Plant Physiology and Biochemistry 136 (2019) 230–244

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Proteomic and metabolomic approaches unveil relevant biochemical changes in carbohydrate and cell wall metabolisms of two blueberry (*Vaccinium corymbosum*) varieties with different quality attributes

M.L. Montecchiarini[a], E. Margarit[a], L. Morales[a], M.F. Rivadeneira[b], F. Bello[b], A. Gollán[b], D. Vázquez[b], F.E. Podestá[a,**], K.E.J. Tripodi[a,*]

[a] Centro de Estudios Fotosintéticos y Bioquímicos (CEFOBI), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000, Rosario, Santa Fe, Argentina

[b] Estación Experimental Concordia, Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Yuquerí, 3200, Concordia, Entre Ríos, Argentina

ARTICLE INFO

Keywords:
Blueberries
Firmness
Cell wall
Metabolites
Fatty acid
Phenolic compounds
Differential proteomic

ABSTRACT

Quality maintenance in rapidly decaying fruit such as blueberries (*Vaccinium corymbosum*) is of essential importance to guarantee the economic success of the crop. Fruit quality is a multifaceted subject that encompasses flavor, aroma, visual and physical issues as main factors. In this paper we report an ample characterization of different biochemical and physical aspects in two varieties (O'Neal and Emerald) of blueberries that differ in firmness, aspect, flavor and harvesting times, at two different phenological stages (fruit set vs. ripe), with the intention of unveiling how the metabolic signature of each contributes to their contrasting quality. To this effect a metabolomic, ionomic and proteomic approach was selected. The results presented here show marked differences in several variables at the two stages and between varieties. Emerald is an early variety with a large, good taste and firm fruit, while O'Neal is soft, medium sized and very sweet. Proteomic data comparison between both cultivars showed that, at fruit set, processes related with the response to inorganic compounds and small molecule metabolisms are relevant in both varieties. However, solute accumulation (mainly amino acids and organic acids), enzymes related with C: N balance, water transport and cell wall recycling are enhanced in Emerald. In ripe fruit, Emerald showed an enrichment of proteins associated with TCA, nitrogen, small molecules and cell wall *in muro* recycling processes, while mannitol and fatty acid metabolism were enhanced in the soft variety. The measured variation in metabolite levels gave strong support to the precedent results. This study suggests that at fruit set, a composite scenario of active metabolic recycling of the cell wall, improved C: N balance and solute accumulation give place to a more efficient carbon and water resource management. During the ripe stage, an increased and efficient *in muro* and metabolic recycling of the cell wall, added to enhanced inositol and secondary metabolism may be responsible for a best turgor conservation in Emerald. These findings may yield clues for improvements in fertilization practices, as well as to assist the guided development of new varieties based on biochemical quality.

1. Introduction

Blueberries are appreciated by their pleasant appearance and flavour, as well as by their high content of bioactive molecules with a wide range of health benefits (Michalska and Łysiak, 2015).

The processes that govern ripening in this fruit are not entirely understood. They generate low levels of ethylene and are thus considered non-climacteric fruit (Frenkel, 1972; Lipe, 1978). One of the most appreciated quality trait is firmness, since a soft fruit would be frequently rejected by consumers. The causes of softening in fruits are diverse and involve events such as dehydration, cell wall dynamic, turgor and membranes damage, among others (Brummell, 2006a; Li et al., 2009; Paniagua et al., 2013; Vicente et al., 2007; Wang et al., 2018; Zoccatelli et al., 2013). In turn, these processes are deeply dependent on environmental conditions (i.e. soil composition, irrigation, climate, precipitation regime) and variety (Prodorutti et al., 2007; Sim et al., 2017; Zapata et al., 2010; Zhao, 2012). These are the major reasons for which literature about calcium fertilization, a frequent

* Corresponding author.,
** Corresponding author.
E-mail addresses: podesta@cefobi-conicet.gov.ar (F.E. Podestá), tripodi@cefobi-conicet.gov.ar (K.E.J. Tripodi).

https://doi.org/10.1016/j.plaphy.2018.12.019
Received 16 October 2018; Received in revised form 13 December 2018; Accepted 19 December 2018
Available online 21 December 2018
0981-9428/ Published by Elsevier Masson SAS.

practice in blueberry cultivation, is not conclusive in terms of softening reduction (Angeletti et al., 2010; Basiouny and Woods, 1992; Berkheimer, E.J., Hanson, 2004; Stückrath et al., 2008).

Some highlights of blueberries production and commercialization in Argentina are relevant in defining the present research goals. For instance, the bulk of annual production is exported fresh and prime fruit have better prices. Thus, both the early production and the excellence in quality attributes are among the most relevant aspect to be considered by farmers and agronomists. Also, the facts that fruit is harvested stepwise due to non-homogeneous ripening, and that occasionally export is done by boat, cause that the time elapsed since the harvest and the arrival to final market location can exceed 30 days in some cases, seriously harming quality on destination. In sum, the above mentioned circumstances stress the need to establish the general quality determinants, and high firmness in particular at a molecular level as a main focus of research. In this sense, it is of interest for researchers involved in crop management assistance to gain knowledge about the molecular basis, i.e. the main compounds and biological processes that are connected with desirable traits. Previous work in which metabolomic and physiologic profiles were analyzed in three blueberries varieties, gave some clues about a few chemical compounds and cell wall linked enzymes activities strongly correlated with fruit firmness (Montecchiarini et al., 2018). For this study, two of these varieties were selected according to their contrasting firmness: 'Emerald', highly productive, with large, good flavoured and very firm fruit and 'O'Neal', an early, very sweet, public cultivar with medium size and less firm fruit (http://www.fallcreeknursery.com/commercial-fruit-growers/varieties).

The main goal of this work is to use both the proteomic and metabolomic approaches to delve into how the differential molecular repertoire in two blueberries varieties at two phenological stages (set and full mature fruit), are linked to their contrasting quality. This information would contribute not only to design suitable crop management programs, but also it may provide agronomists with reliable methods for screening and selection of higher quality varieties.

2. Materials and methods

2.1. Plant material, growth conditions and fruit sampling

Blueberries from 'Emerald' (U.S. Plant Patent 12165 P2) and 'O'Neal' cultivars were collected at local orchards in Concordia (Entre Ríos, Argentina, O'Neal lot Lat S: −31.398364, Long W: −58.107351; Emerald lot Lat S −31.32664, Long W −58.083086) during the morning, in 2016 season. Mature bushes used for field experiments were located in commercial fields, plants were grown on raised pine bark rows with a plant density of 3333 plants/ha. Standard agro-technical procedures were performed during the growing season. For frost protection, overhead sprinklers were used. The sampling dates in each cultivar were at 9 days after full bloom (DAFB) and 80 DAFB, corresponding to fruit set and ripe fruit (full blue fruit), respectively. Emerald blooms in July and harvest season spans from October to December, while O'Neal blooms in late July and is harvested from November to December. Data about precipitation levels, temperature fluctuation and time of harvesting are summarized in Fig. 1. Thirty berries were collected from five different plants of each variety. After manual collection, exocarp and pulp (meso- and endocarp) were carefully separated for mature fruit. Samples were frozen at −80 °C until analysis. The reason to analyse the exocarp and the pulp separately is mainly to dissect the processes governing general quality and firmness in particular in each of these metabolically and anatomical different tissues. All the subsequent determinations were performed on the pulp of ripe berries, while entire fruit was used for fruit set analysis, due to the hindrance presented in the separation of tissues. At least three biological replicates were performed for all measures. Each replica was composed of a pool of three fruits.

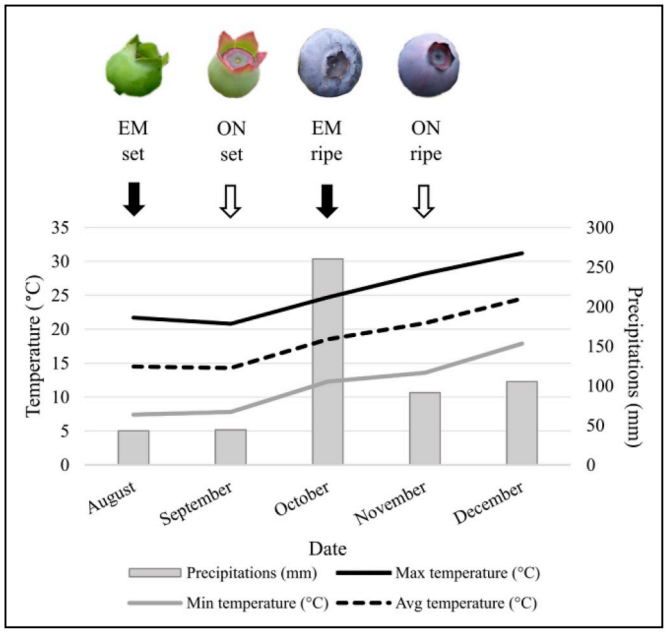

Fig. 1. Precipitation levels and temperature fluctuation during the period of harvesting for each variety at each phenological stage.

2.2. Metabolite purification, derivatization and analysis

Samples were essentially treated as described by Perotti et al. (2011). In brief, 300 mg of frozen tissue were powdered in a mortar with liquid nitrogen, 4.2 mL of cold methanol and 75 µg of ribitol (as internal standard) were added. Preparations were transferred to glass tubes and incubated at 70 °C for 15 min. After the addition of 1.5 mL of chloroform, samples were incubated 5 min at 37 °C. Finally, 3 mL of water were added and extracts were centrifuged at 2200 × *g* and for 15 min. The polar phase (450 µL) was dried in a vacuum centrifuge (CentriVap, Labconco) until complete evaporation. Each pellet was resuspended in 30 µL of freshly prepared 20 mg/mL methoxyamine in pyridine, and tubes were incubated 90 min at 37 °C. Finally, 45 µL of derivatizing reactive, N-methyl-N-trimethylsylil-trifluoroacetamide (MSTFA) were added to each tube and incubated at 37 °C for 30 min. Chromatographic runs were performed by injecting 1 µL of derivatized sample (split: 1:100) in a 30 m long, 0.25 mm thick HP5ms UI GC/MS capillary column using an automatic system coupled to an Agilent simple quadrupole mass spectrometer (5977A). Data were analyzed using the OpenChrom software (http://www.openchrom.net) for peak area determination and the Automated Mass spectral Deconvolution and Identification System software (AMDIS) for compound identification. Chromatograms acquired were analyzed by comparing individual peak areas for each metabolite relative to that of ribitol, the internal standard. Data were revised using the Golm metabolome database from the Golm Metabolomic Institute (Germany) to confirm the identity of the compounds.

2.3. Fatty acid extraction, derivatization and analysis

Total esterified lipids were extracted from fruit as described before (Bligh and Dyer, 1959) with slight modifications. Approximately, 500 mg of tissue were powdered in a mortar with liquid nitrogen and then homogenized with 300 µl of distilled water, to obtain 0.8 mL of homogenate. Samples were transferred to glass tubes containing 6 mL of a methanol: chloroform (2:1) mixture. Each tube was shaken and incubated overnight at −20 °C. After centrifugation at room temperature for 5 min at 800 × *g*, supernatants were transferred to new glass tubes. One mL of chloroform and 1 mL of distilled water were added to

each tube. Samples were centrifuged for 5 min at room temperature at 800 × *g* to facilitate phases separation. The lower phase was conserved and washed twice with 2 mL of 2 M KCl.

Tubes were centrifuged for 5 min at room temperature at 800 × *g*. Finally, after drying the chloroform phase under nitrogen atmosphere, pellets were resuspended with 0.5 mL of sodium methoxide diluted in anhydrous methanol in a 1:8 proportion. After incubation for 30 min at room temperature, 1 mL of 2 M HCl and 1 mL of hexane were added. Finally, the hexane (upper) phase was transferred to a new glass tube for chromatographic analysis. In all cases, 2 µl of derivatized samples were injected in a 30 m × 0.25 mm SUPELCOWAX-10 (Sigma) column coupled to a ThermoQuest mass spectrometer. The run was carried out isothermally for 30 min at 180 °C. Afterwards, the temperature was increased at 12 °C/min to reach 240 °C. Data were collected and analyzed using the Lab Solution software (Shimadzu). In order to identify the different methylated fatty acids, the retention time and peaks obtained in the mass spectrum were compared with true standards (Sigma Aldrich) or with available data in NBS75K (National Bureau of Standards database, Perkin Elmer). After sample analysis and relative amount calculation of each fatty acid, the double bond index (DBI) was obtained. The DBI is a measure of lipid unsaturation and was calculated according to (Zhou et al., 2014) as follows: DBI double bond index = Σ (% unsaturated fatty acid × number of double bonds).

2.4. Alcohol insoluble residue (AIR) determination and ion content analysis

Cell wall polysaccharides as AIR were obtained according to the method described by Angeletti et al. (2010) with the following modifications: 1 g of tissue was ground, homogenized in 3 mL of ethanol and boiled for 45 min to inactivate enzymes. Calcium and other ions were measured in cell wall material as follows: 50 and 15 mg of AIR from set and ripe fruit respectively, were digested with 1 mL of concentrated HNO_3 at 100 °C for 5 h. After sample dilution, ions were quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Perkin Elmer Nexion 350X). Quantification was carried out using standards curves for each compound, results are informed as ng ion/mg AIR.

2.5. Total phenolic compounds determination

The determination of total phenolics content (TPC) was carried out using the Folin-Ciocalteu reagent as described by Velioglu et al. (1998). Powder obtained from 25 mg of frozen tissue was homogenized in 750 µl of buffer (80% methanol, 1% HCl) and it was left to extract for 2 h. After centrifugation (25000 × *g* for 5 min), the supernatant was recovered and a re-extraction of the pellet was carried out. The reaction mixture was prepared with 150 µl of the combined supernatants obtained in the previous step and 150 µl of Folin-Ciocalteu reagent. After 3 min, 500 µl of sodium bicarbonate (20%) were added and the reaction was incubated for 120 min. Finally, absorbance at 730 nm was measured. Results were informed as gallic acid µg/mg of fresh weight. Three technical replicates were performed for each of the three biological replicates employed.

2.6. Total protein extraction and preparation for proteomic analysis

Protein extraction was performed according to Hurkman and Tanaka (1986) with some modifications. Two hundred mg of frozen tissue were ground with liquid nitrogen and homogenized with 2 mL of buffer (0.1 M Tris pH 8.8, 10 mM EDTA, 0.9 M sucrose, 1 mM PMSF, 0.4% (v/v) BME, 5% (p/v) PVPP). Four ml of phenol-Tris HCl (pH 8.8) were added, and the mixture was shaken and incubated at 4 °C for 30 min. After centrifugation at 5000 × *g* for 20 min, the phenolic phase was recovered and mixed with 5 vol of 0.1 M ammonium acetate in methanol. After overnight protein precipitation at −20 °C, the pellet was washed three times with ammonium acetate, and once with 80% (v/v) cold acetone. Pellets were dried and solubilized in 8 M urea. Extractions were made in triplicate for each sample. Protein concentration was assayed with the bicinchoninic acid method (Thermo Scientific Pierce BCA Protein Assay Kit). A calibration curve was carried out using bovine serum albumin as standard.

2.7. Protein identification and analysis

Forty microgram of each protein extract were reduced for 45 min at 56 °C using 10 mM DTT and alkylated for 40 min with 20 mM iodoacetamide at room temperature in the dark. Finally, proteins were precipitated by adding 100% (p/v) trichloroacetic acid to a final concentration of 20%, washed three times with cold acetone, and dried.

Protein preparations were sent to the Proteomics Core Facility of CEQUIBIEM at the University of Buenos Aires. Samples were resuspended in 50 mM $(NH_4)HCO_3$ at pH 8.0, digested overnight with sequencing-grade modified trypsin (Promega) and desalted with ZipTip C18 (Merck Millipore). Proteins were analyzed by nanoHPLC (EASY-nLC 1000, Thermo Scientific, Germany) coupled to a mass spectrometer with Orbitrap technology (Q-Exactive with High Collision Dissociation cell and Orbitrap analyzer, Thermo Scientific, Germany). Peptide Ionization was performed by electrospray. Data were analyzed with Proteome Discoverer 2.1 software (Thermo Scientific, Germany) for identification and area quantitation of each protein. Protein identification was performed using *Vitis vinifera* Uniprot protein collection as a reference (UP000009183; Feb 4, 2017; https://www.uniprot.org/proteomes/UP000009183).

Triplicate area values obtained were checked for missing values, and samples with no values or only one, were replaced with a minimal global one in order to allow comparisons within samples. The Perseus software v1.6.1.3 (Tyanova et al., 2016) was used to perform comparisons among samples area values and statistical tests. Upon analysis, proteins with a $\log_2$ (normalized area ratio) $> |1|$ and p-value < 0.05 were considered as significantly accumulated within each sample comparison.

To characterize the proteins of interest, Uniprot identifiers were assigned to Phytozome IDs using *Vitis vinifera* Genoscope.12X database (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Vviniferav), and used to search for pre-computed *Arabidopsis thaliana* assigned orthologs within this database.

Gene ontology enrichment analysis was carried out using Singular Enrichment Analysis (SEA) through AgriGO v2.0 (http://systemsbiology.cau.edu.cn/agriGOv2/index.php) and the plant GO-slim database as reference. In addition, ShinyGO v0.41 (Ge and Jung, 2018) was also used to perform gene ontology enrichment analysis as well as metabolic pathway networks enrichment through the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways collections for *Arabidopsis thaliana*. In all cases, enrichment studies were statistically tested by the Hypergeometric test performed with multiple testing correction using Hochberg false discovery rate (FDR) correction and a significance level < 0.05 after correction.

2.8. Search strategy for cell wall metabolism-related proteins

In order to explore the nature of enzymes related with cell wall metabolism represented within the complete set of proteins recovered, a keyword search was performed in the *Vitis vinifera* Genoscope.12X proteome (Phytozome) using protein names. This search included alpha and beta galactosidases, endoglucanases, pectinmethylesterases (PME), polygalacturonases (PG), PME inhibitor proteins, PG inhibitor proteins, pectate liases, xyloglucan hydrolases, xylosidases and expansins. Afterwards, their amino acid sequences were retrieved and used to perform a basic local alignment (blastp; minimum e value: 10^{-10}) against the *Vitis vinifera* Uniprot proteome (UP000009183). In this manner, it was possible to assign some missing identities, and to finally search within our proteomics data for cell wall metabolism-related proteins using the identified proteins as bait.

2.9. Statistical procedures

Inferential statistics was carried out applying t-Student test with a significance level of 0.05, in order to identify data with statistically significant differences between varieties in each stage under study. The Sigma Stat Package was used for statistical analysis. Principal component analysis was performed with R package "FactoMineR" and "corrplot".

3. Results

3.1. Metabolite and ion content in blueberries pulp at two maturation stages: fruit set and ripe

In order to detect the vast and diverse collection of molecules that characterize a cellular state, more than one method of analysis is usually required. In this sense, metabolomics is one of the most complex omics approaches, but is probably the best to portray the actual physiological status of the analyzed sample with high fidelity. Thus, by a combination of different techniques, the relative quantification of sugars, sugar alcohols, amino acids, organic acids, total phenolic compounds and fatty acids in the pulp of two blueberry varieties at two different maturation stages was carried out.

Ions quantification was performed in the AIR fraction, enriched in cell wall material. Ion levels were compared for each blueberry variety and are presented as the amount found in Emerald relative to that found in O'Neal (EM/ON ratio) at fruit set (Fig. 2) and in ripe fruit (Fig. 3). Numerical data and statistical analysis is informed in Supplementary Table 1.

It is worth to mention that a number of metabolites, mainly free amino acids, were detected only in Emerald. These were proline (Pro), isoleucine (Ile), methionine (Met), ornithine (Orn), asparagine (Asn), xylose, threonic and galacturonic acid in fruit set; glutamine (Gln) and asparagine (Asn) in ripe stage (Supplementary Table 1).

Organic acids. Citric acid content was significantly higher in O'Neal while malic acid predominated in Emerald at fruit set, as well as shikimic and caffeoylquinic acids (Fig. 2). In ripe fruit, all these compounds were prevailing in Emerald (Fig. 3).

Sugars. No significant changes in the content of the main sugars were observed at fruit set (Fig. 2). Sucrose content was higher in ripe O'Neal, without further variation for the additional major sugars, fructose and glucose (Fig. 3).

Amino acids. Several amino acids, such as alanine (Ala), serine (Ser), valine (Val), threonine (Thr), Gln, aspartate (Asp), glutamate (Glu) and gamma aminobutyric acid (GABA) were more abundant in Emerald at fruit set. In ripe fruit, Ala, Ser, Asp and Glu predominated in Emerald, while Val content was higher in O'Neal (Figs. 2 and 3).

Fatty acids. Major differences between fatty acids content were noticed at fruit set, when levels of 16:0, 22:0, 24:0, 16:1, 18:2 and 18:3 were higher in Emerald (Fig. 2). In ripe fruit, 18:1 level was higher in Emerald, while 18:0 and 17:0 predominated in O'Neal. DBI (double bond index) was calculated from these results and it was found to be higher for Emerald at both phenological stages (Figs. 2 and 3).

Other compounds. The content of a cyclic polyalcohol, inositol, was higher in Emerald at both stages, while mannitol, also a sugar alcohol derived from hydrogenation of mannose, was only detected in ripe fruit and was more abundant in O'Neal. Total phenolic compounds (TPC) content was significantly higher in ripe fruit of Emerald (Figs. 2 and 3).

Ion content in AIR. From the nine ions whose levels were quantified by ICP-MS in the AIR, two are considered as essential macronutrients (calcium and magnesium) and five as essential micronutrients (boron, iron, copper, manganese and zinc) (Benton Jones Jr., 2012). At fruit set, their levels were comparable between both varieties, with the exception made for aluminium, that was almost 3.2 times more abundant in O'Neal, and manganese which was 4.9 times higher in O'Neal. On the other hand, differences found in ripe fruit were significant only for manganese (3 times higher in O'Neal) and iron (2 times higher in Emerald) (Fig. 3). Calcium, frequently associated with cell wall strengthening, did not show significant differences between both varieties at any stage (Figs. 2 and 3, Supplementary Table 1).

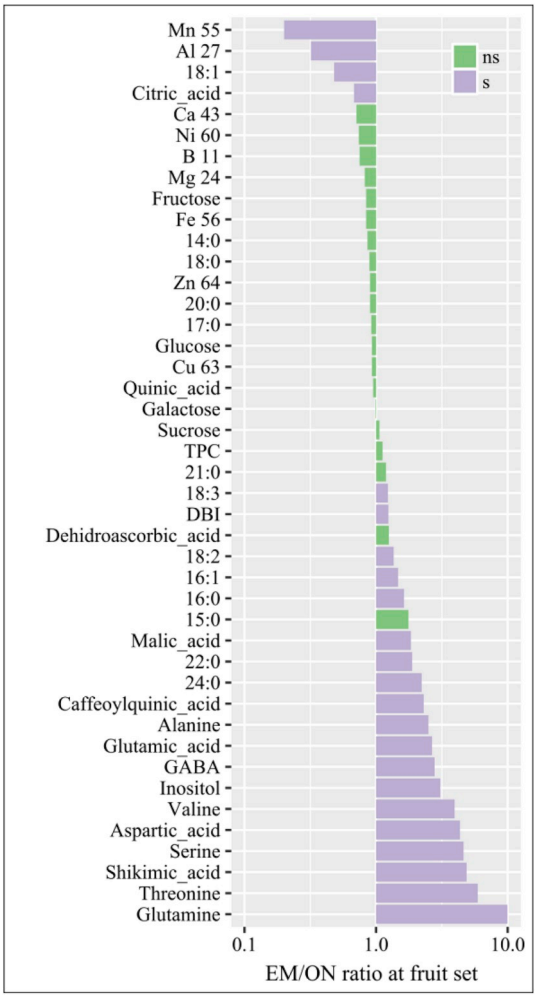

Fig. 2. EM/ON ratio of metabolite, ion, TPC content and DBI at fruit set. X axis has a logarithmic scale. Purple bars indicate that there is a statistically significant difference between varieties ($\alpha = 0.05$, Supplementary Table 1). Graph was constructed using R package "ggplot2". (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

With the aim of gaining insight into the metabolic association with differential traits in both varieties, data from metabolites that fluctuated significantly were subjected to Principal Component Analysis (PCA) (Fig. 4). In this study, metabolites that were undetectable in O'Neal were excluded. Hence, at fruit set, PC1 (90.59%) was able to discriminate both varieties based on the following metabolites: manganese (contribution to individual PC of 4.54) which was predominant in O'Neal; while Ala (4.57), Val (4.71), Ser (4.72), Thr (4.67), malic acid (4.59), Asp (4.45), GABA (4.50), Glu (4.65), Gln (4.71), shikimic acid (4.68), and inositol (4.68) content was more abundant in Emerald. Meanwhile, in ripe fruit, PC1 (89.31%) allowed the separation between varieties, with Ala (4.95), Ser (5.07), Asp (4.88), Glu (4.87), citric acid (4.99), quinic acid (4.95), inositol (5.08) and TPC (5.00) content more abundant in Emerald, while Val (5.04), xylose (4.99), margaric acid (17:0) (4.81), and mannitol (4.72) predominated in O'Neal.

Therefore, at a first glance, the precedent results make it compelling to suggest that some metabolic pathways might be predominant in Emerald at fruit set, in contrast with O'Neal. Proteomic analysis and subsequent data interconnection will shed light on the physiological significance of observed differences between both cultivars.

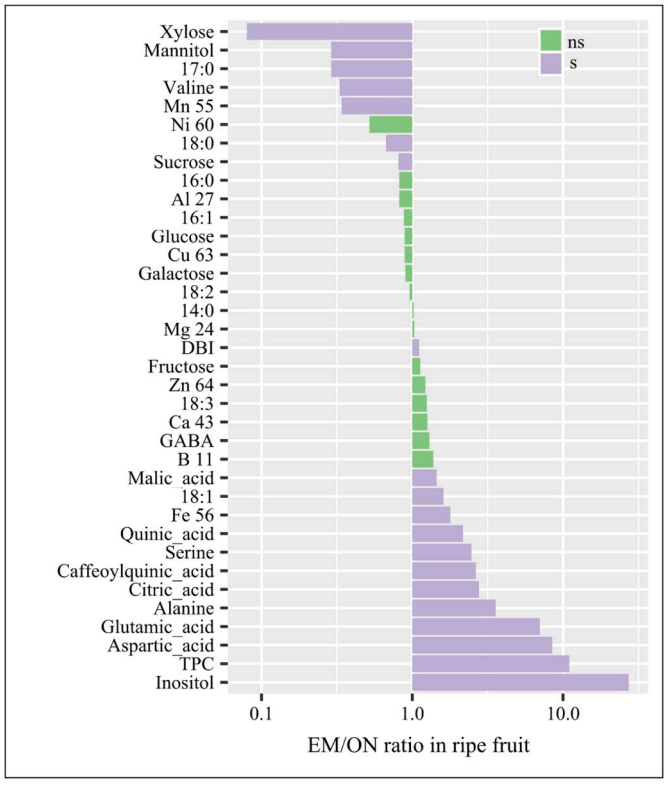

Fig. 3. EM/ON ratio of metabolite, ion, TPC content and DBI in ripe fruit. X axis has a logarithmic scale. Purple bars indicate that there is a statistically significant difference between varieties (α = 0.05, Supplementary Table 1). Graph was constructed using R package "ggplot2". (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Functional characterization and identification of protein differentially expressed between varieties at each phenological stage

Proteomic analysis resulted in a total of 630 different proteins detected for the complete set of samples (data not shown). Comparisons between varieties at the same maturity stage were performed. Proteins whose relative abundance (EM/ON ratio) varied significantly were selected from Volcano plots generated by Perseus software, and their identities assigned based on *Vitis vinifera* proteome database and *A. thaliana* orthology (see materials and methods) (Supplementary Table 2).

Functional analysis and enrichment of biological processes were determined with ShinyGO and AgriGO using *A. thaliana* orthologous gene names. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database, linking genomic and functional information, allowed the systematic analysis of gene functions and metabolic pathways that overrepresented in each analysis (PlantGSEA).

Considering the same developmental stage, the first glaring difference was the magnitude of proteome change in each variety, as judged by the total number of proteins recovered as differentially abundant, being remarkably lower for O'Neal in both cases (Table 1).

During fruit set, 166 (159 non-redundant) proteins were more abundant in Emerald, while 21 (20 non-redundant) were in O'Neal (Table 1). Gene ontology analysis (AgriGO) for Emerald proteins indicated that biological processes terms were enriched in response to stimulus (71), mainly abiotic stimulus (38) and stress response (44)), and also metabolic (115) and cellular processes (128). Among metabolic processes the most represented were biosynthesis of primary metabolites (107), nitrogenated compounds (81) and catabolic processes (30). Cellular processes comprise mainly metabolic aspects such as generation of precursors and energy metabolites (19) and macromolecule metabolism (63). Other processes that appeared represented were gene expression (43), carbohydrate (21) and lipid (12) metabolic processes. Meanwhile, in O'Neal, processes such as response to stimulus (12; abiotic stimulus (5) and response to stress (8)) and post-embryonic development (5) were enriched. Notably, in Emerald 18 proteins clustered in the cell wall cellular component category, while this group was not found in O'Neal. Identity of these proteins is informed in Table 2.

In ripe fruits, 88 (84 non-redundant) proteins whose levels were significantly augmented in Emerald, correlated with metabolic processes (70), from them, cellular (61) and primary (58) metabolism categories were the most enriched. Response to stimulus term (49; abiotic stimulus (23), response to stress (27)) was enriched, as well as nitrogen compound biosynthesis (38), biosynthetic (42) and catabolic processes (16). Other categories were photosynthesis (6), carbohydrate (12) and lipid (11) metabolic processes. Once more, 10 proteins were categorized in Emerald as cell wall cellular component category (Table 2). In O'Neal 35 polypeptides were found, the majority of them were related with cellular processes (25) and metabolic processes (23), with photosynthesis (5) and generation of precursor metabolites and energy (5) as

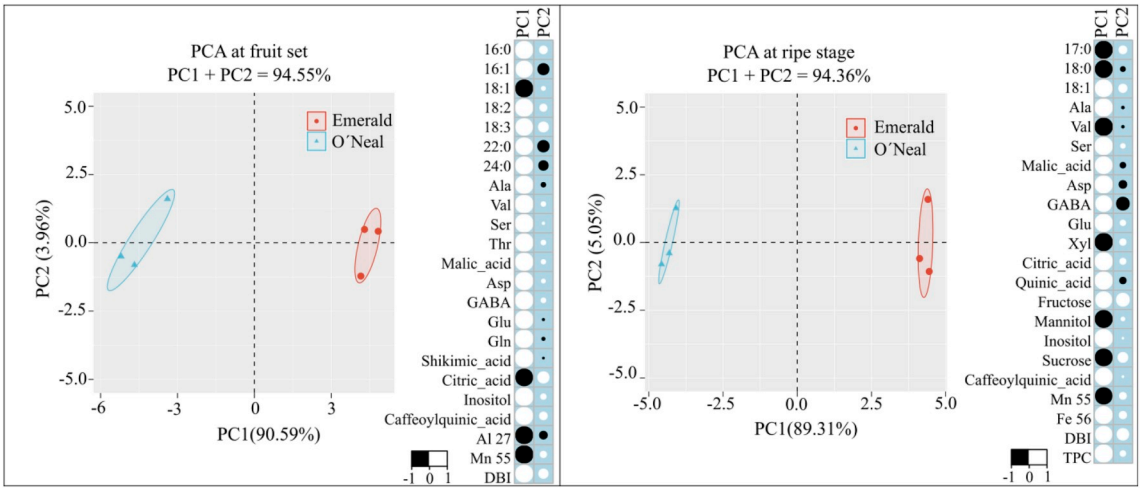

Fig. 4. PCA of data at fruit set and at ripe stage. In each case, only data that showed statistically significant difference between varieties were used. Three independent replicates were analyzed for each variety. The variance explained by each component (%) is given within parentheses. Plots at the right of each PCA indicate the correlation between each metabolite and both of the principal components (PC1 and 2). Positive correlations are displayed in white, while negative correlations in black. The size of the circle is proportional to the correlation coefficient.

Table 1
Functional classification of proteins differentially expressed in Emerald compared to O´Neal. Proteins from Supplementary Table 2 were analyzed with AgriGO. Categories for GO: Biological Processes and GO: Cellular Component (cell wall) increased in each cultivar and phenological stage (FDR < 0.05), are summarized.

Stage: FRUIT SET				Stage: FRUIT SET			
Variety: Emerald				Variety: O´Neal			
GO_acc Biological Process	Term	Number of hits (total: 159)	FDR	GO_acc Biological Process	Term	Number of hits (total: 20)	FDR
GO:0044237	cellular metabolic process	115	5.2e-20	GO:0050896	response to stimulus	12	0.0041
GO:0009987	cellular process	128	1.7e-18	GO:0006950	response to stress	8	0.013
GO:0008152	metabolic process	119	1.9e-15	GO:0009791	post-embryonic development	5	0.02
GO:0044238	primary metabolic process	107	1.9e-15	GO:0009628	response to abiotic stimulus	5	0.043
GO:0006807	nitrogen compound metabolic process	81	1.9e-14	GO:0009987	cellular process	14	0.065
GO:0009058	biosynthetic process	77	2.7e-12	GO:0008152	metabolic process	13	0.089
GO:0006091	generation of precursor metabolites and energy	19	4.8e-12	GO:0007275	multicellular organism development	5	0.09
GO:0044249	cellular biosynthetic process	72	2.8e-11	GO:0032501	multicellular organismal process	5	0.1
GO:0009628	response to abiotic stimulus	38	2.5e-10	GO:0032502	developmental process	5	0.1
GO:0050896	response to stimulus	71	1.4e-09	GO:0048856	anatomical structure development	5	0.1
GO:0009056	catabolic process	30	2.8e-09	GO:0044237	cellular metabolic process	10	0.16
GO:0006412	translation	27	4.8e-07	GO:0009058	biosynthetic process	6	0.33
GO:0006950	response to stress	44	8.5e-07	GO:0044249	cellular biosynthetic process	5	0.46
GO:0044267	cellular protein metabolic process	46	1.5e-06	GO:0006807	nitrogen compound metabolic process	5	0.49
GO:0019538	protein metabolic process	49	2.00E-06				
GO:0005975	carbohydrate metabolic process	21	8.2e-06				
GO:0016043	cellular component organization	32	2.2e-05				
GO:0006139	nucleobase-containing compound metabolic process	42	3.1e-04				
GO:0044260	cellular macromolecule metabolic process	63	4.5e-04				
GO:0043170	macromolecule metabolic process	66	0.001				
GO:0015979	photosynthesis	7	0.0019				
GO:0010467	gene expression	43	0.002				
GO:0034645	cellular macromolecule biosynthetic process	40	0.0037				
GO:0009059	macromolecule biosynthetic process	40	0.0045				
GO:0007275	multicellular organism development	27	0.0096				
GO:0048856	anatomical structure development	29	0.012				
GO:0040007	growth	10	0.012				
GO:0032501	multicellular organismal process	28	0.012				
GO:0032502	developmental process	29	0.015				
GO:0009719	response to endogenous stimulus	18	0.018				
GO:0006629	lipid metabolic process	12	0.021				
GO:0009790	embryo development	8	0.03				
GO:0006810	transport	21	0.049				
Cellular component GO:0005618	cell wall	18	2.2e-07				
Stage: RIPE				**Stage: RIPE**			
Variety: Emerald				**Variety: O´Neal**			
GO_acc Biological Process	**Term**	**Number of hits (total: 84)**	**FDR**	**GO_acc Biological Process**	**Term**	**Number of hits (total: 35)**	**FDR**
GO:0008152	metabolic process	70	8.3e-13	GO:0015979	photosynthesis	5	0.00043
GO:0050896	response to stimulus	49	2.1e-11	GO:0006091	generation of precursor metabolites and energy	5	0.0012

(*continued on next page*)

Table 1 (*continued*)

Stage: FRUIT SET				Stage: FRUIT SET			
Variety: Emerald				Variety: O´Neal			
GO_acc Biological Process	Term	Number of hits (total: 159)	FDR	GO_acc Biological Process	Term	Number of hits (total: 20)	FDR
GO:0044237	cellular metabolic process	61	3.7e-11	GO:0009987	cellular process	25	0.011
GO:0009987	cellular process	67	1.1e-09	GO:0050896	response to stimulus	16	0.011
GO:0044238	primary metabolic process	58	2.2e-09	GO:0044237	cellular metabolic process	21	0.012
GO:0009628	response to abiotic stimulus	23	1.4e-07	GO:0008152	metabolic process	23	0.022
GO:0009058	biosynthetic process	42	1.4e-07	GO:0006950	response to stress	10	0.033
GO:0044249	cellular biosynthetic process	37	8.7e-06	GO:0009628	response to abiotic stimulus	7	0.036
GO:0006091	generation of precursor metabolites and energy	9	9.7e-06	GO:0009058	biosynthetic process	14	0.036
GO:0006950	response to stress	27	9.8e-06	GO:0005975	carbohydrate metabolic process	5	0.036
GO:0006807	nitrogen compound metabolic process	38	9.8e-06	GO:0044249	cellular biosynthetic process	13	0.048
GO:0009056	catabolic process	16	2.0e-05				
GO:0015979	photosynthesis	6	5.0e-04				
GO:0005975	carbohydrate metabolic process	12	5.6e-04				
GO:0006629	lipid metabolic process	11	6.4e-04				
GO:0048856	anatomical structure development	20	2.5e-03				
GO:0016043	cellular component organization	17	2.5e-03				
GO:0032502	developmental process	20	3.1e-03				
GO:0019538	protein metabolic process	24	3.5e-03				
GO:0003006	developmental process involved in reproduction	12	4.2e-03				
GO:0009719	response to endogenous stimulus	13	4.5e-03				
GO:0006139	nucleobase-containing compound metabolic process	23	4.9e-03				
Cellular component GO:0005618	cell wall	10	1.4e-04				

Table 2
Detail of proteins classified in the carbohydrate metabolism (GO: Biological Process) and cell wall (GO: Cellular Component) that are differentially expressed in each phenological stage and variety. The identity (*A. thaliana* orthologous) and a description of these proteins were recovered after proteomic analysis as described in materials and methods.

BIOLOGICAL PROCESS: Carbohydrate metabolic processes	*A. thaliana* orthologous	Description	BIOLOGICAL PROCESS: Carbohydrate metabolic processes	*A. thaliana* orthologous	Description
Stage: FRUIT SET					
Increased in Emerald	AT3G02360	6-phosphogluconate dehydrogenase family protein	*Increased in O'Neal*	AT4G02280	Sucrose synthase 3
	AT3G52990	Pyruvate kinase family protein		AT1G77120	Alcohol dehydrogenase related
	AT3G06580	Mevalonate/galactokinase family protein		AT4G34200	D-3-phosphoglycerate dehydrogenase, chloroplastic
	AT3G22960	Plastidic pyruvate kinase beta subunit 1		AT1G12900	Glyceraldehyde-3-phosphate dehydrogenase, A subunit, chloroplastic
	AT5G15490	UDP-glucose 6-dehydrogenase family protein		AT3G14310	Pectin methylesterase 3
	AT3G55440	Triosephosphate isomerase			
	AT2G28760	UDP-xylose synthase 6			
	AT3G22200	PLP-dependent transferases superfamily protein			
	AT2G22240	Myo-inositol-1-phosphate synthase 2			
	AT5G50850	Transketolase family protein			
	AT5G58330	Lactate/malate dehydrogenase family protein			
	AT5G03650	Starch branching enzyme 2.2			
	AT2G36530	Enolase			
	AT3G02230	UDP-arabinopyranose mutase			
	AT5G13980	Alpha-mannosidase			
	AT3G59480	PfkB-like carbohydrate kinase family protein			
	AT2G01140	Fructose bisphosphate aldolase, chloroplastic related			
	AT4G24620	Phosphoglucose isomerase 1			
	AT3G43190	Sucrose synthase 4			
	AT3G25860	Dihydrolipoamide acetyltransferase component of pyruvate DH complex			
	AT1G59900	Pyruvate dehydrogenase complex E1 alpha subunit			
	AT1G20950	Pyrophosphate-dependent 6-phosphofructose-1-kinase (alfa subunit)			

(*continued on next page*)

Table 2 (*continued*)

BIOLOGICAL PROCESS: Carbohydrate metabolic processes	*A. thaliana* orthologous	Description	BIOLOGICAL PROCESS: Carbohydrate metabolic processes	*A. thaliana* orthologous	Description
CELLULAR COMPONENT: Cell wall	***A. thaliana* orthologous**	**Description**			
Increased in Emerald	AT3G49010	Large subunit ribosomal protein L13e			
	AT5G19760	mitochondrial substrate transporter family protein			
	AT5G07350	Tudor-SN protein 1			
	AT1G45000	AAA-type ATPase family protein/26S proteasome regulatory subunit T4			
	AT3G55440	Triosephosphate isomerase			
	AT5G19780	Tubulin alpha-5			
	AT5G58290	26S proteasome regulatory subunit T3/ regulatory particle triple-A ATPase 3			
	AT2G39730	Rubisco activase			
	AT3G06860	Multifunctional protein 2/Hydroxybutyryl-CoA dehydratase / Crotonase			
	AT3G02230	UDP-arabinopyranose mutase			
	AT2G37620	Actin 1			
	AT5G13980	Alpha-mannosidase			
	AT1G78900	Vacuolar ATP synthase subunit A			
	AT3G06720	Importin alpha isoform 1			
	AT5G42020	Mediator of RNA polymerase II transcription subunit 37A-related			
	AT5G20890	TCP-1/cpn60 chaperonin family protein			
	AT3G58610	Ketol-acid reductoisomerase			
	AT5G15490	UDP-glucose 6-dehydrogenase family protein			
Stage: RIPE FRUIT					
Increased in Emerald	AT5G13870	Xyloglucan endotransglucosylase/hydrolase	*Increased in O'Neal*	AT3G12500	basic chitinase
	AT3G22960	Plastidic pyruvate kinase beta subunit 1		AT1G43670	Fructose-1,6-bisphosphatase
	AT2G45290	Transketolase		AT5G20950	Glycosyl hydrolase family protein/ Beta-D-glucan exohydrolase-like protein
	AT3G55440	Triose-phosphate isomerase		AT3G25860	Dihydrolipoamide acetyltransferase component of pyruvate DH complex
	AT2G22240	Myo-inositol-1-phosphate synthase 2		AT1G42970	glyceraldehyde-3-phosphate dehydrogenase B subunit, chloroplastic
	AT3G43190	Sucrose synthase 4			
	AT3G02230	UDP-arabinopyranose mutase			
	AT5G52920	Plastidic pyruvate kinase beta subunit 1			
	AT1G67280	Glyoxalase/Dioxygenase superfamily protein			
	AT5G03860	Malate synthase			
	AT2G01140	Fructose Bisphosphate aldolase, chloroplastic related			
	AT1G12000	Pyrophosphate-dependent 6-phosphofructose-1-kinase (beta subunit)			
CELLULAR COMPONENT: Cell Wall					

(*continued on next page*)

Table 2 (*continued*)

BIOLOGICAL PROCESS: Carbohydrate metabolic processes	*A. thaliana* orthologous	Description	BIOLOGICAL PROCESS: Carbohydrate metabolic processes	*A. thaliana* orthologous	Description
Increased in Emerald	AT5G13870	Xyloglucan endotransglucosylase/hydrolase			
	AT1G01300	Aspartyl protease-related			
	AT3G55440	Triose-phosphate isomerase / Triosephosphate mutase			
	AT2G02560	Cullin-associated NEDD8- ubiquitin ligase complex			
	AT3G02230	UDP-arabinopyranose mutase			
	AT5G11200	ATP-dependent RNA helicase UAP56/SUB2			
	AT5G42020	Mediator of RNA polymerase II transcription subunit 37A-related			
	AT1G76160	Multi-copper oxidase, subfamily not named			
	AT5G09590	Molecular chaperone DnaK (dnaK)			
	AT1G12000	Pyrophosphate-dependent 6-phosphofructose-1-kinase (beta subunit)			

the most statistically significative terms.

A similar gene ontology analysis was carried out with the ShinyGO resource (see materials and methods), which enables the settlement of more detailed categories, useful to perform a further connection with related metabolomic data. Thus, at fruit set in Emerald, predominated the metabolic processes that included small molecules, carboxylic/organic acids and macromolecular complex subunit organization, as well as biosynthesis of organonitrogen compounds. These results were in coincidence with those obtained by AgriGO. Conversely, in O'Neal, response to inorganic substances, such as metal or cadmium and oxidation-reduction processes were the most dominant (Supplementary Fig. 1). In ripe fruit, Emerald displayed enrichment in carboxylic/organic acids, response to chemical and response to cadmium and metal ions. In ripe O'Neal, the prevalent processes were acyl-CoA and thioester metabolism, small molecules metabolism and response to metal ions (Supplementary Fig. 2).

3.3. Summary of metabolic pathways specific for each variety and stage of development

A further and complementary inspection of KEGG pathways performed with ShinyGO resource (Supplementary Fig. 3) indicated than in Emerald, at fruit set there was an increment in biosynthesis of secondary metabolites and amino acids (Val, Leu, ILe, Gly, Ser, Thr biosynthesis), general carbon metabolism (glycolysis/gluconeogenesis, TCA cycle, pyruvate, carbon fixation) and the proteasome and ribosome involving pathways. Other, less relevant, are the pentose phosphate pathway, carbon fixation, fatty acid, terpenoid, amino and nucleoside sugar metabolisms. In O'Neal, the most important were fatty acid, β-alanine and carbon metabolisms. Others were secondary metabolites, glycolysis/gluconeogenesis, glyoxylate and dicarboxylate pathways.

Metabolic pathways more represented in ripe fruit were, in Emerald, biosynthesis of secondary metabolites, carbon metabolism (like pyruvate and carbon fixation), biosynthesis of amino acids (Arg, Phe, Tyr, Trp), glycolysis/gluconeogenesis (Supplementary Fig. 4). Other were Arg and Pro metabolism, Ala, Asp, Glu metabolism, ascorbate metabolism, fatty acid metabolism and terpenoid acid biosynthesis, amino and nucleoside sugar metabolic pathways. In O'Neal, the main routes were biosynthesis of secondary metabolites, TCA cycle, carbon fixation, pyruvate and fatty acid metabolism, fatty acid biosynthesis.

3.4. Enrichment in proteins related with carbohydrate and cell wall metabolism

Primary cell wall provides to each cell with mechanical integrity, structure and a contact interphase with other cells. In this complex network, three basic groups of components interact: cellulose, hemicelluloses and pectins. Pectins polysaccharides are rich in α-1,4-linked galacturonic acid (GalA) subunits and comprise rhamnogalacturonans (RG) I and II, homogalacturonans (HG), arabinans and galactans (Atmodjo et al., 2013). GalA residues in HG can support different degree of methylation, governed by pectin methyl esterase (PME) activity. Thus, highly unesterified HG can either be cross-linked with Ca^{2+} ions to form an egg-box structure that reinforces the wall, or be substrate for pectinolytic enzymes that stimulate wall loosening. Hemicelluloses include xyloglucans, xylans, glucomannans, arabinoxylans and callose. Xyloglucans have a backbone of β-1,4- linked glucose residues, but also holds short side chains of xylose and galactose. This glycan cross-links with cellulose, strengthening the wall. While cellulose is synthesized directly in the plasma membrane, the other constituents are formed in the Golgi apparatus by diverse glycosyl transferases and, after secretion, suffer modifications catalyzed by apoplastic enzymes (Oikawa et al., 2013). As a result, cell walls are not static entities. On the contrary, during development and cell expansion, a process of constant reorganization, hydrolysis, loosening and polymerization is carried out

(Houston et al., 2016). Likewise, carbohydrate metabolism is crucial at all maturation stages, since it is involved in precursors and energy supply to the growing cell and in general homeostasis of proteins and lipids (Castellarin et al., 2016; Liu et al., 2007; Serrano et al., 2017). Diverse carbohydrates are in turn the raw material for cell wall components biosynthesis. Hence, to better characterize biochemical differences during each phenological stage in both varieties in terms of general fruit quality (and firmness in particular), two gene ontology categories were selected: carbohydrate metabolism (from biological processes GO) and cell wall (from cellular component GO). A complete description of these groups of proteins is given in Table 2.

From this selection, it is notable that, at fruit set, in Emerald several enzymes implicated in glycolysis, cytoskeletal organization, pentose and nucleotide sugars synthesis increase, in comparison with O'Neal. The α-subunit of pyrophosphate-dependent 6-phosphofructo-1-kinase (PFP alpha) was also higher. Notably, the enzyme involved in the committed step of inositol generation, myo-inositol 1- phosphate synthase (IPS) increased, and this is correlated with inositol enhanced levels (Fig. 2). This metabolite, as well as its derivatives, play crucial roles in signal transduction, stress tolerance, phosphate storage, membrane development and the synthesis of ascorbic acid (Conde et al., 2015; Cui et al., 2013; Loewus and Murthy, 2000). UDP-glucose 6-dehydrogenase catalyses the production of UDP-glucuronic acid, providing nucleotide sugars for cell wall polymer synthesis. UDP-arabinopyranose mutase catalyses the reversible conversion of UDP-arabinopyranose to UDP-arabinofuranose and is involved in the biosynthesis of non-cellulosic polysaccharides components of cell wall. It is also notorious an increment in alpha-mannosidase, proteasome and ribosome related proteins, as well as in diverse proteins related with transport. In O'Neal, levels of enzymes that were found to be increased are connected with alcoholic fermentation, pectin demethylation and production of glycerol-3-phosphate, which may be involved in lipid biosynthesis (phospho and acyl lipids). A rise in the content of sucrose synthase (SuSy), two different isoforms for each variety, is probably linked to the predominant nature of blueberry fruit as sink organ, in which this activity is the responsible of sucrose utilization.

When these changes are analyzed in Emerald ripe fruit, a few proteins displayed the same tendency than in the immature stage: pyruvate kinase, triose phosphate isomerase (TPI), transketolase, IPS, aldolase, UDP-arabinopyranose mutase and SuSy. An increment in the content of other enzymes, like xyloglucan endotransglucosylase/hydrolase (XET/H), glyoxalase, malate synthase and the β-subunit of pyrophosphate-dependent 6-phosphofructo-1-kinase (PFP beta) was also observed. In O'Neal, the content of a chitinase was higher than in Emerald, as well as one glycosyl hydrolase, and enzymes related with glycolysis, gluconeogenesis and pyruvate destination.

3.5. Profile of proteins related with cell wall metabolism and water transport in the total set of original data

Since in the precedent analysis only proteins that significantly changed levels by a factor of two or more were considered, and a subgroup was related to cell wall, a further search of proteins related with cell wall metabolism was carried out within original data. In this analysis were included: α and β galactosidases, endoglucanases, pectinmethylesterases (PME), polygalacturonases (PG), PME inhibitor proteins, PG inhibitor proteins, pectate liases, xyloglucan hydrolases, xylosidases and expansins (structural proteins in the cell wall) (Table 3). Aquaporins were added to this analysis as well, considering that water dynamics is deeply related to cell turgor, and hence to firmness, in plant cells (Wong et al., 2018).

From this group of proteins, only the content of three of them varied significantly in one cultivar with respect to the other. No sequence with similarity to PG could be identified. PME protein is more abundant at both stages in O'Neal than in Emerald, but more markedly at fruit set (125 times higher). Xyloglucan endotransglycosidase/hydrolase (XET/H) level increases significantly in ripe Emerald fruit (85.50 times higher). In the case of aquaporins, two proteins from this family could be detected in blueberries, but only one of them showed a significant higher level in Emerald at fruit set (almost 36 times greater than in O'Neal).

Table 3
Identity and pattern of variation of proteins related to cell wall metabolism in Emerald versus O´Neal at both maturity stages. The search of these cell wall related proteins was carried out following the strategy outlined in materials and methods. Numbers indicate ratio between protein levels in Emerald and O'Neal. nc: no change, ns: non significative change.

UNIPROT ID	Description	FRUIT SET	RIPE
		EM/ON	*EM/ON*
A3FA66	Aquaporin PIP14	35.97	nc
A3FA63	Aquaporin PIP11	nc	nc
F6HJ88	Xyloglucan endotransglucosylase/hydrolase	nc	85.50
F6HEX2	α-D-xyloside xylohydrolase/α-xylosidase	nc	1.43 ns
D7SQ37	Xylose isomerase	0.37 ns	0.80 ns
F6HGZ1	Pectin methyl esterase (PME)	0.008	0.45
F6I1A6	β-galactosidase	nc	0.87 ns
D7TXW8	α-galactosidase	nc	nc
D7SN69	UDP-apiose/xylose synthase	nc	nc

4. Discussion

Blueberries of different species and varieties diverge in their quality traits, which in turn have direct impact in fruit shelf life (Lyrene, 2008; Ortiz et al., 2018; Zapata et al., 2010). Many of these structural attributes, related with cell walls and peripheral layers, are settled early in development (Brummell, 2006b; Konarska, 2015; Ng et al., 2015). Emerald is one of the varieties whose cultivated area has been increasing since it was first introduced in the NEA. Two main features influence its settlement as an appreciated cultivar, it is an early variety and it has high firmness (> 1.8N, classification after (Moggia et al., 2017). However, not many studies have been conducted to ascertain the metabolic and physiologic basis of these traits. The main purpose of the precedent study, was to compare this cultivar with O'Neal, as a model of a soft fruit (firmness < 1.6N), at two phenological stages, with the hope to delineate the principal biochemical differences. It is opportune to bear in mind that some of the divergences noticed can be directly connected with the meteorological conditions prevailing when each stage of maturation is reached, which differ between varieties (see Fig. 1). This can be the case for a higher DBI and 18:2 level in Emerald, contributing to balance membranes fluidity in a lower temperature context. However, some metabolites and metabolic routes can be envisaged as molecular signatures of each variety.

4.1. Solutes accumulation, nitrogen: carbon balance and cell wall recycling are enhanced in the firmer variety

Several of the biological processes that prevailed in Emerald at fruit set, are engaged in the biosynthesis of amino acids, organic acids and secondary metabolites that may account for an increased turgor pressure. Indeed, some works point to relations in solute accumulation between the apoplast and symplast of mesocarp cell of grapes as key regulators of turgor pressure during ripening (Wada et al., 2008; Zepeda et al., 2018). An increase in apoplastic solute concentration caused a loss of turgor toward the onset of ripening; the opposite would arise with cytosolic (symplastic) accumulation. GABA and proline, were related with osmotic adjustment in different studies (Bouché and Fromm, 2004; Szabados and Savouré, 2010; Verbruggen and Hermans, 2008), although they also fulfil a range of additional functions. For instance, GABA may contribute to cytosolic pH regulation (Bouché and

Fromm, 2004) or may act as a signal molecule in diverse biotic and abiotic stresses (Bao et al., 2014; Seifi et al., 2013). GABA is also a key metabolite connecting carbon and nitrogen metabolisms that involve cytosolic and mitochondrial reactions, contributing to C:N balance (Plaxton and Podestá, 2006). Among the further roles of proline in plants, it has been proposed to activate the shikimic acid pathway and thus increase secondary metabolites production (Silva et al., 2018). This particular imino acid is a constituent of the cell wall glycosylated family proteins, the so called PRPG or HRPGs, proteins rich in proline and hydroxyproline, with different degree of glycosylation (Kavi Kishor et al., 2015). Malate and citrate accumulate in the vacuole to sustain growth and also play roles in cytosolic pH balance. At the same time, a very active synthesis and turnover of proteins appear to take place, boosted by the increased content in several amino acids and proteins related with their synthesis as well as with proteasome pathway.

A key protein that also plays a role in water-plant status is aquaporin. It belongs to the major intrinsic protein (MIP) family, which include members that transport water, small molecules and elements such as urea, ammonia or boron (Maurel et al., 2015) across membranes. Two classes are broadly distributed in plants, plasma membrane intrinsic proteins (PIP) and tonoplast intrinsic proteins (TIP). In grapes, analysis of microarrays and correlation networks highlighted the strong co-expression relationships within the MIP family and genes involved in processes such as growth, cell division or cell redox homeostasis. Moreover, one of the strongest relationships was found between the MIP family and genes participating in cell wall modification and cell expansion (Schlosser et al., 2008; Wong et al., 2018). In strawberry, two types of aquaporins (PIP1 and 2) showed a differential expression pattern during ripening (Merlaen et al., 2018) and their expression levels were correlated with firmness (Alleva et al., 2010). In the present report, two proteins from the MIP family were detected in blueberries, being one of them almost 36 times more abundant in Emerald at fruit set. In this phenological stage, the main function of these water channels is probably related with the rapidly expanding fruit and diverse solutes accumulation, as was observed in young grape berries (Fouquet et al., 2008). In turn, cells may handle the transport across PIP by regulating their opening or closure in response to environmental conditions (Tournaire-Roux et al., 2003; Uehlein et al., 2008). Although in leaves they were implicated in CO_2 transport, this was not measured in fruit (Terashima and Ono, 2002).

In view that there were no significant differences regarding calcium content (Fig. 1) or in AIR amount (data not shown), it is concluded that at this stage varieties did not differ substantially in cell wall synthesis nor in calcium bridges. Notwithstanding, it is possible that the structure of pectins and hemicelluloses are being intensively remodelled, as suggested by the increase in xylose and galacturonic acid levels (Supplementary Table 1), as well as in enzymes that catalyse the biosynthesis of precursors of cell wall glycans (UDP-xyl synthase, UDP-6PGDH, UDP-arabinose mutase, UDP-glucose 4,6-dehydratase, for rhamnose synthesis), or cell wall hydrolysis (alpha-mannosidase) (Supplementary Table 2). Other hydrolytic enzymes such as α and β galactosidases did not change with respect to O'Neal, while PME protein levels were 125 times lower. PME activity, as mentioned before, generates free carboxylates in galacturonic residues of pectins, which may result in a more porous cell wall when combined with enhanced levels to hydrolytic enzymes; or may increase calcium coordination, strengthening the structure (Jolie et al., 2010). A hint about which processes are taking place can be provided by considering the dynamic nature of the wall. UDP-sugars are direct substrates for *de novo* synthesis of all the glucans present in the cell wall. These carbohydrates constitute approximately the 45% of the total carbon fixed by year (Field et al., 1998), meaning that, far from accomplish only structural roles, cell wall carbohydrates are potential energy source and carbon sink that help to sustain growth and development. A growing body of evidences indicate that these roles are carried out by an intense cell wall recycling in plants (Barnes and Anderson, 2018). As it has been described above, not only the enzymes associated with synthesis, hydrolysis and remodelling of the wall, but also, the metabolites related, have been found increased in Emerald at fruit set. In addition, enzymes from glycolysis and gluconeogenesis (PGM, TPI, PK, enolase, FBPase, PFP alpha) or in pentose phosphate pathway, were increased, supporting a close relation between wall remodelling, precursors for recycling and associated energy supply.

Thus, it is tempting to hypothesize that a combination of an intense cell wall metabolic recycling, increased solute (and compatible solutes) and improved carbon to nitrogen balance, may contribute to a higher efficiency in water and carbon resources management in the firmer variety.

4.2. *In ripe fruit, the firmer variety modulates in muro cell wall recycling, secondary metabolite synthesis and inositol metabolism*

Plants are also able to cleave and reconnect polysaccharides *in muro,* i.e. inside the cell wall, and they perform this through a diverse sort of endo and exo-transglycosylases (Barnes and Anderson, 2018). This is another category of cell wall recycling mechanism, which does not require sugar internalization or its conversion to NDP-sugar substrate and glycosyltransferases activities, as metabolic recycling does. The action of transglycosylases allows to elongate and/or branch xyloglucans, tuning their links and interactions in this way. In ripe Emerald, one xyloglucan endotransglucosylase/hydrolase increased 85.5 times in comparison with O'Neal (Table 2). It belongs to a family of proteins that catalyse endotransglycosylation (XET) and/or xyloglucan endohydrolysis (XEH). These enzymes are involved in the modification of cell wall structure by cleaving and also re-joining xyloglucan molecules in primary plant cell walls. A few studies suggest that some members of this protein family have only the XET activity, being involved in cell wall remodelling (Langer et al., 2018; Nardi et al., 2014). The fact that xylose content was lower, supports the idea that the main activity displayed by this enzyme is as a transglucosylase instead of hydrolase. In addition, the increase in levels of other enzymes such as UDP-arabinose mutase, UDP- glucose 4,6- dehydratase, PFP beta subunit, mannose-1-phosphate guanylyltransferase, TPI and sugar transporters (Supplementary Table 2), suggests that metabolic recycling is also taking place.

In a previous work done with three blueberry varieties, the activities of β-galactosidase (β-gal) and PME in green and ripe fruit (Montecchiarini et al., 2018) were mostly in agreement with enzymes levels reported here (Table 3). In fact, in the less firm variety (O'Neal) a combination of high PME and β-gal activities in ripe fruit suggested that an association of a more soluble pectin in the presence of a high level of a hydrolytic enzyme could be in part the cause of a reduced firmness. Surprisingly, no sequence with similarity with polygalacturonase could be found within the proteomic data. This evidence, as well as the failure to measure PG activity at these stages (data not shown), could be pointing either to a very low activity or to some technical issues in the recovery of this enzyme for the in vitro colorimetric assay. However, other authors reported a failure to detect PG activity in grape berry or detected low transcript levels of related mRNA (Fasoli et al., 2016; Nunan et al., 2001). Moreover, a survey of transcripts of enzymes related to cell wall metabolism in blueberries made it noticeable that polygalacturonases are poorly expressed and only at two intermediate phenological stages, not analyzed here (Rowland et al., 2012). Thus, it is possible that other hydrolytic enzymes could be more relevant for blueberry softening than polygalacturonases.

Secondary metabolites encompass a vast group of specialized compounds synthesized from precursors arising out of primary metabolism. They are bioactive molecules with several health-promoting effects like polyphenols or flavonoids, very abundant in berries (Manganaris et al., 2014; Michalska and Łysiak, 2015). Phenolic compounds are related with diverse functions such as antioxidant capacity, herbivore defence or survival to different environmental conditions (Eichholz et al., 2011;

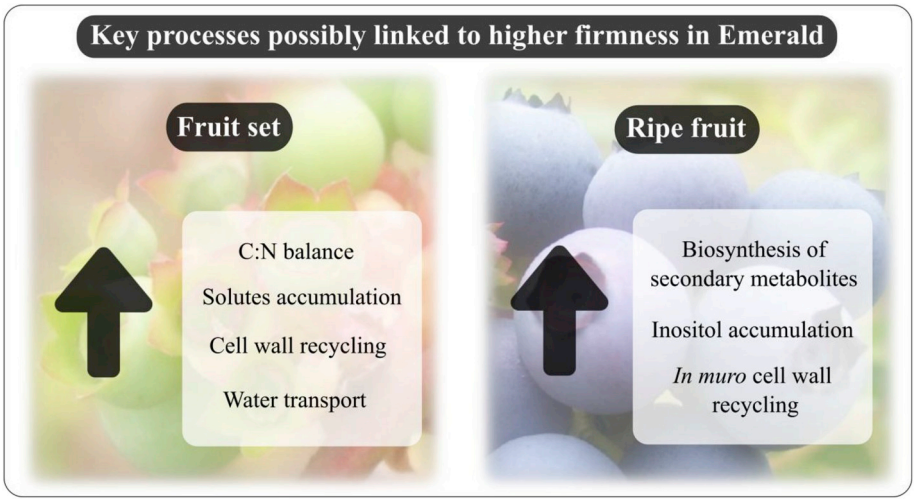

Fig. 5. Differential processes in Emerald. Molecular and metabolic processes that are enhanced in the firmer variety at both phenological stages.

Howard et al., 2003; Keutgen and Pawelzik, 2007). TPC content was higher in Emerald, and two of them, quinic and caffeoylquinic acids, have been identified in the metabolomic study. The accumulation of these compounds is genotype, variety and tissue dependent (Castrejón et al., 2008; Howard et al., 2003; Karppinen et al., 2016; Lee et al., 2014; Mikulic-Petkovsek et al., 2012), also influenced by maturation and field conditions (Teixeira et al., 2013). They are more abundant in exocarp and in the ripe stage and a role of these compounds in negatively regulating PME activity has been proved (Lewis et al., 2008), raising the possibility that they could be in part responsible for enhanced firmness in Emerald.

Myo-inositol, or simply inositol, biosynthesis is carried out by a two-step process in which IPS generates inositol-1-P from 6-phosphogluconate, which is later dephosphorylated by an inositol phosphatase to render inositol (Majumder et al., 1997). The first reaction is considered rate limiting for inositol accumulation. Relative level of this metabolite was higher in Emerald than in O'Neal at both stages, as it was IPS protein, but its role may be different in each stage. This sugar alcohol is related with several functions such as osmotic protection or scavenging of reactive oxygen radicals and in young fruit has been related with maintenance of turgor (Boldingh et al., 2000). In the same way, IPS expression is required for organ development in plants (Chen and Xiong, 2010) and it can be induced by a number of environmental stresses (Munnik and Vermeer, 2010; Valluru and Van den Ende, 2011; Wang et al., 2011). Decay in inositol phosphates pool in seedlings of tomato, mutant in one inositol phosphatase, increased the synthesis of secondary metabolites, although the unambiguous connection with inositol concentration was not established (Alimohammadi et al., 2012).

As mentioned before, changes in the contribution of solute concentration ratio between apoplast and symplast to turgor status in each development stage has been pointed for grape. In blueberry, conductivity measures of total pulp homogenates was significantly higher in ripe Emerald (data not shown) but the implication of this in cell turgor needs further research.

Mannitol is synthesized in source tissues and transported via phloem to sink tissues, as fruit and roots, where it can be stored and metabolized (Patel and Williamson, 2016). It is considered a compatible solute, as other polyols, sugars or amino acids, since its concentration may increase without altering the normal physiology of the cell. Its action is also thought to be due to its antioxidant capacity, as a ROS quencher (Jennings et al., 1998; Meena et al., 2015). Under low osmotic potential, mannitol may protect proteins and cellular structures by interacting with their hydration shell. Likewise, when water potential is low, under salt or drought stress, the damage caused by the increase in ROS concentration may be ameliorated by mannitol. An increase in the content of this metabolite in ripe O'Neal could be a response to a decrease in the water status of the fruit. In fact, precipitation levels in its harvesting period were considerably lower than for Emerald (see Fig. 1).

5. Conclusion

Metabolomic and proteomic studies described here provide useful and complementary information about factors that account for fruit quality in blueberries. Even though high firmness in fruit is the resultant of not fully understood connections between cell wall metabolism, water status and turgor pressure, it is possible to delineate some clues. Taking a soft variety (O'Neal) as a reference, it was possible to describe those compounds and metabolic processes that were most likely connected with general quality in the firmer variety (Emerald). During fruit set, Emerald's higher levels of diverse metabolites, increased content of proteins related with cell wall metabolic recycling and water transport, point to a best handling of carbon, nitrogen and hydric resources at the onset of fruit development. Later on, in ripe fruit, increased content of secondary metabolites (phenolic compounds, quinic acid) and inositol, as well as *in muro* cell wall recycling, emerge as the key events possibly linked with firmness (Fig. 5). Further research is needed to depict the site of solute accumulation and its relationship with cell turgor, for instance, employing specific probes and apoplast/symplast partition. In the same way, new fluorescent oligosaccharides compounds used for pulse-chase and confocal microscopy experiments could pave the way for the understanding of the contribution of cell wall recycling, degradation and sugar salvage to firmness maintenance. Future research will comprise the characterization of the proteome and metabolome of exocarp's tissue, associated with physiologic and structural studies of other intermediate phenological stages.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica to KEJT (PICT 2016-0091) and FEP (PICT 2015-1074). KEJT, FEP and EM are members of the investigator

Career from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). MLM and LM are Doctoral Fellows of CONICET and AG is a Doctoral Fellow CONICET/INTA. FB, MFR and DV are members of the Instituto Nacional de Tecnología Agropecuaria (INTA).

CRediT authorship contribution statement

M.L. Montecchiarini: Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **E. Margarit:** Formal analysis, Software, Writing – original draft. **L. Morales:** Formal analysis, Writing – original draft. **M.F. Rivadeneira:** Formal analysis, Writing – original draft. **F. Bello:** Formal analysis, Writing – original draft. **A. Gollán:** Formal analysis, Writing – original draft. **D. Vázquez:** Formal analysis, Writing – original draft. **F.E. Podestá:** Conceptualization, Data curation, Project administration, Resources, Supervision, Funding acquisition, Formal analysis, Writing – original draft, Writing – review & editing. **K.E.J. Tripodi:** Conceptualization, Data curation, Project administration, Funding acquisition, Resources, Supervision, Formal analysis, Writing – original draft, Writing – review & editing.

Acknowledgments

The authors want to acknowledge the collaboration of Mónica Hourcade and Guillermo Marccuzzi for GC-MS runnings and to phD Ayelén Pagani for ICP-MS ion determinations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.12.019.

References

Alimohammadi, M., De Silva, K., Ballu, C., Ali, N., Khodakovskaya, M.V., 2012. Reduction of inositol (1,4,5)-trisphosphate affects the overall phosphoinositol pathway and leads to modifications in light signalling and secondary metabolism in tomato plants. J. Exp. Bot. 63 825-3.

Alleva, K., Marquez, M., Villarreal, N., Mut, P., Bustamante, C., Bellati, J., et al., 2010. Cloning, functional characterization, and co-expression studies of a novel aquaporin (FaPIP2;1) of strawberry fruit. J. Exp. Bot. 61, 3935–3945.

Angeletti, P., Castagnasso, H., Miceli, E., Terminiello, L., Concellón, A., Chaves, A., et al., 2010. Effect of preharvest calcium applications on postharvest quality, softening and cell wall degradation of two blueberry (Vaccinium corymbosum) varieties. Postharvest Biol. Technol. 58, 98–103.

Atmodjo, M.A., Hao, Z., Mohnen, D., 2013. Evolving views of pectin biosynthesis. Annu. Rev. Plant Biol. 64, 747–779.

Bao, H., Chen, X., Lv, S., Jiang, P., Feng, J., Fan, P., et al., 2014. Virus-induced gene silencing reveals control of reactive oxygen species accumulation and salt tolerance in tomato by γ-aminobutyric acid metabolic pathway. Plant Cell Environ. 38, 600–613.

Barnes, W.J., Anderson, C.T., 2018. Release, recycle, rebuild: cell-wall remodeling, autodegradation, and sugar salvage for new wall biosynthesis during plant development. Mol. Plant 11, 31–46.

Basiouny, F.M., Woods, F., 1992. Effect of chelated calcium on shelf-life and quality of blueberry fruits (Vaccinium ashei Reade). Proc. Fla. State Hortic. Soc. 105, 75–78.

Benton Jones Jr, J., 2012. Plant Nutrition and Soil Fertility Manual, second ed. CRC Press, Boca Raton, FL.

Berkheimer, E.J., Hanson, S.F., 2004. Effect of soil calcium applications on blueberry yield and quality. Small Fruits Rev. Food Prod. Press. Haworth Press. Inc. 3, 133–139.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Boldingh, H., Smith, G.S., Klages, K., 2000. Seasonal concentrations of non-structural carbohydrates of five Actinidia species in fruit, leaf and fine root tissue. Ann. Bot. 85, 469–476.

Bouché, N., Fromm, H., 2004. GABA in plants: just a metabolite? Trends Plant Sci. 9, 110–115.

Brummell, D.A., 2006a. Cell wall disassembly in ripening fruit. Funct. Plant Biol. 33, 103–119.

Brummell, D.A., 2006b. Primary cell wall metabolism during fruit ripening. New Zeal. J. For. Sci. 36, 99–111.

Castellarin, S.D., Gambetta, G.A., Wada, H., Krasnow, M.N., Cramer, G.R., Peterlunger, E., et al., 2016. Characterization of major ripening events during softening in grape: turgor, sugar accumulation, abscisic acid metabolism, colour development, and their relationship with growth. J. Exp. Bot. 67, 709–722.

Castrejón, A.D.R., Eichholz, I., Rohn, S., Kroh, L.W., Huyskens-Keil, S., 2008. Phenolic profile and antioxidant activity of highbush blueberry (Vaccinium corymbosum L.) during fruit maturation and ripening. Food Chem. 109, 564–572.

Chen, H., Xiong, L., 2010. myo-Inositol-1-phosphate synthase is required for polar auxin transport and organ development. J. Biol. Chem. 285, 4238–4247.

Conde, A., Regalado, A., Rodrigues, D., Costa, J.M., Blumwald, E., Chaves, M.M., et al., 2015. Polyols in grape berry: transport and metabolic adjustments as a physiological strategy for water-deficit stress tolerance in grapevine. J. Exp. Bot. 66, 889–906.

Cui, M., Liang, D., Ma, F., 2013. Molecular cloning and characterization of a cDNA encoding kiwifruit l-myo-inositol-1-phosphate synthase, a key gene of inositol formation. Mol. Biol. Rep. 40, 697–705.

Eichholz, I., Huyskens-Keil, S., Kroh, L.W., Rohn, S., 2011. Phenolic compounds, pectin and antioxidant activity in blueberries (Vaccinium corymbosum L.) influenced by boron and mulch cover. J. Appl. Bot. Food Qual. 84, 26–32.

Fasoli, M., Dell'Anna, R., Dal Santo, S., Balestrini, R., Sanson, A., Pezzotti, M., et al., 2016. Pectins, hemicelluloses and celluloses show specific dynamics in the internal and external surfaces of grape berry skin during ripening. Plant Cell Physiol. 57, 1332–1349.

Field, C.B., Behrenfeld, M.J., Randerson, J.T., Falkowski, P., 1998. Primary production of the biosphere: integrating terrestrial and oceanic components. Science 281, 237–240.

Fouquet, R., Léon, C., Ollat, N., Barrieu, F., 2008. Identification of grapevine aquaporins and expression analysis in developing berries. Plant Cell Rep. 27, 1541–1550.

Frenkel, C., 1972. Involvement of peroxidase and indole-3-acetic acid oxidase isozymes from pear, tomato, and blueberry fruit in ripening. Plant Physiol. 49, 757–763.

Ge, S., Jung, D., 2018. ShinyGO: a graphical enrichment tool for animals and plants. bioRxiv. https://doi.org/10.1101/315150.

Houston, K., Tucker, M.R., Chowdhury, J., Shirley, N., Little, A., 2016. The plant cell wall: a complex and dynamic structure as revealed by the responses of genes under stress conditions. Front. Plant Sci. https://doi.org/10.3389/fpls.2016.00984.

Howard, L.R., Clark, J.R., Brownmiller, C., 2003. Antioxidant capacity and phenolic content in blueberries as affected by genotype and growing season. J. Sci. Food Agric. 83, 1238–1247.

Hurkman, W.J., Tanaka, C.K., 1986. Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiol. 81, 802–806.

Jennings, D.B., Ehrenshaft, M., Pharr, D.M., Williamson, J.D., 1998. Roles for mannitol and mannitol dehydrogenase in active oxygen-mediated plant defense. Proc. Natl. Acad. Sci. Unit. States Am. 95, 15129–15133.

Jolie, R.P., Duvetter, T., Van Loey, A.M., Hendrickx, M.E., 2010. Pectin methylesterase and its proteinaceous inhibitor: a review. Carbohydr. Res. 345, 2583–2595.

Karppinen, K., Zoratti, L., Nguyenquynh, N., Häggman, H., Jaakola, L., 2016. On the developmental and environmental regulation of secondary metabolism in vaccinium spp. berries. Front. Plant Sci. 7. https://doi.org/10.3389/fpls.2016.00655.

Kavi Kishor, P.B., Hima Kumari, P., Sunita, M.S.L., Sreenivasulu, N., 2015. Role of proline in cell wall synthesis and plant development and its implications in plant ontogeny. Front. Plant Sci. 6, 544. https://doi.org/10.3389/fpls.2015.00544.

Keutgen, A.J., Pawelzik, E., 2007. Modifications of strawberry fruit antioxidant pools and fruit quality under NaCl stress. J. Agric. Food Chem. 4066–4072.

Konarska, A., 2015. Morphological, anatomical and ultrastructural changes in Vaccinium corymbosum fruits during ontogeny. Botany 93, 589–602.

Langer, S.E., Oviedo, N.C., Marina, M., Burgos, J.L., Martínez, G.A., Civello, P.M., et al., 2018. Effects of heat treatment on enzyme activity and expression of key genes controlling cell wall remodeling in strawberry fruit. Plant Physiol. Biochem. 130, 334–344.

Lee, S., Jung, E.S., Do, S.G., Jung, G.Y., Song, G., Song, J.M., et al., 2014. Correlation between species-specific metabolite profiles and bioactivities of blueberries (Vaccinium spp.). J. Agric. Food Chem. 62, 2126–2133.

Lewis, K.C., Selzer, T., Shahar, C., Udi, Y., Tworowski, D., Sagi, I., 2008. Inhibition of pectin methyl esterase activity by green tea catechins. Phytochemistry 69, 2586–2592.

Li, C., Shen, W., Lu, W., Jiang, Y., Xie, J., Chen, J., 2009. 1-MCP delayed softening and affected expression of XET and EXP genes in harvested cherimoya fruit. Postharvest Biol. Technol. 52, 254–259.

Lipe, J.A., 1978. Ethylene in fruits of blackberry and rabbiteye blueberry. J. Am. Soc. Hortic. Sci. 103, 76–77.

Liu, Y.-B., Lu, S.-M., Zhang, J.-F., Liu, S., Lu, Y.-T., 2007. A xyloglucan endotransglucosylase/hydrolase involves in growth of primary root and alters the deposition of cellulose in Arabidopsis. Planta 226, 1547–1560.

Loewus, F.A., Murthy, P.P.N., 2000. myo-Inositol metabolism in plants. Plant Sci. 150, 1–19.

Lyrene, P.M., 2008. "Emerald" southern highbush blueberry. Hortscience 43, 1606–1607.

Majumder, A.L., Johnson, M.D., Henry, S.A., 1997. 1L-myo-Inositol-1-phosphate synthase. Biochim. Biophys. Acta Lipids Lipid Metab. 1348, 245–256.

Manganaris, G.A., Goulas, V., Vicente, A.R., Terry, L.A., 2014. Berry antioxidants: small fruits providing large benefits. J. Sci. Food Agric. 94, 825–833.

Maurel, C., Boursiac, Y., Luu, D.-T., Santoni, V., Shahzad, Z., Verdoucq, L., 2015. Aquaporins in plants. Physiol. Rev. 95, 1321–1358.

Meena, M., Prasad, V., Zehra, A., Gupta, V.K., Upadhyay, R.S., 2015. Mannitol metabolism during pathogenic fungal-host interactions under stressed conditions. Front. Microbiol. https://doi.org/10.3389/fmicb.2015.01019.

Merlaen, B., De Keyser, E., Van Labeke, M.C., 2018. Identification and substrate prediction of new Fragaria x ananassa aquaporins and expression in different tissues and during strawberry fruit development. Hortic. Res. https://doi.org/10.1038/s41438-018-0019-0.

Michalska, A., Łysiak, G., 2015. Bioactive compounds of blueberries: post-harvest factors influencing the nutritional value of products. Int. J. Mol. Sci. 16, 18642–18663.

Mikulic-Petkovsek, M., Schmitzer, V., Slatnar, A., Stampar, F., Veberic, R., 2012. Composition of sugars, organic acids, and total phenolics in 25 wild or cultivated

berry species. J. Food Sci. 77, 1064–1070.
Moggia, C., Graell, J., Lara, I., González, G., Lobos, G.A., 2017. Firmness at harvest impacts postharvest fruit softening and internal browning development in mechanically damaged and non-damaged highbush blueberries (vaccinium corymbosum L.). Front. Plant Sci. 8, 535. https://doi.org/10.3389/fpls.2017.00535.
Montecchiarini, M., Bello, F., Rivadeneira, M., Vazquez, D., Podestá, F., Tripodi, K., 2018. Metabolic and physiologic profile during the fruit ripening of three blueberries highbush (Vaccinium corymbosum) cultivars. J. Berry Res. 8, 177–192.
Munnik, T., Vermeer, J.E.M., 2010. Osmotic stress-induced phosphoinositide and inositol phosphate signalling in plants. Plant Cell Environ. 33, 655–669.
Nardi, C.F., Villarreal, N.M., Opazo, M.C., Martínez, G.A., Moya-León, M.A., Civello, P.M., 2014. Expression of FaXTH1 and FaXTH2 genes in strawberry fruit. Cloning of promoter regions and effect of plant growth regulators. Sci. Hortic. (Amsterdam). 165, 111–122.
Ng, J.K.T., Schröder, R., Brummell, D.A., Sutherland, P.W., Hallett, I.C., Smith, B.G., et al., 2015. Lower cell wall pectin solubilisation and galactose loss during early fruit development in apple (Malus x domestica) cultivar "Scifresh" are associated with slower softening rate. J. Plant Physiol. 176, 129–137.
Nunan, K.J., Davies, C., Robinson, S.P., Fincher, G.B., 2001. Expression patterns of cell wall-modifying enzymes during grape berry development. Planta 214, 257–264.
Oikawa, A., Lund, C., Sakuragi, S., 2013. Golgi-localized enzyme complGolgi-localized enzyme complexes for plant cell wall biosynthesis. Trends Plant Sci. 18, 49–58.
Ortiz, C., Franceschinis, F., Gergoff Grozeff, G., Chan, H., Labavitch, J., Crisosto, C., et al., 2018. Pre-treatment with 1-methylcyclopropene alleviates methyl bromideinduced internal breakdown, softening and wall degradation in blueberry. Postharvest Biol. Technol. 146, 90–98.
Paniagua, A.C., East, A.R., Hindmarsh, J.P., Heyes, J.A., 2013. Moisture loss is the major cause of firmness change during postharvest storage of blueberry. Postharvest Biol. Technol. 79, 13–19.
Patel, T.K., Williamson, J.D., 2016. Mannitol in plants, fungi, and plant–fungal interactions. Trends Plant Sci. 6, 486–497.
Perotti, V.E., Del Vecchio, H.A., Sansevich, A., Meier, G., Bello, F., Cocco, M., et al., 2011. Proteomic, metabalomic, and biochemical analysis of heat treated Valencia oranges during storage. Postharvest Biol. Technol. 62, 97–114.
Plaxton, W.C., Podestá, F.E., 2006. The functional organization and control of plant respiration. CRC Crit. Rev. Plant Sci. 25, 159–198.
Prodorutti, D., Pertot, I., Giongo, L., Gessler, C., 2007. Highbush Blueberry : cultivation , protection , breeding and biotechnology. Eur. J. Plant Sci. Biotechnol. 1, 44–56.
Rowland, L.J., Bell, D.J., Alkharouf, N., Bassil, N.V., Drummond, F.A., Beers, L., et al., 2012. Generating genomic tools for blueberry improvement. Int. J. Fruit Sci. 12, 276–287.
Schlosser, J., Olsson, N., Weis, M., Reid, K., Peng, F., Lund, S., et al., 2008. Cellular expansion and gene expression in the developing grape (Vitis vinifera L.). Protoplasma 232, 255–265.
Seifi, H.S., Curvers, K., De Vleesschauwer, D., Delaere, I., Aziz, A., Höfte, M., 2013. Concurrent overactivation of the cytosolic glutamine synthetase and the GABA shunt in the ABA-deficient sitiens mutant of tomato leads to resistance against Botrytis cinerea. New Phytol. 199, 490–504.
Serrano, A., Espinoza, C., Armijo, G., Inostroza-Blancheteau, C., Poblete, E., Meyer-Regueiro, C., et al., 2017. Omics approaches for understanding grapevine berry development: regulatory networks associated with endogenous processes and environmental responses. Front. Plant Sci. https://doi.org/10.3389/fpls.2017.01486.
Silva, F.L.B., Vieira, L.G.E., Ribas, A.F., Moro, A.L., Neris, D.M., Pacheco, A.C., 2018. Proline accumulation induces the production of total phenolics in transgenic tobacco plants under water deficit without increasing the G6PDH activity. Theor. Exp. Plant Physiol. 30, 251–260.
Sim, I., Suh, D.H., Singh, D., Do, S.G., Moon, K.H., Lee, J.H., et al., 2017. Unraveling metabolic variation for blueberry and chokeberry cultivars harvested from different geo-climatic regions in korea. J. Agric. Food Chem. https://doi.org/10.1021/acs.jafc.7b04065.
Stückrath, R., Quevedo, R., De La Fuente, L., Hernández, A., Sepúlveda, V., 2008. Effect of foliar application of calcium on the quality of blueberry fruits. J. Plant Nutr. 31, 1299–1312.
Szabados, L., Savouré, A., 2010. Proline: a multifunctional amino acid. Trends Plant Sci. 15, 89–97.
Teixeira, A., Eiras-Dias, J., Castellarin, S.D., Gerós, H., 2013. Berry phenolics of grapevine under challenging environments. Int. J. Mol. Sci. 14, 18711–18739.
Terashima, I., Ono, K., 2002. Effects of HgCl(2) on CO(2) dependence of leaf photosynthesis: evidence indicating involvement of aquaporins in CO(2) diffusion across the plasma membrane. Plant Cell Physiol. 43, 70–78.
Tournaire-Roux, C., Sutka, M., Javot, H., Gout, E., Gerbeau, P., Luu, D.T., et al., 2003. Cytosolic pH regulates root water transport during anoxic stress through gating of aquaporins. Nature 425, 393–397.
Tyanova, S., Temu, T., Cox, J., 2016. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat. Protoc. 11, 2301–2319.
Uehlein, N., Otto, B., Hanson, D.T., Fischer, M., McDowell, N., Kaldenhoff, R., 2008. Function of nicotiana tabacum aquaporins as chloroplast gas pores challenges the concept of membrane CO2 permeability. Plant Cell. https://doi.org/10.1105/tpc.107.054023.
Valluru, R., Van den Ende, W., 2011. Myo-inositol and beyond - emerging networks under stress. Plant Sci. 181, 387–400.
Velioglu, Y.S., Mazza, G., Gao, L., Oomah, B.D., 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J. Agric. Food Chem. 46, 4113–4117.
Verbruggen, N., Hermans, C., 2008. Proline accumulation in plants: a review. Amino Acids 35, 753–759.
Vicente, A.R., Saladié, M., Rose, J.K.C., Labavitch, J.M., 2007. The linkage between cell wall metabolism and fruit softening: looking to the future. J. Sci. Food Agric. 87, 1435–1448.
Wada, H., Shackel, K.A., Matthews, M.A., 2008. Fruit ripening in Vitis vinifera: apoplastic solute accumulation accounts for pre-veraison turgor loss in berries. Planta 227, 1351–1361.
Wang, D., Yeats, T.H., Uluisik, S., Rose, J.K.C., Seymour, G.B., 2018. Fruit softening: revisiting the role of pectin. Trends Plant Sci. 23, 302–310.
Wang, Y., Huang, J., Gou, C.B., Dai, X., Chen, F., Wei, W., 2011. Cloning and characterization of a differentially expressed cDNA encoding myo-inositol-1-phosphate synthase involved in response to abiotic stress in Jatropha curcas. Plant Cell Tissue Organ Cult. 106, 269–277.
Wong, D.C.J., Zhang, L., Merlin, I., Castellarin, S.D., Gambetta, G.A., 2018. Structure and transcriptional regulation of the major intrinsic protein gene family in grapevine. BMC Genomics 19, 248. https://doi.org/10.1186/s12864-018-4638-5.
Zapata, L.M., Malleret, A.D., Quinteros, C.F., Lesa, C.E., Vuarant, C.O., Rivadeneira, M.F., Gerard, J.A., 2010. Estudio sobre cambios de la firmeza de bayas de arándanos durante su maduración. Ciencia, Docencia y Tecnol. 21, 159–171.
Zepeda, B., Olmedo, P., Ejsmentewicz, T., Sepúlveda, P., Balic, I., Balladares, C., et al., 2018. Cell wall and metabolite composition of berries of Vitis vinifera (L.) cv. Thompson Seedless with different firmness. Food Chem. 268, 492–497.
Zhao, Y., 2012. Auxin biosynthesis: a simple two-step pathway converts tryptophan to indole-3-Acetic acid in plants. Mol. Plant 334–338.
Zhou, Y., Pan, X., Qu, H., Underhill, S.J.R., 2014. Low temperature alters plasma membrane lipid composition and ATPase activity of pineapple fruit during blackheart development. J. Bioenerg. Biomembr. 46, 59–69.
Zoccatelli, G., Zenoni, S., Savoi, S., Dal Santo, S., Tononi, P., Zandonà, V., et al., 2013. Skin pectin metabolism during the postharvest dehydration of berries from three distinct grapevine cultivars. Aust. J. Grape Wine Res. 19, 171–179.