieved a better adaptation in response to cold than fruits from to mealy phenotype fruits.

2.3. Effect of cold storage in the metabolic and lipid profile

In order to evaluate changes in the metabolic and lipid profile associated with each phenotype at harvest (E1) and after cold storage (E3), a total of 308 compounds were detected by ALEX-CIS GCTOF MS and HPLC-CSH-ESI QTOF MS/MS. All compounds were found in both phenotypes during E1 and E3 and analyzed by multivariate statistical analysis considering the stage of postharvest and phenotype classes (juicy vs mealy). A PLS-DA analysis was carried out using the juicy fruits as response variable and the identified metabolites as predictor variables. Considering this, the scores plot for the juicy phenotype explained 46.1% variability with two components (Figure 3A). In this projection, the E1 stage (red ellipse) appears to be separated from the E3 stage (green ellipse). Additionally, a Variables Important in Projection (VIP) approach was used to select important features which contribute to this separation (Supplementary Fig. 1A). From this, a total of 25 compounds were obtained and their relative abundance are shown in Figure 3A as a heatmap, where the main compounds that contribute to the separation of stages of postharvest are trehalose-6-phosphate, pyruvic acid, alpha-ketoglutarate, fumaric acid, nicotinic acid, which are presented in high levels at the E1 stage. After cold storage high relative amounts of amino acid as proline, valine, alanine, isoleucine, serine, leucine and phenylalanine and other compounds as 1-kestose and alpha-aminoadipic acid were present in the juicy phenotype. On the other hand, a second PLS-DA analysis was carried out using lipids as predictor variable (Figure 3B). Considering this, the scores plot of the juicy phenotype explained 66.9% of the variation with the two first component (Figure 3B). A total of 25 lipids were identified by VIP analysis (Supplementary Fig. 1B) and showed as a heatmap (Figure 3B). Considering this, the juicy phenotype at E1 stage appears to be rich in lipids as diacylglycerols (DG), phosphatidylcholines (PC) of 32, 33, 34 and 36 carbons, phosphatidylethanolamines (PE) of 34 and 36 carbons, and phosphatidylglycerols (PG) of 32 carbons, and E3 stage showed high levels of triacylglycerols (TAG), phosphatidylcholines (PC) of 38 carbons and digalactosyldiacylglycerols (DGDG).
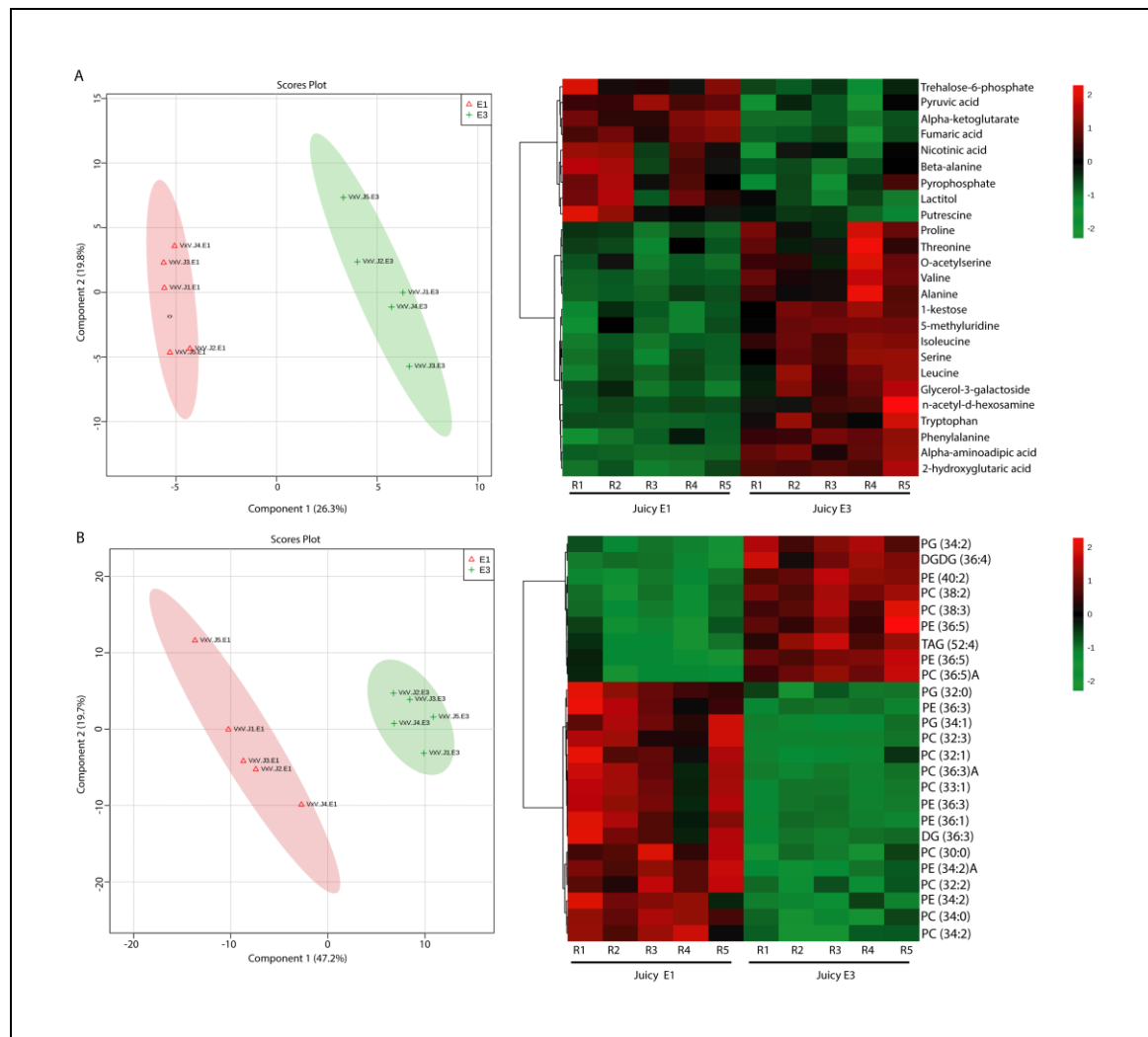
Figure 3. Metabolic and lipid profile of nectarine at E1 and E3 stage of the juicy phenotype. A) Multivariant Analysis for metabolic profile and B) Multivariant analysis for lipid profile. Right panel show Partial Lest Square Regression Discriminant Analysis (PLS-DA). The first component (Component 1) is shown on the x axis and the second component (Component 2) is shown on the y axis. The detected metabolites and lipids were employed as predictor variables and the stage of postharvest as response variable. In the left panel show Heatmap analysis for metabolic and lipid profile showing top 25 compounds identified by PLS-DA VIP. Columns represents technical replicates for each stage (E1: Harvest, E3: After cold storage). The similarity measure employed in order to group the different features was calculated by Euclidean distance and Ward´s linkage.

Biological membranes are crucial for the function of cells in living organism, acting as selective barriers. Their structure and function are not only influenced by membrane proteins, but also by their diverse lipid composition [34]. An increase in the relative content of PC 38:2 was detected only in the juicy phenotype after cold storage (Figure 3B), as seen in Bustamante et al. [35], who also detected high levels of this PC after cold storage. This suggests that high levels of this lipid could be an adaptative response only in the juicy phenotype.

When PLS-DA analysis was performed using the mealy phenotype as response variable, a 54.5% of the variance could be explained with the first two components (Figure 4A), with two ellipses clearly defined. VIP showed the class of compounds associated with the mealy phenotype in both stages of postharvest (Supplementary Fig. 1C), where the E1 stage of postharvest appeared to be rich in trehalose-6-phosphate as the juicy phenotype, and other compounds as arachidic acid, uridine,

nicotinic acid, alpha-ketoglutarate, fumaric acid and pyruvic acid in contrast to E3 stage, which displayer higher abundance of alanine, phenylalanine, proline, isohexonic acid, hexose-6-phosphate, valine, isoleucine, leucine, alpha aminoadipic acid, O-acetylserine, fructose-1-phosphate, serine, 1-kestose, galactonic acid, fructose-6-phosphate, 3,4-dihydroxycinnamic acid, glycerol-3-galactoside and lyxose (Figure 4A, left panel). For the lipid profile (Figure 4B), the mealy phenotype at E1 stage appeared to be rich in only two compounds: phosphatidylcholine (PC) of 32 carbons and monogalactosyldiacylglycerol (MGDG) of 36 carbons. At E3 stage, lipids as triacylglycerols of 52 and 54 carbons, diacylglycerols (DG) of 36 carbons were found in higher abundance.

Some metabolites behave in the same way in response to cold. The accumulation of 1-kestose under stress (Figure 3A and 4A) in both phenotypes, could be a defense response to low temperatures. This function was demonstrated only in studies of grasses (*Lollium perenne* L.) exposed to drought [36] and cold [37, 38]. 1-kestose is a fructan, which may act a rich polyhydroxy compound capable of avoiding water freezing [39] and also can contribute to the osmotic potential in a better way than sucrose and oligosaccharides combined [40]. In onions (*Allium cepa*), fructans provide osmotic adjustment during bulb development, and fructan hydrolysis helping to regulate its turgor in guard cells [41]. Kestose belonging to the inulin-type fructans, can protect structure of membrane under abiotic stresses [42]. Also, high levels of trehalose-6-phosphate were found before cold storage in both phenotypes (Figure 3A, 4A). Trehalose-6-phosphate is an essential signal metabolite in plants, with influence in growth and development that competes with other signaling molecules, including phytohormones [43] It has been proposed to play protective roles under various abiotic stresses, including cold and freezing [44] The protective effects of trehalose is associated to membrane stability and depression in phase transition temperature of biomembranes and remains amorphous even under completely dehydrated conditions [45]. These results suggest that trehalose-6-phosphate could be a protector of the cellular membrane, changings its fluidity and thus protecting the fruit from cold stress.
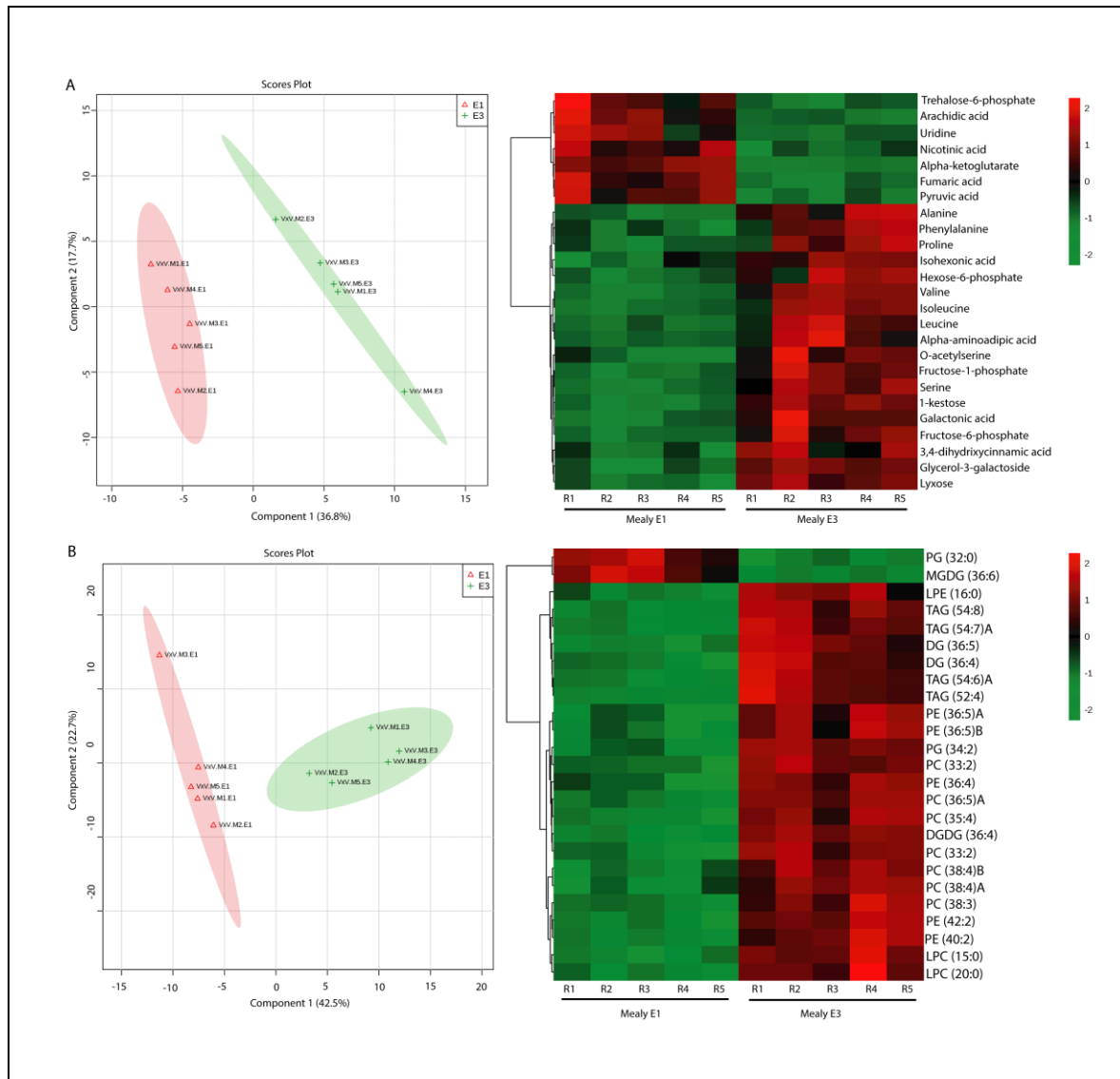
Figure 4. Metabolic and lipid profile of nectarine at E1 and E3 stage of the mealy phenotype. A) Multivariant Analysis for metabolic profile and B) Multivariant analysis for lipid profile. Right panel show Partial Lest Square Regression Discriminant Analysis (PLS-DA). The first component (Component 1) is shown on the x axis and the second component (Component 2) is shown on the y axis. The detected metabolites and lipids were employed as predictor variables and the stage of postharvest as response variable. In the left panel show Heatmap analysis for metabolic and lipid profile showing top 25 compounds identified by PLS-DA VIP. Columns represents technical replicates for each stage (E1: Harvest, E3: After cold storage). The similarity measure employed in order to group the different features was calculated by Euclidean distance and Ward´s linkage.

At harvest (E1), there was already a differential metabolic profile between the phenotypes, being the principal differences are related to higher contents of beta alanine, pyrophosphate, lactitol and putrescine (Figure 3A), in the juicy phenotype. Putrescine with a positive correlation to a reduction in susceptibility to CI [46]. The mealy phenotype was instead rich in uridine (Figure 4A). Uridine is a nucleoside, primarily found in sugar beets (*Beta vulgaris*), sugarcane (*Saccharum officinarum*), tomatoes (*Solanum lycopersicum*), broccoli (*Brassica oleracea*) among others [47]. A previous work reported higher expression of Uridine Diphosphate-glucose PyroPhosphorylase (UGlcPP) in mealy peach fruits. This enzyme plays a key role in carbohydrate metabolism by catalyzing the reversible conversion of glucose-1-phosphate and UTP to UDP glucose and pyrophosphate, respectively. UDP-glucose is the precursor of several nucleotide sugars and is associated with glycan synthesis [48].

High levels of uridine in the mealy phenotype at E1, suggest that this metabolite can alter the correct cell wall composition and structure, affecting fruit texture and could be associated with mealiness phenotype at early stages.

High levels of amino acids such as alanine, leucine, and serine were found only after cold storage in both phenotypes (Figure 3A and 4A). At E3, the mealy phenotype displayed high levels of sugars as fructose 1 and 6 phosphate sugars (Figure 4A). Besides, in response to cold stress in peach (Figure 3A and 4A), the relative abundance of amino acids such as proline, valine, and alanine among others increased not only in the mealy phenotype, but also in the juicy phenotype. The high levels of amino acids after cold storage (Figure 3A and 4A) indicate a possible accumulation of metabolites with a protective function in response to chilling stress. It has been reported that proline is a protective molecule which impart protection against cold stress to many plants not only maintaining its osmolarity but also acting as molecular chaperon hereby stabilizing the structure, and function of important proteins and enzymes [49]. It also protects the plant by maintaining the double strand structure of genetic material and by up-regulating the oxidative stress machinery [50-51]. A positive correlation between the accumulation of endogenous proline and improved cold tolerance has been found mostly in low temperature-insensitive plants such as barley (*Hordeum vulgare*), rye (*Secale cereal*), *Arabidopsis thaliana*, among others [27, 52-53]. The most probable roles of proline are associated to cytosol acidity, regulation of the NAD+/NADH ratio, photochemical activity of the photosystem II in thylakoid membranes and a decrease in lipid peroxidation [54]. This suggest that proline acts a protector non-depending on the phenotype. The relative abundance of alanine increased after cold storage in both phenotypes (Figure 3A and 4A). Previous studies showed that concentrations of alanine increased in CI-sensitive crops during cold storage [55-57], and it was shown that conversion of pyruvate to alanine by glutamate-pyruvate transaminase (GPT) was likely the source of the increased alanine, as levels of pyruvate were found to be very high in chilled cucumbers (*Cucumis sativus*) and eggplants (*Solanum melongena*) while GPT activity was unaffected by the chilling [58].

Others amino acids found in high levels after the cold storage in both phenotypes are isoleucine and valine (Figure 3A and 4A). Previous authors [59], also found a remarkable increase in concentrations of several amino acids, including isoleucine and valine prior to chilling. These results suggest that the accumulation of these amino acids, including proline, could serve as a priming strategy to protect peach fruits from CI damage induced by subsequent chilling stress. Alternatively, the accumulation of isoleucine and valine may serve as substrates for the synthesis of stress-induced proteins and these branched-chain amino acids may act as signaling molecules to regulate gene expression [60]. Another hypothesis is that isoleucine and valine could be critical to maintain protein structure and function under cold-stress conditions, because of their unsubstituted aliphatic side chains with branched alkyl groups [60]. Results suggest that concentration of amino acids increased in response to cold, independent of the phenotype and might be indicative that the metabolism of the fruit is focused on protection, increasing the production metabolites with signaling functions and that participate in the synthesis of proteins related to secondary metabolism.
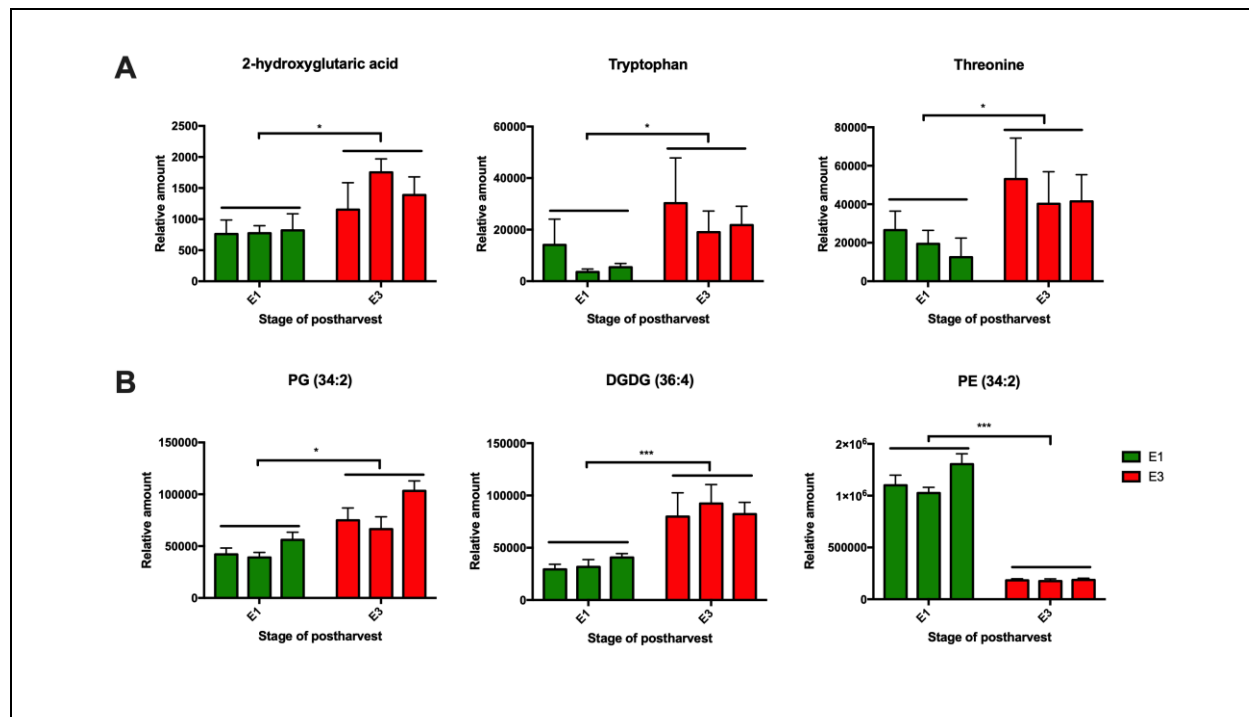
Figure 5. Relative amount of metabolites (A) and lipids (B) at E1 and E3 stages using three VxV siblings tolerant to mealiness (>30% of juice content) from peach segregating population. Each column represents an average of five replicates (n=5), and the bars are the standard deviations. Green box shows E1 stage (harvest) and red box show E3 stage (harvest + 21 days at 0°C). Statistical analysis was performed by t-test using p < 0.05.

2.4 Metabolites and lipids associated to cold stress and possible candidate biomarkers to mealiness

Furthermore, the relative abundance of the top metabolites with differential abundance between the phenotypes in other two individuals from each phenotype (Figure 5 and 6) was assessed and correlated with the previous individuals (Supplementary Fig 2 and 3). High levels of 2-hydroxyglutaric acid, tryptophan and threonine (Figure 5A) after the cold storage were found, as well as high levels of some lipids (Figure 5B) such as PG (34:2) and DGDG (36:4) at E3 stage in juicy phenotype. DGDG is a bilayer lipid that may increase membrane stability [35, 61], suggesting a possible cold tolerance to these individuals. Also, PE (34:2) was present in higher relative amounts at E1 only in the juicy phenotype.

The relative abundance of the top metabolites in the mealy phenotype (Figure 6A), revealed high levels of arachidic acid at E1 stage; and higher levels of fructose 1 and 6 phosphate at E3 stage. It has been extensively reported that high contents of sugars alleviate CI symptoms in peach fruit, because carbohydrates may serve as osmoregulators and cryoprotectans contributing to membrane stability [62-63]. Besides, carbohydrates may act as scavengers of reactive oxygen species and sugar metabolism might provide reducing power to ascorbate-glutathione cycle protecting cells against chilling stress [42, 62-63]. Arachidic acid participate in the biosynthesis of unsaturated fatty. Previous authors proposed fructose 6 phosphate and arachidic acid as physiological marker for incipient chilling injury in tomato [64]. At lipidic level, we found high amounts of MGDG in the mealy phenotype at E1 stage, decreasing to E3 stage (Figure 6B). MGDG is a non-bilayer lipid that can severely destabilize membranes. It has been proposed that an increase in the ratio of bilayer to non-bilayer-forming membrane lipids results in the stabilization of membranes during freezing [35, 61]. The differential levels of MDGD in the mealy phenotype suggest that the membrane stability is altered in response to cold. This phenotype is inverse in the juicy phenotype, where high levels of DGDG at E3 stage were found, suggesting an increase in the membrane stability (Figure 5B). In

addition, it has been shown that an increase in the content of diunsaturated species of PC in rye protoplast leads to an increased tolerance of the plasma membrane against freezing [35,65]. Recent studies under cold stress have reported that besides the increase in the degree of fatty acid unsaturation, the main constituent component of chloroplast-specific lipid is also susceptible, such as a decrease in monogalactosyldiacylglycerol (MGDG) [66-68]. Besides, a notable increase in phosphatidic acid (PA), lysophosphatidylethanolamine (LPE) and lysophosphatidylcholine (LPC) has been reported in response to low temperature [68], being the last one the lipid with the higher abundance in the mealy phenotype after the cold exposure. This changes in the membrane lipids could be an adaptative response to cold. This metabolites and lipids could be studied as possible biomarkers for CI.
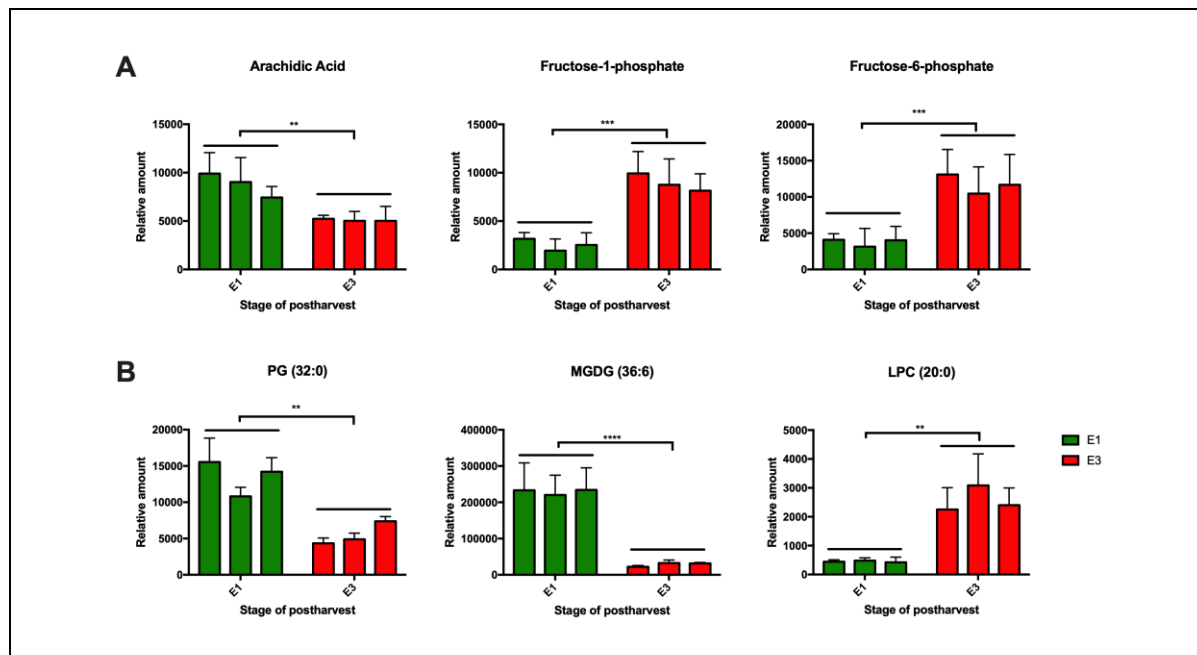

Figure 6. Relative amount of metabolites (A) and lipids (B) at E1 and E3 stages using three VxV siblings susceptible to mealiness (<30% of juice content) from peach segregating population. Each column represents an average of five technical replicates, and the bars are the standard deviations. Green box shows E1 stage (harvest) and red box show E3 stage (harvest + 21 days at 0ºC). Statistical analysis was performed by t-test using $p < 0.05$.

3. Materials and Methods

3.1. Plant Material and phenotyping

Two siblings from the nectarine segregating population (VxV) were selected considering contrasting levels of mealiness susceptibility or juice content after cold storage. The VxV population was obtained from the self-pollination of ´Venus´ nectarine variety, which is located on INIA Rayentué in Chile (34° 32´14´ S, 70° 83´44´ W). This population is freestone, melting and has yellow flesh. Fruits were harvested between mid-December until late January, when their firmness were around 53 N. Two VxV siblings contrasting for mealiness were selected (one sibling juicy and another mealy). A group of harvested fruits (E1) was ripened at 20ºC for eight days, until their firmness was around 4-8 N, corresponding to E2 stage (commercial firmness). Another group of fruits was stored at 0ºC for 30 days corresponding to E3 stage of postharvest. After these 30 days, fruits were kept at 20ºC for seven days until the firmness was around 7 N, corresponding to E4 stage of postharvest. As biological replicates, five fruits from selected contrasting siblings were evaluated at different postharvest stages.   In E1, the parameters evaluated were weight, firmness, background color and soluble solids (SS). For E2 and E4, the parameters evaluated were firmness and juiciness (% of juice), while in E3, only firmness was measured. Flesh firmness was assessed on two paired sides of each fruit after exocarp removal, using a Fruit Pressure Tester fitted with an 8 mm diameter plunger (FT327 and FT011, EFFEGI). Soluble solids content (SS) was determined using a digital refractometer (WINEline HI96811, HANNA). The background color was evaluated using a DA meter (I_{ad}) (SintélelaX FRM01-F) and juiciness was evaluated as previously described by Infante et al. [68]. Briefly with the exocarp previously removed, a piece of mesocarp was taken from each side of the fruit and placed on a previously massed absorbent paper. Then, this paper was folded over the piece of mesocarp and weighed with a balance. The sample was covered with two previously massed absorbent paper and then a mechanical stress was generated on the mesocarp. Finally, these papers were weighed, and the percentage of juice was obtained by the weight difference of the papers. Finally, fruits with 30% or less of juice content were considered as mealy [69]

3.2. Metabolite and lipid extraction

Samples from stages E1 (before cold) and E3 (after cold storage) were used to evaluate their metabolic and lipid profile. Total metabolites were extracted as described by Fiehn et al. [70], where 20 mg of frozen tissue corresponding to mesocarp were placed in a 1.5 mL microtube containing 1 mL of pre-cooled extraction buffer [5:2:2, (v/v/v) of degassed methanol, chloroform and water], and were macerated at 1,750 rpm for 1 minute at 4 °C using a ball mill grinder (MM301; Retsch Corp., Hann, Germany). Tubes were centrifuged for 2 minutes at 14,000 g and 4°C using a bench-top centrifuge (Eppendorf 5415D), discarding the precipitate (plant debris). Five hundred milliliters of supernatant were transferred into a new 1.5 mL microtube and dried using a speed vacuum concentrator (Centrivap cold trap concentrator, Labconco, Kansas, MO, USA). For metabolite derivatization, 20 μL of methoxyamine solution (Sigma-Aldrich, St. Louis, MO, USA) containing 20 mg/mL of pyridine (Sigma-Aldrich) were added to the dried sample tubes and shaken for 90 minutes at 30 °C. To each tube, 91 μL of MSTFA [*N*-Methyl-*N*-(trimethyl-d₉-silyl)trifluoroacetamide] (Sigma-Aldrich) and 10 μL FAME (Fatty Acid Methyl Ester) marker (Supelco C8-C24; Sigma-Aldrich), were added and shaken for 20 minutes at 37 °C. Each prepared sample was transferred to an autosampler vial with micro-insert.

Lipid extraction was carried out as described by Matyash et al. [71]. In shortly, 20 mg of the organic phase was extracted with 1.5 mL of methanol and 5 mL of methyl-tertiary butyl ether (MTBE). Tubes were vortexed for 20 seconds and centrifugated for 2 minutes at 14,000 g. Then the organic phase was separated and was reconstituted in 65 μL of a solution 9:1 (v/v) of methanol and toluene; and N-cyclohexyl-N´-dodecanoic acid urea (CUDA) as internal standard. Each prepared sampled was transferred to a vial with micro-insert.

3.3. Metabolite and lipid measurements

Metabolic analysis was performed by gas chromatography-mass spectrometry (GC-MS) and carried out by the NIH West Coast Metabolomics Center. Data was obtained using the protocol described by Fiehn et al. [70]. Briefly, 0.5 µL of sample were injected in splitless mode into a GC-MS system-mass detector (Leco Pegasus IV mass spectrometer; LECO Corp., St. Joseph, MI, USA). The mass spectrometry was used as follows: mass resolution at 17 spectra per second from 80-500 Da at -70 eV ionization energy and 1800 V detector voltage with a 230 °C transfer line and a 250 °C ion source. Initially, the injection temperature was of 50 °C and ramped to 250 °C by 12 °C per second, while the column temperature started at 50 °C for 1 min and ramped to 330 °C. The GC column was a Rtx-5Sil MS (Restex) of 30 m length, 0.25 mm of internal diameter with 0.25 µm film made of 95% dimethyl/5% diphenylpolysiloxane with a constant flow of 1 mL/min Helium gas as mobile phase. The oven temperature program was of 50 °C for 1 min, then ramped to 20 °C for 1 min and maintained 330 °C for 5 min. Automatic mass spectral deconvolution was performed with peak detection of GC spectrum using the BinBase algorithm (rtx5). The BinBase algorithm was set as: validity of chromatogram (>10 peaks with intensity >10^7 counts s^{-1}), unbiased retention index marker detection (MS similarity z800, validity of intensity range for high m/z marker ions), and retention index calculation by 5th order polynomial regression. Spectra are cut to 5% base peak abundance and matched to database entries from the highest to lowest abundant spectra using the following matching filters: retention index window ± 2,000 units (equivalent to about ± 2 s retention time), validation of unique ions and apex massed (unique ion must be included in apexing masses and present at > 3% of base peak abundance), mass spectrum similarity must fit criteria dependent on peak purity and signal/noise ratios and a final isomer filter. All thresholds reflect settings for ChromaTop v2.32. Quantification was reported as peak height using the unique ion as default, unless a different quantification ion is manually set in the BinBase administration software BinView.

Lipid analysis was carried out by the NIH West Coast Metabolomics Center and performed by Ultra High-Pressure Liquid Chromatography (UHPLC) with charged surface hybrid column and electrospray (CSH-ESI). The detector employed was a quadrupole time of flight with tandem mass spectrometry (QTOF MS/MS). Data was acquired injecting 3 µL of sample in column Waters Acquity UPLC CSH C18 (100 mm length x 2.1 mm internal diameter and 1.7 µm particles) with a mobile phase A of 60:40 v/v acetonitrile:water, 10 mM ammonium formiate and 0.1% of formic acid; and a mobile phase B of 90:10 v/v isopropanol:acetonitrile, 10 mM ammonium formiate and 0,1% of formic acid. The column temperature was 65 °C, the flow rate was 0.6 mL/min and the injection temperature were 4 °C. Data was processed in an untargeted manner by Agilent´s software MassHunter Qualitative in order to find peaks. Peaks features were imported into MassProfilerProfessional for peak alignments and filtering and detect target lipids. Using the MS/MS information and the lipidBlast library was possible to identify lipids with manual confirmation. Then, data was quantified by MassHunter Quantitative software using the unique retention time and the mass/z identification.

3.4. Metabolic pathway assessment

In order to determine enrichment in metabolites when comparing conditions, the first step to characterize pathways, the ChemRICH tool [12] was used following the default conditions. Student´s t-test was performed to evaluate differences between pairs of conditions to be assessed. Metabolites that had no or duplicated Pubchem ID, or that had no InChiKeys, were excluded from this analysis.

3.5. Statistical Analysis

Partial Least Square Regression-Discriminant Analysis (PLS-DA) was performed on the normalized data, using the dataset with results from GC-MS and UHPLC MS/MS analysis using MetaboAnalyst 4.0 (Xia Lab, McGill University, Quebec, Canada). PLS-DA analysis was used with metabolites as predictor variables, while employing phenotype and stage of postharvest as response

variables. To assign an equal variance, all variables were mean centered and weighted by standard deviation. To select important features, Variables Important in Projection (VIP) scores were employed to filter PLS results. These data were analyzed using Student´s t-test statistical tools (p<0.05) to identify compounds with significant differences between E1 vs E3, and mealy vs juicy.

Additionally, in order to validate the metabolites and lipids found in each phenotype, two more individuals with five replicates from each phenotype were used for evaluating the metabolites and lipids that showed a differential abundance in the multivariant analysis. These data were analyzed using Student´s t-test statistical tools (p<0.05) in order to identify statistical differences between the stages of postharvest. In order to correlate differences between samples, we performed a hierarchical clustering analysis using Spearman´s rank correlation and single linkage as clustering algorithm.

4. Conclusions

Our results indicate that fruits from both phenotypes (mealy and juicy) present differences in metabolism before cold storage, probably determining its further cold resistance. After cold storage (E3), metabolism-related to fruit quality like sugar metabolism is partially impaired. Most of the metabolites found in the mealy phenotype and present in lower amounts are related to membrane stability like lipids as MGDG and PG. However, those found in higher amounts are related to cold stress as sugars and LPC. These metabolites and lipids could be used as possible biomarkers for CI.

Supplementary Materials: The following are available online, Figure S1: Variables Important in Projection (VIP) identified by PLS-DA. VIP analysis for Juicy phenotype at E1 and E3 stage of postharvest filtered by metabolites (A) and filtered by lipids (B). VIP analysis for Mealy phenotype at E1 and E3 stage of postharvest filtered by metabolites (C) and filtered by lipids (D). Figure S2: Hierarchical cluster analysis for Juicy phenotype. A) Dendogram show cluster analysis for juicy phenotype samples at E1 and E3 stage for metabolites. B) Dendogram show cluster analysis for juicy phenotype samples at E1 and E3 stage for lipids. Red box represents E1 stage of postharvest and green box represents E3 stage of postharvest. The similarity measure was Spearman´s rank correlation and the clustering algorithm was single linkage. Figure S3: Hierarchical cluster analysis for Mealy phenotype. A) Dendogram show cluster analysis for mealy phenotype samples at E1 and E3 stage for metabolites. B) Dendogram show cluster analysis for mealy phenotype samples at E1 and E3 stage for lipids. Red box represents E1 stage of postharvest and green box represents E3 stage of postharvest. The similarity measure was Spearman´s rank correlation and the clustering algorithm was single linkage.

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