BIOACTIVE COMPOUNDS BIOSYNTHESIS AND METABOLISM IN FRUIT AND VEGETABLES

EDITED BY: Antonio Ferrante, George Manganaris, Maria Manuela Pintado and Alessandra Francini

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BIOACTIVE COMPOUNDS BIOSYNTHESIS AND METABOLISM IN FRUIT AND VEGETABLES

Topic Editors:
Antonio Ferrante, University of Milan, Italy
George Manganaris, Cyprus University of Technology, Cyprus
Maria Manuela Pintado, Catholic University of Porto, Portugal
Alessandra Francini, Sant'Anna School of Advanced Studies, Italy

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EDITORIAL
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Editorial: Bioactive Compounds Biosynthesis and Metabolism in Fruit and Vegetables

Alessandra Francini[1], *Manuela Pintado*[2], *George A. Manganaris*[3] *and Antonio Ferrante*[4]*

[1] Institute of Life Sciences, Scuola Superiore Sant'Anna, Pisa, Italy, [2] Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal, [3] Department of Agricultural Sciences, Biotechnology & Food Science, Cyprus University of Technology, Lemesos, Cyprus, [4] Department of Agricultural and Environmental Sciences—Production, Landscape, Agroenergy, Università degli Studi di Milano, Milan, Italy

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Editorial on the Research Topic

Bioactive Compounds Biosynthesis and Metabolism in Fruit and Vegetables

BIOACTIVE COMPOUNDS BIOSYNTHESIS AND METABOLISM IN FRUIT AND VEGETABLES

Fruit and vegetables are considered to be among the most important sources of bioactive compounds with proven beneficial effect on human diet. Tomato has been evolved as a model crop to study both fruit ripening pattern as well as for understanding how different environmental and agricultural factors can enhance the accumulation of bioactive compounds. The concentration of bioactive compounds is highly dependent on the crop species, cultivar/genotype, agronomic management, preharvest environmental conditions, and postharvest management practices (Toscano et al.). Bioactive compounds in fruit and vegetables are of consumer interest for their potential benefit to the health, especially in counteracting several diseases related to aging and stress. However, the bioactive molecules also have preservation properties that extend the shelf life of the produce. Postharvest technologies and storage conditions can reduce the degradation of bioactive compounds and some industrial operation can even promote their accumulation.

The systematic screening of key bioactive compounds with high content in a wide range of germplasm and the restoration of key genes and gene clusters from wild species and/or landraces both are important for reducing the loss of agro-biodiversity and the creation of a "gene pool" that can be exploited in future breeding programs toward the release of new cultivars with added nutraceutical value (Manganaris et al., 2018). Furthermore, the understanding how the accumulation of bioactive compounds can be enhanced or preserved is crucial for improvement of crop and product quality (Toscano et al.). The availability of advanced molecular tools allows fast and accurate transcriptome profiling that can help in the identification of the main gene clusters that are activated or repressed under different conditions. Such information coupled with the big data from metabolomics studies will be useful both for preharvest and postharvest management of produce with high nutritional value.

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Edited by:
Cai-Zhong Jiang,
USDA-ARS,
United States

Reviewed by:
David Obenland,
San Joaquin Valley Agricultural Sciences Center,
United States

****Correspondence:***
Antonio Ferrante
antonio.ferrante@unimi.it

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IMPACT OF GENOTYPE AND SIGNIFICANCE OF 'OLD-FASHIONED' CULTIVARS ON BIOACTIVE CONTENT

Bioactive compounds show wide variations among different species. Several studies have reported that ancient or old varieties accumulate higher concentration of bioactive compounds that have been linked to their adaptation to different environmental conditions (García-Mier et al., 2013; Manganaris et al., 2018). The accumulation of bioactive compounds can serve as defenses against biotic and abiotic stresses. Local varieties of tomato (*Solanum lycopersicum* L.) grown in Tuscany showed higher polyphenols, flavonoids, and carotenoids compared with commercial ones (Berni et al.). Similar results have been reported in onion (*Allium cepa* L.) and sweet cherry [*Prunus avium* L., (Berni et al.)]. In the meantime, breeding projects are targeting toward the release of new cultivars with enhanced bioactive content, as this is the case for tomatoes (Lenucci et al., 2006). Recently, interest for the black tomatoes has been attributed to the high carotenoid and anthocyanin contents. The 'Sun Black' tomato, a result of a 20-years breeding activity, derived from wild tomato species, such as *Solanum chilense, Solanum cheesmaniae*, *Solanum lycopersicoides,* and *Solanum habrochaites,* through introgression with cultivated genotypes (Mazzucato et al., 2013). A comparative study showed that the anthocyanins-enriched 'Sun Black' tomato had an almost double concentration of phenolics and carotenoids at the ripe stage compared to the wild type. Color is often an indicator of the composition and concentration of the bioactive compounds. In tomatoes and watermelon, the red color is due to the accumulation of lycopene while the yellow color due to β-carotene. The concentration of these two carotenoids can induce different flesh and skin colors (Ilahy et al.). These compounds are also substrates for volatile biosynthesis, contributing to fruit aroma with direct effect on produce quality (Ilahy et al.).

ENVIRONMENTAL CONDITIONS AFFECT THE ACCUMULATION OF BIOACTIVE COMPOUNDS

Environmental conditions can positively or negatively affect the concentration of bioactive molecules in different horticultural produce. Tomato fruits obtained from plants exposed to high salinity conditions (60 or 120 mM NaCl) showed a reduction of antioxidant capacity and several secondary metabolites such as lycopene and phenols (Moles et al.).

Environmental conditions including altitude, temperature, and light can influence bioactive compound accumulation. A study carried out on blueberry (*Vaccinium corymbosum* L.) grown in different altitudes, was shown that lower altitudes induced an early ripening and a higher anthocyanin accumulation (Spinardi et al.).

Light quality can induce the biosynthesis of different secondary metabolites that can have protective functions against biotic and abiotic stresses. Plants exposed to UV-B treatments have increased phenolic compounds in a dose-response manner. In a study performed in peaches (*Prunus persica* L.), UV-B treatments applied for 1 or 3 h enhanced several phenolic compounds. The employment of UV-B was also studied as priming for preventing the development of *Monilinia fructicola* fungus (Santin et al.). Treatments applied for different durations indicated that 1 and 3 h of UV-B treatments increased the phenolics in the fruit except near the inoculation point, while around the inoculation point the effect of UV-B treatments were not always consistent depending also on the effects of fungus, the wounding and their interaction (Santin et al.).

HORMONAL REGULATION OF BIOACTIVE COMPOUNDS

There are plant hormones that have bioactive molecules as precursors such as abscisic acid (ABA), auxin, salicylic acid (SA), and melatonin. ABA biosynthesis is derived from the catabolism of carotenoids, while the auxins, SA, and melatonin are synthetized from the chorismite as their precursor. The connecting molecules may explain the cross-talks among them and their roles in the modulation of the growth and the ripening process of both climacteric and non-climacteric fruits (Pérez-Llorca et al.).

The protection of plant cells from external environments and biotic or abiotic stresses is partly provided by the cell wall and, in several species, by the cuticular waxes. The major components of cuticular wax are very long chain fatty acids and derived compounds (Trivedi et al.). However, the concentration and composition of these molecules in the cuticular waxes varies among species and within cultivars of the same species. Tomato mutants such as NON-RIPENING (*nor*) and RIPENING INHIBITOR (*rin*) have different cuticular waxes (Kosma et al., 2010). These findings suggest that ethylene plays a role in the wax biosynthesis and accumulation. This hypothesis has been confirmed in the apple and orange. At molecular level, it has been shown that many transcription factors are involved in the regulation of wax biosynthesis such as FRUITFULL and TOMATO AGAMOUS-LIKE1 (Trivedi et al.). In particular, the MADS-RIN transcription factor TDR4/FUL1 and its homolog MBP7/FUL2 have high similarity with FRUITFULL of Arabidopsis. This TDR4 transcription factor has been reported to be involved in pigment biosynthesis in tomato fruit. A functional analysis of this gene using virus-induced gene silencing technology (VIGS) demonstrated that the TDR4 gene is effectively involved in the biosynthesis of bioactive molecules. In fact, TDR4-silenced tomatoes showed a strong reduction of amino acids and α-tomatine (Zhao et al.).

The collection of articles in this Research Topic demonstrates that the accumulation of bioactive compounds in produce can derive from different environmental, genetic, and agronomic factors.

AUTHOR CONTRIBUTIONS

All authors planned the structure of the editorial, contributed in its writing, read and approved the submitted version.

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ORIGINAL RESEARCH
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UV-B Pre-treatment Alters Phenolics Response to *Monilinia fructicola* Infection in a Structure-Dependent Way in Peach Skin

Marco Santin[1], *Susanne Neugart*[2,3], *Antonella Castagna*[1]*, *Martina Barilari*[1], *Sabrina Sarrocco*[1], *Giovanni Vannacci*[1,4], *Monika Schreiner*[3] and *Annamaria Ranieri*[1,4]

[1] Department of Agriculture, Food and Environment, University of Pisa, Pisa, Italy, [2] Department of Biological Sciences, Loyola University, New Orleans, LA, United States, [3] Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Großbeeren, Germany, [4] Interdepartmental Research Center Nutrafood "Nutraceuticals and Food for Health," University of Pisa, Pisa, Italy

Phenolic compounds represent a large class of secondary metabolites, involved in multiple functions not only in plant life cycle, but also in fruit during post-harvest. phenolics play a key role in the response to biotic and abiotic stresses, thus their accumulation is regulated by the presence of environmental stimuli. The present work aimed to investigate how different pre-UV-B-exposures can modulate the phenolic response of peach fruit infected with *Monilinia fructicola.* Through HPLC-DAD-MS^n, several procyanidins, phenolic acids, flavonols, and anthocyanins were detected. Both UV-B radiation and fungal infection were able to stimulate the accumulation of phenolics, dependent on the chemical structure. Regarding UV-B exposure, inoculated with sterile water, 3 h of UV-B radiation highest concentration of phenolics was found, especially flavonols and cyanidin-3-glucoside far from the wound. However, wounding decreased the phenolics in the region nearby. When peaches were pre-treated with 1 h of UV-B radiation, the fungus had an additive effect in phenolic accumulation far from the infection, while it had a subtractive effect with 3 h of UV-B radiation, especially for flavonols. Canonical discriminant analysis and Pearson correlation revealed that all phenolic compounds, except procyanidin dimer, were highly regulated by UV-B radiation, with particularly strong correlation for quercetin and kaempferol glycosides, while phenolics correlated with the fungus infection were quercetin-3-galactoside, quercetin-3-glucoside, kaempferol-3-galactoside and isorhamnetin-3-glucoside. Modulation of pathogen-induced phenolics also far from inoculation site might suggest a migration of signaling molecules from the infected area to healthy tissues.

Keywords: flavonol glycosides, ultraviolet radiation, fruit, *Prunus persica*, post-harvest, brown rot

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Alessandra Francini, Scuola Sant'Anna di Studi Avanzati, Italy

Reviewed by:
Andrea Luvisi, University of Salento, Italy
Qingguo Wang, Shandong Agricultural University, China

***Correspondence:**
Antonella Castagna
antonella.castagna@unipi.it

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INTRODUCTION

During their lifespan, fruit have to deal with several biotic (e.g., pathogen infections, herbivore attacks) and abiotic (e.g., water deficiency, UV-B radiation, high/low temperature) stresses. In order to tolerate such environmental adverse conditions, plants have developed fine-tuned responses through the synthesis and accumulation of many phytochemicals (Schreiner and Huyskens-Keil, 2006). Among these, phenolic compounds represent one of the most representative class of

secondary metabolites, widespread in inflorescences, leaves and fruit (Ehlenfeldt and Prior, 2001; Elfalleh, 2012; Chung et al., 2016; Senica et al., 2017). Phenolics fulfill fundamental functions during plant life cycle, e.g., as signaling molecules, phytoalexins, pigments, antioxidants, and flavor-contributors (Ali Ghasemzadeh, 2011). Among phenolics, flavonoids, and phenolic acids play the main role in defensive mechanisms against microbial and fungal attacks (Rauha et al., 2000; Puupponen-Pimia et al., 2001; Rodríguez Vaquero et al., 2007; Tocci et al., 2018). Cushnie and Lamb (2005) reported that flavonoids act as antimicrobials especially by inhibiting the nucleic acid synthesis, the cytoplasmic membrane function and the energy metabolism. Modulation of phenolic compounds is induced not only by biotic, but also abiotic factors, e.g., UV-B radiation (Liu et al., 2011; Castagna et al., 2013; Scattino et al., 2014; Santin et al., 2018). UV-B radiation activates a specific transduction pathway, which leads to the upregulation of genes involved in phenylpropanoid metabolism, altering the phenolic concentration in both vegatative and generative plant organs (Brown et al., 2005; Favory et al., 2009; Scattino et al., 2014). Previous studies revealed that UV-B positively affects phenolic concentration and profile in many different fruit, such as peach (Scattino et al., 2014; Santin et al., 2018), apple (Lancaster et al., 2000; Fiskaa et al., 2007; Falguera et al., 2011) tomato (Castagna et al., 2014) and lemon (Interdonato et al., 2011). Moreover, recent evidences have reported a phenolic class/compound-dependent response toward UV-B radiation in peach skin (Scattino et al., 2014; Santin et al., 2018). In this sense, Santin et al. (2018) found an increase in anthocyanins, flavones and dihydroflavonols according to the UV-B dose given (1.39 or 8.33 kJ m^{-2}), after 36 h of storage. In different peach cultivars and with a 36 h UV-B irradiation, Scattino et al. (2014) showed a different trend of accumulation between hydroxycinnamic acids and flavonols. A positive role of UV-B radiation has been also observed not only as stimulator of antioxidant compounds, but also in extending the shelf-life of post-harvest fruit by lowering the softening process (Scattino et al., 2015). However, effects of UV-B radiation can be very different depending on the UV-B dose given, since a long and intense UV-B exposure might induce non-specific stress responses to the plant, while a mild and short UV-B radiation triggers specific adaptation responses, such as the activation of genes specifically involved in UV-B acclimation (Favory et al., 2009; Jenkins, 2017). However, evidences in literature show that when two stressors are combined, the effects are not simply the sum of both the effects of the two stressors individually (Rizhsky et al., 2004; Mittler, 2006). Indeed, the simultaneous presence of different modifications of environmental conditions might activate different signaling pathways, often with contrasting and complex effects (Asselbergh et al., 2008). Several studies investigated the effect of combination of two or more abiotic stressors on plant phenolics, e.g., heavy metals with high/low temperatures in wheat (Öncel et al., 2000), drought and UV-B exposure in lettuce (Rajabbeigi et al., 2013), UV-C radiation and heat treatment on *Botrytis cinerea* and *Rhizopus stolonifer* growth in strawberry fruit (Pan et al., 2004). However, literature on combined effects of both biotic and abiotic factors on plant phenolics is scarce. Combination of water stress and infection with plant-parasitic nematode in tomato resulted in altering the response of some secondary metabolites among flavonoids and carotenoids, behaving differently from when the two stresses were applied singularly (Atkinson et al., 2011). Studying post-harvest fruit behavior toward combined biotic and abiotic factor is crucial because it reflects better the complex environmental reality that crops has to face daily, with multiple stressors simultaneously. In wheat, it was found that higher mean temperatures recorded over 6 years resulted in a higher susceptibility to many several kinds of infection, such as viral, fungal, and bacterial (Sharma et al., 2007). Similarly, an increased susceptibility toward fungal pathogens was observed in sorghum, common bean and date palm following drought stress (Diourte et al., 1995; McElrone et al., 2001; Suleman et al., 2001; Mayek-Pérez et al., 2002). Abiotic factors might play also a positive role in enhancing the plant defense toward pathogen and pest attacks. It was found that resistance toward *B. cynerea* is increased by water scarcity in tomato fruit (Achuo et al., 2006), and high salinity condition enhanced the resistance toward powdery mildew in barley (Wiese et al., 2004). However, to date very few data are available about the likely positive role of a mild UV-B pre-treatment on counteracting a fungal infection on peach fruit.

One of the most aggressive pathogen for stone fruits is *Monilinia fructicola*, a fungus responsible for the brown rot disease in pre- and post-harvest peach fruit (Guidarelli et al., 2014; Spadoni et al., 2014). Since phenolic compounds play a key role in plant-pathogen interaction (Rodríguez Vaquero et al., 2007; Tocci et al., 2018), and considering the positive effect of UV-B radiation in stimulating phenolic secondary metabolism, this work aimed to investigate whether several different pre-UV-B treatments enhanced the phenolic response induced by the infection by *M. fructicola* in peach fruit.

MATERIALS AND METHODS

Plant Material and UV-B Treatments

Organic peaches (*Prunus persica* cv. Royal Majestic, melting phenotype) were purchased from a local supermarket and brought to the laboratory of the Department of Agriculture, Food and Environment (DAFE), University of Pisa, Pisa (Italy). All peaches were accurately checked and only undamaged ones with homogeneous dimension were used. Fruit were randomly divided into five groups of ten peaches each. UV-B treatments were performed in proper UV-B chambers equipped with three UV-B lamps per chamber (Philips Ultraviolet-B Narrowband, TL 20W/01—RS, Koninklijke Philips Electronics, Eindhoven, The Netherlands; irradiance 1.36 W m^{-2}). A constant temperature of 24°C was set inside the chambers. Five separate UV-B treatments were performed: 0 h (control, UVB-0), 1 h (UVB-1), 3 h (UVB-3), 6 h (UVB-6), and 12 h (UVB-12). The chambers for both control and UV-B irradiated groups were equipped also with photosynthetically active radiation (PAR) lamps, which provided white light for all the duration of the UV-B treatments. After the UV-B irradiation, peaches were kept under PAR up to 24 h (e.g., peaches treated for 1 h UV-B+PAR were kept for 23 h more

under PAR). The experiment was conducted three times, with five individual fruit each time.

M. fructicola Inoculation

M. fructicola 10757, kindly given by Marta Mari (University of Bologna, Italy) was used in this study. The fungus was deposited at the fungal collection of the Department of Agricultural Science, Food and Environment (University of Pisa) on Potato Dextrose Agar (PDA, Difco, USA) agar slants under mineral oil at 4°C and, when needed, grown on PDA plates at 24°C under a 12 h photoperiod per day until sporulation occurred. One-week-old cultures were accurately rinsed with sterile water to obtain the conidia suspension, and conidia concentration was adjusted to 1×10^5 conidia per ml.

Peaches were wounded (∼1 cm long, ∼1 cm deep each wound) with a sterile scalpel and inoculated with 20 μL of *M. fructicola* conidial suspension. Two wounds were made on the UV-B-exposed side of the fruit. For each UV-B treatment, control peaches were inoculated with 20 μL sterile H_2O. Fruits were incubated at 24°C in plastic bags for 24 h, to maintain a high level of humidity until the drop of conidia suspension was absorbed within the wound. After, bags were removed and peaches were left in the incubator for 2 days more. When the necrotic area was clearly visible on inoculated wounds, peach skin from the UV-B-exposed side of the fruit, both inoculated and not inoculated and far and near the necrotic area/wound, was sampled, dipped in liquid nitrogen and lyophilized until further analyses. The ring portion of the skin (∼1 cm wide) just around the necrotic area has been considered as the region "near" *M. fructicola* infected area; a similar ring portion around the not inoculated wound was considered the region "near" to the wound. A circular portion of the skin (∼ 1 cm radius) with margin 3 cm far from the necrotic area margin (or from the uninoculated wounds) was sampled as the "far" region.

Extraction and HPLC-DAD-MS^n Identification of Phenolics

Phenolic compounds were extracted according to Schmidt et al. (2010) with some modification. Lyophilized, ground material (0.02 g) was extracted with 600 μl of 60% aqueous methanol for 40 min at 20°C. The extract was centrifuged at 4,500 rpm for 10 min at the same temperature, and the supernatant was collected in a reaction tube. This process was repeated twice with 300 μl of 60% aqueous methanol for 20 min and 10 min, respectively; the three corresponding supernatants were combined. The extract was subsequently evaporated until dryness and it was then suspended in 200 μl of 10% aqueous methanol. The extract was centrifuged at 3,000 rpm for 5 min at 20°C through a Corning® Costar® Spin-X® plastic centrifuge tube filter (Sigma Aldrich Chemical Co., St. Louis, MO, USA) for the HPLC analysis. Each extraction was carried out in duplicate.

Flavonoid composition (including hydroxycinnamic acid derivatives and glycosides of flavonoids) and concentrations were determined from the filtrate using a series 1100 HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a degasser, binary pump, autosampler, column oven, and photodiode array detector. An Ascentis® Express F5 column (150 mm × 4.6 mm, 5 μm, Supelco) was used to separate the compounds at a temperature of 25°C. Eluent A was 0.5% acetic acid, and eluent B was 100% acetonitrile. The gradient used for eluent B was 5–12% (0–3 min), 12–25% (3–46 min), 25–90% (46–49.5 min), 90% isocratic (49.5–52 min), 90–5% (52–52.7 min), and 5% isocratic (52.7–59 min).

The determination was conducted at a flow rate of 0.85 ml min^{-1} and at wavelengths of 280, 320, 370, and 520 nm. The hydroxycinnamic acid derivatives and glycosides of flavonoids were tentatively identified as deprotonated molecular ions and characteristic mass fragment ions according to Schmidt et al. (2010) and Neugart et al. (2015) by HPLC-DAD-ESI-MS^n using a Bruker amazon SL ion trap mass spectrometer were acquired in negative ionization mode. For the identification of the peaks, data were compared to the literature of the investigated species and their relatives. In the mass spectrometer, nitrogen was used as the dry gas (10 L min^{-1}, 325°C) and the nebulizer gas (40 psi) with a capillary voltage of −3,500 V. Helium was used as the collision gas in the ion trap. The mass optimization for the ion optics of the mass spectrometer for quercetin was performed at m/z 301. The MS^n experiments were performed in auto up to MS^3 in a scan from m/z 200 to 2,000. Standards (catechin, chlorogenic acid, quercetin 3-glucoside, kaempferol 3-glucoside, isorhamnetin-3-glucoside and cyanidin-3-glucoside, Roth, Karlsruhe, Germany) were used for external calibration curves in a semi-quantitative approach. Results are presented as mg kg^{-1} dry weight (DW). Accuracy and precision of the method were evalutated by calculating the limit of detection (LOD) and limit of quantification (LOQ) for the standards of quercetin 3-glucoside (LOD = 0.36 μg g^{-1} DW; LOQ = 1.07 μg g^{-1} DW), kaempferol 3-glucoside (LOD = 0.29 μg g^{-1} DW; LOQ = 0.87 μg g^{-1} DW), and isorhamnetin-3-glucoside (LOD = 0.40 μg g^{-1} DW; LOQ = 1.20 μg g^{-1}). Since catechin, chlorogenic acid and cyanidin-3-glucoside are highly concentrated within peach skin, LODs were not necessary. Reproducibility of the method was assessed by setting the relative standard deviation (RSD) below 5 and 25% for main and minor peaks, respectively. Accuracy was below 2% for all compounds detected.

Statistical Analysis

Data were analyzed by two-way ANOVA followed by Tukey–Kramer *post-hoc* test at the significance level $P \leq 0.05$ in order to evaluate the effect of both UV-B irradiation and fungal infection on each phenolic subclass detected.

Furthermore, data from individual phenolic compounds were subjected to canonical discriminant analysis (CDA) and Pearson correlation to check which experimental conditions were the most effective in determining variations of phenolic concentration, and phenolics were the most discriminant when UV-B was given alone and/or in combination with infection with *M. fructicola*.

All the statistical elaborations were performed using JMP software (SAS Institute, Inc., Cary, NC).

RESULTS

Since *M. fructicola* normally infects peach fruit by penetrating the skin through mechanical damages, both infected and uninfected (control) fruit were wounded and inoculated with either conidia

suspension or sterile water, respectively. For this reason, possible effects of wounding, in addition to UV-B effects and/or fungal effects, were examined separately in the regions near and far from the wound. To isolate the effect of UV-B from the wounding-induced response, the region far from the wound was collected also in uninfected fruit and considered as "only UV-B treated" samples.

Phenolic Compounds

In peach fruit a diversity of phenolic compounds has been identified and quantified including procyanidins, phenolic acids, flavonols, and anthocyanins (**Table 1**). Results about the phenolic subclasses are presented in **Figure 1** and the concentration of each individual phenolic compound is reported in **Table 2**. Phenolic compounds, which represent the sum of all the phenolics detected in this work, resulted to be significantly affected by infection, UV-B and their combination both near and far from the infection site (**Table 2** and **Figure 1A**).

When the region close to the wound was considered, the samples treated solely with UV-B radiation underwent an increase in phenolics concentration with exposure times of 3 h or more. Particularly, the maximum of phenolics detected (3,421 mg kg^{-1} DW) was reached with UVB-3 treatment. In the region far from the wound, the trend of phenolic accumulation was similar to the region near the wounds, if we consider the uninfected samples. In fact, the UV-B radiation positively affected the phenolic concentration in UVB-3-, UVB-6-, and UVB-12-treated samples, with its maximum in UVB-3 exposed fruit (5,895 mg kg^{-1} DW).

The fungus itself did not affect the phenolic concentration far from the necrotic area at UVB-0. However, it induced a significant increase of phenolics (33%) near the infection.

When UV-B was given prior the fungus, the phenolic concentration near the infection was not affected by any of the UV-B pre-treatment except for UVB-6, where the exposure increased phenolics by 55% compared to infected UVB-0. However, when the UV-B-pre-treated fruit are compared with the uninfected ones, UVB-1 and UVB-6 had an additive effect on phenolics concentration (by 21 and 47%, respectively) while UVB-3 had a subtractive effect (−16%). No differences were detected between infected and uninfected UVB-12 samples. The most stimulating treatments for phenolic accumulation far from the necrotic area were UVB-1 (87%) and UVB-3 (102%), although a significant increase was detected also with UVB-6 (41%) and UVB-12 (47%) when compared to infected and UVB-0-treated samples. When results of the combined factors are compared with the correspondent uninfected ones, UVB-1 treatment had an additive effect (+65%) on the phenolic accumulation observed without the fungus, while UVB-3 had a subtractive effect (−21%).

These results suggest a time-dependendent priming effect of UV-B exposure pre-treatment for *M. fructicola* infection.

TABLE 1 | Phenolic compounds identified through HPLC-DAD-ESI-MS^n method.

Phenolic class	Phenolic Compound
PROCYANIDINS	
	Procyanidin dimer
	Procyanidin trimer
PHENOLIC ACIDS	
	Chlorogenic acid
	Neochlorogenic acid
FLAVONOLS	
	Quercetin-3-rutinoside
	Quercetin-3-galactoside
	Quercetin-3-glucoside
	Kaempferol-3-rutinoside
	Kaempferol-3-galactoside
	Isorhamnetin-3-rutinoside
	Isorhamnetin-3-galactoside
	Isorhamnetin-3-glucoside
ANTHOCYANINS	
	Cyanidin-3-glucoside

Procyanidins

The procyanidins concentration was calculated as the sum of procyanidin dimer and trimer, quantified individually. Effect on procyanidins accumulation was different according to the sampled peach area, near or far from the wound/necrotic area (**Figure 1B**).

Near to the not-inoculated wounds, procyanidins concentration increased proportionally with the UV-B dose given, reaching a concentration of 503 mg kg^{-1} DW in the UVB-12 samples. If the region far from the inoculation point is taken into account, the UV-B radiation determined an increase of the procyanidins with UVB-3 (93%) and UVB-6 (67%) doses in uninoculated samples (**Figure 1B**).

The procyanidins were affected by the presence of *M. fructicola* only far from the necrotic area, and both the sampled region resulted to be influenced by either the UV-B treatment or the combined pre-UV-B/inoculation treatment (**Table 3**).

Due to the inoculation with fungus after the UV-B treatment, in the region close to the infection the procyanidins concentration increased significantly only with UVB-6, in which the inoculated fruits showed the highest procyanidin concentration (448.8 mg kg^{-1} DW) among all the inoculated ones. If the uninfected and the infected peaches were compared, they did not differ in terms of procyanidin concentration except for the UVB-12 treatment, where the uninfected ones displayed a significantly higher concentration compared to the corresponding infected fruits (43%).When the individual procyanidins are considered, however, the behavior is different between the procyanidin dimer and the trimer (**Table 2**). The procyanidin dimer in infected samples resulted to be enhanced only for the highest doses tested, UVB-6 and UVB-12, when compared to the infected UVB-0 samples. On the contrary, when the infected and uninfected fruit are compared considering each UV-B pre-treatment, no differences were detected for any UV-B dose except for UVB-12, where the combination of the two factors resulted in a significant decrease of procyanidin dimer. Regarding procyanidin trimer, the UV-B pre-exposure did not alter the fungus-induced response for any of the UV-B

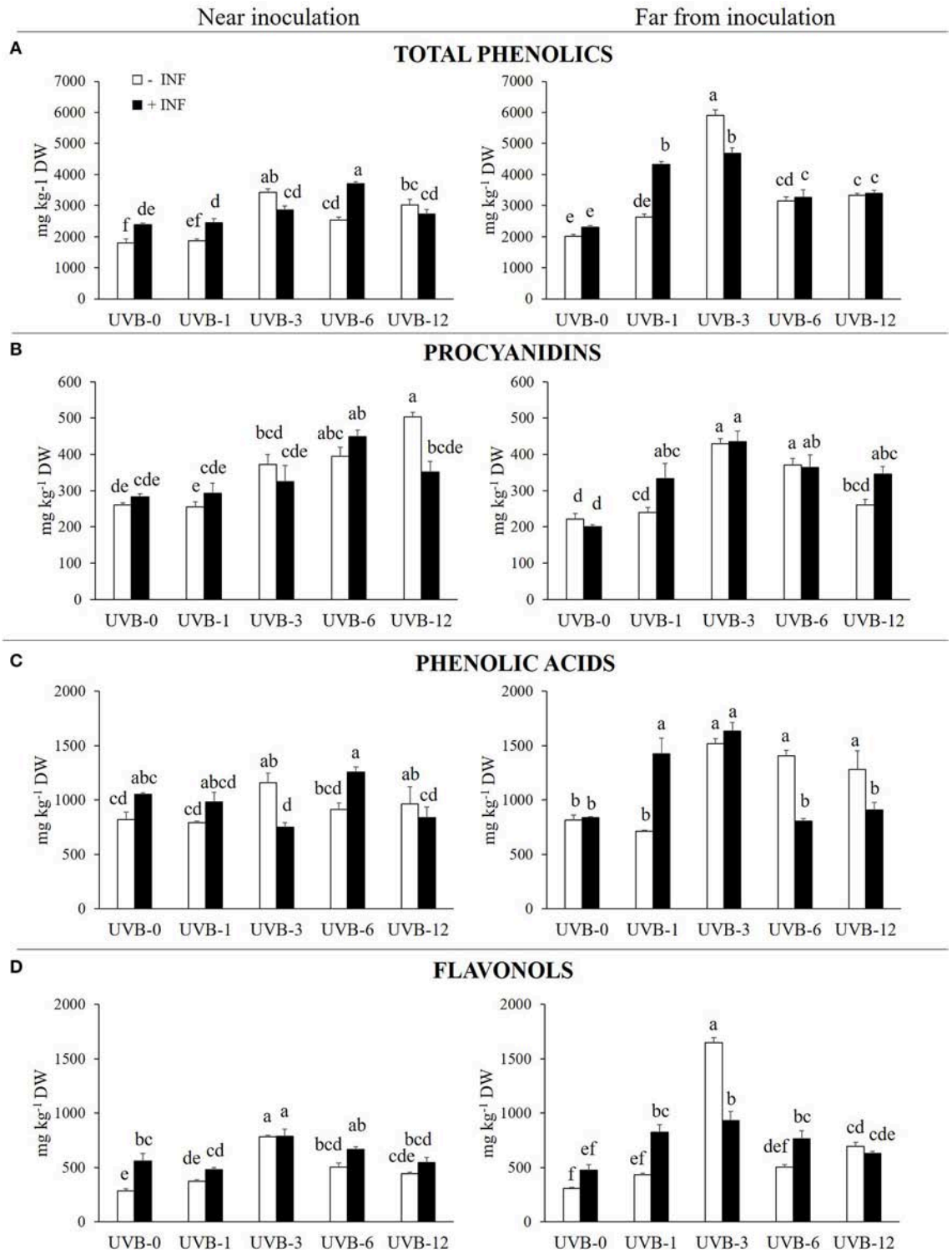

FIGURE 1 | (A) Total phenolics, **(B)** procyanidins, **(C)** phenolic acids and **(D)** flavonols (mg kg^{-1} DW) in the skin of "Fairtime" peaches, near and far from the *M. fructicola* inoculation site, exposed to 0 h (UVB-0), 1 h (UVB-1), 3 h (UVB-3), 6 h (UVB-6), and 12 h (UVB-12) of UV-B radiation. Different letters indicate significantly different values according to one-way ANOVA ($P \leq 05$) followed by Tukey's test.

pre-treatment. Far from the necrotic area, the infected samples displayed a significant accumulation of procyanidins for all the UV-B-dose given, with the maximum with UVB-3 treatment (434.8 mg kg^{-1} DW). Regarding the individual procyanidinds, the procyanidin dimer exhibited the highest concentration in UVB-3, UVB-6, and UVB-12 compared to UVB-0 infected

ones. Generally, the combination of UV-B and fungus resulted in an additive effect for all the UV-B pre-exposures, although such increase was significant only for UVB-12. Contrarily, procyanidin trimer concentration was enhanced in UVB-1, UVB-3, and UVB-12 in comparison to UVB-0 infected fruit. The two factors did not result in altering procyanidin trimer content compared to the correspondent uninfected peaches except for UVB-1, where the combination of the factors had an additive effect on the concentration of such procyanidin.

Phenolic Acids

Regarding the phenolic acids, the values represent the sum of chlorogenic and neochlorogenic acids concentration for each replicate. Their concentration was affected only by UV-B radiation and interaction between infection and UV-B (**Table 3**).

Near the wound, the UV-B treatment itself induced a significant accumulation in phenolic acids concentration only considering the UVB-3 (41%) and UVB-12 (17%) doses (**Figure 1C**). Far from the wound, the UV-B radiation significantly induced an accumulation of phenolic acids in all the UV-B treatments longer than 1 h.

The infection itself did not display any significant modification in phenolic acids concentration, in both near and far regions from the necrotic area.

However, when UV-B was given prior fungal inoculation, 3 and 12 h UV-B doses showed a significant decrease by 35 and 13%, respectively, compared to the corresponding uninoculated samples near the necrotic area. UVB-3 samples also showed a decrease (by 28%) when compared to the inoculated but unirradiated sample. The remaining UV-B treatments, on the contrary, did not show any variation compared to the infected-UVB-0 control. However, an increase in phenolic acids concentration far from the necrosis was detected only with 1 and 3 h of UV-B pre-treatments, by 70 and 95%, respectively, compared to the infected UVB-0 samples. Considering the individual phenolic acids detected, the behaviors described above for both the far and near regions can be particularly valid for chlorogenic acid, mainly because it is 10-times more concentrated than neochlorogenic acid. The neochlorogenic acid concentration near the infection resulted to be increased by UVB-3 and UVB-6 pre-treatments, compared with UVB-0 infected samples. No differences were detected between infected and uninfected samples considering each UV-B pre-exposure, except for UVB-6, where the infection had a significant additive effect to the UVB-6 treatment. Far from the infection, as observed for the total phenolic acids, the neochlorogenic acid was enhanced by UVB-1 and UVB-3 pre-treatments.

Flavonols and Cyanidin-3-Glucoside

Flavonols are considered as the sum of all the individual flavonols detected and listed in **Tables 1**, **2**. Flavonols displayed a similar behavior near and far from the infection, although the entity was different (**Figure 1D**).

In fact, the UV-B radiation determined a significant increase in flavonols concentration in the UV-B-irradiated skin for UVB-3 and UVB-6 by 174 and 76%, respectively, in comparison with uninoculated UVB-0-treated samples. Moreover, in the uninfected peaches, the maximum in flavonols concentration was observed with 3 h of UV-B radiation in both near and far from the wound, reaching values of 782 and 1.646 mg kg^{-1} DW, respectively.

When the fungus was inoculated in the fruit, without UV-B irradiation, the infection determined a significant accumulation (95%) of flavonols close to the necrotic area. However, far from the infection, no significant difference were detected between the inoculated and uninoculated samples (**Table 3**).

When peaches were pre-treated with UV-B and then inoculated with the fungus, for all the UV-B exposures tested the flavonols concentration was similar to the corresponding uninfected samples near the necrotic area. However, when compared with the un-irradiated samples, UVB-3 was the only pre-treatment affecting positively (+41%) the flavonols level. Among the detected flavonols, the effectiveness of the UVB-3 pre-treatment was observed especially for quercetins, which all displayed a significant peak in correspondence to UVB-3 exposure. However, for all of them, such increase was observed already in the UVB-3 without infection, underlying the overwhelming effect of UV-B-induced over the infection-induced effects. Kaempferols, which are the less concentrated flavonols among the ones found, behaved differently between each other. While kaempferol-3-rutinoside exhibited a significant enhancement with UVB-1 and UVB-3 pre-treatments, both compared to the correspondent uninfected and the UVB-0 infected groups, the kaempferol-3-glucoside showed no effect of the UV-B pre-exposure for all the UV-B doses except for UVB-3, where the combination of UV-B and infection negatively affected its concentration compared to the only UV-B-treated one. Isorhamnetin-3-rutinoside concentration, for any pre-treatment, did not change compared to the un-exposed samples. However, the infection following UV-B exposure showed a tendency to increase its concentration for any UV-B pre-exposure compared to the uninfected ones, although it was significant only for UVB-1.

Far from the necrosis, however, the flavonols concentration was significantly higher compared to the UVB-0 infected group for all the UV-B treatments except for UVB-12 (72% for UVB-1; 96% for UVB-3; 60% for UVB-6). However, when compared to the correspondent uninfected samples for each UV-B dose, the infection resulted to positively enhance flavonols concentration only in UVB-1 and UVB-6 (+90 and +52%, respectively), while in UVB-3 the combination of the two factors drastically decreased its concentration (-43%). In UVB-12 pre-treated group, no changes were detected compared to UVB-12 uninfected samples. Since quercetins were the most abundant flavonols detected, the trend observed for the total flavonols is mainly due to the quercetins behavior. They all exhibited a significant peak at UVB-3, compared to UVB-0 infected group, although the presence of the fungus significantly decrease their concentration in comparison to the UVB-3 uninfected ones. While UVB-12 was not effective for all the quercetins, UVB-1 determined an increase only in the quercetin-3-galactoside, while UVB-6 only in the quercetin-3-rutinoside, compared to the UVB-0 infected group. Regarding kaempferols, only kaempferol-3-rutinoside was positively affected by the UV-B pre-treatment,

TABLE 2 | Individual phenolics belonging to procyanidins, phenolic acids, flavonols and anthocyanins subclasses (mg kg^{-1} DW) in the skin of "Fairtime" peaches, near and far from the M. fructicola inoculation site, exposed to 0 h (UVB-0), 1 h (UVB-1), 3 h (UVB-3), 6 h (UVB-6) and 12 h (UVB-12) of UV-B radiation.

			UVB-0	UVB-1	UVB-3	UVB-6	UVB-12
Near							
Procyanidins[a]	Procyanidin dimer	–inf	114 ± 1 e	102 ± 3 e	178 ± 30 cde	293 ± 25 ab	346 ± 5 a
		+inf	109 ± 7 e	153 ± 24 cde	139 ± 30 de	244 ± 9 bc	213 ± 29 bcd
	Procyanidin trimer	–inf	146 ± 6 bcd	153 ± 14 bcd	195 ± 30 ab	102 ± 3 d	157 ± 15 abc
		+inf	174 ± 2 abc	140 ± 14 cd	186 ± 17 abc	205 ± 13 a	138 ± 8 cd
Phenolic Acids[b]	Chlorogenic acid	–inf	731 ± 64 cd	681 ± 12 cd	1040 ± 90 ab	822 ± 58 bcd	1033 ± 43 ab
		+inf	969 ± 22 abc	875 ± 92 abcd	638 ± 38 d	1134 ± 42 a	735 ± 96 cd
	Neochlorogenic acid	–inf	88 ± 8 cd	111 ± 5 abc	118 ± 1 ab	92 ± 11 bcd	101 ± 9 abcd
		+inf	81 ± 5 d	105 ± 5 abcd	114 ± 1 abc	124 ± 3 a	102 ± 2 abcd
Flavonols[c,d,e]	quercetin-3-rutinoside [c]	–inf	47 ± 4 e	58 ± 7 de	120 ± 4 ab	51 ± 5 de	107 ± 2 abc
		+inf	71 ± 3 cde	67 ± 9 cde	146 ± 15 a	120 ± 9 ab	88 ± 15 bcd
	quercetin-3-galactoside [c]	–inf	54 ± 8 c	78 ± 4 bc	212 ± 11 a	143 ± 21 b	89 ± 5 bc
		+inf	144 ± 28 b	110 ± 9 bc	229 ± 20 a	130 ± 10 b	101 ± 3 bc
	quercetin-3-glucoside [c]	–inf	54 ± 7 e	93 ± 8 de	246 ± 16 a	127 ± 11 cde	79 ± 3 e
		+inf	128 ± 24 cde	96 ± 7 de	209 ± 21 ab	194 ± 6 abc	164 ± 32 bcd
	kaempferol-3-rutinoside [d]	–inf	22 ± 2 c	25 ± 1 bc	31 ± 1 abc	24 ± 1 bc	28 ± 1 abc
		+inf	23 ± 3 c	36 ± 1 a	38 ± 5 a	34 ± 1 ab	32 ± 1 abc
	kaempferol-3-galactoside [d]	–inf	3 ± 1 d	5 ± 1 cd	14 ± 1 a	6 ± 1 cd	8 ± 1 bc
		+inf	10 ± 1 b	5 ± 1 cd	8 ± 1 bc	7 ± 1 bcd	7 ± 1 bcd
	isorhamnetin-3-rutinoside [e]	–inf	68 ± 6 c	80 ± 4 bc	80 ± 4 bc	95 ± 2 abc	76 ± 9 c
		+inf	98 ± 16 abc	125 ± 9 a	99 ± 8 abc	120 ± 15 ab	103 ± 3 abc
	isorhamnetin-3-galactoside [e]	–inf	24 ± 1 abc	22 ± 1 c	37 ± 4 ab	27 ± 5 abc	27 ± 2 abc
		+inf	23 ± 3 bc	26 ± 2 abc	30 ± 4 abc	37 ± 1 a	33 ± 3 abc
	isorhamnetin-3-glucoside [e]	–inf	12 ± 2 c	13 ± 1 c	43 ± 9 ab	29 ± 2 bc	27 ± 3 bc
		+inf	06 ± 7 a	16 ± 2 c	29 ± 2 bc	25 ± 1 c	19 ± 2 c
Anthocyanins[f]	cyanidin-3-glucoside	–inf	432 ± 49 d	439 ± 64 d	1108 ± 46 ab	717 ± 16 cd	950 ± 173 bc
		+inf	498 ± 4 d	695 ± 15 cd	996 ± 41 abc	1.331 ± 100 a	992 ± 58 abc
Far							
Procyanidins[a]	Procyanidin dimer	–inf	78 ± 2 de	88 ± 5 cde	170 ± 20 abc	144 ± 1 abcd	51 ± 1 e
		+inf	69 ± 6 de	105 ± 41 bcde	211 ± 23 a	182 ± 23 ab	158 ± 15 abcd
	Procyanidin trimer	– inf	144 ± 14 cd	151 ± 13 cd	259 ± 20 a	227 ± 7ab	209 ± 16 ab
		+inf	132 ± 6 cd	229 ± 2 ab	224 ± 8 ab	181 ± 10 bcd	187 ± 8 bc
Phenolic Acids[b]	Chlorogenic acid	–inf	721 ± 45 c	597 ± 4 c	1320 ± 42 a	1304 ± 49 a	1154 ± 168 ab
		+inf	734 ± 17 c	1269 ± 150 a	1464 ± 85 a	673 ± 21 c	793 ± 71 bc
	Neochlorogenic acid	–inf	93 ± 4 e	114 ± 12 de	197 ± 12 a	103 ± 3 de	125 ± 4 cd
		+inf	104 ± 3 de	156 ± 4 bc	171 ± 10 ab	132 ± 4 cd	114 ± 1 de
Flavonols[c,d,e]	quercetin-3-rutinoside [c]	–inf	59 ± 10 d	95 ± 3 bcd	192 ± 18 a	124 ± 22 bc	130 ± 9 bc
		+inf	86 ± 13 d	103 ± 12 bcd	119 ± 10 bc	138 ± 1 b	86 ± 1 cd
	quercetin-3-galactoside [c]	–inf	57 ± 7 e	79 ± 5 de	496 ± 13 a	102 ± 3 de	179 ± 15 cd
		+inf	101 ± 11 de	250 ± 27 bc	304 ± 50 b	174 ± 27 cd	156 ± 16 cde
	quercetin-3-glucoside [c]	–inf	64 ± 7 d	94 ± 3 d	600 ± 65 a	78 ± 8 d	170 ± 17 bcd
		+inf	128 ± 13 cd	242 ± 24 bc	274 ± 32 b	170 ± 27 bcd	173 ± 2 bcd
	kaempferol-3-rutinoside [d]	–inf	18 ± 1 d	25 ± 1 cd	55 ± 2 a	34 ± 3 bc	32 ± 1 bc
		+inf	19 ± 3 d	33 ± 2 bc	31 ± 1 bc	31 ± 2 bc	37 ± 2 b
	kaempferol-3-galactoside [d]	–inf	5 ± 1 b	4 ± 1 b	36 ± 4 a	4 ± 1 b	6 ± 1 b
		+inf	7 ± 1 b	7 ± 1 b	9 ± 1 b	08 ± 2 b	8 ± 1 b

(Continued)

TABLE 2 | Continued

			UVB-0	UVB-1	UVB-3	UVB-6	UVB-12
	isorhamnetin-3-rutinoside [e]	–inf	67 ± 6 c	92 ± 7 bc	128 ± 7 ab	111 ± 8 bc	107± 5 bc
		+inf	87 ± 18 bc	108 ± 7 bc	108 ± 15 bc	165 ± 21 a	103 ± 2 bc
	isorhamnetin-3-galactoside [e]	–inf	17 ± 1 de	27 ± 2 cd	46 ± 7 a	29 ± 1 cd	32 ± 2 bc
		+inf	13 ± 3 e	36 ± 2 abc	44 ± 2 ab	38 ± 2 abc	33 ± 3 bc
	isorhamnetin-3-glucoside [e]	–inf	18 ± 3 b	16 ± 2 b	92 ± 15 a	18 ± 1 b	36 ± 5 b
		+inf	35 ± 2 b	44 ± 6 b	44 ± 4 b	39 ± 9 b	35 ± 4 b
Anthocyanins[f]	cyanidin-3-glucoside	–inf	672 ± 62 e	1,242 ± 86 bcde	2,302 ± 93 a	871 ± 63 de	1,104 ± 100 cde
		+inf	796 ± 22 de	1,748 ± 178 ab	1,679 ± 263 abc	1,333 ± 168 bcd	1,507 ± 138 bc

Accuracy and precision of the method were evalutated by calculating the limit of detection (LOD) and limit of quantification (LOQ) for the standards of quercetin 3-glucoside (LOD = 0.36 $\mu g\ g^{-1}$ DW; LOQ = 1.07 $\mu g\ g^{-1}$ DW), kaempferol 3-glucoside (LOD = 0.29 $\mu g\ g^{-1}$ DW; LOQ = 0.87 $\mu g\ g^{-1}$ DW) and isorhamnetin-3-glucoside (LOD = 0.40 $\mu g\ g^{-1}$1 DW; LOQ = 1.20 $\mu g\ g^{-1}$1). LODs were not necessary for catechin, chlorogenic acid and cyanidin-3-glucoside, being highly concentrated within peach skin. Reproducibility of the method was assessed by setting the relative standard deviation (RSD) below 5% and 25% for main and minor peaks, respectively. Accuracy was below 2% for all compounds detected.

Mean value (n = 3) ± standard error. For each metabolite values followed by different letters are significantly different according to one-way ANOVA (P ≤ 05) followed by Tukey's test.

[a] *Procyanidins quantified as catechin.*

[b] *Hydroxycinnamic acids quantified as chlorogenic acid.*

[c] *Flavonols quantified as quercetin-3-glucoside.*

[d] *Kaempferol-3-glucoside.*

[e] *Isorhamnetin-3-glucoside.*

[f] *Anthocyanins quantified as cyanidin-3-glucoside.*

regardless the UV-B dose, while kaempferol-3-galactoside did not change at all when compared to the UVB-0 infected samples. As observed for quercetins, the combination of UV-B and infection impacted negatively kaempferols concentration especially in UVB-3, which was the most effective UV-B dose to stimulate flavonols content without infection. Concerning isorhamnetins, the isorhamnetin-3-galactoside was the most responsive one toward the pre-UV-B exposure and the inoculation, since it increased for any UV-B dose tested. However, since no significant differences were detected between infected and uninfected samples for any UV-B duration, the increase observed was likely due to the UV-B treatment over the combination of pre-UV-B and infection. The isorhamnetin-3-glucoside showed no changes between the UVB-0 and any of the UV-B pre-treatment, although the presence of the fungus in UVB-3 group drastically decreased isorhamnetin-3-glucoside concentration in comparison to the UVB-3 uninfected group. Similarly, isorhamnetin-3-rutinoside was not responsive to the combination of UV-B pre-exposure and infection except for UVB-6 dose, where the concentration was significantly higher compared to both the UVB-0 infected and the UVB-6 uninfected groups.

Cyanidin-3-glucoside was the only anthocyanin detected in peach skin (**Tables 1**, **2**). Its concentration was significantly affected by both factors (infection and UV-B) and their interaction (**Table 3**).

The UVB-3 and UVB-12 were the only effective treatments in stimulating its accumulation near the wound in the uninfected samples, with an increase of 156 and 120%, respectively. Far from the inoculation site, similar to what observed near the wound, the cyanidin-3-glucoside concentration reached the maximum in the UVB-3 treatment (2,302 mg kg^{-1} DW). However, all the remaining UV-B treatments resulted to be not significant compared to UVB-0.

TABLE 3 | The two-way ANOVA *P*-values for the effect of infection, UV-B treatment, and of their interactions.

		Two-way ANOVA (*P*)		
		Infection	**UV-B**	**Infection x UV-B**
Total phenolics	Near	0.0002***	< 0.0001***	< 0.0001***
	Far	0.0320*	< 0.0001***	< 0.0001***
Procyanidins	Near	0.2707	< 0.0001***	< 0.0004***
	Far	0.0408*	< 0.0001***	0.0437*
Phenolic acids	Near	0.7581	0.0332*	< 0.0001***
	Far	0.6494	< 0.0001***	< 0.0001***
Flavonols	Near	< 0.0001***	< 0.0001***	0.0179*
	Far	0.8886	< 0.0001***	< 0.0001***
Cyanidin-3-glucoside	Near	0.0005***	< 0.0001***	< 0.0001***
	Far	0.0464*	< 0.0001***	0.0005***

A single asterisk indicates significance at P ≤ 0.05, two asterisks at P ≤ 0.01, and three asterisks at P ≤ 0.001.

The presence of the fungus in UVB-0 samples did not induce any significant change in terms of cyanidin-3-glucoside concentration either near or far from the necrotic area.

In the presence of *M. fructicola*, the significantly effective UV-B exposure were UVB-3, -6, and -12, increasing the cyanidin concentration by 100, 167, and 99%, respectively, compared to the infected UVB-0 samples near the necrosis. However, far from the infection, all the UV-B pre-treatments were found to be effective in increasing the concentration of such anthocyanin.

Canonical Discriminant Analysis (CDA) and Pearson Correlation

Through canonical discriminant analysis (CDA), it is possible to determine whether the biological replicates fit preassigned

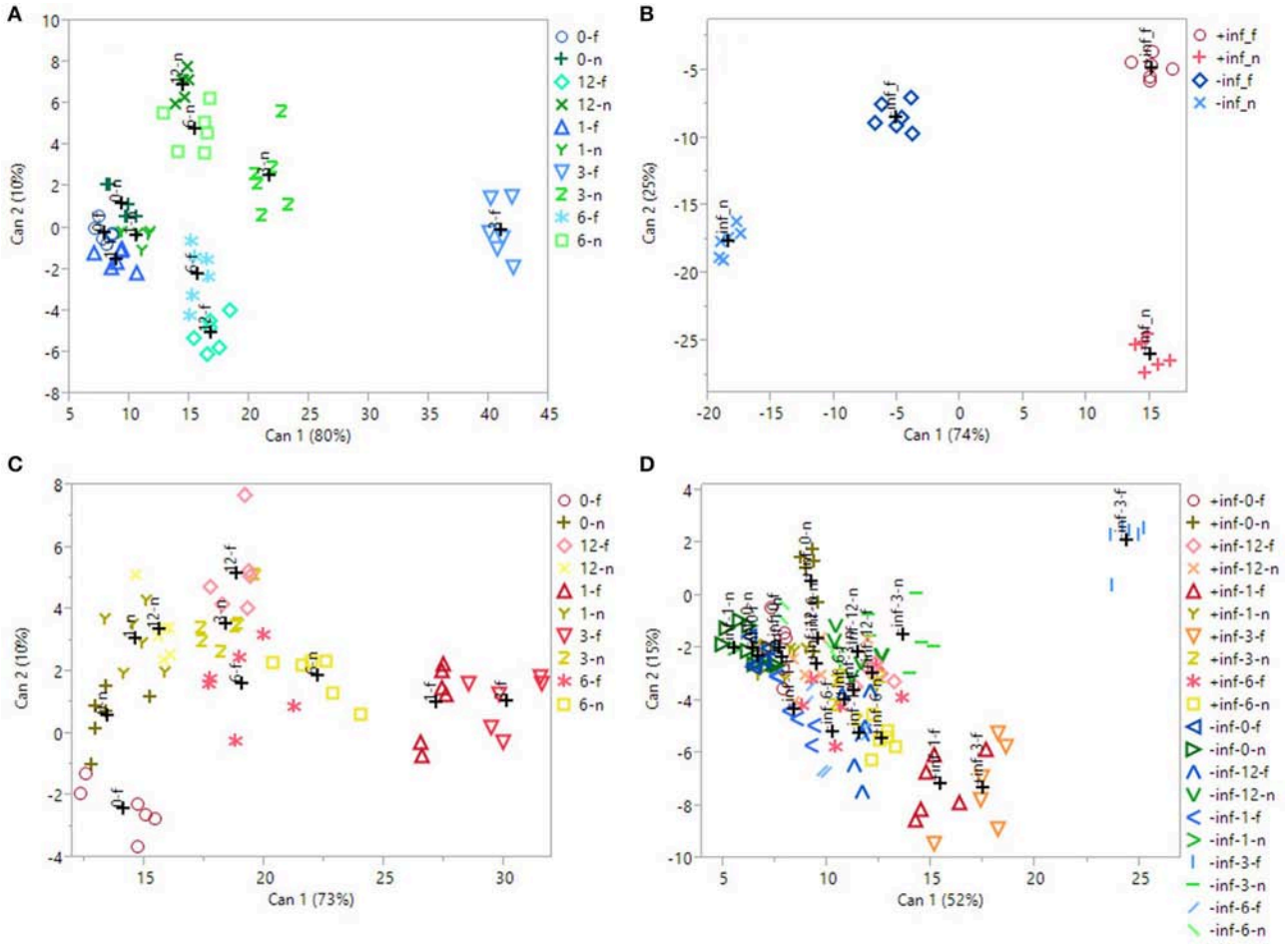

FIGURE 2 | 2D scatterplot of canonical discriminant analysis considering **(A)** the uninfected, UV-B-treated samples; **(B)** the UV-B-untreated samples; **(C)** the infected, UV-B-treated samples; **(D)** all the samples. In **(A,C)**, 0/1/3/6/12_f/n refers to the samples irradiated with UV-B for 0/1/3/6/12 h, considering the region far/near the inoculation point. In **(B)**, ±inf_f/n refers to the infected/uninfected samples, considering the region far/near the inoculation point. In **(D)**, ±inf_0/1/3/6/12_f/n refers to the infected/uninfected samples, irradiated with UV-B for 0/1/3/6/12 h, considering the region far/near the inoculation point. Can 1 and 2 refers to the canonical function 1 and 2, which considers all the variables in order to maximize the separation among the groups.

groups according to all the variables measured. Four different CDAs were performed (**Figure 2**) to check the effectiveness of UV-B and/or fungal infection in separating the groups. Four MANOVA tests (Wilk's Lambda, Roy's Largest Root Test, the Hotelling-Lawley Trace, and the Pillai-Bartlett Trace) were also implemented for each CDA, to check whether the groups were different according to the variables given. For each CDA, all the MANOVA test gave very significant results ($P < 0.0001$), indicating the robustness of the groups separation for each CDA. Furthermore, the Pearson coefficients were determined for each CDA between the concentrations of each phenolic compound and the respective canonical score, to find out which individual phenolics were the main responsible for the groups separation.

The first CDA was performed on just the uninfected samples (**Figure 2A**), to test the effectiveness of the UV-B exposure itself. Canonical function 1 explained the majority of the separation (80%), and the best segregation was visible for the UVB-3 group, both near and far from the wound, especially for the last one. All the other groups overlapped with each other on the left portion of the plot. The Pearson correlation (**Table 4**) revealed that all the phenolics identified in this study were discriminant in this CDA, except of procyanidin dimer.

In **Figure 2B**, only the samples that did not receive any UV-B treatment were considered (UVB-0), to test whether the presence of the fungus, as well as the wounding in uninfected samples, induced an effect in altering phenolic concentration. The CDA were found to be very successful in discriminating the groups, with 74% of segregation explained by canonical function 1, and 25% by canonical function 2. Particularly, the major classification on canonical function 1 is between the infected samples, regardless the sampling region, and the uninfected ones. Furthermore, a clear separation is visible also between the near and far samples of the uninfected ones. According to Pearson correlation (**Table 4**), the most discriminant compounds

were all flavonols. Particularly, the strongest correlation was found for kaempferol-3-galactoside, followed by the quercetin-3-galactoside, the quercetin-3-glucoside and the isorhamnetin-3-glucoside.

Later, the real effect of the pre-UV-B-irradiation in combination with following *M. fructicola* infection was investigated (**Figure 2C**). The 3 h-UV-B exposure resulted to be well-separated also among the samples UV-B-treated and inoculated with *M. fructicola*. In fact, in the CDA including only the fruit that have received both the stressors combined (**Figure 2C**), the UVB-3 treatment, far from the infection, resulted to induce the most remarkable effect in altering phenolics concentration according to canonical function 1 (73% of separation). Immediately after UVB-3 group, UVB-1 one was also well-clustered on the right portion of the plot, supporting the idea of a positive effect of both UVB-3 and UVB-1 exposures on phenolic accumulation. As observed for uninfected samples (**Figure 2A**), also for infected samples (**Figure 2C**) all the other groups are not well-separated and are located almost indistinguishably in the left half of the scatterplot. Interestingly, for both the last CDAs, the UVB—treated groups are the leftmost groups, suggesting that the UV-B treatment, regardless the duration, had a positive effect in increasing phenolics concentration. The Pearson correlation (**Table 4**) indicates as strongly correlated compounds the cyanidin-3-glucoside, the isorhamnetin-3-galactoside, the quercetin-3-glucoside, the quercetin-3-galactoside, the procyanidin trimer, the chlorogenic acid and the neochlorogenic acid.

Finally, in **Figure 2D**, all the samples, regardless the UV-B exposure time, the sampling region or the infection, have been considered for the CDA. Although canonical function 1 explains 52% of the separation, the CDA showed only a partial separation among the groups. In fact, most of the groups are overlapped and located in the left half of the scatterplot. This could be due to overlapping effects of both UV-B treatments, that might be too strong or too weak, and the infection process. However, it is possible to see a good segregation of the uninfected UVB-3 group far from the wound, on the top-right edge of the plot. Other well-separated groups, still on the most positive half of the hyperspace, are the infected UVB-1 and UVB-3 far from the infection, and the uninfected UVB-3 near the wound. The separation of these groups from the others were due to most of the phenolics identified in this study except for the procyanidin dimer and the isorhamnetin-3-rutinoside (**Table 4**).

TABLE 4 | Pearson's correlation coefficients (r) between each phenolic compound detected and the canonical scores for each canonical discriminant analysis (CDA) reported in **Figure 2**.

	Pearson coefficient			
	CDA A	**CDA B**	**CDA C**	**CDA D**
PROCYANIDINS				
Procyanidin dimer	0.19	−0.35	0.25	0.23
Procyanidin trimer	0.63*	0.15	0.74*	0.69*
PHENOLIC ACIDS				
Chlorogenic acid	0.62*	0.42	0.68*	0.62*
Neochlorogenic acid	0.79*	0.12	0.90**	0.82**
FLAVONOLS				
quercetin-3-rutinoside	0.75*	0.55	0.37	0.67*
quercetin-3-galactoside	0.97**	0.62*	0.66*	0.89**
quercetin-3-glucoside	0.93**	0.71*	0.69*	0.88**
kaempferol-3-rutinoside	0.90**	0.02	0.23	0.71*
kaempferol-3-galactoside	0.92**	0.87**	0.09	0.78*
isorhamnetin-3-rutinoside	0.60*	0.37	0.07	0.30
isorhamnetin-3-galactoside	0.70*	−0.30	0.67*	0.69*
isorhamnetin-3-glucoside	0.85**	0.78*	0.17	0.70*
ANTHOCYANINS				
cyanidin-3-glucoside	0.83**	0.43	0.74*	0.82**

CDA A refers to the uninfected and UV-B-treated samples; CDA B refers to just the UV-B-untreated samples (UVB-0); CDA C refers just to the infected and UV-B-treated samples; CDA D refers to all the samples. For each CDA, only canonical scores from canonical function 1, which explains 80% (CDA A), 74% (CDA B), 73% (CDA C) and 52% (CDA D) of the respective separation.

**0.6 > |r| > 0.8: strong correlation.*

***0.8 > |r| > 1: very strong correlation.*

DISCUSSION

Using an HPLC-DAD-MS^n system it was possible to detect many phenolic compounds that belong to several phenolic subclasses, such as procyanidins (procyanidin dimer and trimer), phenolic acids (chlorogenic and neochlorogenic acid), flavonols (quercetin-3-rutinoside. quercetin-3-galactoside, quercetin-3-glucoside, kaempferol-3-rutinoside, kaempferol-3-galactoside, isorhamnetin-3-rutinoside, isorhamnetin-3-galactoside, isorhamnetin-3-glucoside, cyanidin-3-glucoside), and anthocyanins (cyanidin-3-glucoside). All of these compounds have previously been identified in peach fruit (Scattino et al., 2014).

Our aim was to investigate whether the phenolic response to *M. fructicola* infection might be enhanced by a UV-B pre-treatment. For this reason, since this pathogen normally infects peach fruit by penetrating the skin through mechanical damages, both infected and uninfected (control) fruit were wounded and inoculated with either conidia suspension or sterile water, respectively.

Both single UV-B treatment and in combination with a following infection with *M. fructicola*, resulted in significant modulation of phenolic concentration in peach skin, with a differential behavior according to each phenolic compound considered.

When UV-B radiation was given without the infection, 3 h of UV-B exposure seemed to be the best UV-B dose to obtain the maximum phenolic accumulation. This effect, particularly visible on total phenolics, is reflected mainly by flavonols, phenolic acids, cyanidin-3-glucoside and procyanidins far from the wound. UV-B-induced increase in the concentration of phenolic compounds, especially flavonoids has been previously described (Schreiner et al., 2014). Recent evidences in literature suggest that UV-B radiation influences phenolics concentration in peach skin in accordance to the phenolic compound/subclass considered (Scattino et al., 2014; Santin et al., 2018). Indeed,

Santin et al. (2018) found that a 10 and 60 min UV-B treatment determines an increase of specific phenolic compounds, such as anthocyanins, flavones and dihydroflavonols, after 36 h from the treatment. In Scattino et al. (2014), similarly, a different behavior was found between hydroxycinnamic acids and flavonols in response to UV-B radiation in different peach cultivars. In the present study, a UV-B treatment longer than 3 h might cause the activation of generic stress-induced intracellular pathways which might have overlapped the UVR8-mediated signal. Indeed, it might be that a UV-B exposure extended over 3 h have led to an excessive production of reactive oxygen species (ROS), causing the degradation/consumption of the phenolics within the cell in order to avoid potential damages to macromolecules. In fact, the total flavonols decreased of about 36 and 44%, respectively, at 6 and 12 h of UV-B irradiation compared to UVB-3. The UV-B-triggered production of ROS has been observed in tobacco leaves (Czégény et al., 2014), where it was found that UV-B radiation is capable of forming hydroxyl radicals from hydrogen peroxide. Moreover, strong and prolonged UV-B radiations were found to activate generic stress pathways in *Arabidopsis* leaves, thus not inducing phenolic accumulation as specific acclimation effect which are instead favored by short (1–6 h) and mild UV-B exposures (Favory et al., 2009; Jenkins, 2017). The canonical discriminant analysis considering only the UV-B-treated but uninfected fruit (**Figure 2C**) also indicates UVB-3 treatment as the most effective one in stimulating phenolics accumulation, since they are the first two groups on the right side of the plot. Furthermore, regarding total phenolics, as well as for flavonols and the cyanidin-3-glucoside, the concentration detected after UVB-3 treatment was much higher far from the wound than near the wound. In fact, it might be possible that the positive effect of UV-B-radiation in stimulating phenolic accumulation is counteracted by the negative effect of the wound leading to a phenolic depression, which represented a stress for the fruit causing metabolic dysregulations in the region nearby. Another hypothesis could be that, since the region near the infection is likely already colonized by the fungus and will be quicky necrotized, the fruit triggers defense mechanisms in the health tissue (far from the fungus). The first evidence in literature that demonstrated the presence of long-distance signal molecules induced by biotic stresses, which can make plant tissues distant from the infection less susceptible to further biotic attacks, dates back in 1980 (Guedes et al., 1980). This so-called systemic acquired resistance (SAR), involves a wide set of signal molecules, such as salicylic acid, systemin, methyl jasmonate, jasmonic acid, and ethylene, which diffuse from the infection site toward undamaged plant tissues (Enyedi et al., 1992). In addition, several more hormones have been more recently found to be associated with an increased adaptability of the plants toward biotic stresses, such as abscisic acid, auxin, gibberellic acids, cytokinins, and brassinosteroids, whose signaling pathways partially overlap and stimulate the distant and healthy regions of the plant to synthesize defensive compounds to increase its survival chance from eventual pathogen spread on that tissue (Takatsuji and Jiang, 2014). However, such signaling mechanisms have been mostly investigated in plant models, thus knowledge about the pathogen-induced migration of defensive molecules on fruit is scarce. in Among such defense mechanisms, the accumulation of anti-fungal phenolics might also be crucial to limit the infection spreading. The wound effect is visible also through the canonical discriminant analysis (**Figure 2A**), in which the uninfected samples near and far from the wound are clearly separated in the scatterplot considering both canonical function 1 and 2. Previous works investigated the effect of mechanical wounding on fruit and vegetables. For example, it was observed that PAL activity is induced by cutting lettuce leaves in 2 × 2 cm pieces (Saltveit, 2000). However, it was found that phenolics and anthocyanins concentration, as well as the concentration of several other secondary metabolites and the antioxidant capacity, strictly depends on the plant species considered (Fernando Reyes et al., 2006). In fact, it was found that phenolic concentration decreased in zucchini, radish, potato, and red cabbage subjected to shredding process by 26, 7, 15, and 9%, respectively, while it increased in lettuce, celery, carrot, parsnips and sweet potato by 81, 30, 191, 13, and 17%, respectively (Fernando Reyes et al., 2006).

Through CDA considering only the UV-B-exposed peaches (**Figure 2C**), it was possible to observe that between the UVB-3-treated groups, the one sampled far from the wound resulted to be located distantly in the right region of the scatterplot compared to the corresponding group near the wound, indicating a reduction of phenolics concentration close to the wounding site. From the same CDA, it was also possible to confirm the effectiveness of the UVB-3 treatment compared with the other UV-B treatments, since the UVB-3 groups are located in the furthest right portion of the plot, considering canonical function 1.

When infection was given alone, without any UV-B exposure, it determined a modulation in phenolic profile compared to the uninfected ones. Particularly, the infection determined an increase in total phenolics and in flavonols near the infection, although slight but no significant increases were detected for several phenolic classes also far from the infection. It has been stated that phenolics, together with phytoalexins and other plant-defensive secondary metabolites, tend to accumulate in cells surrounding the infection as part of a locally induced defense response (Lattanzio et al., 2006). In this study, the fungus-induced accumulation of phenolics can be observed also in the corresponding CDA (**Figure 2A**), where the infected groups are highly different from the uninfected ones, positioning themselves on the right edge of the plot. Considering the far region, phenolic acids increased in UVB-3, -6, and -12, being the only phenolic subfamily analyzed that did not show a decrease for UV-B treatments longer than 3 h. Phenolic acids represent a crucial junction point in the phenylpropanoid pathway, since they are precursor of most of flavonoids, such as flavonols, anthocyanins and procyanidins. The constantly high level for such UV-B treatments might be due to either a continuous stimulation of their biosynthesis, or their reduced conversion into downstream flavonoids. Especially this last hypothesis might be supported by the fact that flavonols in UVB-6 and UVB-12 samples, as well as procyanidins in UVB-12 samples, decreased to the control level.

The individual phenolics detected that led to the segregation among the infected/uninfected samples, according to the

CDA (**Figure 2A**), were quercetin-3-galactoside, kaempferol-3-galactoside and isorhamnetin-3-glucoside. Involvement of several phenolic subclasses in counteracting fungal infection has been observed in previous studies. Recently, a comparison between the phenolic profile of two apple cultivars, one resistant and one susceptible to blue mold caused by *Penicillium expansum*, revealed that the resistant apple cultivar had higher concentrations of procyanidins, dihydrochalocone, flavonols, and hydroxycinnamic acids (Sun et al., 2017). In bilberry (*Vaccinium myrtillus*) infected by the fungal pathogen *B. cinerea*, an accumulation of several phenolics such as quercetin-3-glucoside, quercetin-3-*O*-α-rhamnoside, quercetin-3-*O*-(4″-HMG)-R-rhamnoside, chlorogenic acid and coumaroylquinic acid was found (Koskimäki et al., 2009). The antifungal role of phenolics was observed also in nectarine and apricot fruits treated with *Sanguisorba minor* extract, where a drastic inhibition of *Monilinia laxa* brown rot was observed due to the high presence of caffeic acid derivatives and flavonoids derived from apigenin, quercetin, and kaempferol in the extract (Gatto et al., 2011).

However, under natural conditions, plants have to face different biotic and abiotic stresses simultaneously, due to their sessile lifestyle, and whose effects are not simply the sum of each individual stressor. For this reason, in this work an attempt was made to apply a combination of a pre-UV-B radiation and a fungal infection, to investigate the responsiveness of phenolic compounds.

When peaches were pre-treated with UV-B and then infected with *M. fructicola*, the scenario changed, and variations were again different according to each phenolic class and individual compound considered. In fact, the UV-B pre-exposure generally induced an accumulation of phenolics compared to the UV-B unexposed samples, especially for UVB-1 and UVB-3 treatments in the region far from the infection. This behavior is particularly visible for flavonols, phenolic acids, cyanidin-3-glucoside and, generally, total phenolics. Such phenolic increment in the far region was not visible in the UVB-1- and UVB-3-treated samples near to the infection probably because the fungus, which already spread and induced brown rot symptoms near the inoculation site, induced partial degradation/consumption of UV-B-induced peach phenolics in the area nearby. This behavior was visible also through CDA (**Figure 2D**), where the only groups well-separated from the others in the most positive region of the scatterplot were UVB-1- and UVB-3-treated samples far from the infection. Considering UVB-1 treatment it is noticeable that the infected samples far from the inoculation site showed a higher phenolic concentration than the corresponding UVB-1-uninfected ones. It is intriguing to note that *M. fructicola* did not induce any change in phenolic concentration unless UV-B radiation was preliminary applied. Similarly, UV-B radiation at the lowest dose (UVB-1) was ineffective in stimulating phenolic accumulation. However, when UVB-1 peaches were infected, phenolics concentration increased. This might be due to an additive effect of the systemic response toward the fungus and the UVB-1 radiation, which itself might have been too mild to induce significant phenolics accumulation. This evidence suggests that signals deriving from the individual factors, too low to induce phenolic biosynthesis, synergically interacted, thus triggering a positive response. Differently from the general trend procyanidins were unaffected by the fungus but only stimulated by UV-B treatments. Regarding UVB-3 treatment, which was the most effective in determining phenolic accumulation without infection, the inoculation of *M. fructicola* resulted in a significant decrease of flavonols and total phenolics far from the infection, while for the other phenolic subclasses no variations were detected. This suggests an impact-specific response in phenolic accumulations. Since UVB-3 was found to be the threshold dose in stimulating phenolic accumulation in absence of fungus or wounding effect, it might be that either the systemic response triggered by *M. fructicola*, or longer UV-B treatments, did not result in enhancing phenolics further. Effectiveness of UVB-3 treatment was observed especially for flavonols, which represent very strong antioxidant compounds among favonoids.

Regarding phenolic acids, it is interesting to notice that, contrarily to what observed when UV-B was given alone, UVB-6 and UVB-12 determined a significant decrease in their concentration as compared to the UVB-3 treatment in the far region. As already stated above, phenolic acids represent precursors for several phenolic subfamilies which might act as defensive compounds also against biotic stresses. Thus, it is likely that such decrease might be due to their utilization in forming antioxidant and antifungal compounds such as procyanidins or flavonols, which in fact remained similar to UVB-3 also for higher doses.

The simultaneous or subsequent presence of different stressors or changes in environmental factors sharing common responses (e.g., induction of phenolic metabolism) may result in a positive or negative effect on metabolite production, as indicated by some results present in literature. Pan et al. (2004) found that the application of UV-C radiation (1.41 kJ m^{-2}) and heat (45°C, 3 h in air), resulted in decreasing phenolics content after 2-days storage at 20°C. Very few previous works investigated the phenolics response to a combined exposure to UV-B radiation and pathogen. In a work by Saijo et al. (2009), the sucrose-induced accumulation of anthocyanins was attenuated by a simultaneous application of bacterial elicitors flg22 and elf18 in Arabidopsis, suggesting a potential subtractive effect of a biotic stress on phenolics production. A previous study in literature found that genes associated with the response of *Nicotiana longisflora* plants to herbivory insect were also induced by UV-B radiation, while, in parsley, pathogen-induced defense responses could inhibit the UV-induced flavonoid biosynthesis (Logemann and Hahlbrock, 2002). Similarly, another work reports a decrease in the UV-B-induced accumulation of flavonols by concurrent application of a bacterial elicitor in *Arabidopsis* cell culture (Schenke et al., 2011). Such a suppression was accompanied by the production of defense-related compounds as phytoalexins and lignin that can limit pathogen spread acting as a structural barrier. Moreover, it is also possible to state that, in the present study, UV-B treatment was the most effective factor, over the fungal infection, in increasing phenolic concentration in peach skin. In fact, the highest phenolics concentration was reached when UV-B radiation was given without fungal infection mainly in the far region. This is particularly valid for flavonols and

cyanidin-3-glucoside, which are among the most antioxidant phenolics in plant kingdom. In the CDA including all the groups considered in this study (**Figure 2B**), the most separated group on the right part of the plot was the uninfected and UVB-3 treated one, far from the wound. This group, among the UVB-3 groups, was the one not affected by stimuli other than UV-B radiation, given at the best dose tested. The overwhelming effect of UV-B radiation compared to the fungal infection was visible also by the Pearson coefficients (**Table 4**). In fact, while the presence of the fungus alone led to the increase of just four out of thirteen phenolics identified, the presence of UV-B radiation alone induced variation of 12 out of 13 phenolics, with very strong correlation values ($|r| > 80$) for most of flavonols and the cyanidin-3-glucoside.

No previous data are reported in literature about the effects of a pre-UV-B exposure on phenolics concentration of peach fruit inoculated with *M. fructicola*. Our study revealed that all the phenolic subclasses identified are enhanced by 1 h- and 3 h-UV-B radiations far from the inoculation point, while near the necrosis the scenario is more complex and depends on the UV-B dose applied and phenolic subclass considered, probably due to an overwhelming effect of both the fungus and the wounding. In fact, a very high conidia concentration was inoculated in this experiment to ensure the development of the infection. However, under environmental conditions, the number of conidia penetrating the fruit and giving rise to the symptoms are supposed to be much less, thus the UV-B-induced phenolic compounds might be able to counteract the fungal spreading. Specifically, in this work an accumulation of chlorogenic acid was observed both near and far from the infection following specific UV-B irradiation doses (e.g., UVB-3), both alone and in combination with the infection. Since the caffeoyl moiety of chlorogenic acid is involved in inhibiting the expression of the *Mf-cut1*, a *M. fructicola* gene encoding a cutinase enzyme, and in preventing cutinase activity as well (Bostock et al., 1999; Wang et al., 2002; Guidarelli et al., 2014), it is likely that the UV-B-induced increase in such phenolic acid might result in contrasting pathogen spreading on the fruit.

This is therefore a preliminary study, and further research is needed in order to understand whether the UV-B radiation can limit the fungal spreading under more realistic condition (inoculation with a lower conidia concentration). Furthermore, investigation on long-distance fungus-induced molecules are encouraged, to unreveal the signaling pathways involved in the phenolic response both at molecular and biochemical levels. Moreover, deeping the knowledge about the relationship between phenolic structure and their effect as protective compounds against fungal infection is strongly recommended.

AUTHOR CONTRIBUTIONS

AR, GV, and MoS designed the research. MaS, SS, SN, AC, and MB carried out the experiments, analyzed the data, and wrote the manuscript. AR, GV, and MoS helped to draft the manuscript and revise the manuscript. All authors read and approved the final manuscript.

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Functional Molecules in Locally-Adapted Crops: The Case Study of Tomatoes, Onions, and Sweet Cherry Fruits From Tuscany in Italy

Roberto Berni[1,2], *Marco Romi*[1], *Claudio Cantini*[2], *Jean-Francois Hausman*[3], *Gea Guerriero*[3] *and Giampiero Cai*[1]*

[1] Department of Life Sciences, University of Siena, Siena, Italy, [2] Trees and Timber Institute-National Research Council of Italy (CNR-IVALSA), Follonica, Italy, [3] Environmental Research and Innovation Department, Luxembourg Institute of Science and Technology, Belvaux, Luxembourg

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***Correspondence:**
Giampiero Cai
giampiero.cai@unisi.it

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The human diet is characterized by highly energetic molecules, but it also requires non-energetic compounds that are equally useful for cell functioning and for preserving the organism's health status. These "functional" molecules are represented by a wide variety of plant secondary metabolites, such as terpenoids, vitamins and polyphenols with antioxidant power. Widespread commercial crop varieties often contain scarce levels of functional molecules, because they have been mostly selected for productivity, rather than for the content of secondary metabolites. Different scenarios (global economic situation, foreseeable environmental changes) are pushing farmers to review the use of high yield crops and to focus on the valorization of locally-adapted plants. This renewed interest is strengthened by the growing need of consumers for functional foods with beneficial effects on human health and by the willingness to promote sustainable low-input agricultural practices exploiting local climate, soil, water, and (micro)biota. Here, we want to discuss a specific case study concerning locally-adapted crops in Tuscany (Italy). Analyses of nutraceutical molecules in locally-grown crop varieties (namely tomatoes, sweet cherries and onions) have shown that they are characterized by substantially higher functional molecule contents than commercial varieties. Our goal is to promote the high-throughput study of locally-adapted varieties to understand, in a medium-term perspective, whether the cultivation of such plants is a valuable support for the diet and an adequate local economic resource. Such plants can provide a boost to the regional economy, by diversifying the local crop-market landscape. Moreover, the exploitation of locally-grown plants results in the manufacture of fully-traceable products (from the raw bioresource to the finished product) with a "0 km" concept that minimizes the C footprint.

Keywords: bioactive molecules, Tuscany, autochthonous ancient varieties, nutraceutics, functional food

FUNCTIONAL MOLECULES IN THE DIET AND THEIR EFFECTS ON HUMAN HEALTH

Plant functional molecules (a.k.a as bioactives or health-promoting compounds) are metabolites produced through the plant secondary metabolism that exert biological effects on humans (Azmir et al., 2013; Berni et al., 2018a). Their role in health protection is one of the most important discoveries of the last decade in the biological, medical, and pharmacological fields. The most noteworthy feature of these health-beneficial molecules is their widespread occurrence in fruits and vegetables. This implies the possibility of assimilating them through the daily diet, thereby avoiding any chemical or industrial process to synthesize them.

Plant secondary metabolites are typically produced in response to changes in the environment or pathogen/herbivore attack and are currently in the spotlight of biotechnology because of their applications in different industrial sectors (i.e., healthcare, cosmetics, to name a few; Guerriero et al., 2018). The chemical structures of plant functional molecules are able to counteract reactive oxygen species (ROS) and reactive nitrogen species (RNS) by acting as natural scavengers (Berni et al., 2018b). A strong body of evidence in the literature has indeed drawn attention on plant secondary metabolites in the light of their antioxidant potential, by focusing on specific classes, namely terpenes, and polyphenols (Ghasemzadeh et al., 2010; Thoppil and Bishayee, 2011).

Terpenes consist of over 40,000 different molecules produced by many organisms, spanning from bacteria, yeasts, fungi and, most notably, plants (Goto et al., 2010). They have been studied for their anti-inflammatory effects (Hortelano, 2009) linked to the regulation of cytokine production in human cells (Ku and Lin, 2013). In the plant kingdom, these molecules are synthesized in large quantities by food crops, such as tomatoes: these vegetables produce large amounts of terpenes in the form of carotenoids and, in particular, of lycopene, a pigment that is responsible for the typical red color (Saini et al., 2015). Lycopene is the most efficient quencher of free radicals with the property of protecting the cellular components against oxidative damage (Nasir et al., 2015).

Polyphenols are the largest group of plant secondary metabolites and are classified into different subsets of compounds, depending on their chemistry (Tsao, 2010; Kabera et al., 2014). Their structure is characterized by aromatic ring(s) linked to hydroxyl groups, which confer antioxidant properties to the molecule (Craft et al., 2012). Particular scientific attention is devoted to the class of flavonoids that are present in common plant food products. Vegetables, such as onions, contain high levels of flavonoids, notably quercetin, that is well known for its applications in cancer prevention (Gibellini et al., 2011). Furthermore, evidence in disease treatment is reported for other flavonoids, such as anthocyanins (Norberto et al., 2013). The role of these molecules is directly linked to the decreased susceptibility in developing cardiovascular diseases (Wallace, 2011).

Red fruits like sweet cherries typically contain high levels of anthocyanins and their dietary intake could help in the prevention of cardiovascular diseases (He and Giusti, 2010; Mirto et al., 2018).

Fruits and vegetables are thus natural sources of different bioactives: in the light of their documented benefit on human health, their intake through the daily diet contributes to boost the natural body defenses.

We have here focused on three examples of crops/fruit tree, i.e., tomatoes, onions, and sweet cherry, which are constituents of the Mediterranean diet and are part of the rich biodiversity of Italian regions. In Tuscany, which is the case study here analyzed, 8 different tomato varieties were previously reported (Naziri, 2009) and specific onion varieties are included in the "PAT" (Prodotti alimentari tradizionali-Traditional food products) by the Ministry of Agricultural Politics (Ferioli and D'Antuono, 2016). The fruits/bulbs of these species are not only consumed fresh, but also dried, or processed as soups/juice/jams/marmalades and canned foods. Additionally, tomatoes and onions are produced for >0,6 million tons and >300,000 tons per year in Italy (statistics from 2010; Sardaro et al., 2013; Caruso et al., 2014) and therefore represent economically-important crops.

NATIVE (LOCALLY-GROWN) PLANTS VS. COMMERCIAL PLANTS

Native crops are wild plants that are consumed for their fruits, leaves, flowers, and seeds and originating in specific geographic areas (Glew et al., 2005). The concept of native crops can be extended to comprise also plants cultivated for several decades in a specific territory and not necessarily originating from the same territory. Native or wild plants have adapted to the territory by establishing a synergy with the local soil and climate. They represent the local germplasm and offer a source of genes and unique phenotypic characters (Berni et al., 2018a). Emblematic is the example of non-commercial apple varieties showing extreme phenotypes, notably russeting (Legay et al., 2015, 2017) and containing molecules with immune-modulatory properties (Andre et al., 2013).

The inexorable industrial development has selected varieties of fruit and vegetables according to the market demand. Varieties that are not productive according to commercial standards are put aside and this leads, indirectly, to a loss of biodiversity (Schmidt and Wei, 2006).

For centuries, wild crops have played a significant role in nutrition, especially during times of weather extremes, thanks to their ability to withstand strong exogenous stresses, thereby representing a key feedstock for human nutrition (Bvenura and Afolayan, 2015). Furthermore, the fruits of such ancient varieties produce a wide range of bioactive molecules that are currently studied for their role in the human diet (Berni et al., 2018c) and as protective molecules against diseases (Francini et al., 2017; Berni et al., 2018b).

The adaptive strategies put in place by these plants to survive in a wild environment (not impacted by any human intervention) translate into an enhanced production of secondary metabolites, such as polyphenols and terpenes. The increased production

of such molecules is likely linked to epigenetic changes that have been induced by the interaction with the environment (Baulcombe and Dean, 2014). Genetic modifications confer to ancient crops unique phenotypic and nutritional features, compared to fruits cultivated for commercial purposes (Legay et al., 2017). In this sense, many authors have focused their attention on the functional molecules derived from autochthonous fruits to shed light on their nutritional and nutraceutical potential in the human diet (Stintzing and Carle, 2004). Ancient fruits are considered as natural antioxidant resources and potential scavengers against oxidative stress. Very interesting, in this respect, are the studies that compare the content of these molecules, expressed in terms of antioxidant potential, in ancient and commercial species. Iacopini et al. (2010) report that the ancient apple varieties they studied contain a higher concentration of antioxidant molecules, with respect to commercial counterparts. We have also recently shown that Tuscan tomatoes sampled in 2016 show higher contents of polyphenols and antioxidant molecules with respect to commercial varieties and that this increase is two- and even three-fold (Berni et al., 2018c). As discussed by other authors, the genotype plays a fundamental role on the final content of functional molecules in fruits (Scalzo et al., 2005), since its interaction with the surrounding environment determines the expression of specific traits (King, 2015). Researches on autochthonous fruits intend to valorize and promote the preservation of these territorial species, by exploiting their nutritional value (Lamien-Meda et al., 2008). Considering the huge spectrum of functional molecules produced, ancient crops can, and should be considered as functional foods and could even be used in support of drug therapies.

THE CASE STUDY OF NATIVE CROPS IN TUSCANY (ITALY): TOMATOES, SWEET CHERRIES AND ONIONS

The region of Tuscany is world renowned as a producer of high-quality products, such as wine and oil. This unrivaled product quality is due to the local production and exclusive use of territorial natural resources (Mangani et al., 2011), as well as to the protection of specific cultivars (Berni et al., 2018a) and to the development of quality labels. The growing interest in these foods is fuelled by their high content in health-beneficial bioactive molecules (Iacopini et al., 2008; Cavallini et al., 2014). The rich composition in bioactives has the potential of making these products key components of a functional diet (Cencic and Chingwaru, 2010) that is able to offer a stronger line of defense against diseases (Pandey and Rizvi, 2009). Thanks to the ability to adapt to the territory, these plants have developed a synergy with the local soil. In this sense, Tuscany has a broad panoply of plant species that constitute the regional germplasm heritage (http://germoplasma.regione.toscana.it/index.php?option=com_content&view=article&id=1&Itemid=127). Most of these plants were commonly used in the past for human consumption or for other uses: fruit harvesting, wood and fodder, to mark the borders and to support other plants and wild animals. Several evidences in the literature indicate that in these woody and herbaceous species the fruits show noteworthy contents of nutritional and functional compounds (Ancillotti et al., 2016). Therefore, analytical studies are necessary to highlight the functional properties of these local species expressed in terms of bioactive compound contents. The case study here reported will shed light on the nutraceutical characteristics of ancient varieties of Tuscan onions, tomatoes and sweet cherries to sensitize the public to a wider use of these varieties that play an important role in the regional biodiversity landscape and that could ultimately improve human nutrition.

Tomatoes are well known for their content in carotenoids, such as lycopene, but also for the occurrence of other molecules, notably flavonoids and hydroxycinnamic acids (García-Valverde et al., 2013). Onions are an excellent source of flavonoids, i.e., myricetin, quercetin and kaempferol. These molecules are typically present in high amount in the fruit flesh; on the contrary, the skins of red onions are rich in anthocyanins (Pérez-Gregorio et al., 2010). Sweet cherries are one of the most abundant red fruits rich in anthocyanins, distributed chiefly in the peel and in the outer layers of the fruit (Kelebek and Selli, 2011). Nevertheless, in some varieties of these fruits, the distribution of anthocyanins is also found in the seeds. Tuscany has classified 8 varieties of tomatoes, 6 of onions and 6 of sweet cherries; these crops are grown in experimental fields (http://www.ivalsa.cnr.it/az-s-paolina.html), which are used to preserve the native genetic resources located in various areas of the region.

The plant material used in the present study was provided by the "Trees and Timber Institute—CNR-IVALSA" (Follonica, Italy). The institute is a germplasm regional bank for the propagation and enhancement of regional genetic resources. Our analysis focused on functional molecules in native varieties collected in 2017, with the aim of comparing their content to that found in the most widespread commercial Italian varieties. All the analyses performed follow the standards used for the determination of functional and nutraceutical molecules, i.e., Ferric Reducing Antioxidant Power (FRAP), Folin–Ciocalteu, aluminum chloride assay, pH differential method (Aramwit et al., 2010; Dai and Mumper, 2010; Jagtap et al., 2010) and confirmed with HPLC analysis (see **Supplementary Material** for a description of the methods used). The results are reported in **Table 1**. Regional varieties showed interesting results, especially when compared with commercial counterparts. Autochthonous fruits contained indeed large quantities of functional molecules (polyphenols, flavonoids, and terpenes) that, in many cases, were higher than those found in commercial fruits (Berni et al., 2018c). Notably, a higher functional molecule content has been demonstrated in other crop landraces (Renna et al., 2014, 2018). These results show that local fruits produce higher amounts of molecules that can be exploited as health-beneficial bioactive compounds (Williamson, 2017; **Figure 1**).

The use of these fruits could represent the next step towards the diversification of the current staple food crop market and a functional diet valorizing the local agrobiodiversity (Cantini et al., 2018). Additionally, such varieties could be the object of high-throughput studies based on—*omics* to uncover the

TABLE 1 | Total content of antioxidants as mmol Fe^{2+} per 100g FW, polyphenols as mg of GAE (gallic acid equivalents) per 100 g of FW, flavonoids as mg of QeE (quercetin equivalents) per 100 g of FW, carotenoids as TCC (total carotenoids content) per 100 g of FW and anthocyanins as CyE (cyanidin-3-glucoside equivalents) per 100g of FW.

Variety names	Antioxidants (mmol Fe^{2+}/100 g FW) ± S.D	Polyphenols (mg GAE/100g FW) ± S.D	Flavonoids (mg QeE/100g FW) ± S.D	Carotenoids (mg TCC/100g FW) ± S.D	Anthocyanins (mg CyE/100g FW) ± S.D
Tomatoes (*Solanum lycopersicum* L.)					
Liscio da Serbo Toscano	0.84 ± 0.04a	74.24 ± 1.7a	12.96 ± 0.2ae	0.80 ± 0.02a	/
Rosso Pitigliano	1.19 ± 0.01b	75.67 ± 1.7a	7.25 ± 0.1b	0.86 ± 0.06ab	/
Quarantino ec Valdarno	1.03 ± 0.02c	90.35 ± 1.1b	20.31 ± 0.5c	0.90 ± 0.04b	/
Fragola	0.64 ± 0.03d	63.68 ± 1.2c	8.83 ± 0.1d	0.87 ± 0.03ab	/
Canestrino di Lucca	0.52 ± 0.04e	53.81 ± 2.5d	7.88 ± 0.1b	0.89 ± 0.01ab	/
Costoluto Fiorentino	1.14 ± 0.03b	76.78 ± 2.4ae	13.42 ± 0.2e	0.75 ± 0.05c	/
Giallo di Pitigliano	0.93 ± 0.02a	79.24 ± 0.7e	21.49 ± 0.4c	0.71 ± 0.07c	/
Pisanello	0.76 ± 0.02f	66.52 ± 0.4c	11.95 ± 0.2a	0.74 ± 0.01c	/
Cuore di Bue (Commercial)	0.48 ± 0.07e	51.35 ± 1.5d	6.58 ± 0.1f	0.69 ± 0.07c	/
Onions (*Allium cepa* L.)					
Maremma	1.84 ± 0.10a	69.25 ± 2.6a	63.2 ± 1.3a	/	7.1 ± 0.8a
Rossa Massese	3.08 ± 0.14b	302.83 ± 3.9b	280.4 ± 3.2b	/	40.2 ± 2.1b
Treschietto	1.18 ± 0.08cde	150.42 ± 4.9c	69.3 ± 1.4c	/	6.8 ± 0.6a
Rossa della Valtiberina	1.15 ± 0.11cd	98.58 ± 3.1d	68.3 ± 3.5c	/	55.5 ± 2.8c
Rossa di Lucca	1.23 ± 0.12d	117.91 ± 4.2e	80.2 ± 2.1d	/	23.3 ± 1.8d
Rossa Fiorentina	0.97 ± 0.09e	89.52 ± 2.9f	60.3 ± 2.9a	/	47.3 ± 2.3b
Tropea (Commercial 1)	0.91 ± 0.02e	55.98 ± 0.9g	24.4 ± 0.9e	/	39.5 ± 2.4b
Giarratana (Commercial 2)	0.83 ± 0.01e	58.76 ± 4.7g	27.9 ± 0.8e	/	/
Sweet cherries (*Prunus avium* L.)					
Carlotta	1.49 ± 0.06a	200.49 ± 15.8a	47.21 ± 2.4a	/	30.80 ± 1.8a
Benedetta	1.51 ± 0.04a	250.91 ± 11.3b	65.33 ± 2.3b	/	27.52 ± 1.8a
Morellona	2.51 ± 0.03b	300.87 ± 13.1c	63.01 ± 3.7b	/	59.41 ± 2.5b
Maggiola	1.92 ± 0.12c	276.71 ± 7.5d	46.41 ± 2.5a	/	33.17 ± 1.7a
Moscatella	1.51 ± 0.05a	215.15 ± 11.9a	44.82 ± 4.8a	/	27.04 ± 2.4ca
Crognola	3.17 ± 0.01d	368.18 ± 5.7e	81.62 ± 3.3c	/	67.75 ± 2.7c
Durone (Commercial)	1.13 ± 0.04e	146.05 ± 2.7f	32.21 ± 2.8d	/	31.65 ± 2.3a

Tomatoes (*Solanum lycopersicum* L.)	Caffeic Acid (μg/g FW) ± S.D	Ferulic Acid (μg/g FW) ± S.D	Chlorogenic Acid (μg/g FW) ± S.D	Naringenin (μg/g FW) ± S.D	Quercetin (μg/g FW) ± S.D	Lycopene (μg/g FW) ± S.D
Liscio da Serbo Toscano	18.75 ± 0.9a	3.89 ± 0.5a	41.23 ± 2.4a	5.48 ± 0.9ad	14.08 ± 2.6a	4.65 ± 0.3a
Rosso Pitigliano	17.21 ± 1.4a	8.25 ± 1.2b	28.26 ± 1.8b	14.24 ± 2.1b	27.45 ± 3.4b	4.23 ± 0.1a
Quarantino ec Valdarno	9.50 ± 0.8b	8.56 ± 1.4b	39.56 ± 2.9a	4.01 ± 0.7a	20.14 ± 2.9c	6.12 ± 0.5b
Fragola	7.45 ± 2.1c	7.81 ± 0.9b	20.14 ± 1.7c	19.12 ± 1.4c	18.01 ± 1.7c	5.06 ± 0.3a
Canestrino di Lucca	8.14 ± 0.8b	3.01 ± 1.2a	19.84 ± 1.9c	6.57 ± 1.2ad	17.12 ± 2.7c	5.89 ± 0.6ab
Costoluto Fiorentino	14.56 ± 1.7d	8.12 ± 1.7b	30.21 ± 2.1b	6.14 ± 0.9ad	9.87 ± 1.5d	4.15 ± 0.2a
Giallo di Pitigliano	8.23 ± 1.2b	9.16 ± 1.9b	18.54 ± 0.7c	8.14 ± 1.9d	10.48 ± 2.7d	4.58 ± 0.5a
Pisanello	19.54 ± 1.4a	2.28 ± 0.9a	16.02 ± 2.4c	3.89 ± 0.4a	10.47 ± 2.8d	6.21 ± 0.7b
Cuore di Bue (Commercial)	5.14 ± 1.2e	0.98 ± 0.8c	13.24 ± 1.8d	2.01 ± 0.2e	6.48 ± 0.7e	4.02 ± 0.1a

Onions (*Allium cepa* L.)	Quercetin (μg/g FW) ± S.D	Myricetin (μg/g FW) ± S.D	Kaempferol (μg/g FW) ± S.D	Peonidin-3-glucoside (μg/g FW) ± S.D	Petunidin-3-glucoside (μg/g FW) ± S.D
Maremma	29.59 ± 0.6a	30.59 ± 7.6a	216.58 ± 8.4a	9.12 ± 0.4a	5.12 ± 1.3a
Rossa Massese	583.77 ± 3.1b	583.77 ± 10.2b	849.14 ± 10.4b	21.64 ± 2.3b	15.24 ± 1.4b
Treschietto	23.54 ± 0.7c	23.54 ± 8.1a	305.68 ± 7.8c	9.57 ± 0.8a	4.91 ± 1.2a

(Continued)

TABLE 1 | Continued

Variety names	Antioxidants (mmol Fe^{2+}/100 g FW) ± S.D	Polyphenols (mg GAE/100g FW) ± S.D	Flavonoids (mg QeE/100g FW) ± S.D	Carotenoids (mg TCC/100g FW) ± S.D	Anthocyanins (mg CyE/100g FW) ± S.D
Rossa della Valtiberina	24.26 ± 0.9c	24.26 ± 9.6a	304.45 ± 8.7c	36.47 ± 1.9c	21.45 ± 1.3c
Rossa di Lucca	183.12 ± 2.1d	183.12 ± 8.2c	251.06 ± 8.1d	10.57 ± 1.1a	7.15 ± 1.9ad
Rossa Fiorentina	22.67 ± 1.2c	22.67 ± 6.4a	221.08 ± 8.9a	31.18 ± 0.7d	20.14 ± 2.1c
Tropea (Commercial 1)	19.86 ± 2.3c	19.86 ± 8.1a	66.68 ± 2.4e	10.96 ± 0.5a	10.24 ± 2.7d
Giarratana (Commercial 2)	20.45 ± 2.5c	20.14 ± 7.4a	87.78 ± 5.6f	/	/
Sweet cherries (*Prunus avium* L.)	**Chlorogenic Acid (μg/g FW) ± SD**	***p*-coumaric acid (μg/g FW) ± SD**	**(+)-Catechin (μg/g FW) ± SD**	**Rutin (μg/g FW) ± SD**	**Cyanidin-3-glucoside (μg/g FW) ± SD**
Carlotta	121.91 ± 0.6a	19.41 ± 02a	25.93 ± 1.9a	30.83 ± 1.3a	51.78 ± 1.8a
Benedetta	81.27 ± 0.4b	16.61 ± 0.3b	201.54 ± 1.4b	53.96 ± 3.4b	33.88 ± 1.5b
Morellona	324.59 ± 0.4c	30.05 ± 0.2c	72.54 ± 2.3c	38.54 ± 1.4c	74.82 ± 1.1c
Maggiola	94.32 ± 0.2d	12.43 ± 0.2d	45.99 ± 1.9d	34.46 ± 1.8c	35.28 ± 1.3b
Moscatella	239.25 ± 01e	10.13 ± 0.3e	34.86 ± 2.7e	27.93 ± 1.6a	33.54 ± 1.6b
Crognola	387.73 ± 07f	28.11 ± 0.3f	163.51 ± 1.4f	95.33 ± 2.1d	151.2 ± 1.2d
Durone (Commercial)	78.25 ± 0.4b	10.85 ± 0.1e	15.85 ± 1.1g	25.65 ± 2.1a	36.27 ± 1.7b

The table also reports the HPLC quantifications of specific secondary metabolites in tomatoes, onions and sweet cherries expressed as μg of component per gram of FW. Values are indicated with the relative standard deviation; different letters refer to statistically significant differences ($p < 0.05$).

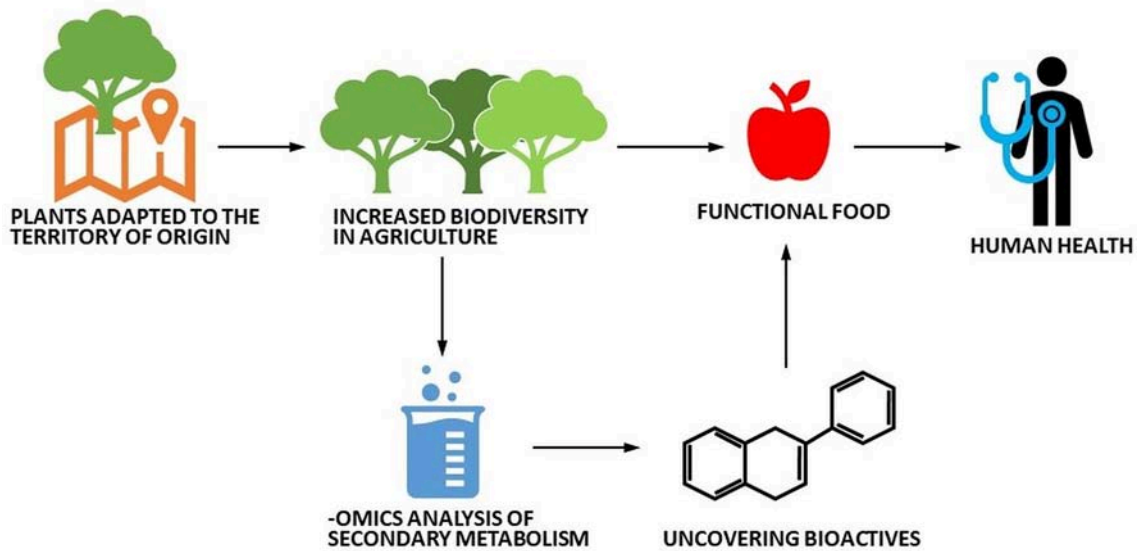

FIGURE 1 | Schematic cartoon depicting the potential valorization route of local ancient plant varieties.

bioactive molecules produced and to test their nutraceutical and health-promoting effects (**Figure 1**).

IS THE CULTIVATION OF LOCAL PLANTS AN ECONOMIC RESOURCE?

Assuming the importance to take functional molecules with the diet, there are contrasting aspects that sometimes make vain the scientific interest on these molecules. One example is the input for the local population to consume local products in opposition to mass-merchandised products, which is often more a cultural than an economic issue. The consumption of products from the local agriculture might be perceived as a return to "old-fashioned" agriculture that is considered less productive and less profitable. Therefore, the consumption of local products is often seen by consumers as the return to an "ancient" agriculture. This may be more a problem for younger

generations than older ones. The new generations have had different cultural backgrounds in which nutrition was not seen as a way to improve health, but essentially as a way to simply take on calories (Popkin et al., 2012). Not going too far into the basic rhetoric about fast-food, this different perception has greatly contributed to affect the trends in agriculture. The consumption of products from the local agriculture also necessarily requires the cultivation of local plants on a large scale. The problem then shifts from the consumer to the farmer and the question is: how ready are farmers to grow local plants instead of more traditional and therefore more commercial ones? This is a very serious question because it implies rethinking on the production yield of these plants. Obviously, no farmer prefers to earn less and it is therefore clear that locally adapted plants must be as productive in economic terms as more traditional ones (Reganold and Wachter, 2016). So far, few researches have been focused on the differences in biodiversity and on the economic benefits between unconventional and conventional crops.

Tuscany has been particularly active in recent years by promoting research and transfer activities in the agro-food sector (Berni et al., 2018a). Through a series of financially-supported projects, Tuscany has actively promoted and supported the research on and the cultivation of autochthonous species rich in functional molecules. An emblematic output of these projects is the manufacture of dark chocolate bars (Toscolata®) functionalized with Tuscan autochthonous food products (Cantini et al., 2018).

In particular, we hereby wish to mention two projects to which we have recently participated: the first concerns the genetic, qualitative and sustainability characterization of products and derivatives from autochthonous Tuscan horticultural crops (BASIQ project: http://www.valdimersegreen.com/basiq/). The second project is about the use of different cultivation practices of wheat to see how it might affect the content of bioactive molecules (INNOVACEREALI project: http://innovacereali.maidicolasovicille.it/).

THE CULTIVATION OF NATIVE PLANTS PRESERVES THE LOCAL AGROBIODIVERSITY

This Perspective paper on the importance of cultivating native species, albeit limited to a specific region of Central Italy, can be enriched by one further final consideration: the cultivation of native plants cannot only preserve the local agrobiodiversity (Berni et al., 2018a), but also contribute to the restoration of original regional habitats. Nowadays, the loss of habitat diversification is a major threat linked to the ever-growing industrialization, climatic changes and land-use. Future forecasts highlight the dramatic scenarios of habitat loss with the increasing use of wild lands for the massive cultivation of man-selected plants (Pereira et al., 2010). Landscape and plant biodiversity are directly linked: the loss of one involves the loss of the other. The cultivation of native crops can play an important role in the preservation of both, while more traditional agricultural practices, especially if intense, can have negative consequences because of the massive use of land resources to promote large-scale products. Therefore, a well-conceived agricultural management, based on the regional valorization of autochthonous species, has been shown to preserve biodiversity and local ecosystems (Tscharntke et al., 2005). Native plants are also useful to maintain the local soil microbiota and composition, thereby preserving an optimal interaction between plants and microorganisms. Lange and colleagues reported the importance of plant diversity in the microbial soil composition with indirect effects also on C storage of the whole ecosystem (Lange et al., 2015).

Based on these evidences, the use of native crops should not aim at massive agricultural production, but rather at maintaining and restoring habitats in regional "niches." From this perspective, the cultivation of local plants has both an economically- and ecologically-relevant impact.

AUTHOR CONTRIBUTIONS

RB, GG, and MR wrote the manuscript and prepared the figures. J-FH, CC, and GC revised the text.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01983/full#supplementary-material

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Unveiling Kiwifruit Metabolite and Protein Changes in the Course of Postharvest Cold Storage

*Anna Maria Salzano[1†], Giovanni Renzone[1†], Anatoly P. Sobolev[2†], Virginia Carbone[3†], Milena Petriccione[4†], Donatella Capitani[2], Monica Vitale[1,5], Gianfranco Novi[1], Nicola Zambrano[5,6], Maria Silvia Pasquariello[4], Luisa Mannina[2,7] and Andrea Scaloni[1]**

[1] Proteomics & Mass Spectrometry Laboratory, Istituto per il Sistema Produzione Animale In Ambiente Mediterraneo, National Research Council, Naples, Italy, [2] Magnetic Resonance Laboratory "Annalaura Segre", Institute of Chemical Methodologies, National Research Council, Monterotondo, Italy, [3] Institute of Food Sciences, National Research Council, Avellino, Italy, [4] Centro di Ricerca per Olivicoltura, Frutticoltura e Agrumicoltura, Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Caserta, Italy, [5] Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, Naples, Italy, [6] Ceinge Biotecnologie Avanzate S. C. a R. L., Naples, Italy, [7] Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza Università di Roma, Rome, Italy

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***Correspondence:**
Andrea Scaloni
andrea.scaloni@ispaam.cnr.it

† These authors have contributed equally to this work

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Actinidia deliciosa cv. Hayward fruit is renowned for its micro- and macronutrients, which vary in their levels during berry physiological development and postharvest processing. In this context, we have recently described metabolic pathways/molecular effectors in fruit outer endocarp characterizing the different stages of berry physiological maturation. Here, we report on the kiwifruit postharvest phase through an integrated approach consisting of pomological analysis combined with NMR/LC-UV/ESI-IT-MS^n- and 2D-DIGE/nanoLC-ESI-LIT-MS/MS-based proteometabolomic measurements. Kiwifruit samples stored under conventional, cold-based postharvest conditions not involving the use of dedicated chemicals were sampled at four stages (from fruit harvest to pre-commercialization) and analyzed in comparison for pomological features, and outer endocarp metabolite and protein content. About 42 metabolites were quantified, together with corresponding proteomic changes. Proteomics showed that proteins associated with disease/defense, energy, protein destination/storage, cell structure and metabolism functions were affected at precise fruit postharvest times, providing a justification to corresponding pomological/metabolite content characteristics. Bioinformatic analysis of variably represented proteins revealed a central network of interacting species, modulating metabolite level variations during postharvest fruit storage. Kiwifruit allergens were also quantified, demonstrating in some cases their highest levels at the fruit pre-commercialization stage. By lining up kiwifruit postharvest processing to a proteometabolomic depiction, this study integrates previous observations on metabolite and protein content in postharvest berries treated with specific chemical additives, and provides a reference framework for further studies on the optimization of fruit storage before its commercialization.

Keywords: kiwifruit, postharvest, metabolomics, proteomics, cold storage

Abbreviations: AA, ascorbic acid; C_2H_4, ethylene; FA, formic acid; MS, mass spectrometry; ROS, reactive oxygen species.

INTRODUCTION

Green-fleshed kiwifruit (*Actinidia deliciosa*) is an economically valuable crop that is highly appreciated for its unique flavor and abundant antioxidants, dietary fiber and amino acids (Tavarini et al., 2008). Generally, fruit is gathered at a physiological ripe stage that, however, does not match to a proper palatable condition, as a result of its firmness and sourness features. Fruit ripening to an edible condition is obtained through a proper berry postharvest management by temperature and chemical treatments, which modulate ethylene (C_2H_4) production and peak respiration. This phase is essential in kiwifruit industry, since it reduces important economic losses due to fruit senescence and optimizes pomological characteristics of the ripe material before its commercialization (Antunes and Sfakiotakis, 2002; Antunes, 2007; Richardson et al., 2011).

It is well known that fruit ripening is regulated by C_2H_4 in climacteric species, while it is virtually independent of this compound in non-climacteric counterparts. In the first ones, ripening parallels a change from a negative feedback regulation (system I) to a positive one (system II) of C_2H_4 production (McMurchie et al., 1972), through C_2H_4-induced expression of 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) and ACC oxidase (ACO) (Tucker, 1993; Lelièvre et al., 1997). C_2H_4 then binds to ethylene receptors and response factors regulating transcription of genes affecting the fruit ripening processes (Yin et al., 2010). Although kiwifruit is considered as a climacteric species, a number of ripening–associated changes occur before system II-dependent C_2H_4 is produced (Richardson et al., 2011; McAtee et al., 2015). In addition, general fruit ripening in cold storage occurs in the lack of measurable C_2H_4 (Hewett et al., 1999; Kim et al., 1999; Ilina et al., 2010). Indeed, a recent transcriptomic study has shown that kiwifruit has both C_2H_4-dependent and low temperature-modulated ripening mechanisms, which are distinct and autonomous (Asiche et al., 2018). These findings provided a foundation in evaluating the whole ripening process in this fruit.

A great challenge in postharvest management of kiwifruit is the occurrence of diseases caused by pathogenic *Botrytis cinerea*, *Botryosphaeria* sp., and *Phomopsis* sp. (Manning et al., 2016). Diseased fruit release infection-induced C_2H_4, which may in turn prompt ripening and feedback regulation of C_2H_4 production in healthy contiguous kiwifruits. In the context of postharvest management, the positive effect of treating kiwifruit by low temperatures (Günther et al., 2015; Park et al., 2015b; Minas et al., 2016), with exogenous ozone (Minas et al., 2012, 2014; Tanou et al., 2015), sodium nitroprusside (Tanou et al., 2015), 1-methylcyclopropene (Mworia et al., 2012; Park et al., 2015a; Thongkum et al., 2018), acetylsalicylic acid (Zhang et al., 2003), C_2H_4 (Hu et al., 2016; Minas et al., 2016; Park et al., 2016) and propylene (Asiche et al., 2016, 2018), or a combination of them (Minas et al., 2014, 2016; Tanou et al., 2015) was assessed, although the latter procedures have found a partial diffusion in kiwifruit industry due to their technology costs. Nevertheless, these studies provided important information on the effect of the application of these postharvest treatments on fruit firmness, respiration, acidity, shelf-life and decay, as well as on ethylene, soluble solid, reducing sugar, starch, antioxidant, and volatile compound content. In some cases, transcriptomic and/or proteomic investigations were also accomplished on the same fruit samples (Zhang et al., 2003; Minas et al., 2012, 2014, 2016; Mworia et al., 2012; Tanou et al., 2015; Asiche et al., 2016; Hu et al., 2016; Thongkum et al., 2018), describing differentially expressed genes and/or represented proteins in treated kiwifruits (with respect to control) that, in the latter case, were identified by MS-based procedures searching the genome of yellow-fleshed kiwifruit (*A. chinensis*) (Huang et al., 2013; Pilkington et al., 2018). Thus, very interesting proteomic studies were performed on berries subjected to treatment with specific chemical additives during postharvest storage at 0°C, which were then removed from these environments and allowed to ripe at 20°C before their proteomic evaluation (Minas et al., 2012, 2014, 2016; Tanou et al., 2015; Ainalidou et al., 2016). These differential investigations lacked information on fruits non-subjected to chemical treatments (control), and were accomplished on samples as obtained at the end of the postharvest process, missing data on the dynamics of protein quantitative changes during cold storage. In addition, they were based on gel silver staining procedures, which suffer of recognized drawbacks due to their limited accuracy in quantitative measurements; the latter were overcome with 2D-DIGE technology (Alban et al., 2003). Chromatography-based metabolomic studies on the same samples were also realized with the aim of evaluating corresponding metabolite concentration variations as result of different postharvest treatments (Günther et al., 2015; Ainalidou et al., 2016).

Proteomic and metabolomic studies reported above represented the interest of fruit industry in understanding active metabolic pathways and molecular processes associated with postharvest berry management. In particular, proteomics has widely been applied in the description of quantitative protein changes occurring during ripening and postharvest phases of apple (Costa et al., 2010; Zheng et al., 2013; Liu et al., 2016), mandarin (Yun et al., 2013), grape (Cai et al., 2014), pear (Pedreschi et al., 2008, 2009; Wang et al., 2017), banana (Li et al., 2015; Du et al., 2016; Xiao et al., 2018), papaya (Huerta-Ocampo et al., 2012; Nogueira et al., 2012), mango (Andrade et al., 2012), peach (Borsani et al., 2009; Lara et al., 2009; Nilo et al., 2010; Zhang et al., 2010, 2011, 2012; Giraldo et al., 2012; Jiang et al., 2014; Lauxmann et al., 2014; Wu et al., 2016; Tanou et al., 2017; Xi et al., 2017) and tomato (Sun et al., 2016). These studies paralleled those on berry physiological development (Palma et al., 2011; Molassiotis et al., 2013), and identified variably-represented metabolic pathways and protein effectors in these climacteric and non-climacteric fruits, rationalizing their grouping on a molecular basis, and linking these compounds to corresponding pomological characteristics, increased respiration rate and C_2H_4 biosynthesis.

Taking advantage of our previous experience in a NMR/LC-UV/ESI-IT-MS^n- and 2D-DIGE/nanoLC-ESI-LIT-MS/MS-based

description of kiwifruit physiological development (Salzano et al., 2018), we used the same procedure to fill the above-mentioned gaps and to describe on a time-course basis proteometabolomic changes in harvest kiwifruit stored at a low temperature, in the absence of chemical additives. Bioinformatic elaboration of resulting data suggested metabolic pathways and molecular processes/interactions affected during different postharvest moments, providing a rationale to corresponding pomological characteristics, and integrating previous proteomic observations on postharvest kiwifruit treated with specific chemicals (Minas et al., 2012, 2016; Tanou et al., 2015; Ainalidou et al., 2016).

MATERIALS AND METHODS

Fruit Sampling and Pomological Measurements

Kiwifruit (*A. deliciosa* cv. Hayward) samples were harvested from a commercial orchard located in Francolise (Caserta, Italy). Fruits were randomly sampled from 10 selected vines at the commercial ripening stage 82 of the BBCH scale (Salinero et al., 2009); they were selected for uniformity and the absence of physical defects/decay. Healthy fruits were stored in a controlled chamber at 4°C, with 85% relative humidity, and removed after 0 (T0), 30 (T1), 60 (T2), and 90 (T3) days of cold storage. At each post-harvest stage, 60 selected fruits were sampled and divided in 3 biological replicates, which were quickly used for the measurement of pomological and qualitative traits (see **Supplementary Material** for details). They were also quickly peeled and their outer pericarp (without inner pericarp containing locules and seeds) was sampled, rapidly cut, frozen in liquid N_2 and stored to −80°C, until used for further metabolomic and proteomic analyses.

NMR Analysis of Metabolites

Extraction of metabolites from outer pericarp samples (about 2 g) taken at different postharvest stages was carried out as previously described (Salzano et al., 2018). Briefly, fruit powder samples were treated with a methanol/chloroform mixture generating corresponding hydroalcoholic and organic extracts (see **Supplementary Material** for details), which were then dried and stored at −20°C. Hydroalcoholic extracts were solved in 0.7 ml phosphate buffer in D_2O containing 2 mM 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP) (used as internal standard). Organic counterparts were solved in 0.7 ml of 2:1 v/v $CDCl_3/CD_3OD$. NMR spectra were recorded at 27°C on a Bruker AVANCE 600 instrument operating under experimental conditions described previously (Salzano et al., 2018) and in **Supplementary Material**. Assignment of ^{1}H spectra of aqueous and organic extracts was achieved as previously reported (Salzano et al., 2018). Metabolite concentrations were derived from the integral values of the corresponding resonances in ^{1}H NMR spectra (**Supplementary Material**). NMR data were subjected to PCA, which was performed using Statistica software for Windows (Statsoft, United States).

LC-UV Analysis of Metabolites Combined With ESI-IT-MS^n

Polyphenolic compounds from kiwifruit samples taken at T0–T3 were extracted as reported previously (Salzano et al., 2018) and in **Supplementary Material**. They were resolved onto C18 Sep-Pak cartridges (Waters, Milford, MA, United States) and analyzed by HPLC-UV with a HP 1110 instrument (Agilent, Palo Alto, CA, United States), monitoring absorbance at 280 nm; column and chromatographic conditions were described previously (Salzano et al., 2018). Identification of phenolic compounds present in the different HPLC fractions was carried out by ESI-IT-MS^n analysis using a Finnigan LCQ DECA XP Max ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, United States), operating as previously described (Salzano et al., 2018). For quantitative analysis, a standard curve for each polyphenol derivative was prepared by using standard compounds over a concentration range of 0.25–5 μg/μl, by means of six different concentration levels and duplicate injections at each level. All samples had three replications and each replicate was measured twice. Results were expressed as mg/kg of fresh weight (FW). Statistical analysis (one-way analysis of variance and multiple mean comparisons Tukey's HSD) of the concentration of individual/total polyphenolic compounds was performed using SPSS Software Package, version 20.0 (SPSS Inc., Chicago, IL, United States).

Protein Extraction and 2D-DIGE Analysis

Kiwifruit outer pericarp samples taken at T0–T3 were extracted for proteins using a modified version of the phenol/SDS-based method (Corrado et al., 2012; D'Ambrosio et al., 2013; Salzano et al., 2018), which were then quantified as reported in **Supplementary Material**. Dried kiwifruit proteins from 3 biological replicates of each postharvest stage (50 μg) were independently solved in 2 M thiourea, 7 M urea and 4% w/v CHAPS, and labeled with 400 pmol of Cy2-, Cy3- or Cy5-dyes (GE Healthcare) using the dye-swapping strategy (Tamburino et al., 2017). Proteins in each biological replicate were then resolved and quantified according to the 2D-DIGE procedure (Alban et al., 2003; Salzano et al., 2018) (see **Supplementary Material** for details). A parallel preparative 2-DE experiment was also performed using 500 μg of unlabeled proteins, which were stained with Sypro Ruby (Thermo Fisher). After spot matching with the master gel from the 2D-DIGE experiment, spots in 2-DE corresponding to those showing quantitative abundance changes in 2D-DIGE were picked with an Ettan spot robotic picker (GE Healthcare), and analyzed for protein identification.

Protein Identification by Mass Spectrometry Analysis

2-DE spots were excised, *S*-alkylated with iodoacetamide, and digested with trypsin (Guarino et al., 2007; Scippa et al., 2010; Tamburino et al., 2017); resulting peptides were resolved on an Easy C18 column (100 mm × 0.075 mm, 3 μm) (Thermo Fisher) and analyzed under a CID-MS/MS data-dependent product ion scanning procedure with a LTQ XL mass spectrometer (Thermo Fisher), as previously reported (Scippa et al., 2010; Corrado et al., 2012; Salzano et al., 2018). Raw MS and MS/MS

data were searched using MASCOT software (v. 2.2.06, Matrix Science, United Kingdom) against the *A. chinensis* protein sequence database (39,040 entries) (Huang et al., 2013), using previously reported (Scippa et al., 2010; D'Ambrosio et al., 2013; Salzano et al., 2018) and default MASCOT parameters. Protein candidates having at least 2 sequenced peptides with an individual peptide ion score >30 and a peptide expectation value <0.05 (consistent with a confidence level >95%) were considered surely identified. Protein assignment was always associated with manual verification. Identified proteins were further filtered according to an EMPAI ratio criterion (EMPAI 1st/EMPAI 2nd > 2). Proteomic data have been deposited to the ProteomeXchange Consortium (Vizcaíno et al., 2016) via the PRIDE partner repository with the dataset identifier PXD011949.

Bioinformatics

Identified proteins were subjected to BLAST sequence homology search against the *Arabidopsis thaliana* protein sequence database TAIR 10 from The Arabidopsis Information Resource repository[1]. Functional categorization of differentially represented proteins (DRPs) was obtained using Mercator pipeline[2] for automated sequence annotation, selecting TAIR 10, SwissProt-UniProtKB plant proteins, KOG clusters and InterPro scan, with a cut-off value of 80. DRPs were assigned to Bevan functional classes (Bevan et al., 1998) using the classification from Mercator. Hierarchical clustering analysis of abundance ratio of DRPs at T0–T3 was performed using Genesis 1.8.1 platform (Sturn et al., 2002). Person's correlation as distance and average linkage clustering were chosen as parameters. Protein interaction networks were obtained with STRING[3] using the *A. thaliana* database.

RESULTS AND DISCUSSION

Based on the above-mentioned considerations regarding the recognition of independent C_2H_4-dependent and low temperature-modulated ripening mechanisms in kiwifruit (Asiche et al., 2018), the widest use of solely temperature-centered storage procedures in the corresponding industry to slow-down the ripening process and extend fruit life (Pranamornkith et al., 2012; Asiche et al., 2017), and the occurrence of previous proteomic studies already describing the effect of combined treatments using both low temperature and chemical additives on berries, which were finally removed from cold storage and allowed to ripe at 20°C (Minas et al., 2012, 2014, 2016; Tanou et al., 2015; Ainalidou et al., 2016), we focused our attention on kiwifruit whose postharvest management was limited to the application of a low temperature (4°C). This temperature value was chosen to avoid any risk of fruit freezing. Cold stored fruit samples were then taken at different times of postharvest treatment (T0–T3) and were subjected to analysis of pomological/compositional characteristics and proteometabolomic composition.

[1]www.arabidopsis.org

[2]http://mapman.gabipd.org/web/guest/app/mercator

[3]http://string-db.org

Results from the analysis of kiwifruit pomological/compositional features at T0–T3 are reported in **Table 1**. These data demonstrated that fruit weight and firmness progressively decreased during cold storage. In particular, weight loss showed a reduction of 1.8, 3.8, and 5.7% after 30, 60, and 90 days of storage, respectively. Our results were in good agreement with previous observations on the same cultivar regarding the effect of cold conditions on fruit weight and firmness (Chiaramonti and Barboni, 2010; Lim et al., 2016; Asiche et al., 2017). On the other hand, we observed a progressive increase of soluble solid content (SSC) (**Table 1**), which reached a value 14.3 °Brix after 90 days. Also in this case, our results were in good agreement with previous investigations (Chiaramonti and Barboni, 2010; Mworia et al., 2012; Asiche et al., 2017), which demonstrated an increase of the SSC value during various regimes of cold storage. Finally, no significant differences were observed in Chroma, Hue angle and protein content values in the range T0–T3 (**Table 1**), confirming previous observations (Ghasemnezhad et al., 2013). Regarding C_2H_4 production in kiwifruit, data reported in **Table 1** show that gas emission was appreciable during the whole period of fruit cold storage. In particular, C_2H_4 was detected even at T0–T1, and followed a production curve resembling that of other climacteric fruits, with low levels in the early storage (T1 and T2), followed by a sharp increase at T3 (climacteric peak). Also in this case, measured data were coherent with previous determinations on kiwifruit samples subjected to cold storage conditions (Chiaramonti and Barboni, 2010; Lim et al., 2016; Asiche et al., 2017).

Metabolomics

We previously used NMR to evaluate metabolite concentration changes during kiwifruit physiological development (Salzano et al., 2018). The same approach was used here to quantify metabolites in hydroalcoholic (free amino acids, organic acids, sugars and others) and organic (fatty acids, phospholipids, sterols, and galactolipids) extracts at different postharvest stages. Almost all metabolites observed in the same extracts during fruit development (Salzano et al., 2018) were also present at T0–T3; unique exceptions were Gln, Asn, Phe, and shikimic acid, which were not detected in this study. Metabolite levels at T0–T3 stages are reported in **Supplementary Figures S1, S2**. Quantitative data were submitted to PCA (**Figure 1**), which provided a general view of metabolite changes associated with postharvest cold storage of kiwifruit. In particular, the first principal component accounted for 37.4% of total variability (**Figure 1A**) and it was strictly associated with postharvest, as shown by grouping of fruit samples according to the postharvest stage along PC1 axis. As observed by the plot of loadings, variables responsible for this trend were sugars (SUCR, AGLC, BGLC, and BFRUPY), AA, Thr, Ile, Val, and DUFA (see the legend to **Figure 1** for abbreviations), which were present at a higher level during the final stages of postharvest (**Supplementary Figures S1, S2**); conversely, Ala and DG showed the highest level at T0. Data on SUCR, AGLC, BGLC, BFRUPY, and AA were coherent with previous determinations on total sugars and AA in postharvest kiwifruit stored at low temperatures (Chiaramonti and Barboni, 2010; Ghasemnezhad et al., 2013). Worth mentioning is the fact that T1 samples

TABLE 1 | Pomological and qualitative traits of kiwifruits taken at postharvest stages T0, T1, T2, and T3 following cold storage at 4°C.

Time storage (days)	Weight loss (%)	Firmness (N)	SSC (°Brix)	Chroma	Hue angle	TP (mg/g FW)	Ethylene concentration (ppm)
0	–	71.2 ± 2.1^{d}	7.4 ± 0.3^{a}	50.8 ± 1.1^{a}	109.2 ± 4.6^{a}	0.59 ± 0.08^{a}	0.02 ± 0.01^{a}
30	1.8 ± 0.4^{a}	45.5 ± 1.5^{c}	10.9 ± 0.8^{b}	51.2 ± 1.5^{a}	110.7 ± 3.3^{a}	0.53 ± 0.09^{a}	0.07 ± 0.02^{b}
60	3.8 ± 0.5^{b}	32.5 ± 1.7^{b}	12.5 ± 0.4^{c}	50.3 ± 1.2^{a}	112.8 ± 3.1^{a}	0.58 ± 0.05^{a}	0.09 ± 0.01^{b}
90	5.7 ± 0.6^{c}	22.3 ± 1.9^{a}	14.3 ± 0.5^{d}	50.7 ± 1.4^{a}	115.3 ± 2.7^{a}	0.52 ± 0.06^{a}	0.39 ± 0.03^{c}

Reported are data on fruit weight loss (%), fruit firmness (N), total soluble solid content (SSC), Chroma, Hue angle, total protein content (TP) and ethylene emission during storage. Reported are mean values ± SD; mean values followed by the same letter do not differ significantly at P = 0.05 (Tukey's Test).

were characterized by distinct PC2 levels in the PCA score plot, due to a particular trend of few metabolites reaching the highest (SFA, MI, and Trp) or the lowest (Asp, Glu, and S7) level at T1. As expected, the trend showed by single metabolites (**Supplementary Figures S1, S2**) confirmed the results of PCA. In detail, AGLC, BGLC, and BFRUPY showed a relevant increase only during the first stage, being relatively constant in the later stages, whereas SUCR showed a constant increment during the whole postharvest period. The level of LA, QA, and CA remained unchanged in the T0–T3 range, whereas a slight increment of AA concentration was observed at T3 stage. Regarding the organic extracts, a constant increment and decrement of DUFA and DG was observed, respectively, whereas the other metabolites remained practically unchanged (**Supplementary Figure S2**).

A quantitative measurement of polyphenolics present in kiwifruit at T0–T3 was achieved through their determination and characterization by HPLC-UV and ESI–ITMS^{n} analysis, respectively. Thus, 9 compounds were identified through the recognition of their diagnostic MS and MS^{n} signals; the classes of phenolic metabolites detected in this study perfectly matched those already described in kiwifruit undergoing physiological development (Salzano et al., 2018). In particular, 4 principal groups were detected, namely phenolic acids (caffeic acid hexoside, *p*-coumaric acid hexoside, ferulic acid hexoside, 2-caffeoyl-3,4-dihydroxybutanoic acid or 4-caffeoyl-2,3-dihydroxybutanoic acid), procyanidins (procyanidins B2 and trimer), flavones (apigenin-*C*-deoxyhexoside), and flavonols (quercetin-3-*O*-glucoside and quercetin-3-*O*-rhamnoside). Total and individual polyphenol amounts in kiwifruit pulp extracts at T0–T3 are shown in **Figure 2**. At T1, kiwifruits showed a slight increase in total phenol content (about 15%, from 8.29 ± 0.45 mg/kg of FW at T0 to 9.54 ± 0.34 mg/kg of FW at T1), which was followed by a slight decrease at T2 and T3 (7.87 ± 0.34 mg/kg of FW and 8.04 ± 0.35 mg/kg of FW, respectively). At the harvest stage (T0), procyanidins were the most predominant molecular class (3.68 ± 0.04 mg/kg of FW), accounting for about 44% of total phenolic content, followed by flavonols, whose total content was 2.09 ± 0.01 mg/kg of FW. Phenolic acids and flavones were found in minor amounts (1.51 ± 0.05 mg/kg of FW and 1.02 ± 0.21 mg/kg of FW, respectively) (**Figure 2**). This trend remained throughout the whole cold storage period (T1, T2, and T3). In agreement with data on total phenol content, above-mentioned polyphenolic classes showed a slight increase after 30 days of cold storage (phenolic acids about +4.6%, procyanidins about +17%, flavones about +32% and flavonols about +9.6%; increase from T0 to T1), which was followed by a certain decrease in the following time (phenolic acids about −34%, flavones about −42% and flavonols about −13,5%; decrease from T1 to T3). Unique exceptions were procyanidins, whose values remained almost constant.

In the whole, this study provided original information on quantitative levels of amino acids, sugars, organic acids, saturated/unsaturated fatty acids, phospholipids, sterols, galactolipids, phenolic acids, procyanidins, flavones, flavonols, and other metabolites in postharvest kiwifruit subjected to cold storage. These results were coherent with previous results on total reducing sugars and AA in postharvest fruit stored under similar experimental conditions (Chiaramonti and Barboni, 2010; Ghasemnezhad et al., 2013; Hu et al., 2016). Eventual discrepancies observed for AA and phenolic compounds may depend on the specific cultivar investigated and/or the non-specific assays authors used, with respect to our compound-oriented determination (Ghasemnezhad et al., 2013; Park et al., 2015b; Lim et al., 2016). In the whole, metabolite changes observed during kiwifruit storage at 4°C were in good agreement with molecular function, as related to fruit physiology (Richardson et al., 2011; Huang et al., 2013). Individual metabolites will be discussed below, together with proteins, corresponding metabolic pathways and related physiological processes.

Proteomics

In order to identify molecular effectors/metabolic pathways deregulated as result of kiwifruit cold storage, protein extracts from fruit samples taken at T0 (reference) and T1–T3 were comparatively evaluated by 2D-DIGE (**Supplementary Table S1**). Corresponding proteomic maps showed the presence of almost 4100 spots occurring within M*r* and p*I* ranges of 10–100 kDa and 3–10, respectively (**Supplementary Figure S3**). These spots were further filtered for abundance fold changes ≥1.5 or ≤−1.5 (T1–T3 vs. T0) and *p*-value ≤ 0.05 (Student's paired *t*-test), ascertaining 311 differentially represented ones (DRSs) associated with cold storage (**Supplementary Figure S3**). Practically, most spots differentially represented at T1–T3 were already present at T0, suggesting that molecular processes modified in the course of kiwifruit cold storage were already active in the corresponding initial phase. Venn diagram showed unique and shared DRSs at the different postharvest stages (**Figure 3**). Hierarchical clustering of spot abundance ratios highlighted that most significant quantitative variations occurred at T3, which corresponded to apex in C_2H_4 emission (**Supplementary Figure S4** and **Table 1**). After running of a

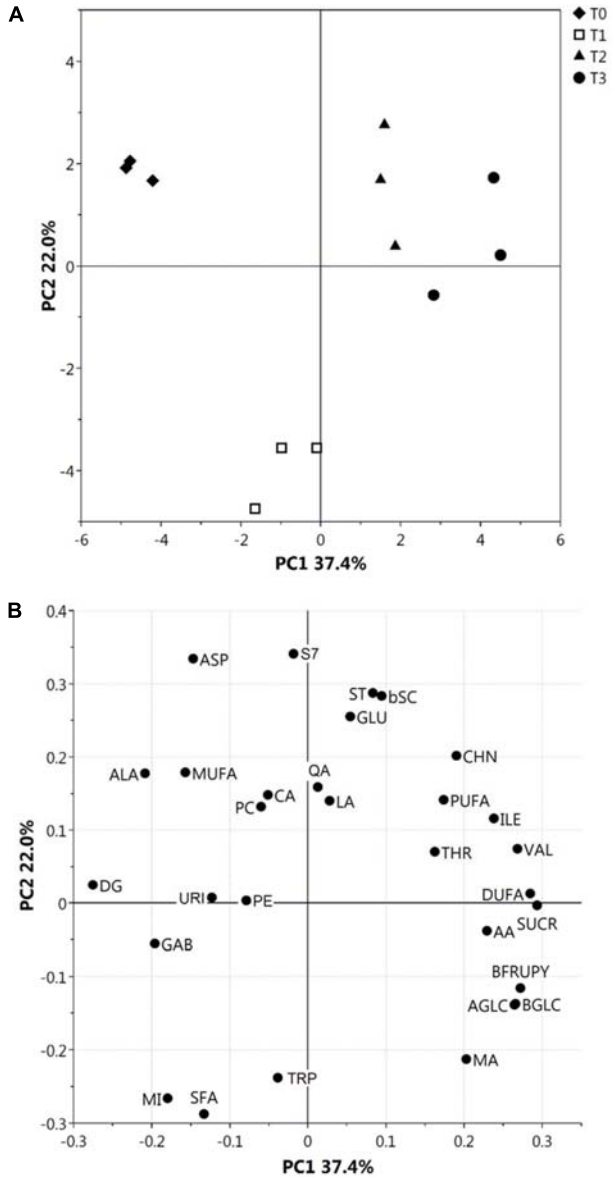

FIGURE 1 | PCA score **(A)** and loading **(B)** plots of metabolite profiles (30 metabolites/metabolite classes) obtained from NMR data related to hydroalcoholic and organic extracts of kiwifruit at different postharvest stages (T0–T3). Results associated with malic acid (MA), citric acid (CA), ascorbic acid (AA), lactic acid (LA), quinic acid (QA), α- and β-glucose (AGLC and BGLC), sucrose (SUCR), β-fructopyranose (BFRUPY), Ala, Thr, Glu, Asp, Val, Ile, Trp and γ-aminobutyric acid (GAB), choline (CHN), uridine (URI), myo-inositol (MI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), digalactosyl-diacylglycerol (DG), stigmast-7-en-3β-ol (S7), stigmasterol (ST), poly-unsaturated fatty acids (PUFA), di-unsaturated fatty acids (DUFA), mono-unsaturated fatty acids (MUFA), saturated fatty acids (SFA), and β-sitosterol plus campesterol (bSC) are shown.

preparative 2-DE, 2D-DIGE software modules allowed matching the corresponding image with that of the analytical counterpart. In 299 cases, MS analysis of the tryptic digest from DRSs led to protein identification (**Supplementary Table S2**). Since some DRSs showed the occurrence of two comigrating proteins within the same spot, results were further filtered as reported in the experimental section to exclude: (i) spots containing molecular species with no coherent quantitative levels in 2D-DIGE; (ii) comigrating proteins having a unique quantitative determination as deduced by 2D-DIGE (**Supplementary Table S3**). This conservative approach limited useful proteomic data to 235 spots, corresponding to 328 non-redundant sequence entries in the kiwifruit genome (Huang et al., 2013). As expected, although significant in number, differentially represented proteins ascertained during cold storage of harvest kiwifruit were lower in number than counterparts in fruit during physiological development (Salzano et al., 2018).

Resultant DRPs were indexed based on Bevan functional cataloging (Bevan et al., 1998) through an initial functional assignment obtained from Mercator software analysis. This analysis automatically attributed a function to all proteins, except for 17 that had not been assigned to any known ontology or function. According to their identity (and incidence > 5% in DRSs), DRPs were related to the Bevan functional category of: (i) disease/defense (38%); (ii) energy (24%); (iii) protein destination and storage (14%); (iv) metabolism (8%); (v) cell structure (4%), underlining the prominent molecular mechanisms modified during kiwifruit cold storage (**Figure 4A**). Distribution of functional groups of DRPs at T1–T3 is reported in **Figures 4B,C**. Among down-represented proteins, most relevant groups were disease/defense (T1, T2, and T3) and energy (T3). Over-represented counterparts were generally more abundant at T3 than at previous stages, most of them belonging to energy, protein destination and storage, disease/defense and metabolism categories. A heat-map picture originated from hierarchical clustering of quantity ratios of DRPs for each functional group during kiwifruit cold storage is shown in **Supplementary Figure S5**; it describes the dynamic expression profile of the various proteins among different storage stages. In subsequent paragraphs dedicated to the most significant protein functional categories, this figure is widely discussed together with corresponding DRPs and metabolites.

A bioinformatic prediction of protein–protein interactions among *A. thaliana* homologs of here-ascertained kiwifruit DRPs revealed a highly-ramified network bridging 98 sequence entries (**Figure 5** and **Supplementary Table S4**), which corresponded to four main functional groups partially structured into three subnetworks. The assemblies of energy (39), protein destination and storage (19), stress/defense (15) and metabolism (12) comprised the highest number of entries. This finding underlined the occurrence of a functional assembly linking together various deregulated metabolic pathways and molecules involved in physiological response of kiwifruit to cold storage. An analysis of the heat-map representation (**Supplementary Figure S5**) of the interacting proteins within the main network showed a general common dynamic trend over time, mainly concentrated at T3. Overall, proteomic results pointed out that various energetic, metabolic and structural processes, together with plant defensive/stress-responsive mechanisms, are temporarily regulated during prolonged kiwifruit cold storage to elicit specific

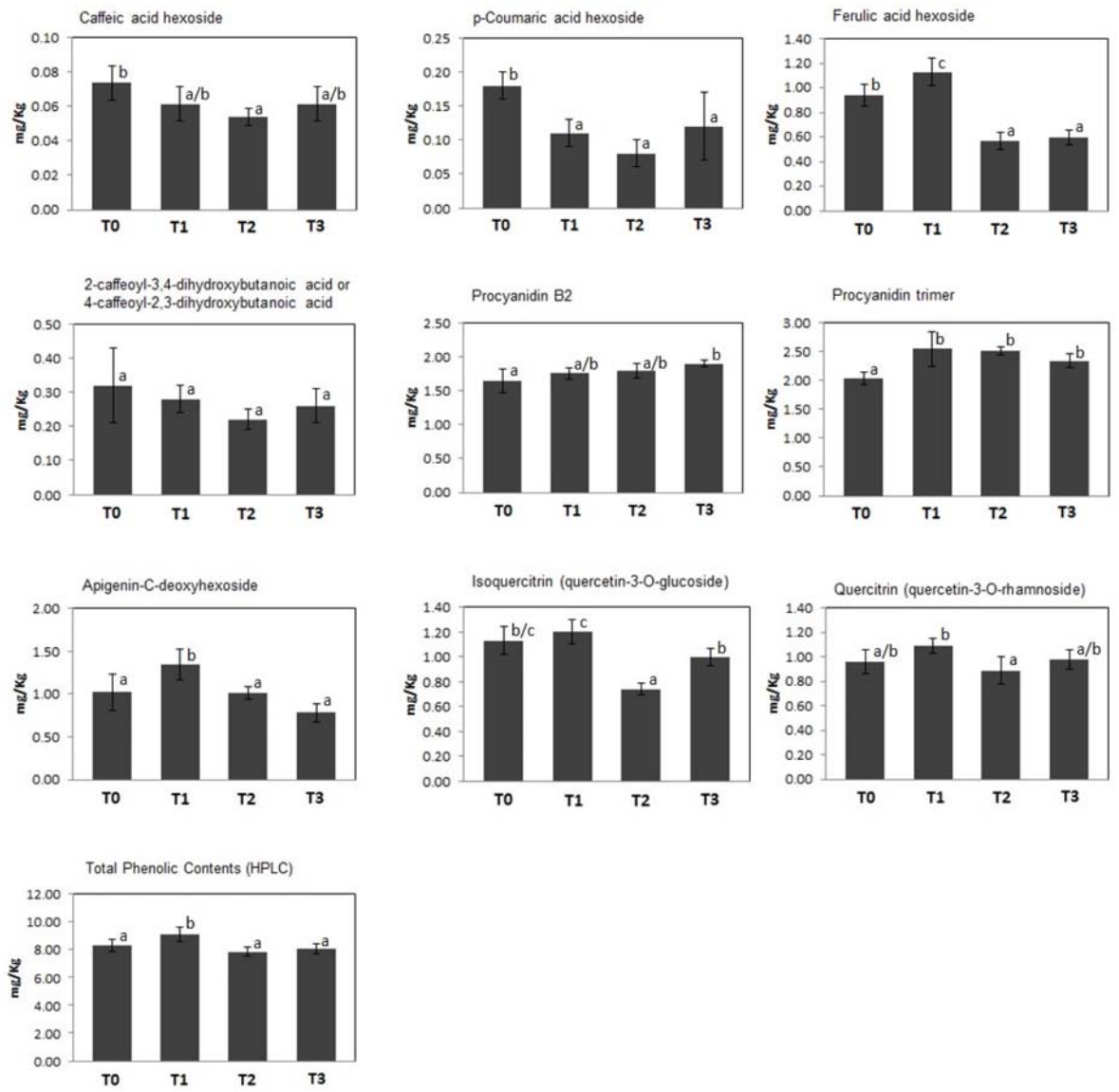

FIGURE 2 | Concentration (mg/Kg FW) of individual and total polyphenols identified in kiwifruit extracts at different postharvest stages (T0–T3). Histograms represent the mean ± SD (n = 6; 3 biological replicates measured twice). Different letters on the bars indicate significant differences of polyphenol concentrations within the postharvest stage (Tukey's test, $p \leq 0.05$).

physiological mechanisms associated with this postharvest management.

Stress/Defense Proteins

Recently, we have demonstrated that kiwifruit physiological maturation is associated with a general increase of defensive proteins, which are synthesized to let the fruit adapt and resist to possible adverse abiotic/biotic stresses (Salzano et al., 2018). These processes are often related to augmented levels of ROS (Bachi et al., 2013) as well as of various antioxidant enzymes, which are induced in berry cells to neutralize the toxic activity of above-mentioned noxious compounds (Rinalducci et al., 2008). Cold storage of harvest kiwifruit induced an opposite quantitative trend for superoxide dismutase [Cu-Zn] (CSD2) and [Mn-Fe] (FSD2), glutathione *S*-transferase (GSTL3), putative glutathione *S*-transferase (GSTF7), abscisic stress ripening protein (AT1G70810), catalase 3 (CAT), quinone oxidoreductase wrbA, dehydrin 2 (COR47) and Cbs domain protein (CBSX3), suggesting that the prominent role of redox stress processes underpin by these proteins during fruit physiological maturation (Jimenez et al., 2002; Palma et al., 2011; Molassiotis et al., 2013) may be reduced in the postharvest cold storage phase. In fact, these components become progressively down-represented at T2 and T3 (**Supplementary Table S3** and **Supplementary Figures S5A,F**). Most of the above-reported proteins are ROS-scavenging species participating in the direct response of plant

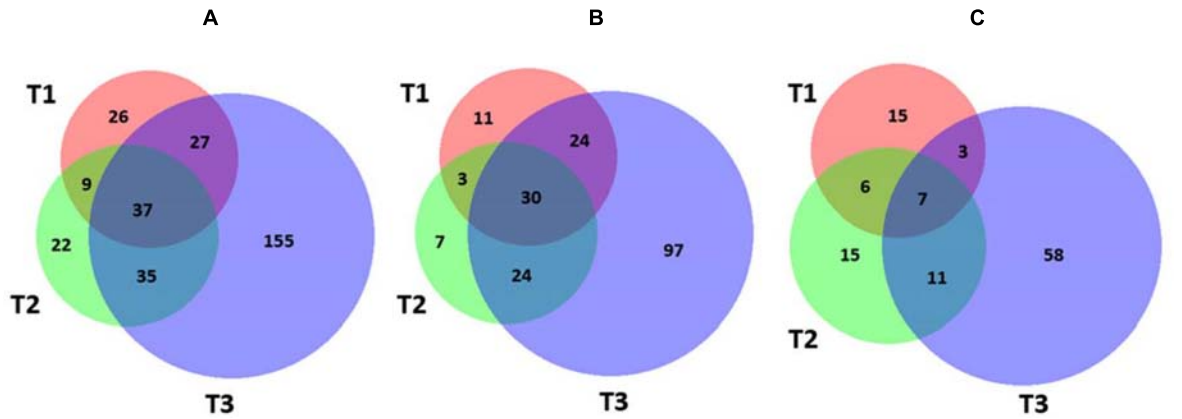

FIGURE 3 | Venn diagram showing differentially represented spots among the different kiwifruit postharvest stages (T1, T2, and T3 in comparison with T0). Diagrams refer to all DRSs **(A)**, those over-represented **(B)**, and down-represented **(C)**, respectively.

cells to these xenobiotics and/or their derivatives (Carey et al., 2007; Kim et al., 2012; Bachi et al., 2013; Liu et al., 2017), enzymes catalyzing the conjugation of these toxic molecules to glutathione (GSH) (Bachi et al., 2013), or effectors regulating the activation of the thioredoxin system controlling H_2O_2 levels (Yoo et al., 2011). On the other hand, a coherent opposite behavior with respect to kiwifruit physiological maturation was also observed for monodehydroascorbate reductase (MDAR1), which showed an augmented representation at T3 (**Supplementary Table S3** and **Supplementary Figure S5A**). Since MDAR1 catalyzes the recycling conversion of monodehydroascorbate into AA (Noctor and Foyer, 1998), its increased levels well correlated with the rise of AA concentration measured during cold storage of harvest kiwifruit (**Supplementary Figure S1**). In this context, worth mentioning is the fact that AA, together with GSH, is an essential component of a dedicated molecular machinery detoxifying H_2O_2 and other peroxides (Noctor and Foyer, 1998), and has been reported to affect Met/C_2H_4 metabolism as well (see below) (Fercha et al., 2013). A similar quantitative profile was also verified for over-representation of cytosolic aldehyde dehydrogenase ALDH7B4 (AT1G54100) (**Supplementary Table S3** and **Supplementary Figure S5G**), which is induced in plants as result of various abiotic stress conditions (Stiti et al., 2011). Measured level of specific proteins mentioned above was in good agreement with that observed in kiwifruit exposed to cold storage under different experimental conditions (Minas et al., 2016), in other climacteric fruits during ripening or cold storage (Nilo et al., 2010; Zhang et al., 2011; Giraldo et al., 2012; Zheng et al., 2013; Li et al., 2015; Du et al., 2016; Wu et al., 2016; Tanou et al., 2017) and in plants exposed to cold stress (Janmohammadi et al., 2015; Die et al., 2016, 2017). Some of the above-mentioned antioxidant proteins occurred in the interaction network shown in **Figure 5**. In the whole, our results suggest that the observed reduced representation of various antioxidant proteins in kiwifruit during cold storage may be associated with compensative regulation mechanisms paralleling the concomitant increased concentration therein of AA and constant representation of polyphenolics (**Figures 1**, **2**), in a condition where redox stress does not seem having a prominent function or, more probably, is balanced by these antioxidant metabolites.

A more variable quantitative trend was observed for proteins involved in defense response to biotic stresses. In fact, while α-toxin, chitinase (HCHIB) and NtPRp27-like protein showed reduced levels at T1–T3, lectin exhibited an opposite condition, and abundant kiwellin, thaumatin-like protein isoforms (OSM34) and major latex-like protein (Bet VI class) presented a mixed profile, also associated with a decreased representation of corresponding proteolytic fragments (**Supplementary Table S3** and **Supplementary Figure S5A**). By eliciting specific activities on the functional integrity of the cell wall (Mauch and Staehelin, 1989; Martínez-Esteso et al., 2009), membrane bilayer (Martínez-Esteso et al., 2009; Draffehn et al., 2013; Offermann et al., 2015) and the ribosomal machinery of exogenous hosts (Hartley and Lord, 2004), most of these proteins protect the fruit from microbial/fungal pathogens, and thus have been grouped as pathogenesis-related proteins (van Loon et al., 2006). These components showed similar quantitative trends in apple, banana, mango, and peach during physiological maturation (Zhang et al., 2011; Andrade et al., 2012; Zheng et al., 2013; Du et al., 2016) or in the same fruits and kiwifruit following cold storage under distinct postharvest conditions (Nilo et al., 2010; Giraldo et al., 2012; Li et al., 2015; Minas et al., 2016). These proteins can induce strong allergic reactions in humans and thus were identified among the most effective allergens in kiwifruit (**Supplementary Table S5**) (Bulley, 2016). Our findings suggest that cold storage of kiwifruit may influence quantitative representation of specific allergens, with important consequences at food consumption level. Thus, they have to be further considered in the development of dedicated studies on postharvest management of kiwifruit.

Protein Synthesis, Destination and Storage

The list of proteins involved in abiotic/biotic stress responses showing a differential representation in harvest kiwifruit after cold storage also included various chaperones, heat shock

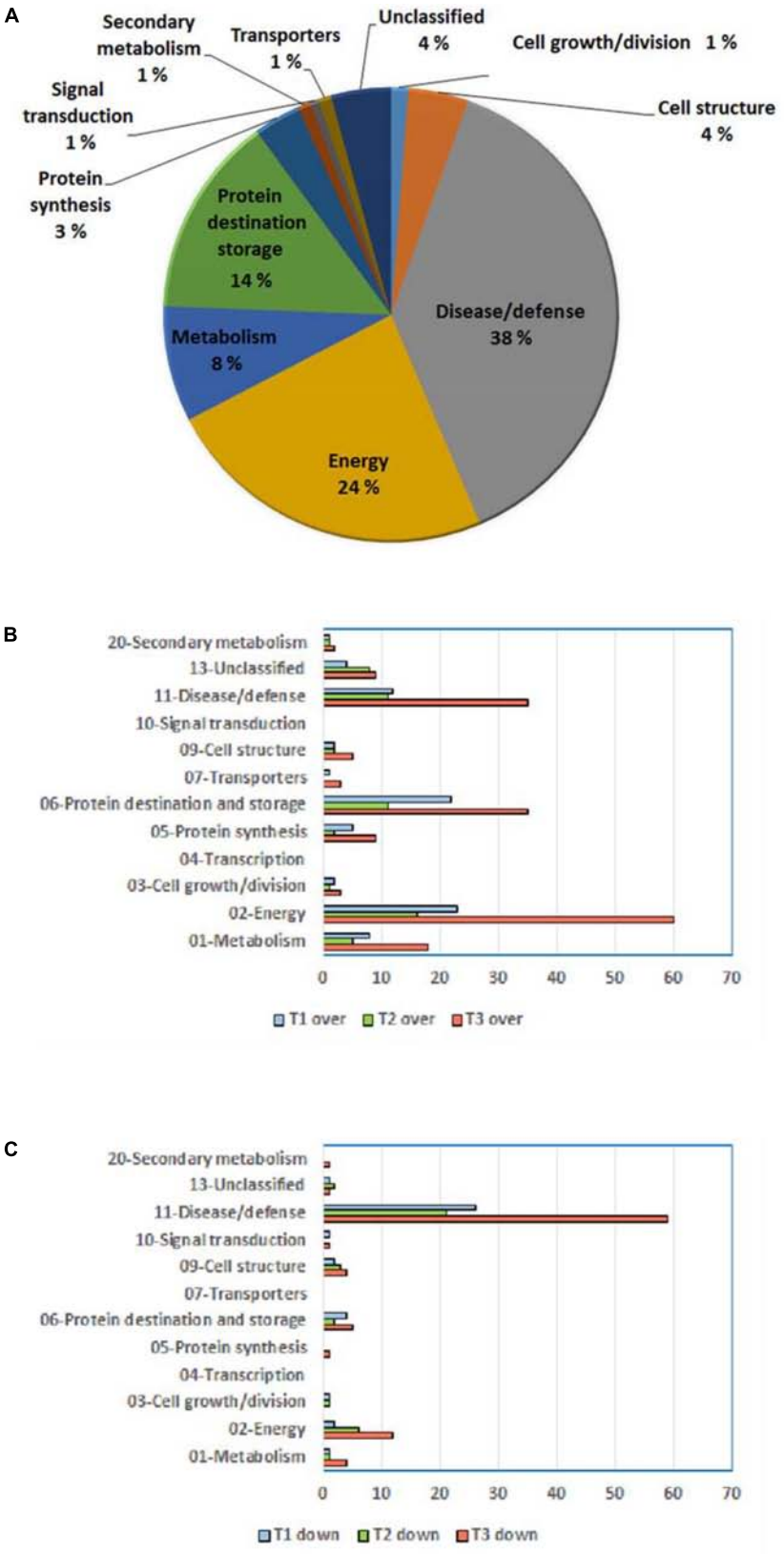

FIGURE 4 | General information on differentially represented proteins (T1, T2, and T3 vs. T0). **(A)** Distribution of DRPs according to Bevan classification (Bevan et al., 1998). Distribution of functional categories of DRPs during different development stages for over-represented **(B)** and down-represented **(C)** proteins.

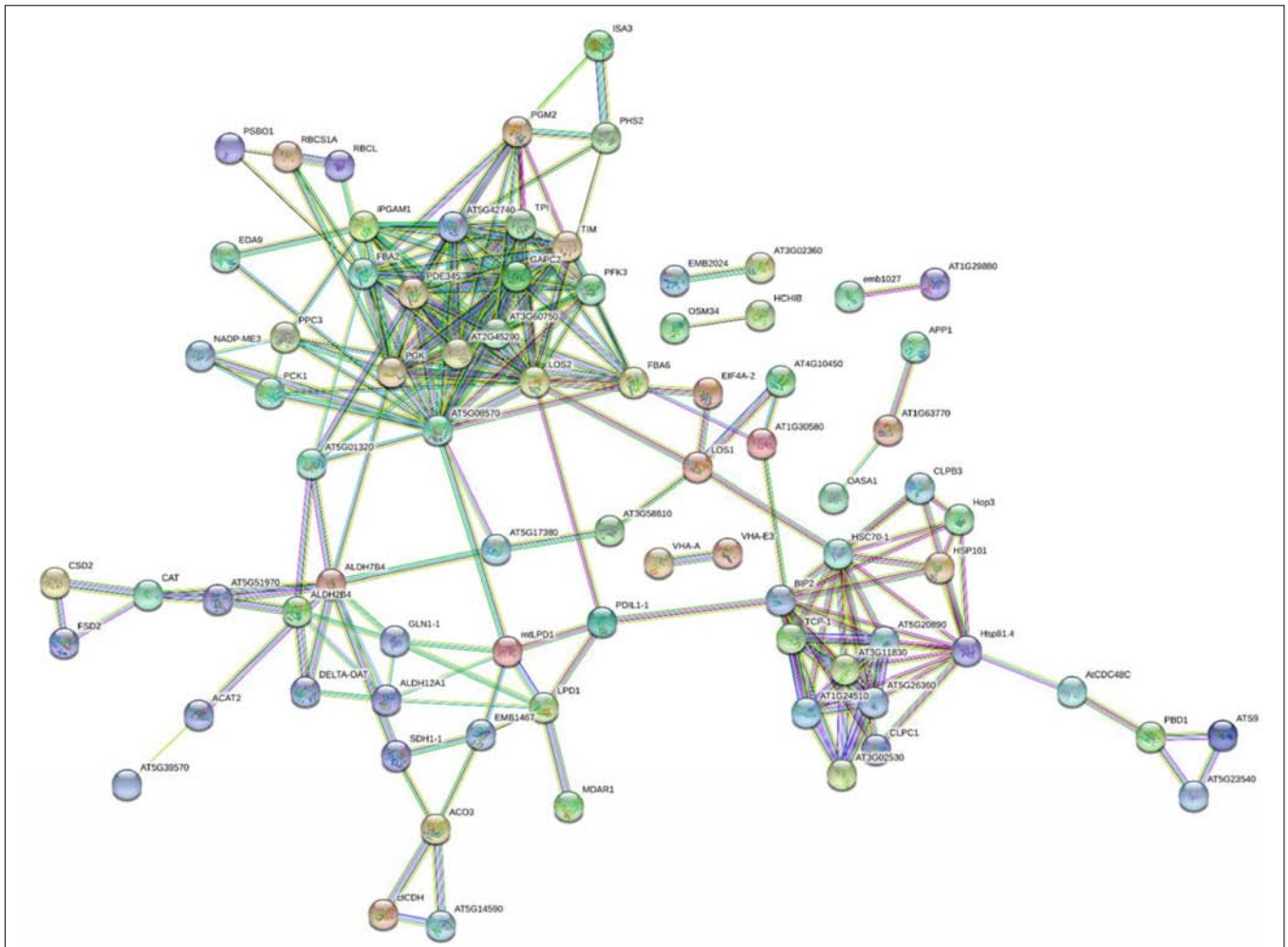

FIGURE 5 | STRING analysis of differentially represented proteins in kiwifruit at T1, T2, and T3 with respect to T0. Only-high confidence interactions (0.7) are evidenced. Abbreviations are reported in **Supplementary Table S4**.

proteins (HSPs) and protein disulfide isomerases (PDIs) that, contributing to a proper polypeptide folding and disulfide pairing, were already described as being involved in plant defense against different environmental challenges (**Supplementary Table S3** and **Supplementary Figures S5A,B**) (Timperio et al., 2008). Whereas protein disulfide isomerase-like (PDIL1-1) and stress-induced phosphoprotein (Hop3) showed reduced levels at T1 and T2, a number of other HSPs and chaperones were over-represented as result of kiwifruit cold storage. In particular, isoforms of HSP 70 kDa protein C (BIP2), HSP 90-2 (HSP81.4), 70 kDa HSP (HSC70-1), chaperone ClpB (CLPB3 and HSP101), and T-complex protein 1 subunits α (TCP-1), β (AT5G20890), γ (AT5G26360), ε (AT1G24510), η (AT3G11830), and ζ (AT3G02530) always showed augmented levels at T1, T2, and/or T3, whereas chaperone ClpB1 (CLPC1) was over-represented for the whole period of investigation. Some of the proteins reported above have already been referred as highly represented in banana, papaya and peach exposed to cold-based storage conditions (Huerta-Ocampo et al., 2012; Li et al., 2015; Wu et al., 2016) or in different plants following various abiotic stresses, including the cold one (Somer et al., 2002; Renaut et al., 2006; Timperio et al., 2008; Janmohammadi et al., 2015; Die et al., 2016, 2017; Muthusamy et al., 2016). They are known to assist polypeptide synthesis and macromolecular structures assembly, and to prevent corresponding misfolding in environmental conditions hampering their function. Above-mentioned species were present as a subnetwork in **Figure 5**, and were linked to other components involved in protein synthesis and protein destination/storage.

To maintain proper cell functioning in an environmental stress condition (cold storage) eventually associated with formation of misfolded/non-soluble polypeptide species, various molecular machineries were also regulated in harvest kiwifruit at T1–T3 to ultimately lead to a condition where optimal protein turnover was ensured, but total protein levels remained unaltered (**Table 1**). In the case of kiwifruit cold storage, these mechanisms seem to include the induction of various proteolytic enzymes, i.e., putative aminopeptidases (AT1G63770), subtilisin-like

proteases (ARA12 and AT5G67090), acylamino-acid-releasing enzyme (AARE) and Xaa-pro aminopeptidase (APP1), and of components of the ubiquitin/26S proteasome machinery, namely 26S proteasome regulatory subunit (AT5G23540) and non-ATPase regulatory subunit (ATS9), which were over-represented at T1–T3 (**Supplementary Table S3** and **Supplementary Figure S5B**). Concomitant repression of components of the ubiquitin-independent 20S proteasome system was also observed, namely subunit β isoforms (PBD1) and ubiquitin-conjugating enzyme MMZ3. In the latter context, worth mentioning is the fact that the 20S proteasome system, through the representation of its components, has already been reported to control the degradation efficiency of the ubiquitin/26S proteasome machinery in kiwifruit and grape (Renaut et al., 2006; Martínez-Esteso et al., 2011; Salzano et al., 2018). Thus, our results suggest that a reduced synthesis of members of the ubiquitin-independent 20S proteasome system, together with induction of components of the ubiquitin/26S proteasome system, may ultimately lead to a promoted activity of the latter in degrading misfolded/non-soluble polypeptide species. The probable occurrence of compensative mechanisms promoting protein synthesis under stressful conditions to maintain total protein levels unaltered fitted with the observation of increased levels of elongation factor isoforms LOS1 (LOS1), eukaryotic initiation factor 4A (EIF4A-1), ATP-binding cassette (ABCF1), Gly-tRNA ligase isoforms (AT1G29880), and Arg-tRNA ligase (emb1027) during kiwifruit cold storage (**Supplementary Table S3** and **Supplementary Figure S5C**). The first protein was already identified as an inducible protein in kiwifruit experiencing C_2H_4 and chilling treatments (Minas et al., 2016). Protein homologs of the above-cited components showed similar quantitative trends in other plants exposed to cold (Renaut et al., 2006; Janmohammadi et al., 2015).

Finally, cysteine proteases cathepsin S (RD21B) and cathepsin B-like (RD21A), also known as actinidin and actinidin Act2a, respectively, showed reduced levels at T1–T3 (**Supplementary Table S3** and **Supplementary Figure S5B**). Actinidins are among the most abundant proteins in physiologically ripe kiwifruit and banana, and were recognized as major allergens therein (**Supplementary Table S5**) (Bulley, 2016; Du et al., 2016). Their quantitative variation during cold storage again suggests that postharvest management of kiwifruit can influence quantitative representation of specific allergens, with important consequences on food allergic properties.

Cell Wall Remodeling Enzymes and Structural Proteins

Above-reported pomological data shows that cold storage of kiwifruit was associated with a reduction of its firmness (**Table 1**), confirming previous studies (Schröder and Atkinson, 2006; Chiaramonti and Barboni, 2010; Lim et al., 2016; Asiche et al., 2017). In agreement with these observations, we ascertained a differential representation of cell wall remodeling enzymes and structural proteins in the corresponding time range, complementing previous biochemical evidences (Martínez-Esteso et al., 2011). In particular, cell wall structural components, i.e., arabinogalactan protein (AT5G11680) and pro-resilin (AT5G39570), and cytoskeletal proteins, namely actin 1 (ACT7), annexin (ANNAT4) and tubulin beta-2 chain (TUB5), showed reduced levels at T2–T3 (**Supplementary Table S3** and **Supplementary Figures S5D,M**). Arabinogalactan protein is a hydroxyproline-rich glycoprotein, heavily modified by arabinose/galactose-rich polysaccharide chains and glycosylphosphatidylinositol anchors, which is cross-linked to pectin and pectin-arabinoxylan to ensure cell wall rigidity (Showalter and Basu, 2016). Above-reported findings were paralleled by over-representation of enzymes regulating cell wall pectin and hemicellulose degradation, namely pectinesterase-2 (PME51), pectinesterase inhibitor (PMEI2), β-xylosidase 4 (XYL4), polygalacturonase-inhibitor protein (PGIP1), putative uncharacterized proteins P0046B10.2-1 (AT3G08030 and AT5G11420) and anthranilate phosphoribosyltransferase (AT1G70570), which in some cases showed increased levels even at T1 or were characterized for their over-representation in T2–T3 range (**Supplementary Table S3** and **Supplementary Figures S5D,F,M**). The activity of AT1G70570 has been reported to be regulated by C_2H_4 (Li et al., 2012). Most of these enzymes have been described to modulate the degree of methylesterification/acetylation and/or (consequent) polymerization of pectin homogalacturonans and hemicellulose xyloglucans (Sénéchal et al., 2014; Zúñiga-Sánchez et al., 2014; Genero et al., 2016). Cell wall-associated AT3G08030 contains a carbohydrate-binding domain and interacts with cell wall polysaccharides (Vázquez-Lobo et al., 2012). Our proteomic observation was in good agreement with previous studies on cell wall remodeling enzymes and structural proteins in cold exposed kiwifruit (Minas et al., 2016; Asiche et al., 2018) and in other climacteric fruits during ripening or subjected to a similar postharvest thermal management (Nilo et al., 2010; Zhang et al., 2011, 2012; Giraldo et al., 2012; Huerta-Ocampo et al., 2012; Du et al., 2016). All these evidences confirmed the occurrence of concomitant, distinct molecular mechanisms regulating cell wall disassembly and fruit softening in the fruit (Bennett and Labavitch, 2008).

Central Carbon and Energy Metabolism

Kiwifruit generally accumulates large amounts of starch during development (Richardson et al., 2011), which is then degraded in the postharvest phase (also during cold storage) (Richardson et al., 2011; Park et al., 2015b; Hu et al., 2016). In this context, progressively increasing SSC values ascertained at T1–T3 (**Table 1**) and corresponding sugar (SUCR, AGLC, BGLC, and BFRUPY) levels (**Supplementary Figure S1**) were coherent with the over-representation of enzymes involved in starch degradation and sucrose metabolism, namely glycogen debranching enzyme (ISA3), α-glucan phosphorylase (PHS2), phosphoglucomutase isoforms (PGM2) and phosphofructokinase (PFK3) (**Supplementary Table S3** and **Supplementary Figures S5F,G**), thus providing a rationale to measured metabolite amounts. Above-cited proteomic changes were paralleled by increased levels of enzymes involved in glycolysis, i.e., glucose-6-phosphate isomerase (AT5G42740), fructose-bisphosphate aldolase isoforms 2 and 3 (PDE345, FBA6, and FBA2), triosephosphate isomerase (TPI), phosphoglycerate

kinase isoforms (PGK), phosphoglycerate mutase isoforms (iPGAM1), enolase isoforms (LOS2) and pyruvate kinase (AT5G08570), and in the citric acid cycle, i.e., dihydrolipoyl dehydrogenase (mtLPD1), aconitate hydratase 2 (ACO3), isocitrate dehydrogenase [NADP] isoforms (CICDH and AT5G14590) and succinate dehydrogenase subunit A (SDH1-1), which were mostly constant at T1–T2 and then over-represented at T3. This ensured active metabolic pathways in harvest kiwifruit providing energy, cofactors and building blocks for fruit survival also during cold storage. Two GTP-binding proteins regulating glycolytic/TCA cycle pathways by a direct interaction with above-mentioned enzymes as well as chloroplast development, membrane fission and sensitivity to hormones were also over-represented in the same time range, namely GTPase obg (AT1G30580) and dynamin (DL1E) (Colaneri and Jones, 2014) (**Supplementary Figures S5E,M**). Regulation of glycolysis and the Krebs cycle pathways in kiwifruit was also ensured through down-representation of triosephosphate isomerase (TIM), glyceraldehyde-3-phosphate dehydrogenase (GAPC2) and dihydrolipoyl dehydrogenase (LPD1) (showing decreased levels at T1 and T3, respectively) (**Supplementary Figures S5F,G**).

Evidences for the activation of alcoholic fermentation during kiwifruit cold storage derived from ascertained over-representation of corresponding enzymes, i.e., Zn-containing alcohol dehydrogenase (AT4G13010), oxalyl-CoA decarboxylase (AT5G17380), and pyruvate decarboxylase 2 (AT5G01320). In particular, increased levels of AT5G01320 were coherent with promoted conversion of pyruvate from glycolysis into toxic acetaldehyde, which then was converted into ethanol by the detoxifying action of augmented AT4G13010, also enabling production of NADH, or transformed into acetate by increased levels of aldehyde dehydrogenase ALDH2B4 (AT3G48000) (Wei et al., 2009; Stiti et al., 2011) (**Supplementary Table S3** and **Supplementary Figures S5F,G**). AT5G01320 and AT4G13010 have already been reported being induced in apple following C_2H_4 treatment (Yang et al., 2016). Conversely, a down-representation of enzymes involved in photosynthesis was observed, namely ribulose bisphosphate carboxylase large chain (RBCL), ribulose bisphosphate carboxylase small chain (RBCS1A), and oxygen-evolving enhancer protein 1 (PSBO1); a similar trend was also observed for plastid-lipid-associated protein (AT4G22240) and chloroplastic outer envelope pore protein 24 (AT1G45170) (**Supplementary Figures S5D,G,M**). These proteins are directly involved or stabilize plastid machineries essential in providing the energetic supply of the fruit and in maintaining its endogenous O_2 balance. Our results suggest that photosynthesis is not a preferential energetic pathway in harvest kiwifruit during cold storage, confirming previous observations based on gene expression data on fruit postharvest at room temperature (Richardson et al., 2011). Nevertheless, energy supply in fruit seemed guaranteed by dedicated compensative mechanisms, as evidenced by increased levels measured for V-type ATP synthase alpha chain (VHA-A), V-type proton ATPase subunit E (VHA-E3) and NADH-ubiquinone oxidoreductase isoforms (EMB1467) isoforms in the T1–T3 range (**Supplementary Table S3** and **Supplementary Figures S5G,L**). Enzymes involved in the C4 cycle, namely malic enzyme (NADP-ME3) and phosphoenolpyruvate carboxylase (PPC3), also showed over-representation at T2 and T3, whereas proteins assisting pentose-phosphate metabolism displayed a mixed quantitative trend overtime, i.e., 6-phosphogluconate dehydrogenase (AT3G02360), 6-phosphogluconolactonase (EMB2024), and transketolase isoforms (AT3G60750 and AT2G45290).

In the whole, the quantitative behavior of specific proteins involved in glycolysis, the Krebs cycle, alcoholic fermentation, energy production and additional carbon metabolism pathways in kiwifruit well paralleled that observed in the same fruit exposed to low temperatures but in different experimental setup (Minas et al., 2016; Asiche et al., 2018), in other fruits during ripening (Borsani et al., 2009; Andrade et al., 2012; Huerta-Ocampo et al., 2012; Nogueira et al., 2012; Yun et al., 2013; Zheng et al., 2013; Du et al., 2016; Xiao et al., 2018) or cold-based postharvest management (Nilo et al., 2010; Li et al., 2015; Wu et al., 2016; Tanou et al., 2017; Wang et al., 2017), or in other plants experiencing cold stress conditions (Janmohammadi et al., 2015; Die et al., 2016, 2017), suggesting the existence of common regulation mechanisms of these metabolic pathways in above-mentioned organisms. Most of the proteins reported above constitute two functional subnetworks linked to each other and to additional ones related to stress response and protein destination/storage (**Figure 5**).

Other Metabolic Enzymes

Significant differences in amino acid content were observed during kiwifruit cold storage. In fact, Val, Ile, Thr, Ala, Trp, Asp, Glu, and GAB showed variable quantitative levels overtime, which were also different with respect to counterparts ascertained during fruit physiological development (**Supplementary Figure S1**). Proteomic results at T1–T3 were frequently indicative of the modulation of the corresponding anabolic/catabolic pathways (**Supplementary Table S3** and **Supplementary Figures S5F–H**). For example, Val and Ile increased during kiwifruit cold storage in parallel to over-representation of Val/Leu/Ile biosynthetic enzymes ketol-acid reductoisomerase (AT3G58610) and 3-isopropylmalate dehydratase large subunit (AT2G05710). Similarly, increased concentration of Trp at T1 was in good agreement with ascertained augmented levels of the biosynthetic enzyme anthranilate phosphoribosyltransferase (AT1G70570) at that time and, at T3, of polyphenol oxidase (NdhS) degrading oxidized protein adducts, which was also possibly explicative for slight augmented concentration of specific polyphenolics at the final stage of kiwifruit cold storage. On the other hand, accumulation of Thr overtime was associated with the decreased representation of L-threonine 3-dehydrogenase (AT5G51970), which is involved in irreversible degradation of this amino acid. More complex was the tentative explanation of Asp, Ala, Glu, and GABA levels in the course of kiwifruit cold storage (**Supplementary Figure S1**), based on multiple (overlapping) metabolic pathways in which these amino acids are involved (Ainalidou et al., 2016), and the limited information on corresponding enzyme representation trends (**Supplementary Table S3** and **Supplementary Figure S5F**).

Finally, the increased levels at T3 of cytosolic cysteine synthase (OASA1), which catalyzes Cys biosynthesis, were associated with its partial involvement in the removal of cyanide formed as result of 1-aminocyclopropane-1-carboxylate-oxidase (ACO)-dependent production of C_2H_4, in agreement with what observed in kiwifruit and other fruits subjected to various postharvest managements (Jost et al., 2000; Du et al., 2016). The quantitative levels of the latter enzyme provided a rationale to the amounts of C_2H_4 measured in the range T0–T3 (**Table 1**), underlying the essential role of ACO4 in C_2H_4 biosynthesis (Atkinson et al., 2011), and the activation of this enzyme in the experimental conditions used in this study for kiwifruit cold storage. In this context, worth mentioning is the fact that our experiments were performed in the absence of C_2H_4 receptors inhibitors (i.e., 1-methylcyclopropene), thus missing the possibility to discriminate between distinct C_2H_4-dependent and low temperature-dependent ripening mechanisms (Asiche et al., 2018). Over-representation of above-mentioned proteins and C_2H_4 well paralleled with a concomitant increase of AA concentration in kiwifruit during cold storage (**Supplementary Figure S3**), further supporting the important relation between AA, ROS, and C_2H_4-regulated enzymes during fruit postharvest (Lum et al., 2016). Some of the above-cited results paralleled what observed in the same fruit exposed to low temperatures but in different experimental setup (Asiche et al., 2018), in other fruits during ripening (Nogueira et al., 2012; Zhang et al., 2012; Zheng et al., 2013; Du et al., 2016) or cold-based postharvest management (Nilo et al., 2010; Tanou et al., 2017), or in plants subjected to cold stress (Die et al., 2016); they were suggestive of specific metabolic steps where regulation of amino acid or secondary metabolite anabolism/catabolism is exerted.

On the other hand, different enzymes involved in purine/pyrimidine catabolism were over-represented in T1–T3 range, namely uricase (urate oxidase) (AT2G26230) and dihydropyrimidase (PYD2) (**Supplementary Figure S5F**), in agreement with analogous reports on other fruits during postharvest management or in plants subjected to cold stress. The first enzyme is involved in uric acid oxidation, thus removing this sparingly soluble plant metabolite, while the second one is involved in uridine degradation, providing a rationale to the decreased levels of this pyrimidine measured overtime (**Supplementary Figure S3**).

Regarding kiwifruit proteins involved in secondary metabolite anabolism/catabolism, proteomic data showed an over-representation at T1–T2 of cinnamyl alcohol dehydrogenase 4 (ATCAD4), as already observed during ripening of banana (Du et al., 2016), whereas 3-ketoacyl-CoA thiolase (ACAT2) and rubber elongation factor (REF) showed increased levels even at earlier stages (**Supplementary Table S3** and **Supplementary Figures S5F,H,M**). ATCAD4 catalyzes NADPH-dependent reduction of caffeyl aldehyde to its respective alcohol in corresponding phenylpropanoid biosynthetic pathway; its abundance in kiwifruit during cold storage was related to the decrease of caffeic acid hexoside concentration ascertained therein (**Figure 2**). REF is a protein assisting poly-isoprene polymerization in the monolayer membrane of rubber, which also presents allergic properties (Berthelot et al., 2012) whereas ACAT2 is involved in isoprenoid/terpenoid pathway that supplies precursors for the biosynthesis of carotenoids, dolichols, and sterols. Over-representation of ACAT2 during kiwifruit cold storage was tentatively associated with increased or, at least, constant concentration of S7, ST, and bSC, respectively (**Supplementary Figure S2**), although a rationale justifying the specific quantitative levels overtime of these sterols was not deduced.

CONCLUSION

By using combined NMR/LC-UV/ESI-IT-MS^n and 2D-DIGE/nLC-ESI-LIT-MS/MS procedures, this study provides a global picture of the metabolite and protein quantitative changes occurring during kiwifruit cold storage, under conditions not using additional treatment with specific chemicals. About 42 metabolites were evaluated, showing in some cases concentration trends similar to that previously reported. In parallel, protein representation results allowed revealing that components related to disease/defense, protein destination and storage, metabolism, energy and cell structure functions were highly affected at specific moments of kiwifruit postharvest management. A number of these components occurred in a predicted functional interaction network that, based on ascertained results, appears to orchestrate protein representation overtime to modulate essential reactions underlying kiwifruit during its postharvest life and/or its adaptation to cold conditions. Most protein quantitative variations occurred in correspondence of the apex in C_2H_4 emission, underlining the prominent role of this phytohormone in fruit physiology. Protein representation trends also provided an explanation to some pomological characteristic and metabolite concentration variations, integrating previous studies on this (Minas et al., 2012, 2014, 2016; Tanou et al., 2015; Asiche et al., 2018) and other fruits (Pedreschi et al., 2008, 2009; Borsani et al., 2009; Lara et al., 2009; Costa et al., 2010; Nilo et al., 2010; Zhang et al., 2010, 2011, 2012; Andrade et al., 2012; Giraldo et al., 2012; Huerta-Ocampo et al., 2012; Nogueira et al., 2012; Yun et al., 2013; Zheng et al., 2013; Cai et al., 2014; Jiang et al., 2014; Lauxmann et al., 2014; Li et al., 2015; Du et al., 2016; Liu et al., 2016; Sun et al., 2016; Wu et al., 2016; Tanou et al., 2017; Wang et al., 2017; Xi et al., 2017; Xiao et al., 2018). Since a number of metabolites and proteins for which a metabolic/functional linkage was hypothesized in physiological ripening of kiwifruit (Salzano et al., 2018) showed a concomitant opposite quantitative behavior with respect to that reported in this study, the results presented here reinforce our previous hypotheses on their mutual functional association. By providing an original proteometabolomic description of harvest kiwifruit during cold storage under conventional postharvest management, this investigation provides a picture of fruit physiology in a condition that is generally adopted from kiwifruit industry, integrates previous important studies on this berry based on different postharvest management procedures (Minas et al., 2012, 2014, 2016; Tanou et al., 2015), and add additional insights on the evaluation of metabolic

pathways and molecular effectors in harvest fruits from other species according to a holistic perspective (Hertog et al., 2011; Pedreschi, 2017).

AUTHOR CONTRIBUTIONS

AMS, GR, VC, MP, APS, LM, and AS designed the experiments, analyzed the data, and wrote the manuscript. AMS, GR, APS, DC, MV, GN, and MSP performed the experiments. VC, MP, NZ, LM, and AS commented on the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00071/full#supplementary-material

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MINI REVIEW
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Biosynthesis, Metabolism and Function of Auxin, Salicylic Acid and Melatonin in Climacteric and Non-climacteric Fruits

*Marina Pérez-Llorca[1], Paula Muñoz[1,2], Maren Müller[1,2] and Sergi Munné-Bosch[1,2]**

[1] Department of Evolutionary Biology, Ecology and Environmental Sciences, University of Barcelona, Barcelona, Spain,
[2] Institute for Research on Nutrition and Food Safety, University of Barcelona, Barcelona, Spain

Climacteric and non-climacteric fruits are differentiated by the ripening process, in particular by the involvement of ethylene, high respiration rates and the nature of the process, being autocatalytic or not, respectively. Here, we focus on the biosynthesis, metabolism and function of three compounds (auxin, salicylic acid and melatonin) sharing not only a common precursor (chorismate), but also regulatory functions in plants, and therefore in fruits. Aside from describing their biosynthesis in plants, with a particular emphasis on common precursors and points of metabolic diversion, we will discuss recent advances on their role in fruit ripening and the regulation of bioactive compounds accumulation, both in climacteric and non-climacteric fruits.

Keywords: auxin, biotrophs, defenses, fruit ripening, maturation, melatonin, post-harvest, salicylates

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***Correspondence:**
Sergi Munné-Bosch
smunne@ub.edu

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INTRODUCTION

Correct progression of fruit ripening is essential to achieve both optimal fruit quality and long shelf life, important traits that determine price markets and final profits. However, ripening is a complex process where many factors are involved, including hormonal control, which regulates biochemical and physiological changes that give final organoleptic and nutritional fruit properties. Traditionally, fleshy fruits have been classified according to there ripening process into climacteric and non-climacteric fruits. Climacteric fruits such as tomatoes or bananas require an upsurge in respiration rate and ethylene production to unleash the ripening process in an autocatalytic response (Barry and Giovannoni, 2007; Liu et al., 2015). Antagonistically, non-climacteric fruits like citrus fruits or grapes do not exhibit a burst neither in ethylene nor respiration prior to ripening onset, and in these fruits, ripening is mainly controlled by progressive accumulation of the phytohormone abscisic acid (ABA; Rodrigo et al., 2006; Castellarin et al., 2011). Nevertheless, new studies are reinforcing the idea that the ripening process is not only governed by the production of one phytohormone but rather seeming to be the result of a controlled hormonal balance (Symons et al., 2012; Teribia et al., 2016; Li et al., 2019).

Auxins, salicylic acid (SA), and melatonin are phytohormones involved in the signaling and regulation of many crucial processes in plants. Auxins have been widely described as growth and development regulators with multiple functions in plants (see Taylor-Teeples et al., 2016); SA triggers the defense response against biotrophic and hemi-biotrophic pathogens (Loake and Grant, 2007) as well as having an important role under abiotic stress (Dong et al., 2014; Wani et al., 2017), flowering and cell cycle control (Carswell et al., 1989; Eberhard et al., 1989, respectively);

and melatoninhas not only been found to have auxin-like functions (Chen et al., 2009; Zuo et al., 2014; Wen et al., 2016) but it also has been suggested to act as a potential antioxidant in some plants (Arnao and Hernández-Ruiz, 2015) and a regulator of plant responses to pathogens (Chen et al., 2018). Interestingly, these three hormones share a common precursor – chorismate -, thus a metabolic cross-talk occurs between them, and a number of genes must be finely regulated to divert chorismate metabolism toward these compounds. During the past few years, several reviews have addressed the biosynthesis and role of auxin (Paul et al., 2012), SA (Asghari and Aghdam, 2010) and melatonin (Feng et al., 2014; Arnao and Hernández-Ruiz, 2018) in fruits. However, their biosynthesis and functions have mostly been described separately. Here, we will discuss the common and differential aspects of the biosynthesis, metabolism and function of auxin, SA and melatonin in the growth and ripening of climacteric and non-climacteric fruits. An emphasis will be put on metabolic diversion key points in their biosynthesis from chorismate, the regulatory role during the ripening of climacteric and non-climacteric fruits, and their role in the modulation of the biosynthesis of bioactive compounds, which largely determines fruit quality.

CHORISMATE-DERIVED PHYTOHORMONES

Chorismate is the final product of the shikimate pathway and plays a key role in the biosynthesis of phytohormones (**Figure 1**). Chorismate gives rise to aromatic amino acids, including tryptophan, and through several reactions, including the conversion of tryptophan to indole-3-pyruvic acid by the tryptophan aminotransferase (TA); it can generate the auxin indole-3-acetic acid (IAA). TA has been proposed as a universal key enzyme to IAA biosynthesis not only in vegetative organs (Enríquez-Valencia et al., 2018) but also in the development of reproductive organs (Reyes-Olalde et al., 2017), including fruit growth and ripening (Estrada-Johnson et al., 2017). By contrast, when isochorismate synthase (ICS) is activated, chorismate can be converted into isochorismate and the latter can be transformed into SA. Despite the fact that this pathway was first identified in bacteria, it is also currently well established in plants (Wildermuth et al., 2001; Uppalapati et al., 2007; Catinot et al., 2008; Garcion et al., 2008; Abreu and Munné-Bosch, 2009). Finally, tryptophan can turn into tryptamine by the enzyme tryptophan decarboxylase (TDC). Although this is another of the multiple routes of IAA biosynthesis (tryptamine turns into indole-3-acetaldehyde and later into IAA), TDC has also been proposed to be the first rate-limiting enzyme in the melatonin pathway (Kang et al., 2007). TDC was first identified in the Apocynaceae family (De Luca et al., 1989) to be later described in a number of plant systems (Byeon et al., 2012, 2014; Zhao et al., 2013; Wei et al., 2018). After decarboxylation, tryptamine is converted into serotonin by the tryptamine-5-hydroxylase, and then serotonin transforms into N-acetyl-serotonin to finally yield melatonin in the cytosol (**Figure 1**).

Key-diverting enzymes that lead to the synthesis of the chorismate-pathway hormones have been recently identified in fleshy fruits (**Figure 1**). *ICS*, which expression has been shown to be crucial for biotic stress tolerance (Garcion et al., 2008; Zeng and He, 2010), has been identified in tomatoes and apples. Zhu et al. (2016) reported the overexpression of *ICS1* (*isochorismate synthase 1*) under cold storage conditions in tomato, while Zhang et al. (2017) found activation of a transcription factor involved in the pathogen-related SA signaling pathway inducing *ICS* expression in apples. Enhanced TDC activity has been reported to occur in unripe pepper fruit upon infection by pathogens – through increased *TDC1* and *TDC2* expression (Park et al., 2013) – and in the growth stage in mulberry fruit (Wang et al., 2016). Finally, TA has been identified in grapevine, both at *pre-* and *véraison* (Böttcher et al., 2013; Gouthu and Deluc, 2015, respectively) as well as during strawberry ripening (Estrada-Johnson et al., 2017). It is noteworthy, however, that the genes and enzymes described in climacteric fruits (i.e., ICS) have not been identified in non-climacteric fruits (i.e., TDC and TA) and vice versa; hence, further studies are imperative to fill these knowledge gaps and better understand how these diversion points are jointly regulated during fruit ripening.

ROLE OF CHORISMATE-DERIVED PHYTOHORMONES IN CLIMACTERIC AND NON-CLIMACTERIC FRUITS

Unraveling the mechanisms of fruit development has been one of the major challenges in recent agronomy research for its economic implications. In this context, phytohormones have been pointed out as accountable drivers of fruit ripening, specially ethylene and ABA in climacteric and non-climacteric fruits, respectively. However, that these phytohormones could regulate fruit development alone was soon proven to be far too simple. After extensive research and with the improvement in analytical chemistry and molecular techniques, several other hormones have been confirmed as potential regulators of fruit development and ripening, including chorismate-derived phytohormones.

Auxins Cross-Talk With Other Hormones During Fruit Set, Growth and Ripening

Auxins are a group of plant hormones that play an essential role in fruit development, both exerting their own influence and modulating expression of other phytohormones. Endogenous contents of IAA are particularly high at fruit set and during initial growth developmental stages, after which IAA amounts tend to decline before ripening onset, both in climacteric (Zaharah et al., 2012) and non-climacteric fruits (Symons et al., 2012; Teribia et al., 2016), with apparently some exceptions, like peaches (Tatsuki et al., 2013) and some plum varieties (El-Sharkawy et al., 2014; **Figure 2A**). It has been demonstrated that IAA is involved in fruit set initiation in combination with gibberellins (Mezzetti et al., 2004; Serrani et al., 2010; Bermejo et al., 2018; Hu et al., 2018). Impairment of IAA biosynthesis or signaling generally leads to fruit parthenocarpy, although it may also result in

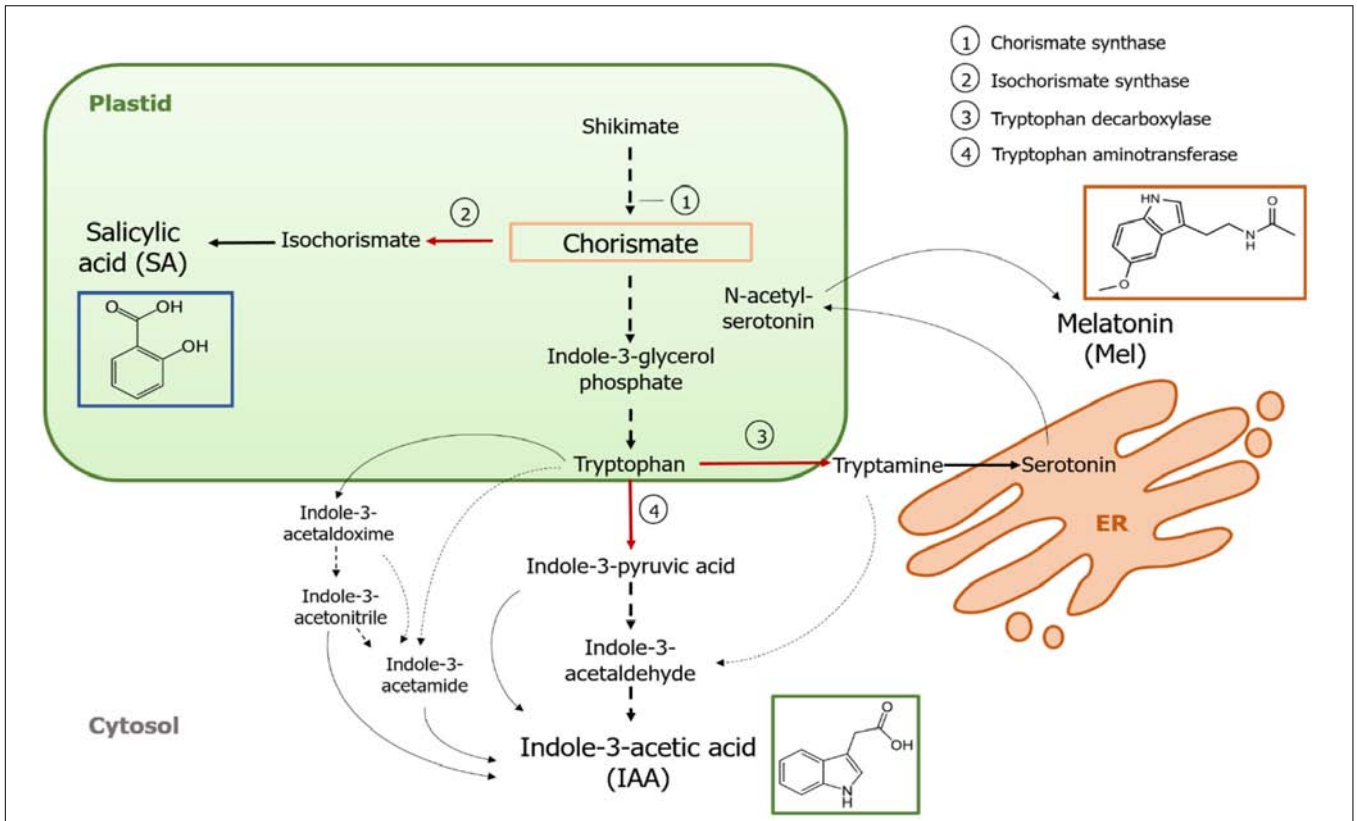

FIGURE 1 | Biosynthesis of indole-3-acetic acid (IAA), salicylic acid (SA), and melatonin (Mel) from chorismate. All three compounds share a common final precursor (chorismate, squared in orange) from the shikimate pathway. Major diversion points are indicated with red arrows and the corresponding enzymes are numbered in a descent divergence order from shikimate. Bold dashed arrows indicate more than one step between compounds. Thin dashed arrows indicate pathways that are still not well defined.

abnormal ripening in some fruits (Wang et al., 2005; Liu J. et al., 2018; Reig et al., 2018). High contents of IAA at initial stages of fruit development promote fruit growth due to auxin implication in cell division in combination with cytokinins and in the control of cell expansion in combination with gibberellins (Liao et al., 2018). During this period, hormonal crosstalk between auxins and gibberellins additionally allows normal fruit shaping in a fine-tuned regulation mediated by Auxin Response Factors (ARFs; Liao et al., 2018; Liu S. et al., 2018).

Reductions of endogenous IAA contents have been reported to occur before the onset of ripening in several fruits. These reductions have been related to IAA conjugation with aspartic acid (IAA-Asp) by IAA-amido synthetases, $GH3_{-1}$ and $GH3_{-2}$ (**Figure 2C**). Indeed, *$GH3_{-1}$ and $GH3_{-2}$* showed higher expression during early fruit development and most particularly during ripening initiation both in climacteric fruits, such as tomatoes (Sravankumar et al., 2018) and apples (Onik et al., 2018), as well as in non-climacteric fruits, like grape berries (Böttcher et al., 2010, 2011) and raspberries (Bernales et al., 2019). Interestingly, grape berries showed enhanced *$GH3_{-1}$* expression after ABA and ethephon application, which could explain the involvement of ethylene in the control of IAA contents after the onset of ripening, even in non-climacteric fruits (Böttcher et al., 2010). In fact, several studies highlight the tight interaction between auxins and ethylene in fruit ripening, with a reciprocal influence between them (Tadiello et al., 2016a; Busatto et al., 2017). For climacteric fruits, increased contents of IAA are necessary to activate expression of ACC synthase genes (*ACS*), encoding ACC synthase, which, in turn, will lead to ethylene production triggering the ripening process (**Figure 2C**; Tatsuki et al., 2013). Fruits like tomatoes (Li et al., 2016) or bananas (Choudhury et al., 2008), which usually experience reduced IAA contents prior to the onset of ripening, show a ripening delay when IAA or analogs are exogenously applied, while fruits like peaches, whose IAA contents increase progressively until fully ripen, show accelerated ripening when auxins are applied (Tadiello et al., 2016b). Therefore, a tight, complex and differential regulation of auxin-ethylene interactions must exist in various fruits during preharvest ripening. Furthermore, IAA contents decrease before ripening onset in some non-climacteric fruits to de-repress *9-cis-epoxycarotenoid dioxygenase* (*NCED*) and start ABA synthesis (**Figure 2C**; Jia et al., 2016). Interestingly, this process is also mediated by IAA conjugation through enhanced $GH3_{-1}$ activity (Böttcher et al., 2010). Nevertheless, Estrada-Johnson et al. (2017) showed that, although IAA contents decrease during strawberry ripening, expression of *FaAux/IAA* and *FaARF* gene families are induced in red receptacles, suggesting the involvement of auxin signaling in fully ripen fruits.

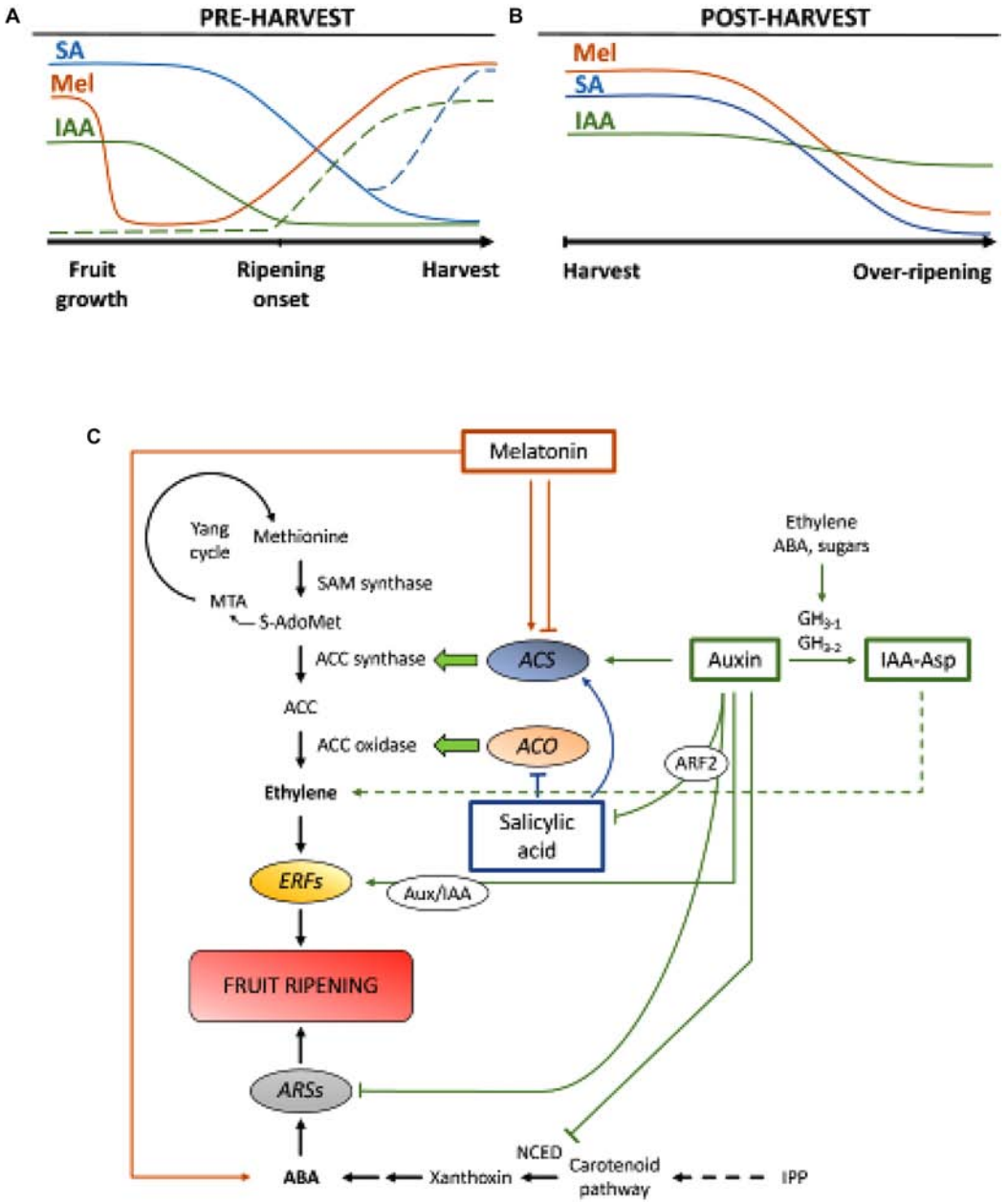

FIGURE 2 | Role of IAA, SA, and Mel during the development of climacteric and non-climacteric fruits. Model summarizing the interactions of IAA, SA, and Mel during the ripening of climacteric and non-climacteric fruits during **(A)** pre- and **(B)** post-harvest. Dashed lines indicate alternative dynamics of phytohormone contents in some fruits (see text for discussion). **(C)** Overview of the interaction of IAA, SA, and Mel with ethylene and abscisic acid (ABA) biosynthesis in climacteric and non-climacteric fruits. Auxin is a positive regulator of ethylene biosynthesis by the activation of ACC synthase genes (*ACS*) and inducing the expression of Ethylene Responsive Factors (ERFs). Auxin also represses ABA production by *9-cis-epoxycarotenoid dioxygenase* (*NCED*) repression and inhibiting ABA-responsive stress genes (*ARS*) by the ARF2 protein. Endogenous IAA contents can be reduced by GH_3 proteins, the synthesis of which is promoted by ethylene, ABA and sugars. Discontinuous line indicates the possible role of IAA by IAA-Asp as a ripening factor enhancing ethylene in some fruits. Melatonin enhances ABA and ethylene production, although ACS can be inhibited depending on melatonin contents. SA acts as an inhibitor of ethylene repressing ACC oxidase (*ACO*) genes, although it can promote the expression of *ACS*. *MTA*, *50-methylthioadenosine; S-AdoMet, S-adenosyl-methionine; SAM synthase, S-adenosyl-methionine synthase; ACC, 1-aminocyclopropane-1-carboxylic acid; IPP, isopentenyl diphosphate*.

During post-harvest, auxin contents usually remain invariant or tend to decrease due to oxidative processes that may give rise to small, but progressive reductions in the endogenous contents of IAA (**Figure 2B**). Auxin treatments after harvest delay over-ripening in some fruits (Chen et al., 2016; Moro et al., 2017) and increase the contents of some organic acids, maintaining fruit acidity (Li et al., 2017), thus suggesting auxin also

plays a significant role in the control of fruit ripening during post-harvest.

Multiple Roles of Salicylic Acid During Fruit Development and Ripening

Salicylic acid is another chorismate-derived phytohormone that has been mostly related to its protective effect under biotic stress to control preharvest and post-harvest losses derived from pathogen fruit infection (Babalar et al., 2007; Cao et al., 2013). In general, endogenous contents of free SA are higher at the beginning of fruit development and then decrease progressively (Oikawa et al., 2015). Lu et al. (2015) reported a secondary increase in SA during the second growth phase of peach fruits. Exogenous application of SA in bananas delayed the ripening process reducing the respiratory burst and the pulp to peel ratio, as well as decreasing activity of enzymes related to cell wall degradation and antioxidant system (Srivastava and Dwivedi, 2000). Moreover, treatment of sweet cherry trees with salicylic and acetylsalicylic acid (ASA) enhanced weight, firmness and color of cherries at commercial stage (Giménez et al., 2014). Exogenous application of SA or metyl salicylate (MeSA) during post-harvest, delayed over-ripening in fruits like kiwis (Zhang et al., 2003), and sweet cherries (Valero et al., 2011). Furthermore, treatments with salicylic or ASA alleviated chilling injury of pomegranates (Sayyari et al., 2011), tomatoes (Aghdam et al., 2014), and avocados (Glowacz et al., 2017). SA also interacts with ethylene through inhibition of ACC oxidase (*ACO*) expression (Shi and Zhang, 2012). Surprisingly, increased contents of *ACS* expression in pears are also found after SA application in a dose-regulated manner (Shi et al., 2013; Shi and Zhang, 2014). Therefore, regulation of ethylene production dependent on SA is finely regulated (**Figure 2C**).

Melatonin: An Emerging Regulator for the Control of Fruit Development

Melatonin is also a plant hormone from the chorismate-derived pathway that has gained much attention in the recent years (Hernández-Ruiz et al., 2005; Arnao and Hernández-Ruiz, 2018; Sharif et al., 2018). Recently, melatonin has been pointed out as an important factor for fruit set, in a similar way to IAA effects (**Figure 2A**; Liu S. et al., 2018). Besides, circadian production of this phytohormone has been observed (Kolář et al., 1997). During on-tree ripening, grapes treated with melatonin showed higher synchronicity and increased weight when fully ripens (Meng et al., 2015). Moreover, another recent study by Xu et al. (2018) described that melatonin could promote grape berry ripening through its interaction with ABA and ethylene, as well as hydrogen peroxide. It has been demonstrated that melatonin confers chilling tolerance during fruit cold storage, which appears to be mostly related to its antioxidant activity (Cao et al., 2018; Aghdam et al., 2019). During post-harvest, tomatoes showed increased ethylene emission after application of melatonin at 50 μM (Sun et al., 2015), contrary to pears, where exogenous application of melatonin at 100 μM inhibited ethylene production (Zhai et al., 2018), or banana fruits, where melatonin at 50–200 μM inhibited the expression of *ACS* and *ACO* (**Figure 2C**; Hu et al., 2017). Although different effects of melatonin application may be related to a dosage effect, further research is needed to better understand the role of melatonin in the control of fruit development.

Cross-Talk of Chorismate-Derived Phytohormones in the Control of Fruit Development

Hormonal crosstalk between chorismate-derived phytohormones has been shown to occur during fruit development and ripening. Breitel et al. (2016) found that auxins can interact with SA through ARF2 (**Figure 2C**) and that overexpression of *ARF2* in tomato resulted in lower contents of SA and a significant ripening delay. This indicates that auxins might be limiting SA production during fruit development, which might explain a trade-off between fruit growth (mediated by auxin) and activation of biotic defenses (mediated by SA). Moreover, exogenous application of SA on papaya fruits resulted in an altered expression of several *IAA* genes, some of them being down-regulated while others up-regulated (Liu et al., 2017). In this case, results were inconclusive of what really occurs endogenously in the putative cross-talk between auxin and SA during fruit ripening and further research is required in this and other climacteric fruits. In any case, it is clear that IAA and SA are closely related, not only metabolically but also functionally. Finally, several studies have reported the influence of melatonin on IAA and SA biosynthesis and/or signaling (reviewed in Arnao and Hernández-Ruiz, 2018), although to our knowledge, none of these studies was performed neither in climacteric nor in non-climacteric fruits.

MODULATION OF BIOACTIVE COMPOUNDS BIOSYNTHESIS BY CHORISMATE-DERIVED PHYTOHORMONES

Bioactive compounds (including phenolic compounds, isoprenoids, and antioxidant vitamins) have not only been widely investigated as responsible for specific organoleptic properties of foods, but also for their protective effects in human cells against oxidative processes in the development of neurodegenerative and cardiovascular diseases and certain type of cancer (Liu et al., 1999; Mueller et al., 2010; Sturgeon and Ronnenberg, 2010; Björkman et al., 2011). Phenolic compounds contribute significantly to imparting specific flavors, such as tannins – responsible for the bitterness or astringency taste of certain fruits, and colors, such as anthocyanin pigments – responsible for red, blue and purple fruit colors (Croteau et al., 2000). The relevance of phenolic compounds for human consumption has been associated with a protective effect against oxidative processes in relation to cardiovascular and central nervous system health as well as a reduced risk for cancers of the gastrointestinal tract (Björkman et al., 2011). Carotenoids, tetraterpenes belonging to isoprenoids, play a role in the protection against photo-oxidative processes and as organic

pigments; they are responsible for the orange-yellow color of fruits (Tapiero et al., 2004). Dietary carotenoids are thought to provide health benefits in decreasing the risk of eye disease and certain types of cancers due to their role as antioxidants (Johnson, 2002). Vitamins C and E, including ascorbic acid and tocopherols, respectively, are essential nutritional quality factors in fruits with many biological activities in humans (Miret and Müller, 2017). Considering the relevant role of all these bioactive compounds as health promoting compounds in fruits, the modulation of their accumulation is of paramount importance in both climacteric and non-climacteric fruits (Vasconsuelo and Boland, 2007). Most of these bioactive compounds are accumulated at high levels during fruit ripening, when less palatable green fruits converse into a nutritionally rich, colored, and tasty fruit (Daood et al., 1996; Ranalli et al., 1998; Dumas et al., 2003; Singh et al., 2011). However, once commenced, ripening cannot be stalled and generally leads to over-ripening that negatively affects the fruit quality (Kumar et al., 2014). Therefore, minimizing post-harvest fruit spoilage while obtaining high contents of bioactive compounds remains one of the biggest challenges to resolve.

Auxin has been identified as an important regulator of carotenoid biosynthesis during ripening of climacteric tomato fruits. Ripening in tomato is associated with the degradation of chlorophyll and the shift of xanthophylls to carotenes (β-carotene and lycopene) (Fraser et al., 1994). IAA appears to delay tomato ripening by repressing ethylene and several upstream carotenoid transcripts including *Psy, Ziso, Pds, Critiso* as well as *Chlases 1–3* and, on the other hand, promoting the accumulation of β-*Lyc1* and *Crtr*-β*1* transcripts, resulting in higher contents of xanthophylls and chlorophyll a (Su et al., 2015). In addition, in some non-climacteric fruits such as cherry and grape berries auxin also seems to modulate anthocyanin biosynthesis and thereby control fruit ripening processes. Teribia et al. (2016) reported a negative correlation between IAA and anthocyanin contents indicating that anthocyanin accumulation starts when IAA contents decrease. Moreover, the application of the synthetic auxin-like compound benzothiazole-2-oxyacetic acid (BTOA) delayed the up-regulation of genes that promote enzymes of the anthocyanin biosynthesis such as chalcone synthase and UDP-glucose:flavonoid 3-*O*-glucosyltransferase in grape berries (Davies et al., 1997).

Pre- and post-harvest treatments with salicylates, including SA and its derivatives ASA and MeSA, have been reported to regulate bioactive compounds accumulation leading to improved antioxidant activity in both climacteric and non-climacteric fruits. Several fruits such as plum, cherry and apricots are known to have a short life with a rapid deterioration in quality after harvest. Therefore, there is a constant search of treatments that improve and maintain fruit quality, and especially the content of bioactive compounds with beneficial health effects. Treatments of plums with SA, ASA, and MeSA resulted in significant higher contents of ascorbic acid, anthocyanins and phenolic compounds both at harvest and after prolonged cold storage (40 days at 4°C; Martínez-Esplá et al., 2017). Similar results could be observed for preharvest treatments with SA, ASA, and MeSA in sweet cherries (Giménez et al., 2014, 2015, 2017). Furthermore, post-harvest treatment with SA, ASA, or MeSA maintained total phenolic contents as well as anthocyanin contents during cold storage in pomegranate (Sayyari et al., 2011), sweet cherry (Valero et al., 2011), cornelian cherry (Dokhanieh et al., 2013), and apricot (Wang et al., 2015). These results suggest that salicylates may be involved in the activation of phenylalanine ammonia lyase, which is the main enzyme involved in the biosynthesic pathway of phenolic compounds (Martínez-Esplá et al., 2017).

Recently, melatonin has been shown to regulate fruit ripening and modulate bioactive compounds. Post-harvest treatments with melatonin in peach fruits increased chilling tolerance by activating antioxidant systems. Chilling injury occurs in peach fruits during low temperature storage (between 2 and 7.6°C) characterized by flesh browning, abnormal ripening and higher sensitivity to decay (Lurie and Crisosto, 2005). Cao et al. (2018) reported that peaches treated with melatonin showed higher transcript abundance of ascorbic acid biosynthesis genes *PpGME, PpGPP*, and *PpGLDH* at seventh and *PpGMPH* at the 21st and 28th day of storage compared to control resulting in increased ascorbic acid contents. Moreover, melatonin induced increases in activities of G6PDH, SKDH and PAL, which are the essential enzymes for phenolic compounds biosynthesis. The authors suggest that melatonin treatment protects peach fruit to a certain degree from chilling injury by specifically activating the biosynthesis of phenolic compounds (Gao et al., 2018). Additionally, a label-free differential proteomics analysis revealed the effect of melatonin on promoting fruit ripening and anthocyanin accumulation upon post-harvest in tomato fruits (Sun et al., 2016). The authors reported that exogenous melatonin increased eight enzymes related to the anthocyanin pathway including inter alia flavonol-3-hydroxylase, flavanone 3 beta-hydroxylase, anthocyanidin synthase/leucoanthocyanidin dioxygenase, and anthocyanidin 3-*O*-glucosyltransferase. Moreover, post-harvest treatment with melatonin increased total phenols and anthocyanins in strawberry fruits (Aghdam and Fard, 2016), and delayed loss of total phenols, flavonoids and anthocyanins in litchi fruits (Zhang et al., 2018). Treatments with melatonin increased the contents of phenols, anthocyanins and flavonoids in grape berries (Xu et al., 2017). Additionally, melatonin has been reported to enhance lycopene accumulation and ethylene production in tomatoes, suggesting that melatonin may increase the content of lycopene by impacting ethylene biosynthesis and signaling (Sun et al., 2015). The participation of ethylene in the melatonin-induced regulation of bioactive compounds has also been observed in grape berries, where double block treatment of ethylene showed reduced effects of melatonin on polyphenol contents (Xu et al., 2017).

CONCLUSION

It is concluded that chorismate-derived phytohormones, including auxin, SA and melatonin, not only share a common precursor but play essential roles in the regulation of fruit growth and ripening. A metabolic and functional cross-talk between them and with other phytohormones occurs in a spatiotemporal manner to finely regulate the development of climacteric and

non-climacteric fruits. The differences in the dynamics within climacteric and non-climacteric fruits evidence that the response of chorismate-derived hormones is not universal but rather strongly species-specific. Furthermore, chorismate-derived phytohormones also modulate the accumulation of bioactive compounds, thus influencing fruit quality. Further research is, however, needed to better understand how (i) other hormones, such as ethylene and ABA, modulate their biosynthesis studying key metabolic diversion points, (ii) they interact at the functional and molecular levels, and (iii) they jointly modulate bioactive compounds biosynthesis and consequently influence not only fruit growth and ripening, but also the quality of climacteric and non-climacteric fruits.

AUTHOR CONTRIBUTIONS

SM-B conceived and designed the review with the help of MP-L. MP-L, PM, MM, and SM-B wrote the manuscript. MP-L prepared figures with the help of PM. All authors contributed to the discussion of ideas, revised and approved the final manuscript.

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MINI REVIEW
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Developmental and Environmental Regulation of Cuticular Wax Biosynthesis in Fleshy Fruits

Priyanka Trivedi[1], Nga Nguyen[1], Anne Linn Hykkerud[2], Hely Häggman[1], Inger Martinussen[2], Laura Jaakola[2,3] and Katja Karppinen[1,3]*

[1] Department of Ecology and Genetics, University of Oulu, Oulu, Finland, [2] Norwegian Institute of Bioeconomy Research, Ås, Norway, [3] Climate Laboratory Holt, Department of Arctic and Marine Biology, UiT the Arctic University of Norway, Tromsø, Norway

The aerial parts of land plants are covered by a hydrophobic layer called cuticle that limits non-stomatal water loss and provides protection against external biotic and abiotic stresses. The cuticle is composed of polymer cutin and wax comprising a mixture of very-long-chain fatty acids and their derivatives, while also bioactive secondary metabolites such as triterpenoids are present. Fleshy fruits are also covered by the cuticle, which has an important protective role during the fruit development and ripening. Research related to the biosynthesis and composition of cuticles on vegetative plant parts has largely promoted the research on cuticular waxes in fruits. The chemical composition of the cuticular wax varies greatly between fruit species and is modified by developmental and environmental cues affecting the protective properties of the wax. This review focuses on the current knowledge of the cuticular wax biosynthesis during fleshy fruits development, and on the effect of environmental factors in regulation of the biosynthesis. Bioactive properties of fruit cuticular waxes are also briefly discussed, as well as the potential for recycling of industrial fruit residues as a valuable raw material for natural wax to be used in food, cosmetics and medicine.

Keywords: fruit, cuticle, cuticular wax, biosynthesis, regulation, temperature, light, bioactivity

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****Correspondence:***
Laura Jaakola
laura.jaakola@uit.no

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INTRODUCTION

The primary surfaces of aerial parts of land plants are covered by a hydrophobic layer called cuticle. The cuticle is composed of polyester cutin and a mixture of lipidic compounds collectively called wax. The chemical composition of cuticular wax varies between species and organs but is also dependent on the developmental stage and environmental conditions (Yeats and Rose, 2013). Cuticular wax appears as amorphous "intracuticular wax" embedded in cutin matrix, that is connected to the polysaccharides on the underlying epidermal cell walls, and as "epicuticular wax" that may exist as crystallized to various micro-morphologies (Koch and Ensikat, 2008; Fernández et al., 2016; Barthlott et al., 2017; **Figure 1**). Cuticle not only provides protection against desiccation but also has a role in plant development and environmental interactions (Yeats and Rose, 2013). In fleshy fruits, cuticular waxes have a crucial role in minimizing water loss/uptake through an often astomatous surface, providing mechanical support, preventing fruit softening, and in resistance to pathogens (Saladié et al., 2007; Martin and Rose, 2014; Wang J. et al., 2014). The cuticle in fruits is usually thicker than in leaves and the epicuticular wax is often visible to the naked eye as a white, dull, or glossy coating. Alterations in cuticular wax biosynthesis, load and composition take place during the fruit development to keep it continuous and adjusted to its tasks.

From a human perspective, fleshy fruits are an indispensable part of a healthy diet and cuticular wax affects important quality traits for consumers, such as fruit color, texture, shelf-life, sensory and nutritional quality, and preventing fruit cracking (Lara et al., 2014; Petit et al., 2017; Chu et al., 2018a; Tafolla-Arellano et al., 2018).

Recent reviews exist concerning cuticular wax biosynthesis in vegetative organs of plants (e.g., Lee and Suh, 2013; Yeats and Rose, 2013; Borisjuk et al., 2014) but also in fruits mainly focusing on cuticle composition (Lara et al., 2015), genetic regulation of cuticle assembly (Hen-Avivi et al., 2014) and role of cuticle in postharvest quality (Lara et al., 2014). The present review compiles the current knowledge on the developmental and environmental regulation of biosynthesis and composition of cuticular waxes in fleshy fruits.

CUTICULAR WAX COMPOSITION AND BIOSYNTHESIS IN FRUITS

The major components of plant cuticular waxes are very-long-chain fatty acids (VLCFAs, typically C_{20}–C_{34}) and their derivatives including alkanes, aldehydes, primary and secondary alcohols, ketones, and esters along with secondary metabolites, such as triterpenoids, sterols, tocopherols, and phenolic compounds (Kunst and Samuels, 2009; Yeats and Rose, 2013). The composition of cuticular wax varies widely among fruit species and cultivars (**Table 1**). While alkanes are common wax components in cuticles of different plant organs, triterpenoids are present especially in fruits (Szakiel et al., 2012). Triterpenoids and *n*-alkanes are the major compounds of cuticular wax in tomato (*Solanum lycopersicum*), apple (*Malus* × *domestica*), Asian pear (*Pyrus* spp.), sweet cherry (*Prunus avium*), peach (*Prunus persica*), and pepper (*Capsicum annuum*) fruits. Also, among wild tomatoes, alkanes are the dominant compounds but the content of triterpenoids varies between tomato species (Yeats et al., 2012). Instead, the cuticular wax in grape (*Vitis vinifera*), olive (*Olea europaea*), persimmon (*Diospyros kaki*), and blueberries (*Vaccinium* spp.) contain high amounts of triterpenoids but only traces of alkanes (**Table 1**).

Apart from alkanes and triterpenoids, many fruits have high proportions of other components in their cuticles. A recent study indicated high levels of primary alcohols and tocopherols in the cuticular wax of some pear cultivars (Wu et al., 2017, 2018). Plum (*Prunus domestica*) and some apple cultivars show high proportion of secondary alcohols in fruit cuticle, while tomato cuticle contains significant amounts of polyunsaturated constituents, including alken-1-ols and alkenes (Kosma et al., 2010). Aldehydes are abundant only in cuticles of some fruits, such as cucumber (*Cucumis sativus*), cranberry (*Vaccinium macrocarpon*), and *Citrus* fruits. Cuticular wax of bayberry (*Myrica pensylvanica*) uniquely consists of glycerolipids while blueberries contain high levels of β-diketones (**Table 1**).

Many of the cuticle properties are affected by the composition of wax. For example, wax composition rather than cuticle thickness has been indicated to affect water transpiration rate (Riederer and Schreiber, 2001). The presence of long-chain alkanes and aldehydes has been found to increase water impermeability of fruit cuticles, while triterpenoids and sterols have opposite effects (Vogg et al., 2004; Leide et al., 2007; Parsons et al., 2012; Wang J. et al., 2014; Moggia et al., 2016). Instead, triterpenoids were shown to enhance mechanical strength of persimmon fruit cuticle by functioning as nano-fillers (Tsubaki et al., 2013). Wax composition also affects epicuticular wax micro-morphology (Koch and Ensikat, 2008). Alkanes, aldehydes and alcohols were shown to promote the formation of epicuticular wax crystals in orange (*Citrus sinensis*) and apples (Liu et al., 2012, 2015; Yang et al., 2017).

The knowledge of cuticular wax biosynthesis has mainly been gained from the studies in *Arabidopsis* leaves, but also from tomato fruit owing to its thick, astomatous, easy-to-isolate cuticle and availability of mutants (Bernard and Joubès, 2013; Lee and Suh, 2013; Hen-Avivi et al., 2014). During recent years high-throughput sequencing has facilitated the identification of candidate genes involved in the fruit cuticle formation and wax biosynthesis in addition to tomato (Mintz-Oron et al., 2008; Matas et al., 2011) in apple (Albert et al., 2013; Legay et al., 2015), mango (*Mangifera indica*, Tafolla-Arellano et al., 2017), sweet cherry (Alkio et al., 2012, 2014), orange (Wang et al., 2016), pear (*Pyrus pyrifolia*, Wang Y. et al., 2014), and bayberry (Simpson and Ohlrogge, 2016).

The cuticular wax components are biosynthesized in the epidermal cells of fruit peel. The biosynthesis of aliphatic wax constituents utilizes C_{16} and C_{18} fatty acids produced by *de novo* synthesis in plastids (**Figure 1**). These precursors are elongated to C_{20}–C_{34} VLCFAs in endoplasmic reticulum (ER) by the fatty acid elongase (FAE) complex with β-ketoacyl-CoA synthase (KCS) as the rate-limiting enzyme of the complex (Kunst and Samuels, 2009; Yeats and Rose, 2013). Tomato *lecer6* mutant has shown that *KCS* plays a key role in wax aliphatic compound biosynthesis and determines the chain-length of VLCFAs in tomato fruit (Leide et al., 2007). The resulting VLCFAs can be converted into primary alcohols and esters by acyl reduction pathway or aldehydes, alkanes, secondary alcohols and ketones by decarbonylation pathway (Kunst and Samuels, 2009). In decarbonylation pathway, *CsCER1* and *CsWAX2* (*CER3*) of cucumber and *PaCER1* of sweet cherry was recently shown to play important roles in alkane biosynthesis (Alkio et al., 2012; Wang et al., 2015a,b), while *CsCER3* was linked to aldehyde biosynthesis in orange fruit (Wang et al., 2016). Also *CsCER4* linked to wax biosynthesis was recently identified in cucumber (Wang W. et al., 2018). Wax triterpenoids and sterols are biosynthesized from squalene produced from mevalonate (MVA) pathway followed by modifications into various compounds (Sawai and Saito, 2011; Thimmappa et al., 2014; **Figure 1**).

DEVELOPMENTAL REGULATION OF FRUIT CUTICULAR WAX FORMATION

Tomato is a model species for studying regulation of fleshy fruit development and ripening (Karlova et al., 2014). During the last decades, intensive studies in tomato performed in cuticle formation indicate connections in regulatory network between

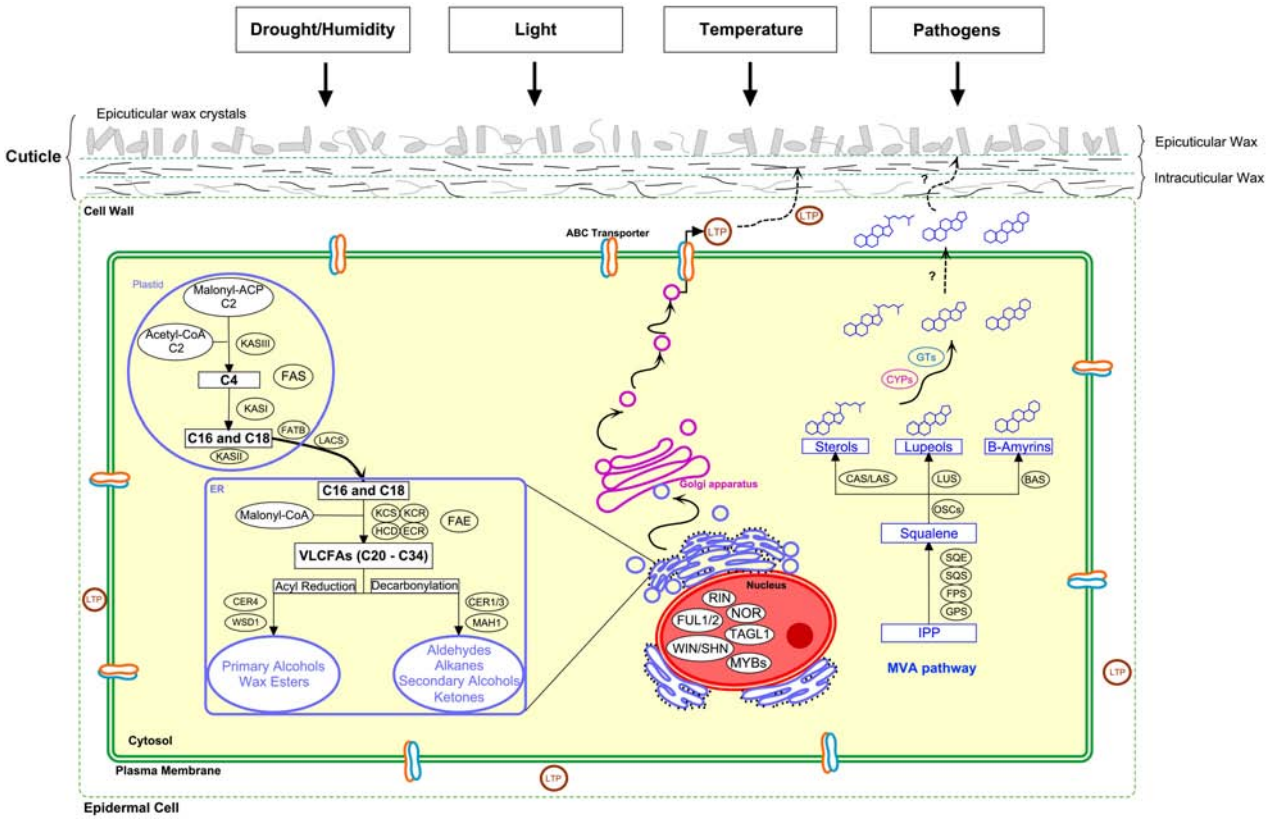

FIGURE 1 | Cuticular wax biosynthesis and interacting environmental factors. Cuticle has an important role as water barrier and in environmental interactions. Biosynthesis of aliphatic wax compounds starts with the generation of fatty acids in plastid by fatty acid synthase complex (FAS). The C_{16} and C_{18} precursors are hydrolyzed by acyl-ACP thioesterase (FATB) and converted to CoA thioesters by long chain acyl-CoA synthase (LACS) before transferred to endoplasmic reticulum (ER). In the ER, fatty acids are extended to very-long-chain fatty acids (VLCFAs) by fatty acid elongase (FAE) complex enzymes β-ketoacyl-CoA synthase (KCS), β-ketoacyl-CoA reductase (KCR), β-hydroxyacyl-CoA dehydratase (HCD), and enoyl-CoA reductase (ECR). VLCFAs are modified to primary alcohols by fatty acyl-CoA reductase (CER4) and further to wax esters by wax synthase (WSD1) through acyl reduction pathway. Decarbonylation pathway produces aldehydes, alkanes, secondary alcohols and ketones by enzymes including fatty acyl-CoA reductases (CER1/3) and a midchain alkane hydroxylase (MAH1). The compounds are transported to the plant surface through Golgi network and ABC transporters and by lipid transfer proteins (LTPs). Wax triterpenoids and sterols are derived from squalene that is produced from isopentenyl diphosphate (IPP) through mevalonic acid (MVA) pathway by geranyl pyrophosphate synthase (GPS), farnesyl pyrophosphate synthase (FPS), squalene synthase (SQS), and squalene epoxidase (SQE). Squalene is cyclized by oxidosqualene cyclases (OSCs) including cycloartenol synthase (CAS), lanosterol synthase (LAS), lupeol synthase (LUS), and β-amyrin synthase (BAS) to produce sterols, lupeols, and amyrins, respectively, which are modified by cytochrome P450 monooxygenases (CYPs) and glycosyltransferases (GTs) before transported to plant surface. TFs important for cuticle development are shown in the nucleus. Modified according to Kunst and Samuels (2009); Sawai and Saito (2011); Lee and Suh (2013); Yeats and Rose (2013); and Thimmappa et al. (2014).

cuticle and fruit development. Transcription factors (TFs) NON-RIPENING (NOR), and RIPENING INHIBITOR (RIN) are important regulators of fruit ripening, but tomato *nor* and *rin* mutants also show altered fruit cuticular wax profile from early stage throughout the fruit development (Kosma et al., 2010). In addition, other ripening regulators, including FRUITFULL (FUL1,2) and TOMATO AGAMOUS-LIKE1 (TAGL1), have been linked to fruit cuticle development (Bemer et al., 2012; Hen-Avivi et al., 2014; Giménez et al., 2015).

In climacteric fruits, including tomato and apple, plant hormone ethylene acts to initiate and co-ordinate ripening processes, while in many non-climacteric fruits abscisic acid (ABA) has been shown as ripening inducer (Cherian et al., 2014; Karppinen et al., 2018). Both ethylene and ABA signaling seems to play important roles in fruit cuticle biosynthesis (Ziv et al., 2018). Studies have indicated that ethylene accelerates cuticular wax accumulation in orange and apple (Ju and Bramlage, 2001; Cajuste et al., 2010; Li et al., 2017). The *Arabidopsis* members of the SHINE (WIN1/SHN1) clade of ethylene responsive factors (ERFs), transducing signal from ethylene, are well-characterized regulators of the cuticular wax biosynthesis (Aharoni et al., 2004; Broun et al., 2004). In tomato, *SlSHINE3* (*SlSHN3*) was shown to regulate fruit cuticle formation and cuticular lipid biosynthesis (Shi et al., 2013). Also the expression of sweet cherry, apple and mango homologs for *WIN1/SHIN1* coincided with fruit cuticle deposition (Alkio et al., 2012; Lashbrooke et al., 2015b; Tafolla-Arellano et al., 2017). Downstream to *SlSHN3*, MYB TF *SlMIXTA* has been shown to regulate fruit cuticle assembly in tomato (Lashbrooke et al., 2015a; Ewas et al., 2016). Recently, a grape berry-specific

TABLE 1 | The main cuticular wax compound classes in various fleshy fruits at mature stage and changes during fruit development.

Species	Main compound classes*	References
Tomato (*Solanum lycopersicum*)	Alkanes (*n*-hentriacontane, *n*-nonacosane) ∼, triterpenoids (amyrins) ∼	Bauer et al., 2004; Leide et al., 2007, 2011; Saladié et al., 2007; Mintz-Oron et al., 2008; Kosma et al., 2010; Petit et al., 2014
Wild tomato (*Solanum* spp.)	Alkanes (*n*-hentriacontane, *n*-nonacosane), triterpenoids (amyrins), esters	Yeats et al., 2012
Eggplant (*Solanum melongena*)	Alkanes (*n*-hentriacontane), alkanoic acids	Bauer et al., 2005
Apple (*Malus* × *domestica*)	Triterpenoids (ursolic acid) ↓, alkanes (*n*-nonacosane) ↓, primary and secondary alcohols ↑	Belding et al., 1998, 2000; Ju and Bramlage, 2001; Verardo et al., 2003; Legay et al., 2017; Yang et al., 2017; Leide et al., 2018
Asian pear (*Pyrus* spp.)	Alkanes (*n*-hentriacontane, *n*-nonacosane) ↓, triterpenoids (α-amyrin) ↑, primary alcohols (triacontanol, triacontane-1,30-diol) ↑, fatty acids ↑	Yin et al., 2011; Li et al., 2014; Heng et al., 2017; Wu et al., 2017, 2018
European pear (*Pyrus communis*)	Alkanes (*n*-hentriacontane), primary alcohols (triacontanol, triacontane-1,30-diol)	Wu et al., 2018
Sweet cherry (*Prunus avium*)	Triterpenoids (ursolic acid) ↓, alkanes (*n*-nonacosane) ↑, fatty acids	Peschel et al., 2007; Belge et al., 2014a; Rios et al., 2015
Peach (*Prunus persica*)	Triterpenoids (ursolic acid, oleanolic acid), alkanes (*n*-tricosane, *n*-pentacosane)	Belge et al., 2014b
Plum (*Prunus domestica*)	Secondary alcohols, alkanes (*n*-nonacosane)	Ismail et al., 1977
Grape (*Vitis vinifera*)	Triterpenoids (oleanolic acid) ↓, alcohols ↓	Radler, 1965; Comménil et al., 1997; Casado and Heredia, 1999; Pensec et al., 2014
Orange (*Citrus sinensis*)	Triterpenoids (friedelin, lupeol) ↑, aldehydes ↑, alkanes (*n*-hentriacontane) ∼, fatty acids ↓	Sala et al., 1992; Liu et al., 2012; Wang J. et al., 2014; Wang et al., 2016
Satsuma mandarin (*Citrus unshiu*)	Aldehydes (octacosanal) ↑, triterpenoids (friedelin) ∼, alkanes (*n*-nonacosane) ∼, fatty acids ∼	Sala et al., 1992; Wang J. et al., 2014
Grapefruit (*Citrus paradisi*)	Triterpenoids (friedelin), aldehydes	McDonald et al., 1993; Nordby and McDonald, 1994
Olive (*Olea europaea*)	Triterpenoids (oleanolic acid) ↓, primary alcohols ↑, fatty acid derivatives ∼	Bianchi et al., 1992; Huang et al., 2017
Persimmon (*Diospyros kaki*)	Triterpenoids (ursolic acid, oleanolic acid), alkanes, alcohols	Tsubaki et al., 2013
Pepper (*Capsicum annuum*)	Triterpenoids (amyrins), alkanes (*n*-hentriacontane)	Bauer et al., 2005; Kissinger et al., 2005; Parsons et al., 2012, 2013
Cucumber (*Cucumis sativus*)	Alkanes (*n*-nonacosane), aldehydes, fatty acids	Wang et al., 2015a,b
Blueberry (*Vaccinium corymbosum*)	Triterpenoids (ursolic acid, oleanolic acid) ∼, β-diketones ↓	Chu et al., 2017, 2018b
Blueberry (*Vaccinium ashei*)	Triterpenoids (ursolic acid) ↑, β-diketones ↓	Chu et al., 2017, 2018b
Cranberry (*Vaccinium macrocarpon*)	Triterpenoids (amyrins), aldehydes	Croteau and Fagerson, 1971
Bayberry (*Myrica pensylvanica*)	Glycerolipids (triacylglycerol, diacylglycerol)	Simpson and Ohlrogge, 2016

**Proportional change in cuticular wax during fruit development is indicated when information available. ↑, increased proportion; ↓, decreased proportion; ∼ no clear trend. The main compound(s) indicated in parentheses when information available.*

ERF *VviERF045*, resembling SHINE clade members, and *Malus* AP2/SHEN member *McWRI1* were indicated in regulation of cuticular wax biosynthesis (Leida et al., 2016; Hao et al., 2017). A connection between ABA and cuticular wax biosynthesis was demonstrated in orange fruit (Wang et al., 2016). In cucumber, ABA was shown to induce gene expression involved in cuticle alkane biosynthesis (Wang et al., 2015a,b).

Due to the multiple tasks, maintaining intact cuticle over the fruit development is necessary, but challenging, due to rapid and extensive surface expansion. Cuticular wax deposition starts early in fruit development (Comménil et al., 1997; Casado and Heredia, 2001; Curry, 2005; Domínguez et al., 2008). However, the pattern of wax load varies markedly between species (in contrast to cutin load) and indicates separately regulated wax biosynthesis from cutin biosynthesis (Wang et al., 2016). In many fruits, including apple (Ju and Bramlage, 2001; Lai et al., 2016), orange (Liu et al., 2012; Wang et al., 2016), pear (Li et al., 2014), blueberries (Chu et al., 2018b), bayberry (Simpson and Ohlrogge, 2016), and mango (Tafolla-Arellano et al., 2017), cuticular wax load increases during the fruit development leading to a thick cuticle at maturity. Furthermore, in many fruits, modification of the wax chemical profile and cuticle accumulation, even after harvest has been reported (Ju and Bramlage, 2001; Belge et al., 2014a,b; Tafolla-Arellano et al., 2017; Yang et al., 2017). Tomatoes also have a thick cuticle at maturity but there are clear cultivar-specific variations in cuticle development (España et al., 2014). In cherry tomatoes, cuticular wax is deposited early in fruit development (Domínguez et al., 2008), while in medium-sized tomatoes, such as "Micro Tom" and "Ailsa Craig," the wax amount reaches its maximum level at orange-colored stage (Leide et al., 2007; Mintz-Oron et al., 2008) and in some other cultivars the wax amount increases continuously toward the fruit maturity (Bauer et al., 2004). In tomato, all the wax compound classes, except branched alkanes, accumulate during the cuticular

wax load (Leide et al., 2007; Mintz-Oron et al., 2008; Kosma et al., 2010). However, in many cases, the continuous wax load leads to changes in the cuticular wax profile during the fruit development (**Table 1**). For example, in apple, hydrocarbons and triterpenoids predominate in cuticles of young fruits while fatty acids, alcohols and esters contribute mostly to the wax increase during fruit ripening increasing wax greasiness (Ju and Bramlage, 2001; Yang et al., 2017).

High cuticular wax deposition rate at the early stages of fruit development followed by reduction at later stages has been described for sweet cherry (Peschel et al., 2007; Alkio et al., 2012; Lai et al., 2016) and grape (Comménil et al., 1997; Becker and Knoche, 2012; Pensec et al., 2014). The decrease in sweet cherry wax load toward fruit maturity was mainly attributed to the decrease in triterpenoids (Peschel et al., 2007). Similarly, the total triterpenoids decreased during the development of grape berries (Pensec et al., 2014). The role of cuticle as a mechanical support at fruit ripening is important when degrading cell walls cannot sustain the fruit internal pressure. Thus, the inability of the wax deposition to keep in the pace with surface expansion makes ripening fruits vulnerable for micro- and macro-cracking leading to uncontrolled water movement and fungal infections (Comménil et al., 1997; Børve et al., 2000). Cracking is a serious problem in many fruit species, such as tomato and cherries (Domínguez et al., 2012). Recently, an association between cuticular *n*-nonacosane level and cracking tolerance among sweet cherry varieties was described by Rios et al. (2015). Failure in cuticle deposition associated with micro-cracking can cause formation of russeting, a common disorder in fruits, such as apples and pears (Khanal et al., 2013). Improper cuticular wax deposition was shown to be accompanied by the decreased expression of wax biosynthetic genes and *MdSHN3* TF in russeted apples (Lashbrooke et al., 2015b; Legay et al., 2015, 2017).

ENVIRONMENTAL REGULATION OF FRUIT WAX BIOSYNTHESIS AND COMPOSITION

Being a protective barrier on fruit surface, cuticle has a crucial role in the tolerance to various environmental stresses (**Figure 1**), including osmotic stress (Shepherd and Griffiths, 2006; Xue et al., 2017). Both drought stress and humidity have been shown to affect cuticle deposition. In general, a decrease in cuticle deposition has been detected in plants under high humidity (Tafolla-Arellano et al., 2018). In tomato fruit, decreased cuticle thickness was detected in high humidity, but had no effect on wax accumulation (Leonardi et al., 1999; Domínguez et al., 2012). Instead, plants adapted to water deficit conditions usually have well-developed cuticles in fruits (Crisosto et al., 1994; Barker and Procopiou, 2000; Xue et al., 2017). Regulation of cuticular wax biosynthesis in response to drought stress has been most intensively studied in *Arabidopsis* but also in tomato and cucumber (Xue et al., 2017). In tomato, overexpression of *SISHN1* TF induced expression of wax biosynthetic genes leading to enhanced cuticular wax deposition and drought-tolerance compared to control plants (Al-Abdallat et al., 2014). In cucumber, the expression of fruit-specific cuticular wax genes *CsCER1* and *CsWAX2* increased under drought and salinity stresses (Wang et al., 2015a,b). Furthermore, transcriptome level studies in drought-sensitive cucumber variety suggested that the decreased expression of cutin, suberin, and wax biosynthetic genes might be responsible for sensitivity to drought (Wang M. et al., 2018).

Both light and temperature can directly change the morphology and properties of fruit epicuticular wax (Schirra et al., 1999; Charles et al., 2008). For example, a post harvest heat treatment at 38°C was shown to affect the structure of the epicuticular wax in apple (Roy et al., 1994). However, temperature changes can also modify the biosynthesis of fruit cuticular waxes. Since wax layer is important in maintaining postharvest quality (Lara et al., 2014; Chu et al., 2018a), most temperature treatments have been performed on postharvest fruits. In *Malus* fruits, low temperature treatment (+4°C) increased the thickness of cuticular wax compared to control fruits and up-regulated the expression of *McWRI1*, *McKCS*, *McLACS*, and *McWAX* leading to the accumulation of alkanes (Hao et al., 2017). Similarly, expression of cucumber fruit-specific *CsCER1* and *CsWAX2* were induced by low temperature (Wang et al., 2015a,b). Changes in fruit cuticular wax content and composition during cold storage have also been reported for blueberries (Chu et al., 2018b), Asian pears (Wu et al., 2017), grapefruit (*Citrus paradisi*, Nordby and McDonald, 1991), and sweet cherries (Belge et al., 2014a).

Cuticle is the first barrier to receive light radiation. The increase in thickness of the cuticular wax layer as a response to higher irradiation has been shown in many plant species (Shepherd and Griffiths, 2006; Tafolla-Arellano et al., 2018). In grape berries, the cuticle amount was reported to be higher in sun-exposed berries compared to berries developed in canopy shade (Rosenquist and Morrison, 1989). Also, the spectral quality of light affects the cuticular wax biosynthesis and several reports show that cuticular wax plays a role in the protection against damaging UV-light. Irradiation with enhanced UV-B or UV-C has been demonstrated to increase total amount of cuticular wax and alter wax composition (Tafolla-Arellano et al., 2018). Monochromatic far-red light was shown to stimulate the cuticular wax biosynthesis increasing hydrophobicity of the wax in both tomato and bell pepper fruits during storage (Cozmuta et al., 2016a,b). In grapefruit and mango, interaction of light and temperature conditions affected fruit cuticle accumulation and cuticular wax composition considering difference between fruits growing in interior or exterior canopy (McDonald et al., 1993; Léchaudel et al., 2013).

BIOACTIVITY AND COMMERCIAL POTENTIAL OF WAXES

Cuticle serves as a primary defense against pathogens and affects susceptibility of fruits to pathogens (Comménil et al., 1997; Saladié et al., 2007; Shi et al., 2013). It was shown in sweet orange and pepper that fruits respond to fungal infections by increasing the cuticle load (Kim et al., 2004; Marques et al., 2012). Agudelo-Romero et al. (2015) reported

that grape berries infected with *Botrytis cinerea* accumulated saturated long-chain fatty acids with simultaneous up-regulation of genes related to lipid and wax biosynthesis, including acyl-CoA synthetases (LACSs). A transcriptome analysis of *Colletotrichum gloeosporioides* infected tomato fruits showed activation of genes linked to the formation of cuticular wax VLCFAs (Alkan et al., 2015). Also, a contact of orange fruit with yeast *Kloeckera apiculata* was shown to trigger biosynthesis of cuticular waxes and expression of *CsKCSs* leading to increased wax hydrophobicity and changes in wax morphology (Liu et al., 2014).

In addition to cuticles acting as physical barriers, recent findings suggest that cuticle composition rather than thickness determines fruit susceptibility to pathogens (Reina-Pinto and Yephremov, 2009; Ziv et al., 2018). Fruit cuticular waxes are especially rich sources of triterpenoids, which have clear bioactive properties, such as anticancer, anti-inflammatory, antimicrobial and cardioprotective (Dzubak et al., 2006; Szakiel et al., 2012). He and Liu (2007) isolated triterpenoids from apple peels and reported antiproliferative activity against human cancer cells. The antifungal activity of Asian pear fruit cuticular wax was associated with *n*-alkanes, fatty acids along with triterpenoids (Yin et al., 2011; Chen et al., 2014; Li et al., 2014).

Plant cuticles potentially offer a natural alternative for synthetic waxes. Industrial leftover material in particular, such as peels from juice production, provides raw material for isolating fruit wax compounds. For example, extraction of apple peel pomace using supercritical fluid extraction (SFE) demonstrated the reuse potential of juice industry leftovers as a source for value-added wax (Li et al., 2015). Recently, Tedeschi et al. (2018) demonstrated the utilization of fatty acids from tomato pomace waste for production of packaging films. Thus, fruit cuticular waxes from industrial waste can provide sources for bioactive compounds and biodegradable products for the use in pharmaceuticals, cosmetics, packaging, nanocoatings, and the food industry.

AUTHOR CONTRIBUTIONS

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Inside and Beyond Color: Comparative Overview of Functional Quality of Tomato and Watermelon Fruits

*Riadh Ilahy[1], Imen Tlili[1], Mohammed Wasim Siddiqui[2], Chafik Hdider[1] and Marcello Salvatore Lenucci[3]**

[1] Laboratory of Horticulture, National Agricultural Research Institute of Tunisia (INRAT), University of Carthage, Tunis, Tunisia, [2] Department of Food Science and Postharvest Technology, Bihar Agricultural University, Bhagalpur, India, [3] Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università del Salento (DiSTeBA), Lecce, Italy

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***Correspondence:**
Marcello Salvatore Lenucci
marcello.lenucci@unisalento.it

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The quali-quantitative evaluation and the improvement of the levels of plant bioactive secondary metabolites are increasingly gaining consideration by growers, breeders and processors, particularly in those fruits and vegetables that, due to their supposed health promoting properties, are considered "functional." Worldwide, tomato and watermelon are among the main grown and consumed crops and represent important sources not only of dietary lycopene but also of other health beneficial bioactives. Tomato and watermelon synthesize and store lycopene as their major ripe fruit carotenoid responsible of their typical red color at full maturity. It is also the precursor of some characteristic aroma volatiles in both fruits playing, thus, an important visual and olfactory impact in consumer choice. While sharing the same main pigment, tomato and watermelon fruits show substantial biochemical and physiological differences during ripening. Tomato is climacteric while watermelon is non-climacteric; unripe tomato fruit is green, mainly contributed by chlorophylls and xanthophylls, while young watermelon fruit mesocarp is white and contains only traces of carotenoids. Various studies comparatively evaluated *in vivo* pigment development in ripening tomato and watermelon fruits. However, in most cases, other classes of compounds have not been considered. We believe this knowledge is fundamental for targeted breeding aimed at improving the functional quality of elite cultivars. Hence, in this paper, we critically review the recent understanding underlying the biosynthesis, accumulation and regulation of different bioactive compounds (carotenoids, phenolics, aroma volatiles, and vitamin C) during tomato and watermelon fruit ripening. We also highlight some concerns about possible harmful effects of excessive uptake of bioactive compound on human health. We found that a complex interweaving of anabolic, catabolic and recycling reactions, finely regulated at multiple levels and with temporal and spatial precision, ensures a certain homeostasis in the concentrations of carotenoids, phenolics, aroma volatiles

and Vitamin C within the fruit tissues. Nevertheless, several exogenous factors including light and temperature conditions, pathogen attack, as well as pre- and post-harvest manipulations can drive their amounts far away from homeostasis. These adaptive responses allow crops to better cope with abiotic and biotic stresses but may severely affect the supposed functional quality of fruits.

Keywords: antioxidants, aromas, biosynthetic pathways, carotenoids, *Citrullus lanatus*, *Solanum lycopersicum*, phenolics, vitamin C

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) and watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai var. *lanatus*] fruits enter frequently in our diet as fresh or processed products contributing to the intake of antioxidants and high nutritional value bioactives. Although botanically distant, both species show strong similarities in the chemical profiles of some secondary metabolites, especially the carotenoid pigments of ripe fruits. Among these, lycopene represents the compound to which both fruits owe the health-promoting popularity; nevertheless, many other molecules may synergistically contribute to their functional quality and antioxidant properties. With a global production of 183 and 119 million tons, tomato and watermelon fruits are among the main vegetable crops grown and consumed all over the world, and constitute the main sources of dietary lycopene in eastern and western cultures. Their importance on a global level greatly exceeds that of other lycopene containing fruits characterized by lower production (13 and 9 million tons for grapefruit and papaya, respectively) and per capita consumption (FAO STAT, 2019[1]).

Besides, tomato fruit color mutations have phenotypic equivalents in watermelon, suggestive of a similar regulation of pigment and related metabolites' biosynthesis and accumulation. Actually, several endogenous and exogenous factors affect the amounts and profiles of all these compounds and cross-talk to finely regulate their metabolic pathways, though they are, in turn, influenced by the different ripening physiology and developmental programs of the two fruits.

Although a wide scientific literature is available on the functional properties and secondary metabolism regulation of tomato and watermelon fruits, only recently some studies comparatively evaluated the development of carotenoid pigmentation during ripening (Lewinsohn et al., 2005b; Tadmor et al., 2005) or the alterations of carotenoid profiles during processing (Aguiló-Aguayo et al., 2009; Aganovic et al., 2017). However, in most cases, the other classes of bioactives received little or no consideration. Here, a comparative overview of the recent finding on the biosynthesis, accumulation and regulation of carotenoids, phenolics, aromas and vitamin C is given in order to emphasize the main similarities/differences between the two fruits at full maturity and during ripening and to highlight the rising concerns about the possible harmful effects of excessive bioactive assumption on health.

[1]http://www.fao.org/faostat/en/#data/QC

CAROTENOID IN TOMATO AND WATERMELON FRUITS

Chemical Features and Functions

Carotenoids are natural tetraterpenoid pigments largely produced by plants, algae, phototropic bacteria and some mycetes (Enfissi et al., 2017). In green plant tissues, their biosynthesis takes place within chloroplasts, mostly in the inner envelope and the thylakoid bilayer. Chloroplasts accumulate high levels of lutein, β-carotene, violaxanthin, and neoxanthin, but the green of chlorophylls masks their characteristic yellow/orange color (Sun et al., 2018). Within chloroplasts, carotenoids play essential roles as accessory pigments to maximize light harvesting efficiency of photosystems, and act as chemical buffers against photo-oxidation of the cell constituents detoxifying the triplet state chlorophyll molecules and the highly reactive oxygen species (ROS) produced by photosynthesis (Hashimoto et al., 2016).

In Angiosperms, carotenoids are also responsible for the bright yellow to red pigmentation of many organs, especially flowers and fruits, and are the precursors for the synthesis of scents and flavors involved in the attraction of pollinators and seed dispersors. Non-photosynthetic tissues may accumulate carotenoids within chromoplasts, organelles specialized in their massive biosynthesis and sequestration (Sun et al., 2018). Moreover, carotenoids are intermediates of the synthesis of abscisic acid (ABA) and strigolactones (SLs), key phytohormones regulating plant development and environmental stress responses (Al-Babili and Bouwmeester, 2015; Hou et al., 2016).

Besides their central functions in plants, carotenoids are also essential for human nutrition and health. They provide dietary sources of provitamin A and reduce the incidence of some chronic and age-related pathologies, acting as antioxidants and/or via other, yet not fully understood, molecular mechanisms. These embrace the modulation of gene expression, the regulation of cell metabolism and hormone production, as well as the promotion of immune responses and cell gap-junctional communication (Perveen et al., 2015). Carotenoid content and profiles have become, hence, actual quality traits for many horticultural species, directly influencing crop productivity, industrial demand, consumer appeal, nutritional quality and health promoting properties (Yuan et al., 2015).

The intense research resulting from the multifaceted interest on these pigments triggered the identification and characterization of all genes and enzymes involved in carotenoid biosynthetic and catabolic core reactions (Nisar et al., 2015). It is now well established that the enzymes involved in carotenoid

metabolism are encoded by nuclear genes, synthesized in the cytosol, translocated within plastids, and sorted to specific organelle sub-domains depending on plastid type and morphology (Sun et al., 2018). In chloroplasts, sub-plastidial proteomic studies located most carotenogenic enzymes in the envelopes, except for violaxanthin de-epoxidase (VDE), which was associated to thylakoids, and zeaxanthin epoxidase (ZEP), found in both membrane systems (Ytterberg et al., 2006; Joyard et al., 2009). Carotenoid metabolites and enzymes were also detected in thylakoid-associated plastoglobules (PGs), lipoprotein particles identified as a site of carotenoid breakdown for apocarotenoid production and trafficking (Rottet et al., 2016).

Regarding chromoplasts, the information on enzyme sub-organelle location is much more fragmented; however, ζ-carotene desaturase (ZDS), lycopene β-cyclase (LYCB), and two β-carotene hydroxylases (BCH1/2) were identified in PGs isolated from red-bell peppers chromoplasts, suggesting a specific function in carotenoid biosynthesis, beyond the known role as storage/sequestering compartments (Siddique et al., 2006).

Biosynthesis

Although in higher plants the biosynthesis of carotenoids occurs via the general isoprenoid pathway (**Figure 1**), their amount, composition and ratio are amazingly reliable in the photosynthetic tissues of different species, while large variation exists in non-green tissues even within the same crop (Yuan et al., 2015). In some fruits, the rate of carotenoid synthesis increases substantially during ripening alongside their hyper-accumulation into pigment-bearing sequestration sub-compartments, and results in dramatic changes in tissue colouration according to specific genetic programs, as well as in response to molecular, developmental and environmental stimuli. The ripe berries of tomato and the fleshy endocarp of watermelon peponides are clear examples of this variability, as a wide range of fruit colors and shades characterize both species (**Figure 2**).

Ordinary red tomato and watermelon cultivars appear to have a similar carotenoid biosynthetic pathway leading to the synthesis and accumulation of large amounts of lycopene as major fruit pigment (Tadmor et al., 2005). Lycopene is the linear carotene responsible of the distinctive color of both fruits at full ripeness and has aroused considerable interest as health-promoting phytochemical, because its dietary intake positively correlates with lowered risk of coronary heart disease, prostate, and lung cancers (Carluccio et al., 2016; Bruno et al., 2018; Rao et al., 2018).

In most plants, lycopene is an intermediate in the biosynthesis of other carotenoids and is cyclized at both ends by specific lycopene cyclase enzymes (LYCB/CYCB and LYCE) to α- and/or β-carotenes. However, in tomato and watermelon fruits, the downstream gene expression of *LYCB*/CYCB and *LYCE* is downregulated resulting in the break of the metabolic flux with the consequent accumulation of the upstream product (Lv et al., 2015; Enfissi et al., 2017). Lycopene accounts, in fact, for more than 85% of total carotenoids in many red-ripe tomato cultivars and for even higher percentages (>90%) in red-fleshed watermelons. In both fruits much lower concentrations of β-carotene (<10% and <5%, respectively) are typically found, while the content of other carotenes and xanthophylls is almost negligible (Tadmor et al., 2005; Perkins-Veazie et al., 2006; Tlili et al., 2011a; Liu et al., 2015).

Fruit Concentration and Distribution

Genotype is a major determinant of the extent of variability in the content of carotenoids of ripe fruits in both tomato and watermelon. Lycopene and β-carotene levels in the range of 10–150 and 3.0–12.5 mg/kg fresh weight (fw), respectively, are common in ordinary red tomato cultivars; similarly, in most red-fleshed watermelon lines of commercial importance, lycopene varies between 30 and 70 mg/kg fw and β-carotene between 1.2 and 10.2 mg/kg fw (Perkins-Veazie et al., 2001, 2006; Ilahy et al., 2018). It is worthwhile mentioning that the mean lycopene concentration of watermelon (48.7 mg/kg fw) is about 40% higher than the mean for conventional red-ripe raw tomato (30.3 mg/kg fw). This indicates both species as comparable dietary sources of this powerful functional compound, although tomato and/or its numerous industrial products enter almost daily in the diet of most populations all around the world, while watermelon consumption is generally restricted to the summer season. Furthermore, a study on healthy subjects revealed that lycopene from untreated watermelon juice is just as bio-available as that from tomato juice subjected to heat, a treatment assumed to improve lycopene bioavailability (Edwards et al., 2003).

The introgression of spontaneous or induced color mutations is widely used to increase the levels and diversify the profile of carotenoids of tomato and watermelon and contributes to broaden the high variability characterizing these fruits. Many monogenic mutations affecting fruit carotenoid pigmentation have been isolated in tomato since the late 1940s and their molecular basis have been elucidated. Some of them trigger a deeper red colouration of the ripe fruits by increasing lycopene content. Several high-lycopene (HLY) tomato lines have been developed so far by introgression of *high-pigment* (*hp*) and *old-gold* (*og*) mutations (Ilahy et al., 2018). *Hp* tomato mutants all share a common phenotype characterized by enhanced plastid number and size, and consequential increased pigmentation of both unripe and ripe fruits, but they result from mutations of different genes. *Hp-1* and *hp-2* mutants carry single nucleotide alterations in the tomato *UV-damaged DNA-binding protein 1* (*DDB1*) and *DE-ETIOLATED1* (*DET1*) loci, respectively (Mustilli et al., 1999; Lieberman et al., 2004). Both mutations determine an exaggerated phytochrome-mediated response to light resulting in increased levels of carotenoids, mainly lycopene, and other antioxidant molecules (phenols, flavonoids, and vitamin C) in the ripe fruits, compared to their near isogenic wild-type counterparts (Liu et al., 2004; Levin et al., 2006). *Hp-3* mutants, instead, are not photomorphogenic, but harbor allelic mutations in the zeaxanthin epoxidase (*ZEP*) gene leading to a drastic reduction in the conversion of zeaxanthin to violaxanthin, and ultimately synthesis of ABA, whose deficiency is proposed to cause the enlargement of plastid compartment size by increasing their division rate (Galpaz et al., 2008). *Og* and *old-gold crimson* (og^c), are both recessive null alleles of the *Beta* (*B*) locus coding for a tomato chromoplast-specific form of β-cyclase (CYCB) that increase ripe fruit lycopene concentration at expense of

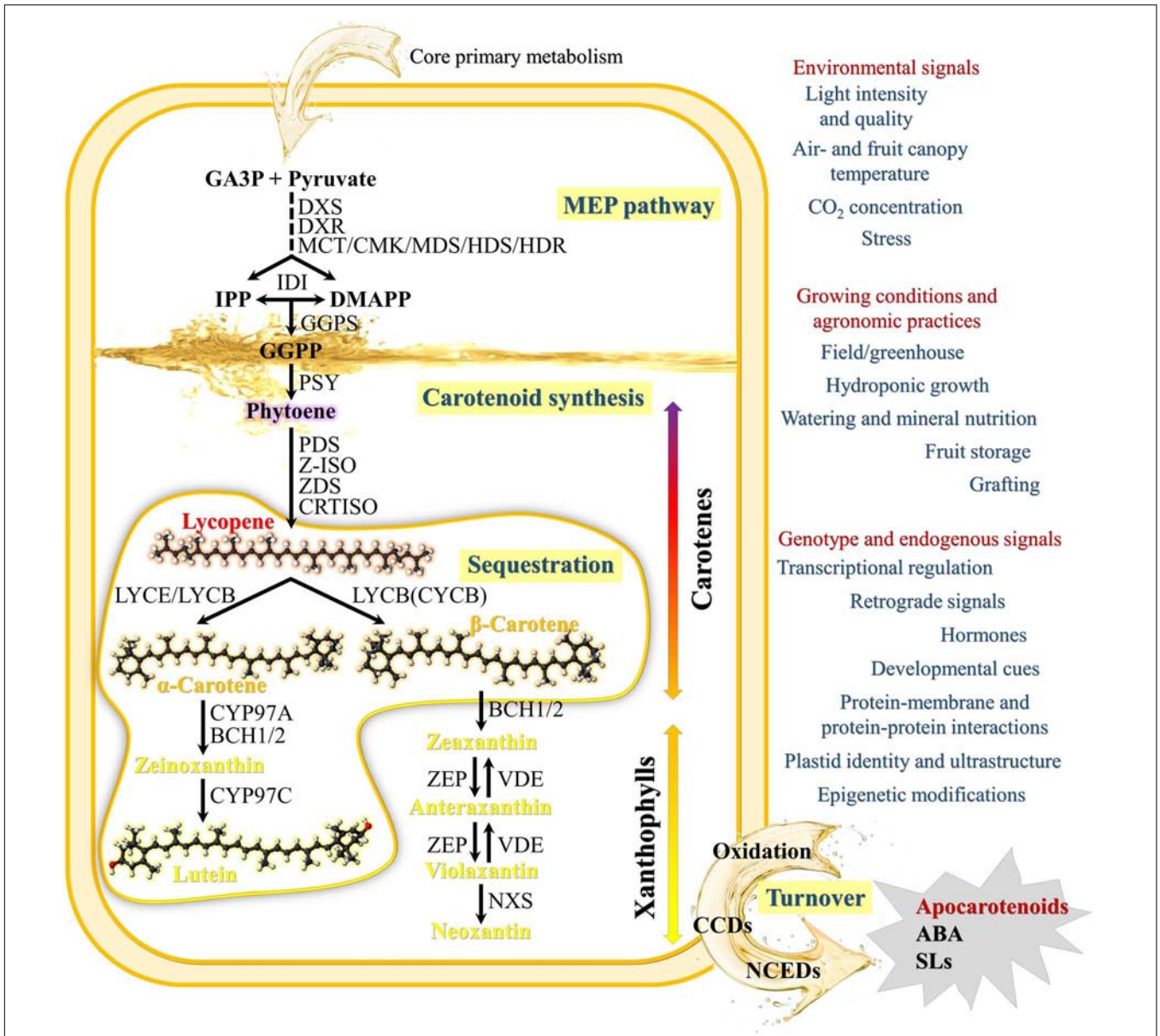

FIGURE 1 | Schematic Carotenoid Metabolic Pathway and main factors affecting carotenoid synthesis and accumulation. The pathway shows the primary steps found in nearly all plant species. The synthesis of carotenoids from core primary metabolism initiates via the plastid-localized 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, leading to the production of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), central precursors of other isoprenoid metabolites including tocopherols, chlorophylls, quinones, gibberellins (GA), and monoterpenes. The first step of the MEP pathway involves the transketolase-type condensation of pyruvate and D-glyceraldehyde 3-phosphate (GA3P) to 1-deoxy- D-xylulose 5-phosphate (DXP) and is catalyzed by the enzyme DXP synthase (DXS). MEP is subsequently formed via an intramolecular rearrangement and reduction of DXP by the enzyme DXP reductoisomerase (DXR). MEP is then converted in IPP and DMAPP by a number of consecutive steps catalyzed by five independent enzymes: 2-*C*-methyl-D-erythritol 4-phosphate cytidyltransferase (MCT), 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase (CMK), 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS), 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (HDS), and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR). IPP isomerase (IDI) catalyzes the isomerization of the relatively un-reactive (IPP) to the more-reactive DMAPP. Geranylgeranyl diphosphate (GGPP), the immediate C_{20} ubiquitous isoprenoid precursor in the synthesis of all plastid carotenoids, is generated by geranylgeranyl diphosphate synthase (GGPS) that catalyzes the condensation of three IPP and one DMAPP units. The first committed step in carotenoid biosynthesis is the condensation of two molecules of GGPP by phytoene synthase (PSY) to form the C_{40} carotenoid phytoene as a 15-*(Z)* isomer. Phytoene is converted to all-*trans*-lycopene by sequential desaturation and *Z-E* isomerization reactions. In plants, at least four enzymes are required: phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), ζ-carotene isomerase (Z-ISO) and carotenoid isomerase (CRTISO). PDS/ZISO and ZDS/CRTISO constitute metabolic units involved in the steps catalyzing the synthesis of 9,9′-di-*(Z)*-ζ-carotene, and *all(E)*-lycopene, respectively (Fantini et al., 2013). Cyclization of lycopene with lycopene ε- (LCYE) and β-cyclases (LCYB) is a crucial branch-point in carotenoid metabolism and generates carotenoid diversity distinguished by different cyclic end groups. In one branch, a single enzyme, lycopene β-cyclase (LCYB or CYCB in tomato fruits), introduces a β-ring at both ends of lycopene to form β-carotene in

(Continued)

FIGURE 1 | Continued
a sequential two-step reaction. The first dedicated reaction in the other branch, leading to lutein, requires both LCYE and LCYB to introduce one β- and one ε-ring into lycopene to form α-carotene. α-carotene and β-carotene are further hydroxylated to produce xanthophylls (e.g., lutein and zeaxanthin). α-carotene is acted upon by β-ring hydroxylases (CYP97A and BCH1/2) to form zeinoxanthin, which is then hydroxylated by an ε-ring hydroxylase (CYP97C) to lutein. β-carotene can be hydroxylated in a two-step reaction to zeaxanthin, with β-cryptoxanthin as an intermediate product by BCH1/2. Zeaxanthin epoxidase (ZEP) hydroxylates β-rings of zeaxanthin in two consecutive steps to yield antheraxanthin and then violaxanthin. Zeaxanthin can be epoxidized to violaxanthin, and a set of light- and dark-controlled reactions known as the xanthophyll cycle rapidly optimize the concentration of violaxanthin and zeaxanthin in the cell through the action of zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), respectively, via antheraxanthin. Violaxanthin is converted to neoxanthin by neoxanthin synthase (NXS), which represents the final step in the core carotenoid biosynthetic pathway. Catabolism of carotenoids proceeds by their cleavage by enzymes of the carotenoid cleavage dioxygenase family (CCDs) and 9-(*Z*)-epoxy-carotenoid dioxygenase family (NCEDs) to produce various apocarotenoids, phytohormones (ABA and SLs) and isoprenoid volatiles. In addition to enzymatic cleavage, oxidation via peroxidases/lipo-oxygenases or non-enzymatic photochemical oxidation of carotenoids have also evidenced especially in photosynthetic tissues under high light stress and suggested to mediate carotenoid homeostasis.

β-carotene. In *og* tomatoes, the lack of feedback inhibition by β-carotene (or one of its metabolites) might further increase the activity of the upstream enzymes during ripening resulting in lycopene hyper-accumulation, as suggested by Bramley (2002). Depending upon mutation, HLY red-ripe tomato lines contain variable amounts of lycopene and β-carotene, but usually higher (up to twofold) than the respective ordinary control cultivars (Ilahy et al., 2018).

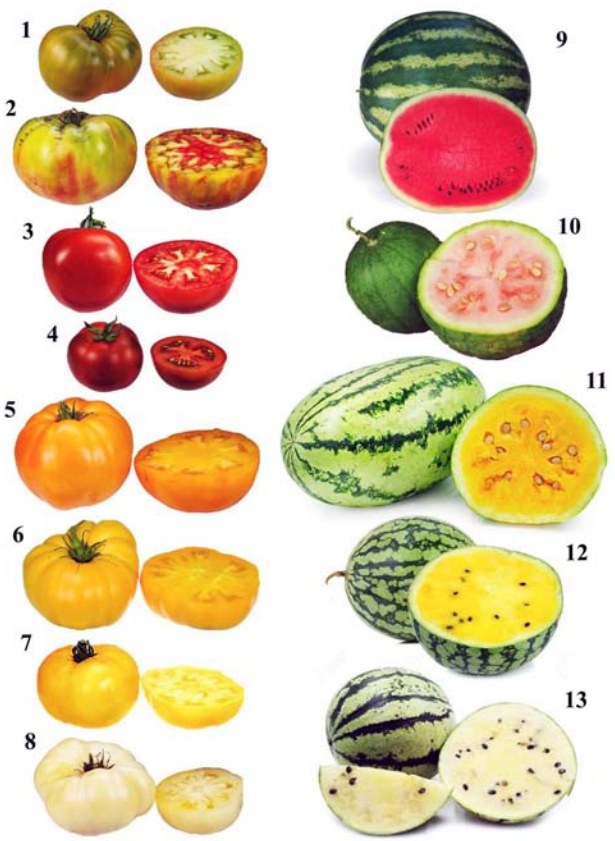

FIGURE 2 | Example of color variability in tomato and watermelon. 1, Cherokee Green (green); 2, Ananas Noir (multicolor); 3, Primo Red (red); 4, Geronimo (dark red); 5, Black Velvet (orange); 6, Orange Slice (yellow); 7, Lemon Boy (bright yellow); 8, White Wonder (white); 9, Crimson Sweet (red); 10, Malali (salmon), 11, Tendersweet (orange); 12, Yellow Doll (yellow); 13, White Wonder (white).

Along with red cultivars, many white, yellow, and orange tomato heirloom or hybrid genotypes have been described and characterized for their diverse carotenoid profiles and contents. Most of these color variants are the results of mutations in single genes coding for enzymes involved in different steps of the carotenoid biosynthetic pathway. Some white and yellow tomato genotypes show a strong reduction (or even a complete lack) of carotenoids, due to a loss-of-function mutation of the *PSY-1* (known as *yellow-flesh*, locus *r*) gene. This determines a block in the first committed step of the biosynthetic pathway and results in the pale-yellow flesh, and more intensely yellow-colored skin phenotype typical of the ripe fruits (Lewinsohn et al., 2005b). Different orange cultivars were found to accumulate poly*(Z)*-lycopene (pro-lycopene), β-carotene or δ-carotene instead of *all(E)*-lycopene. Pro-lycopene accumulation in *tangerine* (*t*) tomatoes is due to a mutation in the *CRTISO* gene coding for a carotenoid isomerase catalyzing the desaturation of carotenoids from the poly*(Z)*- to the *all(E)*-configuration (Isaacson et al., 2002). The accumulation of β- or δ-carotene is, instead, the result of the upregulation of one of the two lycopene cyclase genes. In particular, the dominant *Beta* (*B*) mutation is responsible of the upregulation *CYCB* leading to the cyclization of lycopene to β-carotene, while *Delta* (*Del*) mutants result from an upregulation of *LCYE*, involved in the synthesis of δ-carotene (Ronen et al., 2000).

Like tomato, several deep red-fleshed watermelon cultivars have been bred. These lines show high lycopene contents ranging from 80 to over 120 mg/kg fw. Further, watermelon also exhibits a wide range of fruit color phenotypes [canary yellow, salmon yellow, orange, deep-red, and white (**Figure 2**)] supposed to result by the expression of orthologous of the *og*, *r*, *t*, *Del,* and *B* tomato mutations (Tadmor et al., 2005; Zhao et al., 2013). However, this categorization, based exclusively on the HPLC carotenoid profiles, was shown to be often inconsistent. In the orange-fleshed watermelon cultivar NY162003, for example, β-carotene accounts for more than 99%, resembling an extreme case of tomato *B* mutation. Similarly, the deep-red HLY "Moon and Stars" cultivar has a phenotype corresponding to the *og* tomato mutant. However, the *CYCB* gene known to control colouration in tomato *B* and *og* mutants has not been detected in watermelon, thus the above-mentioned phenotypes are likely based on different molecular mechanisms (Bang et al., 2010; Lv et al., 2015). At this regard, Bang et al. (2007) proposed *LCYB* as the color-determining gene between canary yellow and red flesh varieties. The authors suggested that a single-nucleotide mutation

in the *LCYB* gene introduced an amino acid replacement in the catalytic site, significantly impairing the activity of the enzyme with a consequent lycopene accumulation and appearance of the red phenotype. The active (wild) form of the enzyme is, instead, responsible for the canary-yellow phenotype. In yellow-fleshed watermelons, neoxanthin, violaxanthin, luteoxanthin and neochrome were found to be the predominant carotenoids indicating that, in the absence of an upstream blockage of lycopene cyclization, carotenoid metabolism proceeds rapidly toward the synthesis of xanthophylls (Bang et al., 2010; Lv et al., 2015). In watermelon, the enzymes carotenoid cleavage dioxygenases (CCDs) and 9-*(Z)*-epoxy-carotenoid dioxygenases (NCEDs), involved in the catabolic pathway, are candidate to play a fundamental role in fruit color determination. In fact, although, the up-/down-regulation of the carotenogenic genes direct the biosynthetic flow to specific carotenoids, the regulation of the catabolic genes affects the degradation/storage of the end-products. Accordingly, a high expression of *NCED* genes, particularly in the advanced phases of fruit development and ripening was demonstrated in pink, yellow, and white watermelon lines, leading to the cleavage of at least part of the synthesized xanthophylls and contributing to the observed reduced carotenoid content of these genotypes (Kang et al., 2010). Similar results were described in tomato, where fruit-specific RNAi-mediated suppression of *SINCED1* increased the accumulation of upstream compounds in the carotenoid pathway (Sun et al., 2012).

In non-red watermelon lines, the reduced carotenoid content has been, also, attributed to a generalized reduced transcription of most genes involved the biosynthetic pathway. For example, the transcript levels of almost all carotenogenic genes (except *LCYB* and *ZEP*) of the white genotype "ZXG507" were found lower than those of all the assayed colored cultivars at the same developmental stage, while the *NCED1* transcripts increased dramatically in the late ripening stages (Lv et al., 2015).

Fruit dimensions also seem to affect carotenoid content in tomato, with small fruited red-ripe cherry tomatoes generally showing higher lycopene and β-carotene contents than cluster, elongate and salad types (Leonardi et al., 2000; Lenucci et al., 2006). Instead, no systematic reports on different sized red watermelon genotypes are currently available. Nevertheless, it has been generally reported that seedless mini watermelon cultivars have higher amounts of lycopene (>50.0 mg/kg fw) than seeded open pollinated large sized ones (Perkins-Veazie et al., 2001). Furthermore, ploidy, often associated to changes in fruit dimensions, was found to affect lycopene content. Polyploids, especially triploids, had higher levels of lycopene than their diploid progenitors, and triploids tended to contain more lycopene than tetraploids (Liu et al., 2010).

Fractionate analyses of the peels, pulp and seeds isolated from red-ripe berries of different tomato cultivars revealed substantial differences among fractions. The peels showed up to fivefold more lycopene and β-carotene than pulp, while seed fractions were almost devoid of both carotenoids (Ilahy and Hdider, 2007; Chandra and Ramalingam, 2011; Chandra et al., 2012; Ilahy et al., 2016a). Similarly, topological differences on lycopene concentration were reported by Tlili et al. (2011b) in the full-ripe endocarp of different red watermelon cultivars, with the stem-end and the heart areas showing significant higher values than the blossom-end and the peripheral areas, and rind being completely devoid of the linear carotene, suggesting a spatial regulation of carotenoid metabolism.

Besides the genetic potential, many authors highlighted a large variability in lycopene and β-carotene concentrations and ratios in dependence of environmental factors, agro-technical processes, ripening stage, harvest and postharvest manipulations both in tomato and watermelon fruits (Abushita et al., 2000; Dumas et al., 2003; Perkins-Veazie and Collins, 2004; Lenucci et al., 2009; Siddiqui et al., 2018).

Accumulation Factors and Regulation

In tomato, light radiation intensity and quality, air- and fruit canopy temperature, CO_2 concentration and growing system (irrigation, fertilization, grafting, etc.) have been demonstrated to significantly affect fruit carotenoid concentration and confer prospects for enhancing their accumulation without resorting to metabolic engineering (Pogonyi et al., 2005; Brandt et al., 2006; Dorais, 2007). Light and circadian rhythm are known to alter the expression of nearly all MEP genes and several carotenoid synthesis and catabolism genes (Liu et al., 2004; Cordoba et al., 2009). Tomato fruits subjected to high irradiance and high temperature have demonstrated an increased metabolism of carotenoids, probably in relation to the protective role of carotenoids against the resulting oxidative stress, and/or the capacity of dissipating excess absorbed energy in the xanthophyll cycle (Cocaliadis et al., 2014). Oxidative stress in tomato fruit increases co-ordinately with fruit ripening and reaches a peak at the final stages thus triggering metabolic changes and fruit softening (Jimenez et al., 2002). It is also known that red-light and far-red light, respectively, stimulate and inhibit carotenoid accumulation in tomato by a mechanism likely involving phytochrome receptors (Alba et al., 2000; Giovannoni, 2001; Schofield and Paliyath, 2005). In ripening tomato, the content of lycopene and β-carotene positively correlated with an increase of photosynthetically active radiation (PAR) and more precisely with exposure to blue-light (Gautier et al., 2004).

Light also has a major role in modulating the developmental programs determining plastid identity and ultrastructure. Plastid type and sub-organelle compartmentalization not only influence carotenoid profile and storage capacity but also play a central role in controlling the activity of PSY and other enzymes of the metabolic pathway. During tomato fruit ripening, light stimulates the differentiation of chromoplasts and induces carotenoid biosynthesis, while in non-photosynthetic tissues of carrot roots, it promotes chloroplasts differentiation instead of chromoplasts. Furthermore, tomato fruit carotenoid biosynthesis seems adjusted to actual ripening progression by a light-dependent mechanism involving the endogenous shade signaling components of chloroplasts. During chloroplast-to-chromoplast transition the controlled chlorophyll degradation reduces the self-shading effect and promotes the turnover of a transcription factor (*Pif1a*) that directly repress PSY1 expression, boosting carotenoid biosynthesis as ripening progress (Llorente et al., 2017). As far as we know, there are no

specific reports on the effects of light on the biosynthesis of carotenoids in watermelon fruit tissues. Nevertheless, light intensity, temperature, and irrigation were reported to alter lycopene content by 10–20% in ripe watermelon fruits (Perkins-Veazie et al., 2001; Leskovar et al., 2004).

In tomato fruits, lycopene biosynthesis is strongly inhibited at temperatures below 12°C and completely blocked above 32°C (Dumas et al., 2003). An increase of lycopene cyclization to β-carotene, resulting in a decrease of lycopene content, was observed in fruits exposed to temperatures above 35°C (Hamauzu et al., 1998). Elevated CO_2 levels also decrease significantly the lycopene content in greenhouse-grown tomatoes (Helyes et al., 2011). The authors proposed that the elevated air CO_2 concentration might support the plants to cope better with environmental stresses, reducing the need of activating the stress response systems, including the synthesis of carotenoids. Postharvest ripening of tomato fruits at high CO_2 atmospheres significantly prevented the rise in ethylene (ET) production, and slowed down lycopene biosynthesis and chlorophyll degradation, suggesting an alternative mechanism by which CO_2 can indirectly affect carotenoid accumulation (Sozzi et al., 1999). Field experiments showed that a shift from 20 to 37°C of on-vine watermelon fruits did not result in color reduction as in tomato, indicating that, in this thermic range, lycopene synthesis is not affected by temperature (Vogele, 1937). Additionally, watermelon stored at 21°C gained 11–40% in lycopene and 50–139% in β-carotene, indicating active carotenoid biosynthesis as observed in tomato during off-vine ripening, whereas, little change in carotenoid content was reported at temperatures below 13°C (Perkins-Veazie et al., 2006).

Growing systems also affect the carotenoid content of tomato and watermelon. Field-grown tomatoes generally have higher levels of lycopene than greenhouse-grown tomatoes, in which it ranges between 1 and 108 mg/kg fw (Sahlin et al., 2004). As far as we know, no literature is available at this regard for watermelon. The effects of mineral nutrition on lycopene levels in tomato and watermelon are yet no conclusive, as the response is strongly dependent on rate, genotype, growing conditions and growth stage. In tomato, potassium (K) and/or phosphorous (P) supplementation in either soil or soilless (hydroponic) cultivation significantly increased, up to 30%, lycopene and total carotenoid content of ripe fruits (Dumas et al., 2003; Serio et al., 2007; Almeselmani et al., 2009; Bidari and Hebsur, 2011). Conversely, little or no increase in lycopene with increased soil K or P rates was reported in tomato and watermelon fruits by Fontes et al. (2000) and Perkins-Veazie et al. (2003). The ratio of K and nitrogen (N) has been also shown to affect the content of total carotenoids and lycopene of hydroponically grown tomatoes (Kaur et al., 2018). K concentration has been proposed to play an indirect role in the process of carotenoid biosynthesis in tomato by activating several of the enzymes regulating the metabolism of carbohydrates and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, leading to an increase of the precursors of isopentenyl diphosphate (IPP) (Fanasca et al., 2006).

The response to water amount and quality on lycopene content depends on crop and germplasm. Some tomato cultivars showed a decrease of lycopene with decreased soil water, while in other it was increased. Salinity (0.25% NaCl w/v) significantly improved lycopene content of tomatoes, but the response was genotype dependent (De Pascale et al., 2001; Krauss et al., 2006). Evidence suggests that osmotic and/or salt stress cause ET synthesis, which is a main positive regulator of lycopene synthesis in tomato (Wu and Kubota, 2008).

No significant differences in lycopene content were reported in watermelon irrigated at different regimes, while it positively correlated with salinity increase (Ban et al., 2014). Conversely, a decrease in the expression of *PSY*, *PDS*, *ZDS*, *LCY*-β genes was reported at increasing salt concentrations (between 0 and 200 mM NaCl) by Cheng et al. (2015).

Grafting is used widely with tomato and watermelon to improve water and mineral nutrition of plants, enhance the tolerance to soil-borne diseases (e.g., *fusarium* wilt) and abiotic stress, as well as to increase yield and fruit quality (Kyriacou et al., 2017). Complex interactions between rootstock type, growing season, salinity, and genotype on carotenoid concentration, particularly lycopene, have been reported in both vegetable crops, leading to conflicting results (Fekete et al., 2015; Marsic et al., 2018).

Despite the common trait of synthesizing and accumulating lycopene, red tomato and watermelon show similarities and differences in the mechanisms that implement and regulate the process. First, a polyphyletic origin of the trait has been proposed based on the different fruit color of the putative wild ancestors of the domesticated tomato and watermelon. *Solanum pimpinellifolium* L., the progenitor of modern tomato, has intensely colored red berries (Rick, 1995), *C. lanatus* var. *citroides* (L.H. Bailey) Mansf., the supposed ancestor of watermelon, has, instead, white fleshed fruits (Navot and Zamir, 1987), suggesting that the genetic changes resulting in the red-flesh trait occurred after watermelon had been domesticated (Tadmor et al., 2005).

A further manifest difference between tomato and watermelon lies in the color transition during fruit ripening, which is the result of different developmental programs of chromoplasts differentiation. Ripening tomatoes undergo a marked green-to-red color change, due to the interconversion of chromoplasts from pre-existing photosynthetic chloroplasts (Suzuki et al., 2015). Green-unripe tomato fruits show, in fact, the typical carotenoid profile of photosynthetically active tissues (Bramley, 2002). In ripening tomato fruits, chloroplast-to-chromoplast interconversion is marked by a burst of lycopene synthesis and its massive accumulation as large crystals within membrane-shaped structures, accompanied by the simultaneous breakdown of thylakoid membrane, the *de novo* formation of membranous carotenoid-sequestrating substructures within chromoplasts, and the increase in the number and size of plastoglobules (Simkin et al., 2007; Bangalore et al., 2008). The white flesh of young watermelon fruits contains, instead, only trace amounts of carotenoids because chromoplasts differentiate from non-photosynthetic plastids, possibly directly from undifferentiated proplastids (Wang et al., 2013). Ordinary and HLY tomatoes showed a similar pattern of change in lycopene and β-carotene during ripening, with a sharp increase in the synthesis of these carotenoids during the transition from the green to the breaker stage of ripening, suggesting that the mechanisms regulating

the process are conserved among genotypes (Hdider et al., 2013). A similar trend was reported in different commercial and newly HLY developed watermelon lines by Tlili et al. (2011a), with the burst in carotenoid biosynthesis occurring between the white-pink and the pink stages of ripening. As previously told, the regulation of chromoplast biogenesis plays a crucial role in providing a site not only for active carotenoid biosynthesis but also for carotenoid storage. In chromoplasts, various lipoprotein substructures (e.g., globules, crystals, membranes, fibrils, and tubules) sequester carotenoids. Based on the pigment-bearing sequestration substructures, chromoplasts are classified into five major types as globular, crystalline, membranous, fibrillar, and tubular (Egea et al., 2010; Li and Yuan, 2013; Schweiggert and Carle, 2017). Usually more than one kind of chromoplasts co-exists in a species. Tomato has globular chromoplasts characterized by abundant plastoglobules. Crystalline chromoplasts typically over-accumulate lycopene and β-carotene as red/orange crystals, and are abundant in tomato, and watermelon (Jeffery et al., 2012; Zhang et al., 2017).

Moreover, tomato fruits are climacteric, thus ripening processes, including lycopene synthesis and accumulation, are dependent on ET; watermelon, instead, is non-climacteric. These differences are reflected by the mechanisms controlling the biosynthesis and accumulation of carotenoids in the two crops.

The carotenoid metabolism and accumulation in tomato and watermelon fruits is temporally and spatially regulated by systematic and sophisticated mechanisms and is dependent upon several factors besides the modulation of the pathway gene expression (Nisar et al., 2015; Yuan et al., 2015). Plants have evolved, in fact, diverse strategies to regulate, at multiple levels, carotenoid metabolism in response to developmental programs, environmental factors, and metabolic signals. Transcriptional regulation certainly provides the first layer of control in the biosynthesis and accumulation of specific carotenoids during fruit ripening of both species. The transcription of many genes of the MEP, carotenoid biosynthetic and catabolic pathways have been found to be up- or down-regulated during tomato and watermelon fruit ripening (Apel and Bock, 2009). In red conventional cultivars of tomato and watermelon the transcription of precursor (upstream) carotenogenic genes encoding for specific fruit isoforms of DXS, GGPS, PSY, PDS, ZDS, CRTISO are up-regulated during ripening and parallel the synthesis and accumulation of lycopene (Fraser et al., 2002; Grassi et al., 2013). In tomato, plastid isopentenyl diphosphate isomerase (IDI) was shown to play an important function in carotenoid biosynthesis, thus highlighting its role in optimizing the ratio between IPP and dimethylallyl diphosphate (DMAPP) as precursors for different downstream isoprenoid pathways (Pankratov et al., 2016). The expression of downstream *LCY-E* and *LCY-B* genes is, instead, dramatically decreased, or maintained at low levels, during ripening (Ronen et al., 1999; Tadmor et al., 2005; Kang et al., 2010; Zhang et al., 2013). Cyclization of lycopene is a key regulatory branching point of carotenogenesis in both crops, since *LCYB/CYCB* and *LCYE* are involved in channeling lycopene into the downstream pathway. In the red-fleshed watermelon cultivar Dumara, for example, a constant low expression level of both *LCYB* and *LCYE* was reported and related to the biogenesis of chromoplasts from non-photosynthetic plastids. In fact, in contrast to tomato, no chloroplast-to-chromoplast transition occurs in watermelon flesh, thus the metabolic flux toward cyclic carotenes and xanthophylls, which are present in significant quantities in the purified chloroplasts of unripe tomatoes (Lenucci et al., 2012; Ilahy et al., 2014), can be permanently maintained at low levels during the entire process of fruit development and ripening. Nevertheless, significant differences in *LCYB* expression patterns were reported among red watermelon genotypes and between these and non-red watermelons (Guo et al., 2015). Besides, in the cultivar Dumara, the high expression levels of downstream *BCH* and *ZEP*, whose expression increased early during fruit ripening and remained stable over time, may help maintain the amounts of γ- and β-carotene at low levels as intermediate metabolites (Grassi et al., 2013). Accordingly, the lack of both zeaxanthin and violaxanthin, products of *CHYB* and *ZEP* activities, in the watermelon carotenoid profiles at any stage of ripening supports the hypothesis of their rapid turnover toward the synthesis of phytohormones and/or other carotenoid derived signaling molecules (Kang et al., 2010). The steady-state level of carotenoids is, in fact, the result of the equilibrium between synthesis and degradation (Li and Yuan, 2013). Thus, the catalytic activity of CCDs is critical in regulating carotenoid accumulation. In tomato, *CCD1* expression has been associated with the emission of isoprenoid volatiles, including neral [*(Z)*-citral], geranial [*(E)*-citral], and farnesyl acetone, whose production is correlated to carotenoid levels (Klee and Giovannoni, 2011; Ilg et al., 2014). Similarly, different members of the NCED family have been shown to increase during tomato and watermelon ripening.

In most plants, phytohormones and transcription factors operate in concert to finely tune the expression of carotenogenic genes (Zhong et al., 2013). Phytohormones, including ET, auxin, ABA, gibberellic acid (GA), jasmonic acid (JA) and brassinosteroids (BR), as well as non-enzymatic oxidation carotenoid-derived compounds have been proposed to directly or indirectly regulate ripening and carotenoid accumulation in tomato fruits (Ma et al., 2009; Havaux, 2014; Liu et al., 2015; Su et al., 2015; Cruz et al., 2018). Among them, ET plays a central role. In tomato fruits, the onset of ripening is triggered by a dramatic increase in ET production, correlating with the rapid accumulation of β-carotene and lycopene, and the expression of *SlPSY1* and *SlPDS* is dependent on ET (Marty et al., 2005). Following the application of various hormone-like substance to tomato fruits at the mature-green ripening stage, Su et al. (2015) demonstrated that auxin application, as indole-3-acetic acid (IAA), retards tomato ripening by affecting a set of key regulators, such as *Rin*, ET and ABA, and key effectors, such as genes for lycopene and β-xanthophyll biosynthesis and for chlorophyll degradation, thus suggesting that carotenoid accumulation during tomato fruit ripening is modulated by the IAA-ET balance. Recently, ET and auxin have been proposed to be involved as part of the light signaling cascades controlling tomato fruit metabolism, providing a new crosstalk between light signaling, plant hormone sensitivity and carotenoid metabolism in ripening tomato fruits (Cruz et al., 2018). At the best

of our knowledge, the role of phytohormones in carotenoid biosynthesis has not been thoroughly investigated in watermelon fruits. However, although ET biosynthesis is not essential for watermelon fruit ripening, varying patterns of production have been reported, indicating that ET and/or a modulated sensitivity to the hormone might participate in physiological changes during watermelon fruit development (Perkins-Veazie et al., 1995). Indeed, many non-climacteric fruits, including watermelon, are highly sensitive to exogenous ET (Chen et al., 2018).

Recently, ABA has been shown to be involved in the regulation of watermelon fruit ripening, but if it is directly involved in carotenoid synthesis and accumulation is still not known (Wang et al., 2017).

Several ripening-related regulatory factors, particularly those involved in ET production and sensing, were found to finely control the carotenoid levels in tomato fruits (Cazzonelli and Pogson, 2010; Enfissi et al., 2010; Liu et al., 2015). These include the *RIPENING INHIBITOR (RIN-MADS)*, *TOMATO AGAMOUS-LIKE 1 (TAGL1)*, *ETHYLENE RESPONSE FACTOR 6* (*ERF6)*, *APETALA2a (AP2a)*, *MC*, *NON-RIPENING* (*NAC-NOR*), *DE-ETIOLATED1* (*DET1)*, *UV-DAMAGED DNA BINDING PROTEIN1* (*DDB1)*, *CULLIN-4 (CUL4)*, *COLORLESS NON RIPENING* (*CNR*), *GOLDEN 2-LIKE (GLK2)*, *HB-1*, *UNIFORM RIPENING (U)/GLK2, FRUITFULL 1/2* (FUL1/TDR4; FUL2/MBP7), and *B-BOX20* (*BBX20*) transcriptional regulators (Vrebalov et al., 2002; Manning et al., 2006; Lin et al., 2008; Itkin et al., 2009; Chung et al., 2010; Powell et al., 2012; Pan et al., 2013). In watermelon although homologous genes of most tomato transcription factors have been identified their transcription often showed different levels than in tomato, suggesting that while a common set of metabolic and regulatory genes is conserved and influences carotenoid accumulation during development and ripening, specific regulatory systems may differ possibly related to the different ripening physiologies of climacteric and non-climacteric fruits (Grassi et al., 2013).

Transcriptional regulation is not the only regulatory system directing carotenoid biosynthesis and accumulation in plants; post-translational and epigenetic mechanisms (Zhong et al., 2013; Arango et al., 2016) provide other adaptive layers of control. However, there is a great lack of knowledge about the identity of these processes both in tomato and watermelon (Lu and Li, 2008). Protein-membrane and protein–protein interactions, as well as feedback and feedforward control mechanisms (Luo et al., 2013; Chayut et al., 2017; Enfissi et al., 2017) have been proposed to modulate the levels and activities of carotenogenic enzymes in different species, including tomato. A tomato STAY-GREEN protein, *SlSGR1*, was shown to regulate lycopene accumulation during fruit ripening by directly inhibiting PSY1 activity. The formation of membrane-bound multi-enzyme complexes acting in sequence (metabolons) also facilitates metabolite channeling to drive flux toward completion (Ruiz-Sola et al., 2016). In tomato, the levels of different carotenoid metabolites exert a feedback and feedforward regulation of carotenoid pathway enzymes, acting predominantly at the level of PSY1 and cyclases (Kachanovsky et al., 2012; Enfissi et al., 2017). Light signals mediated by fruit-localized phytochromes as well as the physical sequestration of carotenoids in plastid sub-compartments from downstream enzymes have been also reported to affect the activity of the enzymes involved in carotenoid metabolism (Nogueira et al., 2013; Gupta et al., 2014). The extent to which parallel mechanisms operate in watermelon fruits is still unknown, but highly probable.

PHENOLICS IN TOMATO AND WATERMELON FRUITS

Chemical Features and Functions

Phenolics represent an important class of secondary metabolites broadly distributed in the plant kingdom, characterized by the presence of mono- or poly-hydroxylated aromatic rings (Dai and Mumper, 2010). Tens of thousands of diverse molecules, with the number incessantly increasing, ranging from hydrophilic, lipophilic to insoluble structures, have been identified in plants. Some occur as low molecular-weight monomers, other are dimerized, polymerized or even highly polymerized to large complex compounds. Based on the number of C-atoms and basic arrangement of carbon skeletons in their chemical structure, phenolics are classified in groups and subgroups (**Figure 3**) with different characteristics and distribution (Alu'datt et al., 2017).

The complexity of functions of phenolics within plants is still poorly understood, however, fundamental roles as antioxidants, structural polymers, coloring pigments, chemoattractants or repellents to pollinators or pests, UV-screens, signaling compounds in symbiosis initiation and plant–microbe interactions and defense weapons against aggressors have been suggested (Dai and Mumper, 2010). Besides, phenolics are responsible of various organoleptic traits in fruits and vegetables and have stimulated substantial attention as health-promoting compounds because of their antioxidant, anti-inflammatory, anti-allergic, anti-atherogenic, anti-thrombotic and anti-mutagenic effects (Cory et al., 2018; Shahidi and Yeo, 2018). The dietary intake of phenolics positively correlates to a reduced incidence of many chronic pathologies, including cardiovascular, neurodegenerative and neoplastic diseases. They seem also involved in the modulation of the human immune system by affecting the proliferation of white blood cells and the production of cytokines or other defense factors (Olivares-Vicente et al., 2018). Accordingly, breeders, growers, and processors are increasingly searching for tools to improve the profile and enhance the content of specific phenolics in newly released elite cultivars to respond for the growing interest of consumers for high quality fruits and vegetables.

Biosynthesis

Plant phenolics arise biogenetically from a complex network of routes based principally on the shikimate/phenylpropanoid pathway or the "polyketide" acetate/malonate pathway (Cheynier et al., 2013). The shikimate pathway converts carbohydrates in the aromatic amino acids phenylalanine and tyrosine, the main substrates for the synthesis of hydroxycinnamic (*p*-coumaric, caffeic, ferulic and sinapic) acids (**Figure 4**). The limiting reaction

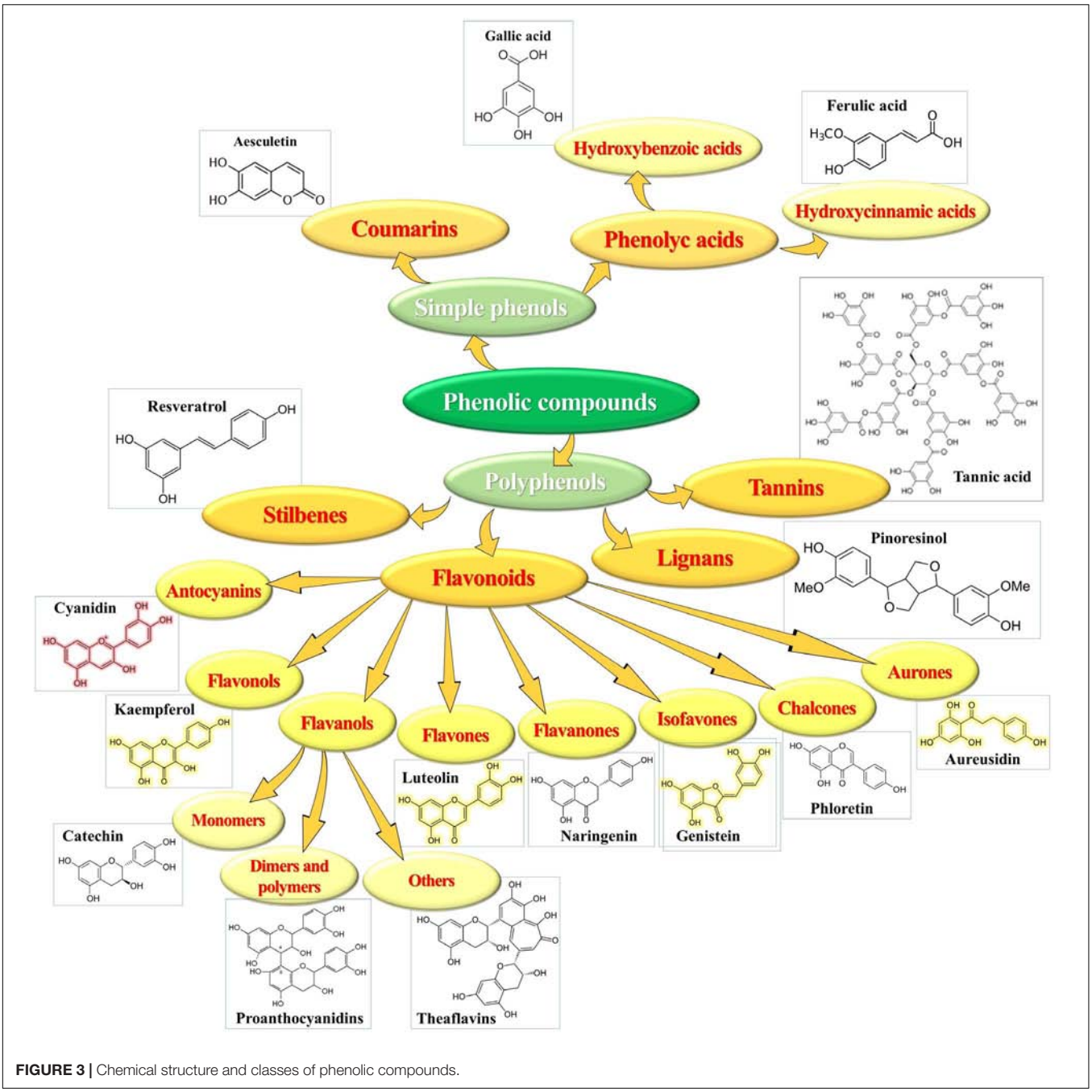

FIGURE 3 | Chemical structure and classes of phenolic compounds.

that interlocks the primary and secondary metabolisms is catalyzed by phenylalanine ammonia lyase (PAL) that deaminates phenylalanine to cinnamic acid. Cinnamic acid is hydroxylated at the C4-position by the enzyme cinnamate 4-hydroxylase (C4H) to *p*-coumaric acid that, in turn, receives another hydroxyl at the C3-position by the activity of *p*-coumaroyl ester 3-hydroxylase (C3′H) to caffeic acid. Ferulic acid is biosynthesized from caffeic acid by the action of the enzyme caffeate *O*-methyltransferase (COMT). *p*-Coumaric acid can also be directly produced from tyrosine deamination, though this reaction, catalyzed by tyrosine ammonia lyase (TAL), is barely used by plants. *p*-Coumarate is converted to its CoA ester by the 4-coumarate:CoA ligase (4CL); this step is a central point of branching of the phenylpropanoid pathway (Cheynier et al., 2013).

Regarding flavonoid synthesis (**Figure 5**), one aromatic ring and its side chain arises from phenylalanine, while the other arises from acetyl-CoA via the acetate/malonate pathway: the *p*-coumaroyl-CoA reacts with three molecules of malonyl-CoA under the action of the key enzyme chalcone synthase (CHS) to produce naringenin chalcone. This is prone to isomerization, hydroxylation and reduction/oxidation reactions to give primary aglycone flavonoids. The action

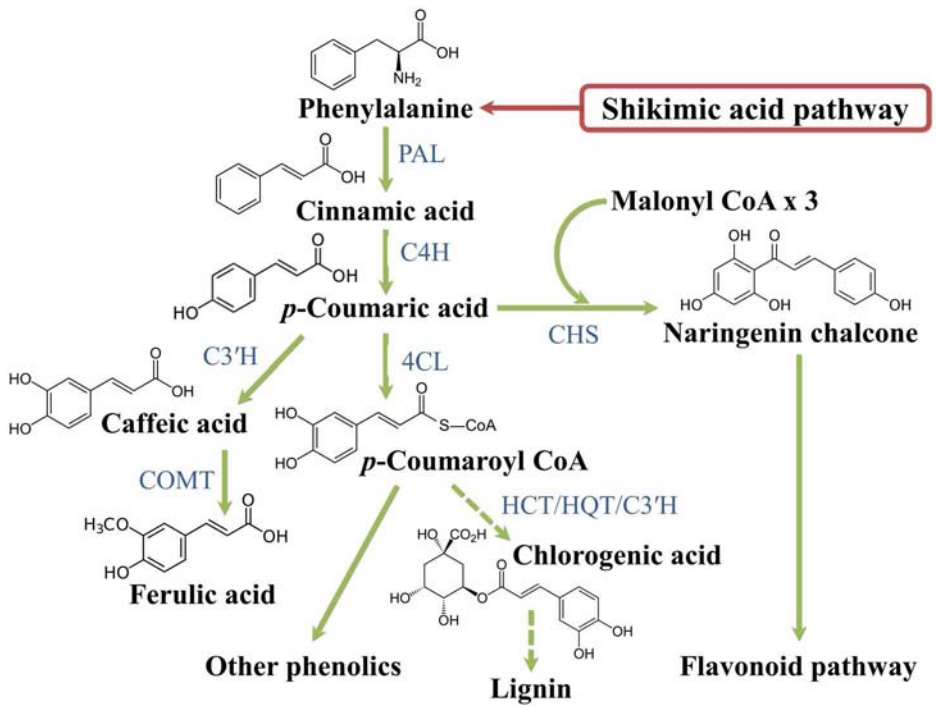

FIGURE 4 | Phenylpropanoid biosynthetic pathway. 4CL, 4 coumarate CoA ligase; C3'H, *p*-coumaroyl ester 3 hydroxylase; C4H, cinnamate 4 hydroxylase; CHS, chalcone synthase; COMT I, caffeic/5-hydroxyferulic acid *O*-methyltransferase; HCT, hydroxycinnamoyl transferase; HQT, hydroxycinnamoyl CoA:quinate hydroxycinnamoyl transferase; PAL, phenylalanine ammonia lyase.

of glycosyltransferases, methyltransferases and acyltransferases further increases the heterogeneity of flavonoids (Saltveit, 2009).

Fruit Concentration and Distribution

Ordinary red tomato and watermelon cultivars appear to have similar phenolic and flavonoid levels and, likely, analogous regulation mechanisms leading to the synthesis and accumulation of significant amounts of these compounds during ripening. The total phenolic content of different red tomato and watermelon cultivars is in the range 166–770 and 137–260 mg Gallic Acid Equivalent (GAE)/kg fw, respectively, with flavonoids [40–260 and 111–176 mg Rutin Equivalents (RE)/kg fw] substantially contributing to the total (Slimestad et al., 2008; Tlili et al., 2010, 2011a,b; Choudhary et al., 2015; Ilahy et al., 2018). Nevertheless, phenolic concentrations and profiles are strongly dependent on genotype, ripening stage, environmental factors, as well as pre- and post-harvest manipulations (Dumas et al., 2003; Ilahy et al., 2016b, 2018). This variability was confirmed also in HLY tomato cultivars, showing higher levels of phenolics and flavonoids than the ordinary tomato cultivars.

Besides, phenolic concentrations vary widely within the different fruit fractions (Ilahy et al., 2016a). Tomato peels showed 2–4 and 1.4–2.3 times higher total phenolic and flavonoid contents with respect to pulp. Tlili et al. (2011b) also evidenced a significant variation between the stem-end, blossom-end, heart and peripheral areas of ripe watermelon fruits in the concentrations of phenols and flavonoids.

Based on multiple analytical methods, Slimestad and Verheul (2009) reported the presence of 114 different phenolic molecules in ordinary red tomato fruits. These included mainly phenolic acids and flavonoids, indicated as the most represented groups in the ripe fruits. Phenolic acids contribute more than 70% to the total phenolics of red-ripe tomato fruits. They are distributed in the exocarp (peel), mesocarp and endocarp tissues primarily as chlorogenic acid (Moco et al., 2007), though the presence of *p*-coumaric, caffeic, ferulic, and sinapic acids has also been reported (Long et al., 2006). Flavonoids comprise mainly the flavanone naringenin and its isomer chalconaringenin (46%), followed by the flavonols quercetin (39%), myricetin (9%) and kaempferol (6%) (Haytowitz et al., 2018). Rutin (quercetin-3-*O*-rutinoside) is the major glycosylated flavonoid in ripe tomato fruits, although the flavonol quercetin 3-*O*-(2″-*O*-β-apiofuranosyl-6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) and the dihydrochalcone phloretin 3′,5′-di-*C*-β-glucopyranoside often show equivalent levels. More than 95% of flavonols (rutin and kaempferol) is in the peels (Stewart et al., 2000; Ilahy et al., 2016a), where concentrations up to 1840 mg/100 g dry weight (dw) of the stilbenoid phytoalexin resveratrol and related glycosides have been detected, with little change during fruit ripening and a maximum at 4 weeks post breaker (Ragab et al., 2006). Besides, it has been reported that specific flavonoid molecules may be synthesized in the tomato peel under particular light conditions, suggesting an important role of irradiance quality and intensity in determining the level and the profile of phenolic compounds in fruits (Slimestad and Verheul, 2009).

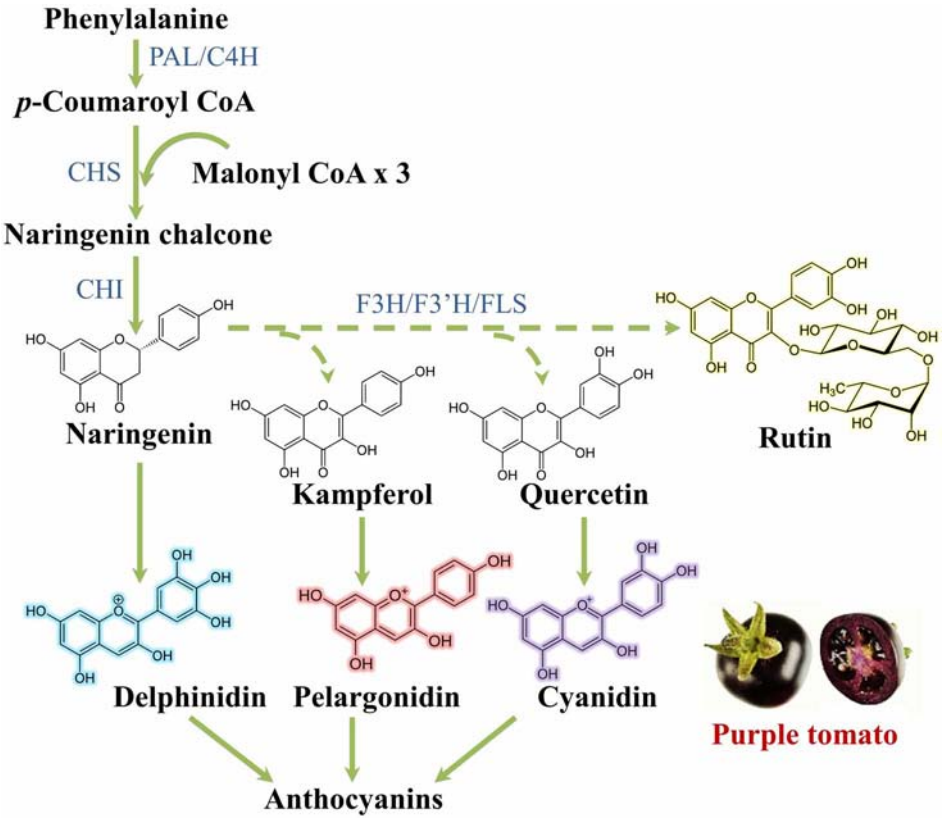

FIGURE 5 | Flavonoid biosynthetic pathway. C4H, cinnamate 4 hydroxylase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavanone 3′-hydroxylase; FLS, flavonol synthase; PAL, phenylalanine ammonia lyase.

While tomatoes have the genes for producing anthocyanins, they are typically not expressed in the fruit resulting in a negligible concentration of these health-promoting phenolic pigments in fruits. Recently, a "purple" tomato, highly enriched with anthocyanins, was produced by the ectopic expression of two selected transcription factors from snapdragon. The content of anthocyanins in the whole fruit was about 5.2 g/kg dw with little difference between pulp and peels. Petunidin-3-(*trans*-coumaroyl)-rutinoside-5-glucoside and delphinidin-3-(*trans*-coumaroyl)-rutinoside-5-glucoside were the most abundant anthocyanins, contributing up to 86% to the total. These fruits were found to extend the life of cancer-susceptible mice, suggesting that they have additional health-promoting effects (Su et al., 2016).

Luteolin and chlorogenic acid are the main phenolics detected in watermelon fruits (Fu et al., 2011). Interestingly, a combination of both molecules is regarded as prospective treatment against the inflammatory propagation in patients affected by rheumatoid arthritis (Lou et al., 2015). Abu-Hiamed (2017) evaluated the levels of flavonoids in watermelon external and internal pulp, as well as in the rind fraction. Rutin (1.66 mg/100 g fw) was detected exclusively in the internal pulp, while any of the tested fractions revealed the presence of quercetin and kaempferol, even though USDA flavonoid database reports the presence of kaempferol in a concentration ranging between 0 and 1.81 mg/100 g fw (Haytowitz et al., 2018). Chlorogenic, vanillic and sinapic acids are, instead, the main phenolics found in the rind of watermelon fruits (Mushtaq et al., 2015).

Extensive variability in phenolics and flavonoids levels has been highlighted during tomato and watermelon ripening. Ilahy et al. (2011) evidenced a complex and genotype-dependent pattern of changes in the amount of total phenolics and flavonoids among different cultivars of tomatoes, including both ordinary and HLY *hp-2* lines. The total phenolic content in the tomato cultivar HLY18 peaked (310 mg GAE/kg fw) at the orange-red stage, whereas cultivar HLY13 exhibited two consecutive peaks at the green and orange–red stages (223 and 240 mg GAE/kg fw, respectively). However, at the same ripening stages, the traditional cultivar Rio Grande showed the lowest total phenolics content (113 and 138 mg GAE/kg fw, respectively). Besides, flavonoid concentration peaked at the green-orange ripening stage in both HLY cultivars, but at the green stage in cultivar Rio Grande. Although the content of flavonoids was unchanged at advanced ripening stages, their levels were higher in HLY cultivars than in the traditional tomato cultivar Rio Grande regardless the stage of ripening.

Tlili et al. (2011a) focused on the dynamic of total phenols and flavonoids of different field grown watermelon cultivars (Crimson sweet, Dumara Giza, P503 and P403) throughout the ripening stages. Total phenols and flavonoids changed

significantly during ripening. Crimson Sweet, Dumara and P503, P403 and Giza exhibited a peak of phenols at the red-ripe stage. Regarding flavonoid, a linear increase was observed during ripening of cultivar Crimson Sweet with more complex dynamic of change for the other cultivars.

Accumulation Factors and Regulation

Being massively involved in signaling and response processes, the metabolism of phenolics in plants is severely affected by exogen stimuli (Rivero et al., 2001). Environmental factors have a strong influence over genotype. Fully ripe watermelon fruits grown in Southern Italy had 77–121% and 33–74% higher levels of phenols and flavonoids than those, of the same cultivars, grown in the North of Tunisia following identical agronomic procedures (Tlili et al., 2011a). Watermelon fruit quality is also prone to the influence of pre-harvest procedures and agro-technical processes such as irrigation, fertilization, grafting, and growing method (conventional/organic farming). Rivero et al. (2001) found that thermal stress induces a massive phenolic accumulation in tomato and watermelon fruits by activating the biosynthetic and inhibiting the oxidation pathways. This mechanism operates below 15°C to overcome chilling stress in watermelon and above 35°C in tomato to counteract heat stress and can be regarded as an acclimation response to extreme temperatures.

Storage conditions significantly affect tomato phenolics determining an increase or decrease depending on genotype, pre- and post-harvest handling (Ilahy et al., 2018). Nevertheless, some procedures have been proposed to preserve or increase the levels of total phenols during tomato storage including brassinolide, high-voltage electrostatic field, direct-electric-current and delactosed whey permeate treatments (Dannehl et al., 2011; Aghdam et al., 2012; Ilahy et al., 2018). Studies on the effect of postharvest procedures on the phenolic and flavonoid content of watermelon are lacking. Nevertheless, Perkins-Veazie and Collins (2004) reported possible alteration of the general antioxidant component by temperature and storage.

At the gateway from primary metabolism, PAL is known to play a pivotal role in the pathway regulation. During plant growth and development, as well as under biotic and abiotic stress conditions, the expression/activity of different PAL gene/protein is, in fact, highly modulated and controlled by internal and external signals in stringent correlation with fluctuations in phenolic concentrations. Tomato contains a surprisingly large family of PAL genes (∼26 copies/diploid genome) widely dispersed in the genome. Nevertheless, differently to watermelon and other plants, in which many of the PAL gene loci are expressed in response to alternative stimuli, only a single sequence is expressed (Chang et al., 2008). In watermelon, of the 12 *PAL* genes identified, 6 are moderately or strongly expressed in the fruits, particularly in the pulp, suggesting their potential regulative roles in the *de novo* synthesis of phenolics and other secondary metabolites (Dong and Shang, 2013).

Redundant but distinctive *cis*-regulatory structures for stress responsiveness have been identified in watermelon and tomato PAL genes through promoter motif analysis. Feedback effects or post-translational modifications are also involved in PAL regulation. Furthermore the differential subcellular distributions of PAL isoforms and the formation of metabolons with C4H, facilitating metabolite channeling to *p*-coumaric acid, might provide an extra and unsuspected level of metabolic regulation, allowing precursors partition into different branch phenylpropanoid pathways (Boudet, 2007).

More than sixteen cytochrome P450 monooxygenases, including C4H and C3'H, have been recognized as important regulators in phenolic metabolism by irreversibly channeling 4-coumaroyl-CoA flow into specific pathway branches, contributing thus to the diversity and flexibility of phenolic metabolism. In particular, putative *cis*-acting elements have been identified in the promoter region of C4H genes, further suggesting a coordinated transcriptional regulation of the expression of the enzyme, repeatedly identified as rate limiting (Boudet, 2007). Schijlen et al. (2004) pointed out the presence of a well-synchronized control of the biosynthetic genes at transcriptional level, mediated by various transcription factors, outlining the levels of each compound to attain within plant cells. These phenols and flavonoids regulatory genes are tissue- and stress-dependent and have been classified in two families; the first with sequence homology to proto-oncogene c-MYB protein, and the other similar to the basic-Helix–Loop–Helix (bHLH) protein encoded by the proto-oncogene c-MYC (Mol et al., 1998).

Conjugation and compartmentalization processes have also a role in determining the levels of phenolics in plants. Members of the multi-antimicrobial extrusion protein family in synergism with vacuolar sorting proteins, glutathione *S*-transferases and ERFs (ERF1 and ERF4) have been proposed to finely orchestrate phenolic sequestration within the vacuole in a tomato introgression line carrying a 32 cM single homozygous chromosome segment from *Solanum pennellii* characterized by increased levels of total phenolics (Di Matteo et al., 2013).

Future investigations are required to better elucidate the complex regulation of phenylpropanoid pathway.

AROMA VOLATILES IN TOMATO AND WATERMELON FRUITS

Chemical Features and Functions

Fruits synthesize, store and release various volatile molecules perceived by humans as aromas by interacting with specific receptors of the olfactory epithelium. Aromas constitute a huge heterogeneous class of chemicals carrying alcoholic, aldehydic, ketonic, acid, ester and ether functional groups inserted on saturated/unsaturated, straight/branched or cyclic structures (Schwab et al., 2008). Some (primary odorants) bind/activate a single receptor and define one odor individually; other (secondary odorants) interact with multiple receptors, leading to a more complex smell perception (Baldwin et al., 2000). Aroma is essential for fruit flavor, and along with sugars and acids, noticeably affects food consumer acceptance and choice; thus, it is gaining growing consideration as major quality attribute of fresh and processed fruit products (Wang et al., 2016).

Tomato and watermelon fruit volatile profiles are extremely complex. In tomato, more than 400 molecules have been

identified. However, only few (∼30) are assumed to effectively contribute the typical aroma of the fresh fruits and processed products based on concentration (>1 ppb), perception threshold (the level at which a compound can be detected by smell) and positive Log odor units (the logarithm of the ratio of the concentration of a component in a food to its perception threshold). According to Petro-Turza (1987) and Buttery et al. (1998), *(Z)*-3-hexenal, β-ionone, hexanal, β-damascenone, 1-penten-3-one, 3-methylbutanal, *(E)*-2-hexenal, *(Z)*-3-hexenol, 6-methyl-5-hepten-2-one, methyl salicylate and 2-isobutylthiazole are the major contributors driving the aroma of fresh, ripe tomato, though others may embellish the overall flavor as background notes. In watermelon Nijssen (1996) documented the presence of 75 different chemicals: 23 alcohols, 21 aldehydes, 12 furans, 8 ketones, 7 hydrocarbons, 2 lactones, 1 acid, and 1 oxide. However, those mostly contributing to the pleasant aroma notes of the ripe fruits include C6 and C9 aldehydes [*(Z)*-3-nonenal, *(Z,Z)*-3,6-nonadienal, *(E,Z)*-2,6-nonadienal, hexanal, nonanal, *(E)*-2-nonenal, *(E)*-6-nonenal], ketones (6-methyl-5-hepten-2-one, geranyl acetone), alcohols [*(Z)*-3-nonenol, *(Z,Z)*-2,6-nonadienol, hexanol, nonanol, *(Z)*-6-nonenol, *(E,Z)*-2,6-nonadienol] and their esters (Fredes et al., 2016). Watermelon fruits may also develop off-flavors such as *(E)*-6-nonenol responsible for the pumpkin-like fragrance (Kyriacou and Rouphael, 2018).

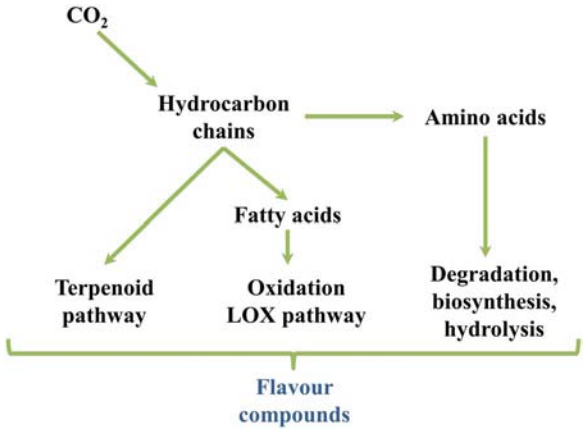

FIGURE 6 | Overview of flavor compounds biosynthetic pathways.

Biosynthesis, Fruit Profiles and Regulation

Despite the relatively low rate of DNA sequence diversity within the cultivated germplasm, significant variation in the amounts and profiles of volatiles among genotypes and fruit fractions has been reported for both tomato and watermelon. A surprisingly large chemical diversity in volatile emissions (up to 3,000-fold change, expressed as ng/g fw/h) across 152 heirloom tomato cultivars was observed by Tieman et al. (2012). Regardless of cultivars, peels and pulp seem the main fractions influencing tomato fruit aroma, followed by the locular fluid and, barely, seeds (Buttery et al., 1998; Wang et al., 2016). Similarly, Lewinsohn et al. (2005a) found different aroma profiles in two red-fleshed watermelon cultivars, with Moon and Star showing significantly higher monoterpenes and norisoprenes than Crimson Sweet, and 6-methyl-5-hepten-2-one as predominant volatile molecule (157 ng/g fw). Geranial [*(E)*-citral] was, instead, the main aroma detected in Crimson Sweet (16 ng/g fw). Beaulieu and Lea (2006) identified 59 volatile compounds (12 for the first time) in five different fully ripe triploid seedless red watermelon cultivars. The authors confirmed that the characteristic aroma of freshly cut fruits is associated mainly with alcohols, aldehydes and ketones, accounting up to 81.6% of the total volatile compounds, and highlighted some differences between conventional seeded and seedless cultivars. No acid and ester volatiles were detected, in fact, in any of the seedless watermelon cultivars assayed, possibly related to the minimal production of ET, typical of these genotypes, required to initiate the cascade of enzymatic reactions leading to the synthesis of these aromas. Among seedless cultivars, Pure Heart was the only synthesizing hexanal as major aroma volatile, confirming the importance of genotype as major determinant of aroma composition (Beaulieu and Lea, 2006).

The production of volatile aromas in tomato and watermelon, as well as in most other fresh fruits and vegetables, occurs through different metabolic pathways, but primarily via the catabolism of fatty acids, amino acids, glycosides, carotenoids and terpenoids (**Figure 6**) (El Hadi et al., 2013). Various enzymatic modifications (hydroxylation, acetylation, methylation, etc.) further increase the chemical complexity of the synthesized compounds. Besides, some volatiles, absent in the intact fruit, are produced as a result of tissue disruption occurring when the fruits are cut, squeezed or homogenized (Buttery et al., 1998).

Although the use of fast-evolving analytical tools is deeply affecting our knowledge on aroma biosynthesis, many steps have not been elucidated, yet. However, most are thought to be identical in different crops (El Hadi et al., 2013). Free fatty acids, particularly polyunsaturated linoleic (C18:2) and linolenic (C18:3), are the main precursors of most fruit aroma volatiles via α- and β-oxidation in intact fruits, or the LOX pathway in fresh-cut fruits and juices (**Table 1**) (Schwab et al., 2008). Two different LOX (9-LOX and 13-LOX), together with hydroperoxide lyases (HPLs) and a hydroperoxy cleavage enzyme, convert linoleic and linolenic acids to hexanal and *(Z)*-3-hexenal, respectively, through 9- and 13-hydroperoxy-intermediates. Hexanal and *(Z)*-3-hexenal are reduced to hexanol and *(Z)*-3-hexenol, respectively, by the reductase enzyme alcohol dehydrogenase (ADH). Further isomerization of *(Z)*-3-hexenal to *(E)*-2-hexenal can occur either enzymatically or non-enzymatically (Baldwin et al., 2000). In tomato, five LOX genes (Tomlox A, B, C, D, and E) are expressed during fruit ripening. Their suppression severely alters aroma and flavor synthesis suggesting them as a target for genetic modification (Pech et al., 2008). Furthermore, antisense targeting of phospholipase D (PLD-α), a key enzyme that initiates membrane deterioration and leads to the loss of compartmentalization and homeostasis during fruit ripening and senescence, yielded important quantities of the LOX-derived aldehydes *(E)*-pentenal and 2-hexenal after

TABLE 1 | Overview of carotenoids-, fatty acids- and amino acids-derived aroma and flavor compounds biosynthetic pathways. LOX, lipoxygenase.

Compounds	Reactions	Additional information	Final aromatic products
Carotenoids			
Phytoene and phytofluene	Cleavage catalyzed by a family of carotenoid cleavage dioxygenases (CCDs)	Acyclic volatiles	Farnesylacetone Dihydroapofarnesal Geranylacetone 6-Methylhept-5-en-3-one
Lycopene, prolycopene, neurosporene and δ-carotene			Neral Geranial 6-Methyl-5-hepten-2-one 2,6-Dimethylhept-5-1-al 2,3-Epoxygeranial (*E,E*)-Pseudoionone
β-carotene		Cyclic volatiles	β-Ionone β-Cyclocitral Dihydroactinidolide
δ-carotene			α-Ionone
Fatty acids	β-Oxidation	Aroma volatiles in intact fruits	C_1 to C_{20} straight-chain alcohols, aldehydes, ketones, acids, esters and lactones
	LOX	Aroma volatiles in fresh-cut fruits	
Amino acids (alanine, valine, leucine, isoleucine, phenylalanine, and aspartic acid)	Deamination/transamination, decarboxylation, oxide-reduction, and esterification		Alcohols, carbonyls, aldehydes, acids and esters

tomato tissue disruption probably due to the accumulation of high substrate levels of the LOX pathway indicating the existence of very intricate regulatory mechanisms (Oke et al., 2003).

Two isoforms of ADH have been identified in tomato fruits: *LeADH2* has been found to be particularly active during ripening for the synthesis of *(Z)*-3-hexenol.

Several amino acids (alanine, valine, leucine, isoleucine, and phenylalanine) are involved in the production of aroma volatiles through complex reactions of deamination/transamination, decarboxylation, oxide-reduction and esterification (**Table 1**) (Schwab et al., 2008). This occurs typically in the fruit during ripening rather than upon cell disruption. Decarboxylases and deaminases transform the amino acids in aldehydes, whose low and high levels have been strictly correlated to pleasant and unpleasant fragrances, respectively (Tadmor et al., 2005). The antisense down-regulation of the decarboxylase gene in tomato yield a limited synthesis of phenylacetaldehyde and phenylethanol, while its overexpression increases phenylethanol, phenylacetaldehyde, phenylacetonitrile, and 1-nitro-2-phenylethane levels, significantly affecting tomato aroma (Tieman et al., 2006).

Glycoside hydrolysis also leads to the release of volatile aromas such as 3-methylbutyric acid and β-damascenone, phenylacetaldehyde, 2-phenylethanol, linalool, linalool oxides, hotrienol, α-terpineol, 4-vinylguaiacol, 4-vinylphenol, furaneol (Buttery et al., 1998).

Carotenoids are precursors of some important C8, C13, C18 linear and cyclic isoprene flavor volatiles through a pathway involving three steps: an initial oxidative cleavage by CCDs to apocarotenoids, an enzymatic transformation in polar aroma precursors, and finally the acid-catalyzed conversion to volatiles. The synthesis of carotenoid-derived volatiles occurs late during fruit ripening and is a good indicator to monitor the process (Auldridge et al., 2006). In tomato, two similar genes (*LeCCD1A* and *LeCCD1B*) were reported to encode for CCDs able to cleave multiple linear and cyclic carotenoids preferably at the 9,10 (9′,10′) locations leading to the synthesis of β-ionone and geranylacetone, major contributors of fresh tomato aroma, representing, therefore potential targets to genetically modify fruit aroma synthesis (Simkin et al., 2004).

Finally, some volatiles (e.g., α-copaene, linalool, neral, geranial, methyl salicylate, eugenol, benzaldehyde, and guaiacol) are formed from terpenoids (C10 and C15), related to lignin or of unknown origin, but most do not seem to contribute to the aroma of either tomato or watermelon fruits.

Volatile and aroma profiles of fruits are continuously changing as several endogenous and exogenous factors/stimuli affect their synthesis and accumulation, generally, the same responsible of fluctuations in their originating precursors, including ripening stage, growing system and conditions and pre- and post-harvest practices (Baldwin et al., 2000). Although their importance and potential practical application in controlling a chief fruit quality attribute, there is a lack of knowledge about the cross-talk of the different factors in determining the potential of aromas in tomato and watermelon (El Hadi et al., 2013).

During tomato and watermelon ripening a series of qualitative and quantitative biochemical changes occur and affect the volatile profile of fruits. Several authors reported a general increase in aroma concentration along with both natural and artificial ripening, especially through the breaker to red stage transition, however, the expression patterns and concentrations of the individual compounds follows a complex and largely genotype-dependent pattern of change, with some remaining stationary or even decreasing (Renard et al., 2013). It is well known that early harvest of tomatoes before full ripeness impairs volatile production, resulting in poor flavor quality fruits. Fruits harvested 1-day before the breaker stage revealed significantly higher levels of 1-penten-3-one, *(Z)*-3-hexenal, 6-methyl-5-hepten-2-one, 2-isobutylthiazole and geranylacetone than those harvested 5-day before the breaker stage (Maul et al., 1998). Baldwin et al. (1991) assessed aroma volatiles in two tomato cultivars (Sunny and Solar Set) at five ripening stages. Hexenal, hexenal acetone, 6-methyl-5-hepten-2-one, geranylacetone, *(Z)*-3-hexenol, acetaldehyde, *(Z)*-3-hexenal, *(E)*-2- and 2-isobutylthiazole peaked at the turning, pink, or red-ripe stages. Eugenol concentrations decreased during

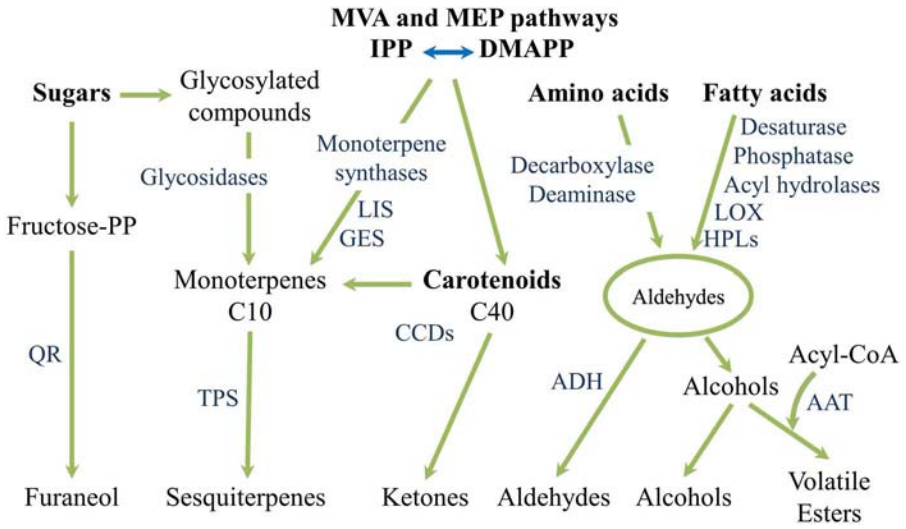

FIGURE 7 | Overview of the fatty acids-, amino acids- and carotenoids-derived aroma and flavor compounds biosynthetic pathways. AAT, alcohol acyl-transferases; ADH, alcohol dehydrogenase; CCDs, carotenoid cleavage dioxygenases; DMAP, dimethylallyl diphosphate; GES, geraniol synthase; HPLs, hydroperoxide lyases; IPP, isopentenyl pyrophosphate; LIS, S-linalool synthase; LOX, lipoxygenase; QR, quinone oxidoreductase; TPS, terpene synthase.

ripening regardless the cultivar and 1-penten-3-one decreased only in the cultivar Sunny. Ethanol and *(E,E)*-2, 4-decadienal concentrations remained unchanged or little fluctuated during ripening. Except for ethanol and hexanal in mature tomato fruits, Solar Set tomato fruits showed higher flavor content than Sunny. Accordingly, the activity of the tomato microsomal LOX increased between the green and turning stage and decreased as the fruit reached full ripeness.

It has also been reported that aroma volatiles differ between vine and off-vine ripened tomato fruits. Benzaldehyde, citronellyl propionate, citronellyl butyrate, decanal, dodecanal, geranyl acetate, geranyl butanoate, nonanal, and neral are synthesized and emitted at higher levels in vine-ripened tomato fruits than in off-vine ripened fruits, which in turn release increased levels of other compounds including butanol, 2,3-butanedione, isopentanal, isopentyl acetate, 2-methyl-3-hexanol, 3-pentanol and propyl acetate (Madhavi and Salunkhe, 1998; Ilahy et al., 2018). The development of off-flavors is associated with the increased productions of 2-methyl-1-butanal, mostly in artificially ripened tomato fruits.

It is worthwhile to mention that since key aroma volatiles in watermelon and tomato derive from carotenoid catabolism, differences in carotenoid profile underlie differences in their composition (Lewinsohn et al., 2005a; Liu et al., 2012; Kyriacou and Rouphael, 2018). Lewinsohn et al. (2005a) comparatively evaluated the volatile profiles of tomato and watermelon genotypes bearing different carotenoid biosynthetic gene mutations (*r*, *t*, *Del*, and *B*) revealing a strong overlap in the two crops for the same fruit flesh colouration and proposing that color differences induce similar profile changes in both tomato and watermelon fruits. Thus, ripe fruits of the red tomato and watermelon cultivars share the same major norisoprenoid volatiles 6-methyl-5-hepten-2-one, farnesyl acetone, pseudoionone and geranial; the yellow ones have farnesyl acetone as main isoprenoid volatile. High concentrations of farnesyl acetone, geranyl acetone and 6-methyl-5-hepten-2-one characterize orange *tg* tomato mutant and its watermelon equivalent Orangelo. In the orange *B* tomato and watermelon genotypes the main volatiles were, in descending order, dihydroactinidolide, farnesyl acetone, 6-methyl-5-hepten-2-one, β-ionone, β-cyclocitral, geranial, 2,3-epoxygeranial, neral, pseudoionone and dihydro-apo-*(E)*-farnesal. In the *Del* mutants, the main volatiles were 6-methyl-5-hepten-2-one, farnesyl acetone, α-ionone and geranial. Cyclic aroma compounds such as β-ionone, β-cyclocitral and dihydroactinidolide are the result of β-carotene cleavage, α-ionone derive, instead, from δ-carotene. Acyclic aroma volatiles are produced from the oxidative cleavage of phytoene, phytofluene, lycopene, pro-lycopene, and neurosporene (**Table 1** and **Figure 7**).

Field grown tomatoes have higher levels of aroma volatiles than greenhouse-grown fruits, while other pre-harvest factors such as fertilization and watering all influence volatile composition depending on the levels.

Postharvest handling have a deep impact on the aroma compounds of fresh tomato and watermelon fruits. Tomato fruits mild chilling and refrigeration lead to significant changes in the levels of 3-methylbutanal, linalool, guaiacol, hexanol, *(E)*-2-hexenal and *(E)*-3-hexenol, possibly due to a decrease of fruit ADH activity (Sanchez et al., 2009; El Hadi et al., 2013). Controlled atmosphere reduces the emitted volatiles from fresh product following the inhibition of ET production (Mattheis et al., 2005). In addition, the application of methyl jasmonate was reported to alter the biosynthesis of different aroma components and minimize aroma post-harvest depletion in climacteric and non-climacteric fruits (De la Peña et al., 2010; El Hadi et al., 2013).

The composition of aroma volatiles in tomato and watermelon fruits differs between intact fruit, sliced fruits and homogenates.

After tomato tissue disruption, some compounds appear [e.g., *(E)*-2-pentanal and geranial], other increase [e.g., *(Z)*-3-hexenal, *(E)*-2-hexenal, hexanal, *(E)*-2-heptenal, 1-penten-3-one, 1-penten-3-ol, geranylacetone, 2-isobutylthiazole, 1-nitro-2-phenylethane] and other remain unchanged [3-methylbutanol, pentanol, *(Z)*-3-hexenol, hexanol, 6-methyl-5-hepten-2-one, phenylacetaldehyde and 2-phenylethanol] (Baldwin et al., 2000).

Studying the quality characteristics of watermelon slices of the seedless cultivar Sugar Heart stored under modified atmosphere conditions at 5°C, Saftner et al. (2006) found a general decrease in the concentration of most volatiles, except *(Z)*-6-nonen-1-ol, 6-methyl-5-hepten-2-one, 6-methyl-5-hepten-2-ol, *(Z)*-6-nonenal, *(E)*-2-nonenal, nonan-1-ol, β-cyclocitral and geranyl acetone whose levels remained unchanged or transiently increased. The accumulation of *(Z)*-6-nonen-1-ol is of particular importance as it has been associated with the pumpkin-like off odor. The authors also reported that whole fruit pre-treatments with 1-methylcyclopropene could prevent ET-induced adulterations of watermelon slices volatile aromas, but not the development of off-odors.

The pH, also, has been demonstrated to play a fundamental role on the stability of fresh watermelon juice aromas; acid values in the range 3.0–5.6 promote the development of off-flavors, while the juice is more stable at pH between 6.0 and 7.0 (Yajima et al., 1985).

Acting to promote the transcription and translation of numerous ripening-related genes, ET has an important role in aroma volatile development in both tomato and watermelon fruits (Wyllie et al., 1998). ET is known as lipoxygenase activator, promoting the synthesis of the fatty acid derived volatiles as reported for tomato and climacteric and non-climacteric melon cultivars (Obando-Ulloa et al., 2008). ET also affect amino acid and carotenoid derived volatiles by controlling the synthesis of their direct precursor, in fact the synthesis of phenylalanine, leucine, and isoleucine, as well as some steps of the carotenoid metabolic pathway, are strongly under ethylene control during ripening (Klee and Giovannoni, 2011).

VITAMIN C IN TOMATO AND WATERMELON FRUITS

Chemical Features, Distribution and Functions

Ascorbic acid (AsA), also known as Vitamin C, is a multi-functional metabolite common in all photosynthetic eukaryotes and essential for plant growth and development. Besides its role in photosynthesis as enzyme cofactor, AsA is a major hydrophilic antioxidant able to efficiently scavenge free radicals and reduce high oxidation states of iron to Fe^{2+}. In fact, it is easily oxidized with loss of one electron to monodehydroascorbic acid (MDHA) and then, losing a second electron, to dehydroascorbic acid (DHA) (Smirnoff, 1996). In addition, AsA and DHA can act as signaling agents mediating the response to biotic and abiotic effectors, including pathogens, ozone, oxidizing agents, and water loss (Mellidou et al., 2012).

Ascorbic acid occurs in all plant cell organelles/compartments at intracellular concentrations of 2–25 mM and appears differentially distributed depending on subcellular localization and tissue. In *Arabidopsis* and *Nicotiana* mesophyll cells, the highest AsA levels were found in the cytosol, followed by peroxisomes, nuclei, mitochondria, chloroplasts and vacuoles (Smirnoff, 1996). It has also been detected in the apoplast (along with an extracellular isoform of ascorbate oxidase), where represents the only significant redox buffer and appears involved in the non-enzymatic scission of plant cell wall polysaccharides by triggering the *in situ* production of hydroxyl radicals via the Fenton reaction. This phenomenon is particularly important during fleshy fruit ripening and has been proposed to play a role in tomato softening (Dumville and Fry, 2003; Zechmann, 2011). DHA is also detectable in plant tissues, but its role is controversial as some authors assume it comes from the artifactual oxidation of AsA occurring during sample processing. However, the presence of AsA and DHA uptake/efflux high affinity carriers on plant cell membranes argues in favor of defined physiological functions. Besides, the uptake of AsA by chloroplasts is controlled by DHA concentrations (Smirnoff, 2018).

In humans, AsA is an essential micronutrient required for normal metabolic functioning of the body, especially for collagen, carnitine and neurotransmitters biosynthesis. Many health benefits have been attributed to ascorbic acid such as antioxidant, anti-atherogenic, anti-carcinogenic, immunomodulatory, etc. However, lately the health benefits of ascorbic acid have been the subject of debate and controversies. Humans and other primates have lost the ability to synthesize vitamin C, thus, it must be introduced with the diet. The Recommended daily intake (RDI) for ascorbic acid for non-smoking adults is set to be 75 and 90 mg/day for females and males, respectively (Monsen, 1996).

Fruit Concentration, Distribution, and Regulation

Tomato and watermelon fruits are good sources of vitamin C accumulating, on average, 12.6 and 8.1 mg/100 g fw as total AsA, respectively[2] though, in both crops, genotype, ripening stage, growing season and pre/post-harvesting practices have been reported to strongly affect its levels (Isabelle et al., 2010; Ilahy et al., 2011, 2016b, 2018; Tlili et al., 2011a,b). In the ordinary tomato cultivar Rio Grande, for instance, a significant variation of AsA and DHA concentrations in the range 67.8–108.0 mg/kg fw and 73.4–152.0 mg/kg fw was reported during ripening (Ilahy et al., 2011), while HLY tomato cultivars showed broader intervals (55.8–180 mg/kg fw and 37.8–213 mg/kg fw for AsA and DHA, respectively). The dynamic of change of total vitamin C (AsA + DHA) was also genotype-dependent, with some cultivars (Rio Grande, HLY18, and HLY13) peaking at the orange-red ripening stage, while Lyco2 showing two peaks at the green–orange and red-ripe stages (Siddiqui et al., 2018). Differences in the total vitamin C and AsA accumulation profiles during ripening were also reported between the "low-AsA" tomato cultivar Ailsa Craig and the "high-AsA" cultivar

[2]https://ndb.nal.usda.gov/ndb

Santorini, although both displayed a characteristic peak at the breaker stage (Mellidou et al., 2012).

Al-Harbi et al. (2017) suggested that grafting is ineffective in improving vitamin C content in tomato fruits. A 14–20% decrease was, instead, reported by Di Gioia et al. (2010) in open field grown fruits of the heirloom "Cuore di Bue" cultivar grafted onto two interspecific (*S. lycopersicum* × *Solanum habrochaites*) rootstocks (Beaufort F1 and Maxifort F1). Mycorrhiza treatment applied at sowing and planting during two consecutive seasons determined an increase of vitamin C content under dry conditions and a decrease under wet conditions (Nemeskéri et al., 2019). Helyes et al. (2019) focused on the simultaneous effect of three water supply regimes and treatments with different concentrations of the bio-fertilizer Phylazonit on the physiology and quality of the processing tomato cultivar Uno Rosso. The authors revealed highly significant effects of water supply regimes and bio-fertilization on the content of AsA that increased from 221.8 to 369.1 μg/g fw.

Recently, Ilahy et al. (2019) suggested that tomato cultivars carrying *hp* mutations could be used in organic farming to overcome the general observed antioxidant content and functional quality decline in traditional tomato cultivars. It has been reported that storage influence tomato health-promoting properties negatively or positively according to storage duration and conditions. Ascorbic acid content was found to decrease during storage and the greater the levels of different bio-actives initially accumulated in the fruits, the lower is the effect of the storage on ascorbic acid content (Siddiqui et al., 2018).

Similar to tomato, total vitamin C exhibited a cultivar-dependent dynamic of change during watermelon fruit ripening. The highest amount of total vitamin C was recorded at the red-ripe and pink stages in Crimson sweet and P503 cultivars, respectively, but at the white stage in Giza, Dumara and P403 cultivars. In the flesh of red-ripe fruits, total vitamin C values ranged from 119.7 to 204.0 mg/kg fw in the cultivars P403 and Giza, respectively. Vitamin C levels also varied in relation to the growing location, in fact, Giza, Dumara and P403, exhibited lower total vitamin C content when grown in Southern Italy than in Northern Tunisia (Tlili et al., 2011a,b).

Choo and Sin (2012) assayed the AsA content in the flesh of different flesh-colored watermelons. Yellow cultivars were found to have significantly lower AsA levels (52.1 mg/kg fw) than red ones (86.3 mg/kg fw). Otherwise, Isabelle et al. (2010) found higher ascorbic acid content in yellow than in red watermelon ripe fruits. These differences have been attributed to the strong influence by genotype, environmental conditions, harvest and post-harvest practices on AsA anabolic, catabolic and recycling pathways. It has also been reported that AsA content of the watermelon rind and seed were significantly lower compared with the pulp (Johnson et al., 2013).

Tlili et al. (2011b) reported significant variations in total vitamin C content between watermelon cultivars and sampling areas within each cultivar with values ranging from 105 to 240 mg/kg fw in Aramis and Dumara, respectively. Besides, a genotype-dependent effect of sampling area on the total vitamin C content was noticed with the highest values recorded for peripheral and stem-end areas and the lowest obtained for heart and blossom-end areas.

Although data of the effect of growing season and pre/post-harvesting practices on total vitamin C in watermelon cultivars are scarce, Leskovar et al. (2004) reported that vitamin C and lycopene are not affected by water stress in watermelon fruit. Proietti et al. (2008) demonstrated that dehydroascorbate and vitamin C contents were increased, respectively, by 13 and 7% in grafted compared to ungrafted plants.

Hdider et al. (2019) recently reviewing the available data regarding the nutritional composition, antioxidant properties and health benefits of watermelon fruits, reported that temperature and postharvest storage conditions and duration are the most important factors to extend shelf-life and conserve quality of fresh watermelon including vitamin C content.

Even if alternative routes involving uronic acids, L-gulose and myo-inositol may contribute to enhance AsA levels in higher plants, D-mannose/L-galactose (Smirnoff-Wheeler) pathway (**Figure 8**) represents the primary biosynthetic route in most crops, including tomato (Wheeler et al., 2015). The pathway appears regulated at both transcriptional and post-transcriptional levels; however, the knowledge on the mechanisms that control AsA synthesis and metabolism is still limited. Based on metabolite analyses, non-labeled and radiolabelled substrate feeding experiments, enzyme activity measurements and gene expression studies conducted on different crop species, several key regulatory components of the pathway have been proposed. These include GDP-D-mannose pyrophosphorylase (GMP), GDP-D-mannose-3,5-epimerase (GME), GDP-L-galactose phosphorylase (GGP), L-galactose-1-P phosphatase (GPP), L-galactose dehydrogenase (GDH) and L-galactonolactone dehydrogenase (GLDH). Phosphomannose isomerase (PMI) and phosphomanno mutase (PMM), the enzymes catalyzing the early steps of the pathway, instead, do not seem determinant in modulating AsA level (Ioannidi et al., 2009; Mellidou et al., 2012; Mellidou and Kanellis, 2017; Tao et al., 2018).

Various regulatory proteins and transcription factors have been proposed to positively or negatively affect the content of AsA. For instance, HD-ZIP1 increases the transcription of GME, GPP, GGP, and GLDH; ERF98 that of GMP, GME, GGP, GLDH and GDH, CML10, and CONJAK1/2 stimulate PMM and GMP activity, respectively, and VTC3 influences positively GGP posttranslational modification. AMR1 and CSN5B have been proposed to negatively affect the post-translational modulation of GMP and the transcription of all intermediate enzymes of the D-mannose/L-galactose pathway (Mellidou and Kanellis, 2017).

In tomato, GPP appears as the main regulatory enzyme of the D-mannose/L-galactose pathway. Its expression is ripening and ethylene regulated, and affected by light and stress conditions (Ioannidi et al., 2009). GPP is also subjected to translational regulation through a *cis*-acting upstream open reading frame in the 5′-UTR that represses GGP translation under high AsA concentration allowing a rapid and feedback responsive control of its biosynthesis under demanding conditions (e.g., high light, low temperatures) (Bulley et al., 2012; Mellidou et al., 2012; Mellidou and Kanellis, 2017).

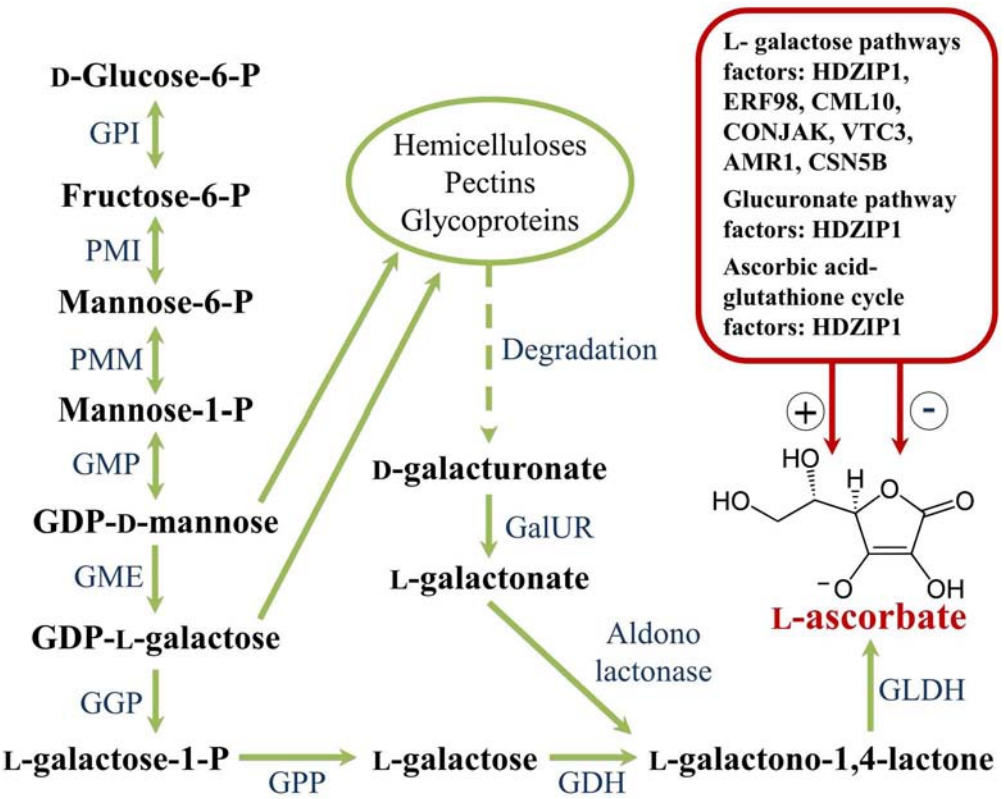

FIGURE 8 | Key biosynthetic pathways of ascorbic acid in plants. GalUR, D-galacturonic acid reductase; GDH, L-galactose dehydrogenase; GGP, GDP-L-galactose phosphorylase; GLDH, L-galactonolactone dehydrogenase; GME, GDP-D-mannose-3,5-epimerase; GMP, GDP-D-mannose pyrophosphorylase; GPI, glucose-6-phosphate isomerase; GPP, L-galactose-1-P phosphatase; PMI, phosphomannose isomerase; PMM, phosphomanno mutase.

The regulatory role of the other enzymes of the pathway is still controversial in tomato fruits. For example, GLDH gene expression followed an opposite profile when compared with the corresponding melon gene, which accumulated during ripening, coinciding with elevated levels of AsA (Pateraki et al., 2004), indicating different means of regulating AA synthesis in these two fruit.

In red-ripe tomato fruits, similarly to other crop species including strawberry, apple, orange, and grape, a less important alternative pathway operates in maintenance of AsA pools. This pathway uses galacturonate as a substrate of aldono lactonase (AL) to give L-galactono-1,4-lactone that is subsequently converted to AsA by GLDH. Galacturonate is a pivotal compound with double importance since it is also the elementary unit forming cell wall pectins and as AsA primer (Mellidou and Kanellis, 2017).

The ascorbate pool undergoes turnover in plants. The final stable products of AsA catabolism differ between species and in tomato are represented by oxalate, threonate and oxalyl threonate (Smirnoff, 2018). The rate of degradation and/or the capacity for AsA regeneration are also responsible for the control of AsA levels in tomato fruits. After oxidation to MDHA and DHA AsA is regenerated by the ascorbate-glutathione cycle and, particularly, the intervention of glutathione, DHA and MDHA reductases (GSHR, DHAR, and MDHAR, respectively) (Foyer and Noctor, 2011; Mellidou and Kanellis, 2017). Within this cycle, the HD-ZIP1 transcription factor has been reported to influence positively the transcription of both key enzymes DHAR and MDHAR. It also enhance the transcription of oxygenase (MIOX), enzyme involved in the synthesis of D-glucuronic acid from myo-inositol. These mechanisms of regulation have been proposed to be cultivar-dependent. In fact, in the low-AsA cultivar Ailsa Craig, this alternative route of AsA biosynthesis may supplement biosynthesis via L-galactose, while in the high-AsA cultivar Santorini, enhanced AsA recycling activities appear to be responsible for AsA accumulation in the later stages of ripening.

At the best of our knowledge, there are no specific reports on AsA regulation in watermelon fruits. Nevertheless, foliar application of gibberellic acid was reported to increase the AsA content in other Cucurbitaceae as also reported for tomato fruits (Basu et al., 1999).

HARMFUL EFFECT OF EXCESS BIOACTIVE COMPOUNDS ON HUMAN HEALTH

Consuming plants for their presumed health benefits has occurred since early civilizations. Phytochemicals are generally

thought to be safe for consumption because they are produced naturally. However, this is not always the case as many natural compounds found in several commonly consumed plants are potential deleterious for health, especially if assumed in excessive amount. Most plant bioactive compounds are plant secondary metabolites and some exert potent antioxidant activities and/or disease-combatting abilities when consumed within specific doses (Bruno et al., 2018; Siddiqui et al., 2018).

Based on the rising interest of costumers for natural and isolated bioactive compounds of plant origin, and the consequential risk of excessive intake, there is an urgent need to evaluate their real or potential functionality on human health and/or wellbeing and develop scientifically based recommendations for a safe intake.

Although the increasing evidence regarding the negative correlation between fruits and vegetables dietary consumption and the incidence of various chronic diseases, the observed effects are hardly attributable to a single compound or class of compounds (Erdman et al., 2009; Russo et al., 2017; Durante et al., 2019). Recently, various researchers reported a double-edged sword function (antioxidant/pro-oxidant activity) for many phytochemicals, including lycopene, phenols and flavonoids (Skibola and Smith, 2000; Tokaç et al., 2013; Bacanli et al., 2017) depending on various factors (Fernando et al., 2019). Generally, high concentrations, low pH and/or the presence of redox-active transition metal ions, causes certain phytochemicals to exhibit pro-oxidant activity (Eghbaliferiz and Iranshahi, 2016).

Clinical trials using different isolated molecules, including β-carotene, vitamin E and vitamin C, resulted disappointing (Bjelakovic et al., 2008; Erdman et al., 2009). Hennekens et al. (1996) reported that long-term supplementation with β-carotene has no beneficial effect on the incidence of malignant neoplasms and cardiovascular diseases. Unlike essential nutrients, bioactive compounds act through various and complex mechanisms to confer, often, a similar outcome. The body defense system is poly-sided, consequently the treatment with an isolated molecule will probably arise little effect on the incidence of chronic disease except in the case of early deficiency (Erdman et al., 2009).

Additionally, it has been reported that bioactives acts in synergy. According to Erdman et al. (2009), lycopene may synergize with other antioxidant compounds leading to the supposed and/or observed protective effect in some of the cardiovascular or cancer protection and hence it might not be the only important phytochemical in tomato and watermelon fruits. Besides, the importance of the catabolic products of lycopene (lycopenoids) on health is emerging due to growing evidences on their direct effect on gene expression and modulation (Erdman et al., 2009). It has been demonstrated that at concentrations ranging from 0.5 to 8 μM, lycopene does not induce DNA impairment, however, above 12 μM, it seems to prompt DNA damage in human cells (Tokaç et al., 2013; Bacanli et al., 2017).

Many flavonoids mimic mutagenic agents when ingested at high/cytotoxic levels. Quercetin was demonstrated to induce frame-shift mutation and DNA pair-base substitutions as well to prompt chromosome abnormalities and exert pro-oxidant activity (Skibola and Smith, 2000). Arif et al. (2015) focused on the cellular DNA damage initiated by different flavonoids and concluded that myricetin exhibited the highest level of DNA degradation followed by fisetin, quercetin, kaempferol, and galangin. Scheffler et al. (2018) reported that catabolism of AsA and DHA generates highly reactive carbonyl-intermediates able to induce glycation of cell proteins, an irreversible and relatively slow process thought to underlie the functioning of the biological clock that controls aging and involved in the onset of several age-related and degenerative diseases. Recently, Parent et al. (2018) reported that vitamin C intake is ineffective in prostate cancer prevention.

Therefore, it is important to focus accurately on dose-response relationship between ingested single/group of antioxidant and reactive oxygen species balance in human body in order to develop scientifically based recommendations for a safe intake. Future intervention studies had better to emphasize, the cumulative, synergistic and antagonistic effect of the entire bioactive pool present in fruits and vegetables rather than simply concentrating on the effect of single phytochemical compound without sufficient consideration to the potential of associated compounds.

CONCLUSION

Tomato and watermelon functionality resides deeply inside the presence of high levels of carotenoid pigments but it goes far beyond color as many other botanicals synergistically contribute to the hailed health-promoting properties of both fruits. The quantities and profiles of all these bioactive compounds, as well as their metabolic pathways and regulatory mechanisms, show similarities and differences between the two crops but, concurrently, appear substantially genotype-dependent despite the low level of genetic diversity in the cultivated germplasm.

A complex interweaving of anabolic, catabolic, and recycling reactions, finely regulated at multiple levels and with temporal and spatial precision, ensures a certain homeostasis in the concentrations of carotenoids, phenolics, aroma volatiles and AsA within the fruit tissues. Nevertheless, several exogenous factors including light and temperature conditions, pathogen attack, as well as pre- and post-harvest manipulations can drive their amounts far away from homeostasis. These adaptive responses allow crops to better cope with abiotic and biotic stresses but may severely affect the supposed functional quality of fruits.

Although the knowledge on the cross-talk between the plethora of stimuli and effectors involved in the regulation mechanisms is still limited, tomato and watermelon have been subjected to successful technological interventions aimed at fortifying and/or diversify the health-promoting attributes of the fruits either through metabolic engineering or the manipulation of pre- and post-harvest factors. However, based on the safety concerns recently raised, particularly about the seemingly indiscriminate addition of botanicals to foods, the real or potential functionality of these nutritionally fortified fruits must be supported by intervention studies on the cumulative, synergistic and antagonistic effect of their entire bioactive phytocomplex.

AUTHOR CONTRIBUTIONS

RI and ML conceptualized the idea of this review. RI, IT, ML, and MS scanned the literature, retrieved and processed manuscript referenced in the review, and wrote the manuscript. RI, ML, and CH critically reviewed the manuscript and enriched the key parts of the manuscript. All authors contributed to the revision of the manuscript prior to submission.

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Metabolomic and Transcriptomic Analyses Reveal That a MADS-Box Transcription Factor *TDR4* Regulates Tomato Fruit Quality

Xiaodan Zhao[1], Xinyu Yuan[2], Sha Chen[3], Da-Qi Fu[2] and Cai-Zhong Jiang[4,5]**

[1] School of Food and Chemical Engineering, Beijing Technology and Business University, Beijing, China, [2] Laboratory of Food Biotechnology, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China, [3] Institute of Traditional Chinese Medicine, China Academy of Chinese Medical Sciences, Beijing, China, [4] Department of Plant Sciences, University of California, Davis, Davis, CA, United States, [5] Crops Pathology and Genetics Research Unit, United States Department of Agriculture, Agricultural Research Service, Davis, CA, United States

Tomato fruit ripening is a complex process, which determines the formation of fruit quality. Many factors affect fruit ripening, including environmental conditions and genetic factors. Transcription factors (TFs) play key roles in regulating fruit ripening and quality formation. Current studies have found that the *TDR4* gene is an important TF for tomato fruit ripening, but its effects on fruit metabolism and quality are less well studied. In this study, suppression of *TDR4* gene expression obtained through virus-induced gene silencing (VIGS) technology resulted in an orange pericarp phenotype. Transcriptomic analysis of *TDR4*-silenced fruit showed changes in the expression of genes involved in various metabolic pathways, including amino acid and flavonoid biosynthesis pathways. Metabolomic analysis showed that levels of several amino acids including phenylalanine and tyrosine, and organic acids were reduced in *TDR4*-silenced fruit, while α-tomatine accumulated in *TDR4*-silenced fruit. Taken together, our RNA-seq and metabolomics analyses of *TDR4*-silenced fruit showed that *TDR4* is involved in ripening and nutrient synthesis in tomato fruit, and is therefore an important regulator of fruit quality.

Keywords: transcription factors, virus-induced gene silencing, *TDR4*, fruit quality, metabolomic, transcriptomic

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****Correspondence:***
Xiaodan Zhao
docdan@126.com
Cai-Zhong Jiang
cjiang@ucdavis.edu

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INTRODUCTION

Fruit is an important source of human healthy diet which can provide vitamins, minerals, and a wide range of bioactive compounds, including antioxidant carotenoids and various polyphenols (Seymour et al., 2013). The quality and nutrition of fresh fruits are gradually formed during ripening. Studying the molecular mechanism of fruit ripening is an important way to understand the formation of fruit quality. Fruit ripening is a complex biological process to form delicious and nutritious fruits for attracting animals to eat and spread seeds (Martel et al., 2011). Some general ripening-associated changes take place among some fruit species, including the cell wall degradation for fruit softening, alteration of the composition and levels of secondary metabolites, such as pigments, flavors, and aromas during fruit ripening (Martel et al., 2011). These changes are influenced by multiple genetic and biochemical pathways that are regulated by several critical transcription factors (TFs) (Giovannoni, 2007).

The tomato (*Solanum lycopersicum*) is the main horticultural crops and it is hot popular food for consumers. Tomato is considered as an ideal model material for studying fleshy fruit ripening (Klee and Giovannoni, 2011; Karlova et al., 2014). In climacteric fruits, including tomatoes, increased ethylene production is required for the onset of ripening (Barry and Giovannoni, 2007). During fruit development and ripening, the biosynthesis and signal transduction of ethylene are both regulated by several TFs, including RIPENING INHIBITOR (MADS-RIN), COLORLESS NON-RIPENING (CNR), NON-RIPENING (NOR), TOMATO AGAMOUS-LIKE1 (TAGL1), NOR-like1, and APETALA2a (AP2a) (Vrebalov et al., 2002, 2009; Manning et al., 2006; Giovannoni, 2007; Karlova et al., 2011; Gao et al., 2018b, 2019). TDR4/FUL1 and its homolog MBP7/FUL2 are MADS-box family TFs with high sequence similarity to *Arabidopsis* FRUITFULL. In contrast to the above-mentioned TFs, TDR4/FUL1 and MBP7/FUL2 do not regulate ethylene biosynthesis but affect fruit ripening in an ethylene-independent manner (Bemer et al., 2012). A previous study revealed that TDR4/FUL1 mRNA and protein accumulate during ripening in tomato fruit, while MBP7/FUL2 mRNA and protein accumulate during the pre-ripening stage and throughout ripening process (Shima et al., 2013). RNAi-silencing of each of the *FUL* homologs independently results in very mild changes to tomato fruit pigmentation, while the silencing of both genes results in an orange ripe fruit with highly reduced levels of lycopene, suggesting that FUL1/TDR4 and FUL2/MBP7 possess redundant functions in fruit ripening (Bemer et al., 2012). The expression of genes involved in cell wall modification, cuticle production, volatile production, and glutamate accumulation was also altered in *TDR4* silencing tomato fruit (Bemer et al., 2012). Chromatin immunoprecipitation coupled with microarray analysis (ChIP-chip) revealed that FUL homologs take part in many biological processes through the regulation of ripening-related gene expression, both in cooperation with and independent of RIN (Fujisawa et al., 2014).

In order to further study the effect of TDR4 on tomato quality metabolism, we utilized virus-induced gene silencing (VIGS) to silence *TDR4* in tomato fruit. Analysis of transcripts and metabolites of *TDR4*-silened fruit indicated that it was involved in the metabolism of several amino acids and biosynthesis of secondary metabolites, altering fruit nutrient levels and flavor. The result shows that *TDR4* regulates the nutrient levels and quality of tomato fruit.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tomato plants (*S. lycopersicum* "Ailsa Craig") were planted in commercial tomato-cultivated soil and grown under standard glasshouse conditions of 16-h day length and 25°C, with a night temperature of 18°C with 75% relative humidity. Flowers were tagged at 1 day post-anthesis (DPA). Ten plants are for control and 10 plants were used to silence *TDR4* gene; each plant was no less than 15 fruits.

Vector Construction

The tobacco rattle virus (TRV)-based vectors pTRV1 and pTRV2 were used for VIGS. To construct a pTRV2-*TDR4* recombinant, a 360-bp *Eco*RI/*Bam*HI-containing DNA fragment of the *TDR4* gene, corresponding to nucleotides 323–682 (NM_001247244.2), was amplified from tomato fruit complementary DNA (cDNA) using primers *TDR4*-VIGS-For and *TDR4*-VIGS-Rev (**Supplementary Table S1**). The resulting products and pTRV2 vector were digested with *EcoRI/BamHI* and ligated by T4 ligase.

Agro-Infiltration

The VIGS assay was carried out as previously described (Fu et al., 2005) with slight modification. All plant inoculations were performed using a 1:1 (v/v) mixture of two *Agrobacterium tumefaciens* GV3101 cultures, one containing the pTRV1 vector and the other containing the pTRV2 or pTRV2-derived vector. Bacterial clones were grown overnight at 28°C in Luria-Bertani medium containing 10 mM MES and 20 mM acetosyringone with kanamycin, gentamycin, and rifampicin antibiotics. They were then harvested and transferred to the infiltration medium [10 mM $MgCl_2$, 10 mM MES (pH 5.6), 200 mM acetosyringone] to a final OD_{600} of 6.0. For co-infiltration studies, 1:1 mixtures of pTRV1, pTRV2-00, or pTRV2-*TDR4* were used. The *Agrobacterium* mixture was injected into the carpopodium of the tomato fruit at 7–10 DPA after pollination using a 1-ml syringe with a syringe needle. The control fruits were infected by *A. tumefaciens* containing a pTRV2 empty vector, and the *TDR4*-silenced fruits were infected by *A. tumefaciens* containing a pTRV2-TDR4 vector. Each infected fruit was not less than 100 from 10 different plants.

RNA-Seq and Data Processing

Total RNA was extracted from the fruit pericarp of TRV2-00 infected control fruits and TRV2-*TDR4* silenced fruits (three biological replicates in which each sample was collected from six different fruits) using a RNeasy MiniKit (Qiagen, Hilden, Germany) (Wang et al., 2016). RNA integrity was evaluated on 1% agarose gels stained with ethidium bromide (EB). RNA concentrations were measured using a Nano Photometer® spectrophotometer (Implen, CA, United States). cDNA libraries were generated using the NEBNext® Ultra RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, United States) following the manufacturer's instructions. Briefly, mRNA was enriched using oligo (dT)-attached magnetic beads. Fragmentation was performed by divalent cations in NEBNext First Strand Synthesis Reaction Buffer. These fragments were used to synthesize first-strand cDNA using random hexamer primers and M-MuLV Reverse Transcriptase. Then, second-strand cDNA synthesis was achieved using DNA Polymerase I and RNase H. Exonuclease/polymerase activities were used to convert overhangs into blunt ends. In order to select cDNA fragments of the appropriate size, library fragments were purified with the AMPure XP system (Beckman Coulter,

Beverly, MA, United States). USER Enzyme (New England Biolabs) was subsequently used with size-selected, adaptor-ligated cDNA. Then, PCR was carried out with Phusion High-Fidelity DNA polymerase, universal PCR primers, and Index (X) Primer. Finally, PCR products were purified, and library quality was evaluated on the Agilent Bioanalyzer 2100 system (Palo Alto, CA, United States). Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the HiSeq 4000 PE Cluster Kit (Illumina, San Diego, CA, United States) according to the manufacturer's instructions. Then, library preparations were sequenced on an Illumina HiSeq 4000 platform, and 150-bp paired-end reads were generated.

We used cutadapt[1] and the FASTX-Toolkit[2] to trim raw reads in order to remove barcode and adaptor sequences, and the resulting clean reads were checked for quality using a threshold of Q < 20. Clean reads from each library were aligned to the tomato reference genome (SGN release version SL2.50[3]) using TopHat[4]. We used Cufflinks[5] to assemble reads with fewer than two mismatches. Differentially expressed genes (DEGs) between pTRV2-*TDR4* andpTRV2-00 were identified with the following criteria: fold-change ≥ 2 and Q-value < 0.05. Clean reads of RNA-seq were deposited in the National Center for Biotechnology Information Sequence Read Archive[6] under accession number SRP201254.

Gene Enrichment and Pathway Analysis

To assess the distribution of DEG functions, Gene Ontology (GO) enrichment analysis was performed using WEGO[7]. FASTA format files containing DEG cDNA sequences were obtained using Perl scripts, and then pathway analysis was conducted with the Kyoto Encyclopedia of Genes and Genomes (KEGG) in KOBAS[8], based on native BLAST tools and organism annotation libraries.

Validation of RNA-Seq by Quantitative Real-Time PCR(qRT-PCR)

Total RNA (2 μg) from three biological replicates was purified and reverse-transcribed into cDNA using TranScript One-step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) with oligo(dT). Then, qRT-PCR was

[1]https://pypi.python.org/pypi/cutadapt/
[2]http://hannonlab.cshl.edu/fastx_toolkit/download.html
[3]ftp://ftp.sgn.cornell.edu/tomato_genome
[4]http://ccb.jhu.edu/software/tophat/index.shtml
[5]http://cole-trapnell-lab.github.io/cufflinks/
[6]https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP201254
[7]http://wego.genomics.org.cn/
[8]http://kobas.cbi.pku.edu.cn/download.php

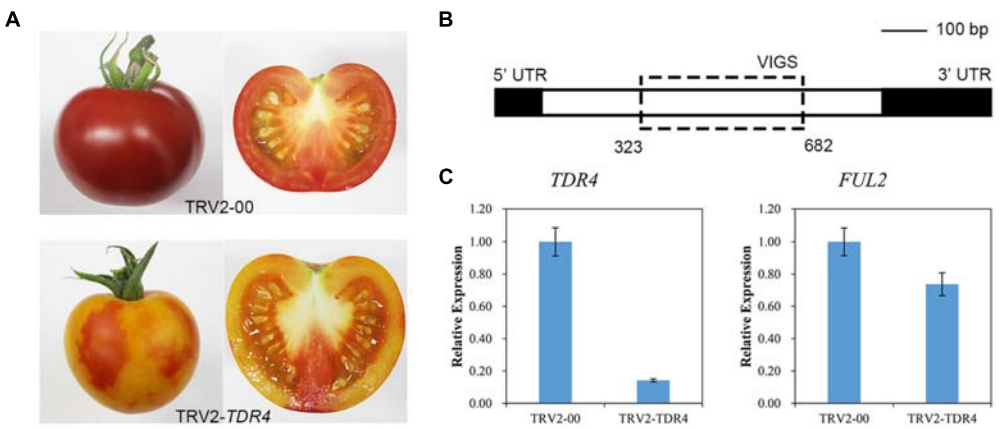

FIGURE 1 | VIGS in tomato fruit. **(A)** Phenotype of *TDR4*-silenced tomato fruit. TRV2-00 was used as a control. **(B)** Schematic representation of the structure of the *TDR4* coding sequence and the positions of the fragments used for VIGS. **(C)** Silencing efficiency of *TDR4* and *FUL2* genes.

TABLE 1 | Summary of clean read counts and percentage of unique mapped reads.

Sample	Clean reads left/right	Left unique mapped	Right unique mapped	Unique aliment
TRV2-00-1[a]	21377728 (100%)	18987592 (88.8%)	18084744 (84.6%)	17254526 (80.7%)
TRV2-00-2	23506338 (100%)	21188488 (90.1%)	19683578 (83.7%)	18860690 (80.2%)
TRV2-00-3	22897923 (100%)	20740458 (90.6%)	18994540 (83.0%)	18160445 (79.3%)
TRV2-TDR4-1	21079921 (100%)	19277894 (91.5%)	17904806 (84.9%)	17144044 (81.3%)
TRV2-TDR4-2	19561207 (100%)	17455736 (89.2%)	15730401 (80.4%)	14925877 (76.3%)
TRV2-TDR4-3	22102725 (100%)	19841339 (89.8%)	17906852 (81.0%)	17006260 (76.9%)

[a] *Sample labels 1, 2, and 3 indicate three biological replicates.*

performed with SYBR Green PCR SuperMix (TransGen Biotech) on a Bio-Rad Real-Time PCR System CFX96 (Bio-Rad, Hercules, CA, United States), using a tomato actin gene as a reference gene. All primer sequences are listed in **Supplementary Table S1**. The reaction proceeded as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The fluorescence signal was monitored automatically at each cycle. Relative expression levels of specific mRNAs were measured using the $2^{-\Delta\Delta Ct}$ method. Standard errors were calculated based on a minimum of three biological replicates.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis

Frozen TRV2-00 and TRV2-*TDR4* tomato pericarp samples (six biological replicates) were milled into powder using a mortar

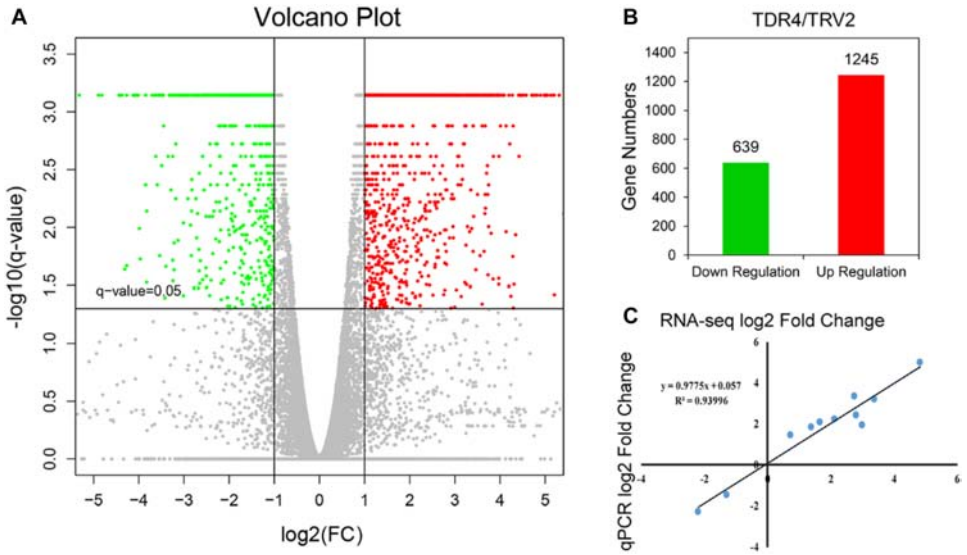

FIGURE 2 | Global overview of DEGs between *TDR4*-silenced and control tomato fruit. **(A)** Volcano plot of DEGs. Spots above the threshold line (Q-value = 0.05) indicate significant DEGs. Genes for which expression in *TDR4*-silenced fruit was less than half that in control fruit, with a Q-value < 0.05, are shown in green, while those for which expression in *TRD4*-silenced fruit was more than two fold that in the control group are shown in red. Genes in gray were neither up- nor downregulated. **(B)** Number of down- (639) and upregulated (1245) genes. **(C)** Expression levels of 11 genes as determined by qRT-PCR are closely correlated with those according to RNA-seq.

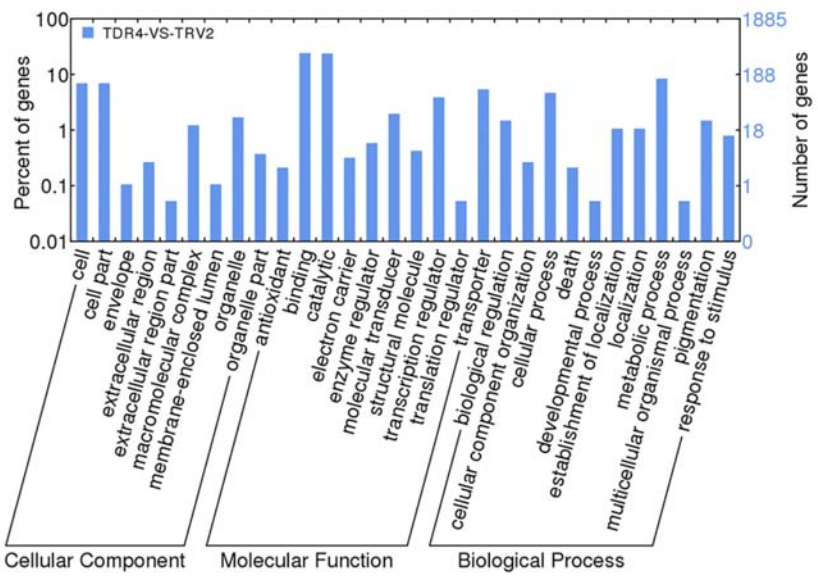

FIGURE 3 | GO analysis of DEGs between *TDR4*-silenced and control tomato fruit according to WEGO.

and pestle, and the powder from each sample was weighed. Next, 100 mg was suspended in either 1.0 ml pure methanol or 1.0 ml 75% aqueous methanol for the extraction of lipid-soluble and water-soluble metabolites, respectively. Both types of methanol contained 20 mg l^{-1} lidocaine and 20 g l^{-1} CHAPS. The suspensions were vortexed and then extracted at 4°C overnight. Following centrifugation at 12,000 × *g* for 10 min, the supernatants of both the lipid-soluble and water-soluble metabolites were collected and mixed in a ratio of 1:1 before being filtered through a 0.45-μm membrane and subjected to LC-MS analysis.

A high performance liquid chromatography unit, equipped with a photodiode array detector (HPLC-20A, Shimadzu, Japan), was used to analyze the metabolites in the tomato extract. The separation of metabolites was carried out under the following conditions: column: Eclipse XDB-C_{18} (3.0 mm × 50 mm); solvent A: water with 0.2% formic acid; solvent B: acetonitrile; gradient program: 95:5 A:B (v/v) at 0 min, 5:95 A:B at 12 min, 5:95 A:B at 15 min, 95:5 A:B at 15.1 min, 95:5 A:B at 22 min; flow rate: 0.2 ml min^{-1}; temperature: 45°C; injection volume: 2 μl. The masses of the eluted compounds ranging from 50 to 1500 $m\,z^{-1}$ were monitored with a Triple Quad LC/MS equipped with an electrospray ionization (ESI) source.

Quantitative detection was performed using an UHPLC-ESI-QQQ-MS (Agilent 1290 and 6460 triple quadrupole mass spectrometry series). An ESI source working either in positive or negative ion mode was used for all MS analyses, with nitrogen as the drying agent. The MS conditions in positive mode were as follows: HV voltage: 4000 kV; capillary: 7 μg; nozzle voltage: 500 V; delta EMV: 300 V; gas flow: 5 l min^{-1}; gas temperature: 400°C; sheath gas flow: 11 l min^{-1}. Collision energy was optimized based on the standards. Helium was used as the collision gas for collision-induced dissociation (CID). Quantification was performed using the multiple reaction monitoring (MRM) mode under unit mass-resolution conditions. The data were processed with MassHunter software.

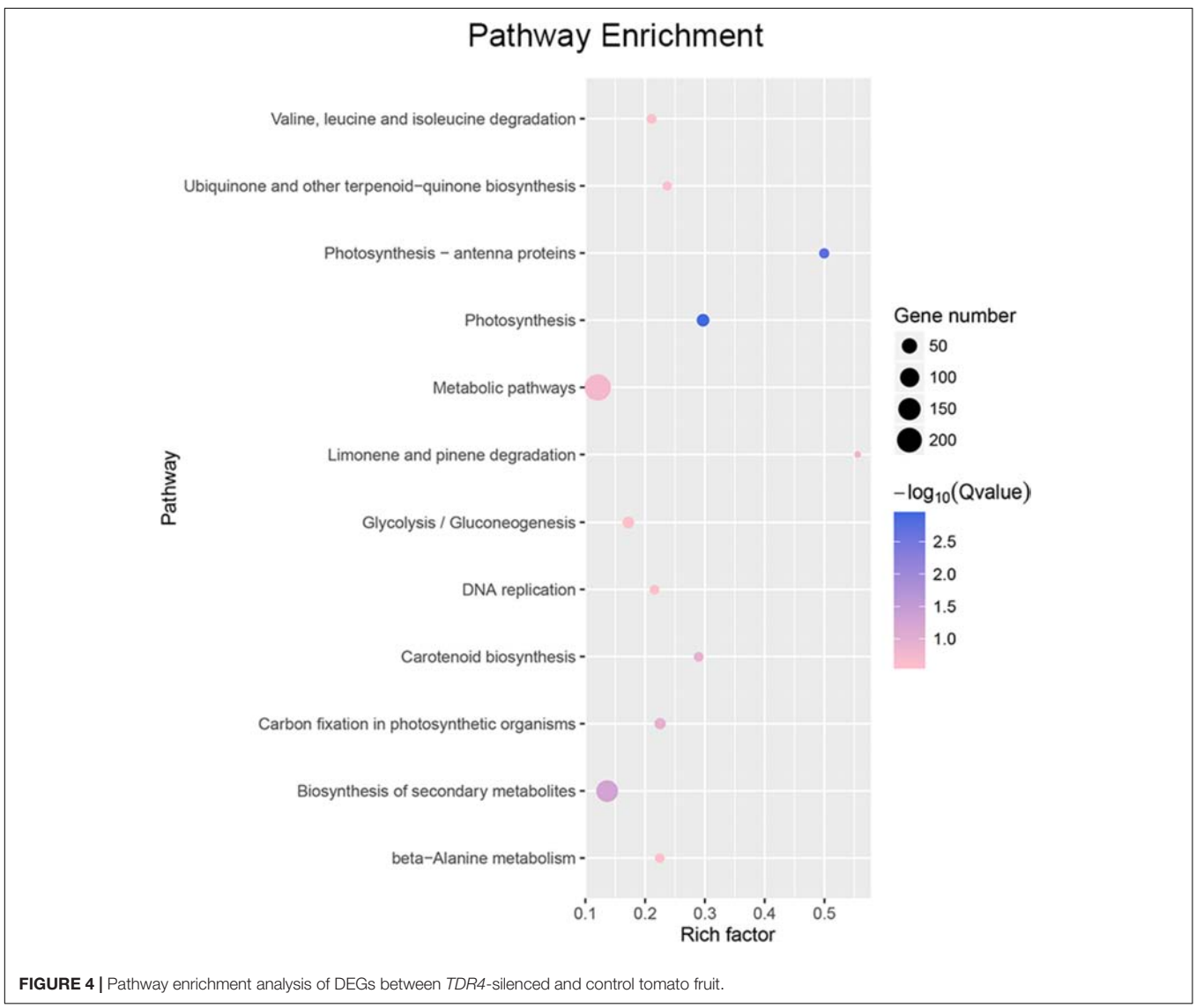

FIGURE 4 | Pathway enrichment analysis of DEGs between *TDR4*-silenced and control tomato fruit.

RESULTS AND DISCUSSION

Silencing of *TDR4* Inhibits Tomato Fruit Ripening

To silence *TDR4* gene and analyze its effect on tomato fruit metabolism, a mixture of *Agrobacterium* cultures containing pTRV-*TDR4* and pTRV1 was injected into the carpopodium of the tomato fruit at 7–10 days after pollination using a 1-ml syringe with a needle. Around 35 days post-Agro-injection, approximately 35 tomato fruits injected with pTRV-*TDR4* failed to turn red and developed an orange phenotype at the red ripening (RR) stage. All control fruit injected with *A. tumefaciens* containing pTRV1 and pTRV2-00 turned red normally, like the wild-type fruit (**Figure 1A**). Quantitative real-time PCR (qRT-PCR) was performed to confirm *TDR4* silencing. The primers that anneal to the *TDR4* gene outside the region targeted for silencing were used (**Figure 1B**). In pTRV-*TDR4* injected fruits, the *TDR4* message was reduced by more than 80% compared with the TRV injected controls (**Figure 1C**). The level of *actin* gene RNA was similar in TRV-*TDR4* and TRV alone injected tissue and served as an internal control for RNA quality and RT-qPCR. Based on the above results, we can conclude that the *TDR4* gene has been successfully silenced in tomato fruit and *TDR4*-silenced fruits can be used for subsequent studies on the effects of *TDR4* gene on tomato fruit metabolism.

Global Overview of RNA-Seq Profile of *TDR4*-Silenced Tomato

To test the molecular consequences of silencing the *TDR4* gene in *TDR4*-silenced fruit, we compared the gene expression levels in the pericarp of *TDR4*-silenced fruit with that in control pericarp at red stage using strand-specific mRNA sequencing. The result showed that all clean reads were mapped and aligned against the tomato reference genome (ITAG2.4). Within each file, 79.1 ± 2.1% of the reads were uniquely aligned, suggesting that the sequencing results were effective and reliable (**Table 1**).

Using cutoff criteria with an expression ratio of ≥2 and $P < 0.05$ between TDR4-silencd and control tissues, analysis of DEGs revealed that 1245 genes were upregulated while 639 genes were downregulated in the *TDR4*-silenced fruits compared with that in control fruit (**Figure 2**). To provide an overview of the role of *TDR4*, we evaluated the DEGs using GO and KEGG pathway enrichment analyses. GO analysis indicated that *TDR4* silencing affected multiple metabolic pathways including 9 cellular component GO terms, with cell and cell part being the most enriched terms; 10 molecular function terms, with binding and catalytic being the most enriched; and 11 biological process terms, with metabolic process being the most enriched (**Figure 3**). KEGG pathway enrichment analysis showed that *TDR4* was involved in photosynthesis and the biosynthesis of secondary metabolites (**Figure 4**). Eleven genes related to fruit ripening and nutrient metabolism were selected for qRT-PCR validation

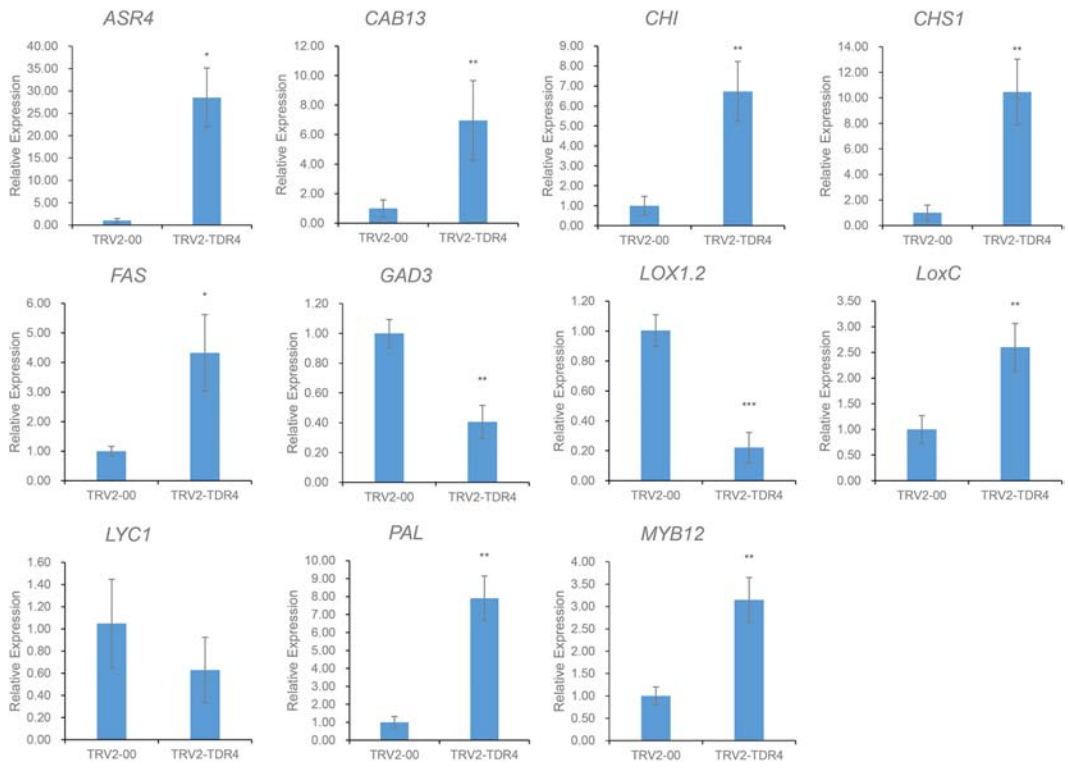

FIGURE 5 | Expression levels of 11 genes according to qRT-PCR. Error bars indicate the standard deviation of three biological replicates. Asterisks indicate significant differences as determined by Student's *t*-tests (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

of the RNA-seq data. The RT-qPCR results were consistent with the sequencing data, indicating the reliability of the sequencing results (**Figure 5**).

Analysis of Metabolites in Tomato Fruit Samples

To examine metabolic changes in the *TDR4* silenced fruit, LC-MS/MS metabolite analysis was performed in *TDR4*-silenced and control tomato fruits. The result indicates that 50 metabolites were identified in *TDR4*-silenced tomato fruit. According to a two-way analysis of variance (ANOVA), 17 of these metabolites significantly differed in abundance between control (TRV2-00) and *TDR4* silenced (TRV2-*TDR4)* fruit tissues (**Table 2**). All differential metabolites were further classified into four groups: amino acids, organic acids, phenolics, and solanum alkaloids. Possible pathways for each metabolite were determined by searching the KEGG database (**Figure 6**). These results suggest that the silencing of *TDR4* altered the tomato fruit metabolism.

Silencing of *TDR4* Alters Fruit Metabolism

Amino acids are primary metabolites that contribute to the flavor and nutritional value of tomato fruits. In *TDR4*-silenced fruit, levels of three amino acids were significantly reduced, including L-tyrosine (–65%), L-phenylalanine (–75%), and L-glutamic acid (–47%) (**Table 2**). KEGG pathway analysis revealed that the metabolic pathway of aromatic amino acids, such as phenylalanine and tyrosine, biosynthesis, and glutamate metabolism were also altered in *TDR4*-silenced fruits compared to control fruit (**Figure 4**), indicating that *TDR4* gene plays a role in the accumulation of certain amino acids during tomato fruit ripening, which contributes to flavor formation of tomato fruit. Tomato fruit synthesizes flavor and nutrients during its ripening, and ripening process is regulated by ripening-related TFs, such as *AP2a* and *Rin* TFs, so these TFs may also participate in the regulation of flavor synthesis. The majority of the amino acids (15 out of 22) were present at significantly lower levels in the AP2i fruits than in the wild type, and the most dramatic reductions were in β-Ala, Ile, Met, Phe, and Trp (Karlova et al., 2011). Rin protein can target the promoter of *TomloxC* and *ADH2* genes, which encode lipoxygenase (LOX) and alcohol dehydrogenase, respectively, and are critical for the production of characteristic tomato aromas derived from LOX pathway (Qin et al., 2012).

TABLE 2 | Relative quantitation of metabolites in *TDR4*-silenced and control tomato fruit.

Analytes	Ratio (TDR4-silenced/control)	*P*-value[a]	Pathway
L-Tyrosine	0.32	3.28E-02	Biosynthesis of amino acids
L-Phenylalanine	0.25	4.69E-02	Biosynthesis of amino acids
L-Glutamic acid	0.53	8.29E-04	Biosynthesis of amino acids
Glutathione	0.53	2.50E-08	Glutathione metabolism
Eriodictyol chalcone	3.06	1.94E-03	Flavonoid biosynthesis
5-Caffeoylquinic acid	0.39	3.39E-04	–
α-Tomatine	7.69	1.32E-14	–
Benzoic acid	3.89	4.00E-09	Phenylalanine metabolism
Malic acid	1.30	1.28E-06	Citrate cycle (TCA cycle)
Citric acid	0.90	2.38E-04	Citrate cycle (TCA cycle)
Kaempferol-3-rutinoside	0.41	8.87E-03	–
4-Aminobenzamide, allopurinol, hypoxanthine	0.73	2.41E-02	–
Quercetin-hexose-deoxyhexose, -pentose	0.72	2.88E-02	–
$C_{11}H_{23}O_{12}P$	0.43	2.51E-03	–
$C_{16}H_{20}O_{10}$	1.52	1.88E-02	–
$C_{34}H_{46}O_{14}$	1.38	3.40E-03	–
$C_{14}H_{20}N_2O_3$	0.27	2.04E-02	–

[a] *Pvalues calculated using Student's t-tests.*

Phenylalanine is an important precursor of many aroma volatiles and flavonoids. For example, 2-phenylacetaldehyde and 2-phenylethanol are derived from phenylalanine (Tieman et al., 2006); both of these have pleasant fruity, floral odors, and important biological functions in plants (Knudsen et al., 1993). They attract mammals and other seed dispersers and exhibit antimicrobial properties (Goff and Klee, 2006).

RNA-seq result show that two previously unreported genes (*Solyc11g066890* and *Solyc06g050630*), that encode prephenate dehydratase proteins, were significantly upregulated in *TDR4*-silenced fruit (**Supplementary Table S3**). These are probably involved in the first step of the sub-pathway that synthesizes L-phenylalanine or L-tyrosine, respectively, from L-arogenate. A gene (*Solyc10g038080*) encoding a shikimate dehydrogenase appears to be downregulated in *TDR4*-silenced fruits (**Supplementary Table S2**), which may contribute to a reduction in shikimate, an important precursor of L-phenylalanine and L-tyrosine.

Benzoic acid, synthesized from trans-cinnamate, is hypothesized to be the functional group in salicylic acid, and its derivatives are assumed to be involved in inducing stress tolerance in plants (Senaratna et al., 2003). In our study, the content of benzoic acid increased (3.89-fold) (**Table 2**) in *TDR4*-silenced fruit, along with the expression of two phenylalanine ammonia-lyase (PAL) genes. PALs are key enzymes in plant metabolism, catalyzing the first step of the sub-pathway that synthesizes trans-cinnamate from L-phenylalanine. One of the two upregulated genes is *PAL5* (**Supplementary Table S2**), which is strongly expressed in old leaves and flowers and may function in response to biotic and abiotic stresses (Guo and Wang, 2009; Puthoff et al., 2010). Our findings suggest that the *TDR4* gene negatively regulates the expression of *PALs* to inhibit the synthesis of benzoic acid in tomato fruit.

Glutamic acid is the most abundant amino acid in the diet, and a high level of free glutamate in some foods results in an umami taste (e.g., tomatoes, mushrooms, cheeses) (Bellisle, 1999). During tomato ripening, the glutamic acid content rises dramatically (Bemer et al., 2012). In our study,

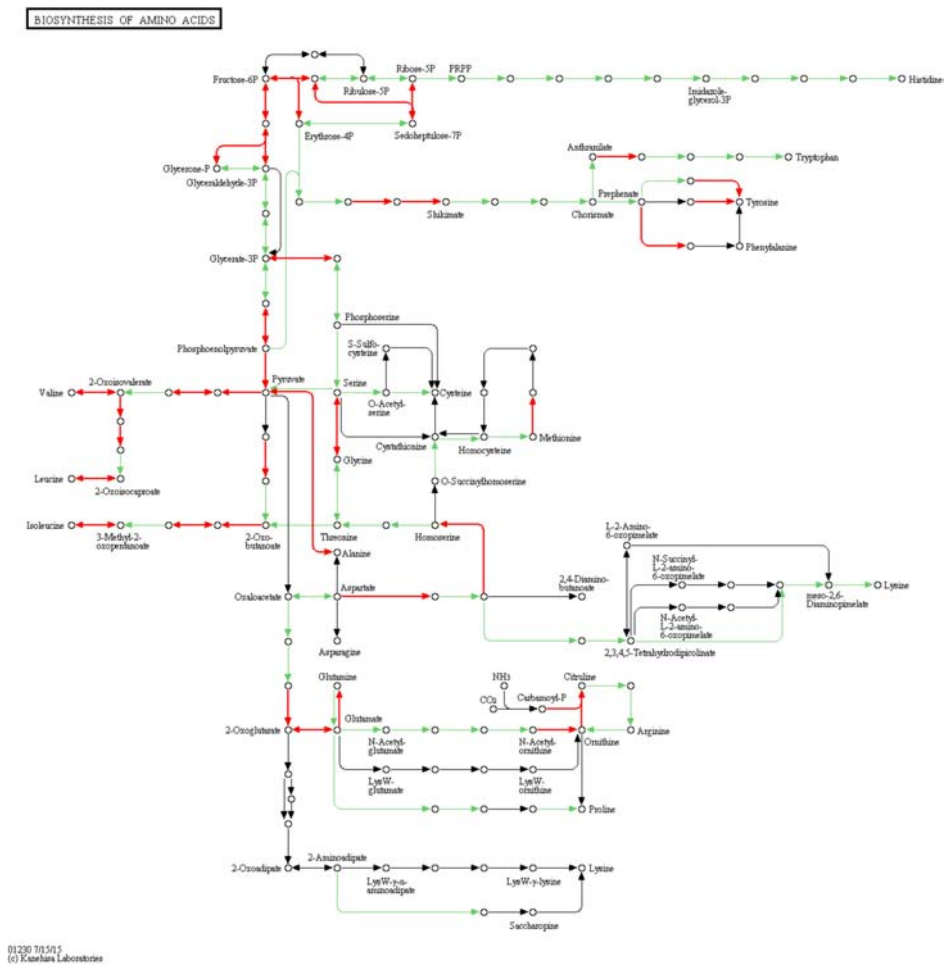

FIGURE 6 | Schematic diagram of amino acid biosynthesis pathway. Diagram constructed using KEGG pathway analysis. Circles represent metabolites, and arrows indicate steps. Red arrows indicate steps catalyzed by DEGs.

the expression levels of five related genes were significantly different in *TDR4*-silenced and control tomato fruit, and four of these were upregulated, including glutamate dehydrogenase (*GDH1*) (1.11-fold), glutamine synthetase (*GS*) (6.04-fold), glutamate decarboxylase 1 (*GAD3*) (2.20-fold), and a gene encoding isocitrate dehydrogenase (1.99-fold) (**Supplementary Table S2**). GDH1, which acts in the mitochondria, catalyzes the reversible amination of 2-oxoglutarate to L-glutamic acid (Ferraro et al., 2015). GS is a chloroplast glutamine synthetase that assimilates ammonia into glutamine (Perez-Rodriguez and Valpuesta, 1996), which is a metabolic intermediate in the synthesis of other nitrogen-containing compounds in plants (Perez-Rodriguez and Valpuesta, 1996). GAD3 converts L-glutamic acid to γ-aminobutanoic acid (GABA). The increased transcript abundances of these genes indicate the acceleration of glutamate metabolism in *TDR4*-silenced fruit.

Glutathione (GSH) is a tripeptide (γ-glutamylcysteinylglycine) that exists in a broad range of organisms, from bacteria to humans (Frendo et al., 2013). In humans, GSH plays an important role in the metabolism and detoxification of cytotoxic and carcinogenic compounds and reactive oxygen species (ROS) (Knapen et al., 1999). In plants, GSH is crucial for plant development and the plant response to the abiotic and biotic environment, and it is also involved in the detoxification of xenobiotics (Frendo et al., 2013; Yu et al., 2013). In our analysis of tomato fruit, *TDR4* silencing resulted in a significant reduction in glutathione (−47%) (**Table 2**). KEGG pathway analysis showed that the *GST* gene, encoding glutathione S-transferase, was upregulated, which would promote the conversion of glutathione to glutamate.

Eriodictyol chalcone is a type of flavonoid. It has been reported that eriodictyol chalcone accumulates predominantly in the tomato peel and exhibits the highest accumulation at the breaker stage, gradually decreasing during ripening (Iijima et al., 2008). Tomato *SlAN11* regulates flavonoid biosynthesis (Gao et al., 2018a). In our study, the content of eriodictyol chalcone was significantly increased in *TDR4*-silenced fruit (3.06-fold) (**Table 2**), compared to that in controls. KEGG pathway analysis showed that flavonoid biosynthesis was altered in *TDR4*-silenced fruit, which was consistent with the observation that chalcone synthase 1 (*CHS1*) and chalcone synthase 2 (*CHS2*) were upregulated (**Supplementary Table S2**).

5-Caffeoylquinic acid (chlorogenic acid) is one of the most abundant and widespread soluble phenolics among vascular plants (Mahesh et al., 2007). Evidence suggests that it can protect plant cells against oxidative stress, and plays a role in resistance to phytopathogens (Mahesh et al., 2007). The existence of another route involving the direct 3′-hydroxylation of *p*-coumaryol quinic acid was first suggested in carrot cell cultures, and studies of the impact of the expression of the hydroxycinnamoyl quinic acid gene in tobacco and tomato plants demonstrated that this route may be predominant in the Solanaceae family (Niggeweg et al., 2004). Analysis of *TDR4*-silenced tomato fruit revealed a significant reduction in 5-caffeoylquinic acid levels (–61%) (**Table 2**).

α-Tomatine is an anti-nutritional factor for humans (Friedman, 2013). In tomato, α-tomatine is present at high concentrations during the mature green (MG) stage and dramatically decreases during fruit ripening (Mintz-oron et al., 2008). In the present study, levels of α-tomatine were significantly elevated in *TDR4*-silenced fruit (7.69-fold) (**Table 2**). At the same time, the expression level of *GAME11* was increased in *TDR4*-silenced fruit (**Supplementary Table S3**). It was reported that using VIGS technology to silence *GAME11*a putative dioxygenase in the cluster resulted in a significant reduction in α-tomatine levels and accumulation of several cholestanol-type steroidal saponins in tomato leaves (Itkin et al., 2013), which was consistent with our results. Putatively, GAME11 catalyzes the closure of the E-ring of 22,26-dihydroxycholesterol to form the furostanol-type aglycone (Itkin et al., 2013). In summary, the silencing of *TDR4* promotes α-tomatine biosynthesis by enhancing the expression of *GAME11* and TDR4 is a negative regulator of *GAM11* gene.

CONCLUSION

The silencing of *TDR4* using VIGS resulted in a non-ripening phenotype with an orange pericarp. RNA-seq analysis of *TDR4*-silenced fruit showed the altered expression of genes involved in various metabolic pathways. Analysis of metabolites by LC-MS/MS showed reductions in several amino acids as well as the accumulation of α-tomatine in *TDR4*-silenced fruit. These results suggest that *TDR4* regulates the accumulation of nutrients and flavor in tomato fruit via the transcriptional regulation of target genes.

AUTHOR CONTRIBUTIONS

XZ designed the experiments and wrote the manuscript. XY and SC did the experiments. D-QF analyzed the data. C-ZJ revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00792/full#supplementary-material

TABLE S1 | Oligonucleotide primers used in the study.

TABLE S2 | Representative DEGs in *TDR4*-silenced tomato fruits.

TABLE S3 | The DEGs in *TDR4*-silenced tomato fruits.

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Nutraceutical Characterization of Anthocyanin-Rich Fruits Produced by "Sun Black" Tomato Line

Federica Blando[1], Helge Berland[2], Gabriele Maiorano[1], Miriana Durante[1], Andrea Mazzucato[3], Maurizio E. Picarella[3], Isabella Nicoletti[4], Carmela Gerardi[1], Giovanni Mita[1] and Øyvind M. Andersen[2]*

[1] Institute of Sciences of Food Production, (ISPA), CNR, Lecce, Italy, [2] Department of Chemistry, University of Bergen, Bergen, Norway, [3] Department of Agriculture and Forest Sciences, University of Tuscia, Viterbo, Italy, [4] Institute for Biological Systems (ISB), CNR, Rome, Italy

Tomato (*Solanum lycopersicum* L.) is one of the most cultivated vegetable in the world and it represents a large source of bioactive compounds, including carotenoids and polyphenols (phenolic acids and flavonoids). However, the concentration of flavonoids in tomato is considered sub-optimal, particularly because anthocyanins are not generally present. Therefore, this crop has been the object of an intense metabolic engineering in order to obtain anthocyanin-enriched tomatoes by using either breeding or transgenic strategies. Some wild tomato species, such as *S. chilense* and *S. cheesmaniae*, biosynthesize anthocyanins in the fruit sub-epidermal tissue, and some alleles from those genotypes have been introgressed into a new developed purple tomato line, called "Sun Black" (SB). It is a tomato line with a purple skin color, both in green and in red fruit stages, due to the biosynthesis of anthocyanins in the peel, and a normal red color pulp, with a taste just like a traditional tomato. SB is the result of a breeding programme and it is not a genetically modified (GM) product. We report the chemical characterization and structure elucidation of the attractive anthocyanins found in the peel of SB tomato, as well as other bioactive compounds (carotenoids, polyphenols, vitamin C) of the whole fruit. Using one- and two-dimensional NMR experiments, the two main anthocyanins were identified to be petunidin 3-*O*-[6″-*O*-(4‴-*O*-*E*-*p*-coumaroyl-α-rhamnopyranosyl)-β-glucopyranoside]-5-*O*-β-glucopyranoside (petanin) and malvidin 3-*O*-[6″-*O*-(4‴-*O*-*E*-*p*-coumaroyl-α-rhamnopyranosyl)-β-glucopyranoside]-5-*O*-β-glucopyranoside (negretein). The total anthocyanins in the whole ripe fruit was 1.2 mg/g dry weight (DW); 7.1 mg/100 g fresh weight (FW). Chlorogenic acid (the most abundant phenolic acid) was 0.6 mg/g DW; 3.7 mg/100 g FW. The main flavonol, rutin was 0.8 mg/g DW; 5 mg/100 g FW. The total carotenoid content was 211.3 μg/g DW; 1,268 μg/100 g FW. The total phenolic content was 8.6 mg/g DW; 52.2 mg/100 g FW. The vitamin C content was 37.3 mg/100 g FW. The antioxidant activities as measured by the TEAC and ORAC assays were 31.6 and 140.3 μmol TE/g DW, respectively (193 and 855.8 μmol TE/100 g FW, respectively). The results show the unique features of this new tomato genotype with nutraceutical properties.

Keywords: tomato, tomato breeding, bioactive compounds, anthocyanins, functional food

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Eugenio Butelli, John Innes Centre (JIC), United Kingdom

****Correspondence:***
Federica Blando
federica.blando@ispa.cnr.it

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INTRODUCTION

The tomato (*Solanum lycopersicum* L.) is one of the most cultivated vegetables in the world, whose production was around 182 million tons (FAOSTAT)[1] in 2017. The average annual consumption of tomato fruit is 18 kg per capita in Europe and 8 kg in the US, with regular dietary intake in fresh, cooked and processed form, depending on the local habit (1). The tomato fruit has a high nutritional value, thanks to the presence of numerous antioxidant compounds such as carotenoids, phenolic compounds, vitamin C and E. These compounds are supposed to be responsible for the beneficial effects on health, such as reduced risk of inflammatory processes, cancer and cardiovascular diseases (2–5). Since tomatoes are an important component of the Mediterranean diet, probably their consumption contributes to the decreased risk of some chronic diseases in the Mediterranean area (6). However, among phenolic compounds, one sub-class of these secondary metabolites is scarce in tomato, i.e., flavonoids, whose concentration is considered sub-optimal, particularly because anthocyanins are not generally present in the fruit. Therefore, tomato is an ideal candidate for anthocyanin enrichment due to its widespread consumption over the world, year-round.

A great variation in size, shape and color exists in the modern tomato varieties. Some cultivated varieties show dark skin color ("black" or "purple") as a result of mutations in carotenoid biosynthesis or chlorophyll breakdown (7). The red color of the tomato fruit results from the accumulation of lycopene, the most present carotenoid in the fruit. Conventional tomato genotypes accumulate anthocyanins on vegetative tissues, but not on fruit (8). On the contrary, the fruit of different wild *Solanum* species, closely related to cultivated tomato, accumulates anthocyanins in the peel (7, 9).

Anthocyanins belonging to the flavonoid family are a group of natural pigments represented by over 700 different molecular structures responsible for the red-blue color of many fruits and vegetables (10), having strong antioxidant properties (11). Anthocyanins are of particular interest to the agri-food industry, because of their potential health benefits, as well as their unique feature to confer vibrant colors to a variety of products (12–14). Recent studies using purified anthocyanins or anthocyanin-rich extracts on *in vitro* experimental systems have shown anti-inflammatory and anti-atherosclerotic effects (15–17). *In vivo* animal experiments and human clinical trials have given reason of the efficacy and biological activities of anthocyanins in the prevention of cardio-vascular disease (CVD) and cancer [(18–20) and references therein].

Giving these premises, it is not surprising that in the last 20 years there has been an increasing interest in developing highly consumed food, such as tomato, rich in flavonoids and particularly anthocyanins. To this purpose, transgenic approaches have been applied to modify the biosynthesis of phenylpropanoids, in order to alter the flavonoid composition of the tomato fruit. The ectopic expression in tomato of specific transcription factor (TF) genes from maize (*Lc* and *C1*), lead to 60% increase of flavonol (kaempferol) content at the whole fruit level (8). Conversely, the overexpression of TFs *Del* and *Ros1* from snapdragon (*Antirrhinum majus* L.), under the fruit-specific *E8* promoter resulted in high anthocyanin accumulation throughout the fruit of Micro-Tom, a model cultivar for tomato research (21). The same approach, using TFs *Del* and *Ros1* from *A. majus*, as used by in Butelli et al. (21) was applied to an Indian commercial cultivar (22). Recently, Scarano et al. (23) reported the engineering of a new tomato line, named "Bronze," expressing the TFs *Del* and *Ros1* from *A. majus* (inducing anthocyanin biosynthesis), *MYB12* from *A. thaliana* (regulating flavonol biosynthesis), and the biosynthetic gene *StSy* (from *V. vinifera*) controlling the production of resveratrol.

A strategy more likely to receive consumer acceptance is the conventional breeding using interspecific crosses with wild *Solanum* species, which transfers to cultivated varieties the ability to produce anthocyanins in the fruit. Some wild tomato species, such as *S. chilense*, *S. cheesmaniae*, *S. lycopersicoides,* and *S. habrochaites*, biosynthesize anthocyanins in the sub-epidermal tissue of the fruit, and some alleles from those genotypes have been introgressed into cultivated genetic backgrounds. At Tuscia University (Viterbo, Italy), a 20-years breeding activity has produced new tomato lines by intervarietal crossing or backcrossing mutant genes (for fruit color) to commercial cultivars used as recurrent parents (24). In particular, the combination of different alleles, responsible for the anthocyanin biosynthesis (*Aft* and *atv*), led to select a line (*AftAft/atvatv*) with a "purple" skin color, due to the biosynthesis of anthocyanins in the peel, having still a red colored flesh and a taste just like a traditional tomato (25). This selection was named "Sun Black" due to the requirement of light for anthocyanin accumulation, as phytochromes are known to be involved in light-mediated regulation of anthocyanins biosynthesis (26). The molecular nature of the introgressions underlying the "Sun Black" phenotype has been recently clarified. A dominant gene located on Chr10 and encoding an *R2R3-MYB* TF activating anthocyanin biosynthesis is thought to underlie the *Aft* variant (27), although it is still uncertain which of the three paralogs located in the involved genomic region is the actual responsible for the phenotype (28). More recently, the identity of the *atv* variant has been also identified as a mutation of an *R3-MYB* located on Chr7 and acting as a repressor of anthocyanin biosynthesis (29, 30).

We report here the chemical characterization and structure elucidation of the anthocyanins found in the peel of "Sun Black" tomato, as well as nutraceutical features of the whole fruit.

MATERIALS AND METHODS

Plant Material (Genetic Characteristics of SB Tomato)

Purple tomatoes were bred by crossing a line carrying the *Anthocyanin fruit* introgression (*Aft*, LA1996) and the *atroviolaceum* mutant line (*atv*, LA0797) as described (25).

A stable *AftAft/atvatv* purple line (line V710448, called "Sun Black," SB) was selected in parallel with a stable

[1]FAOSTAT. Available online at: http://www.fao.org/faostat/en/#home

line with red fruits (*aftaft/AtvAtv*) (line V710445, hereafter referred to as WT, standing for "wild type") used as a wild type reference. The two lines were selected for having the same vegetative and fruit characteristic, except for the fruit color phenotype. The term "Sun Black" has been protected as a trademark and the cultivars selected from the Tuscia University breeding programme are now commercialized (cv "Solenero" and others). The genetic combination giving rise to anthocyanin accumulation on the tomato peel is the same exploited in other commercial genotypes, such as cv "Indigo Rose" (https://extension.oregonstate.edu/news/purple-tomato-debuts-indigo-rose). Differently from "Indigo Rose" which is an indeterminate genotype, the SB line is a semi-indeterminate genotype.

Plants of the two lines were grown in an unheated tunnel in Viterbo, Italy (42°25′07″ N, 12°06′34″ E) arranged in twin rows and grown with the same agronomic techniques and inputs, corresponding to the standard agronomic practices for fresh market tomatoes. All genotypes were grown on tutors; axillary shoots were systematically removed, and the canopy reduced to expose the fruits to natural light. Plants were left to open pollination to set fruits.

Reagents and Standards

Reagents were purchased from various suppliers as follows: Authentic standards of kuromanin (cyanidin 3-*O*-glucoside chloride), chlorogenic acid (3-caffeoylquinic acid), gentisic acid, rutin (quercetin 3-*O*-rutinoside) (Extrasynthèse, Genay, France); the standard for carotenoids (lutein, carotene, α-carotene, β-carotene and lycopene) were purchased from CaroteNature (Lupsingen, Switzerland); gallic acid, Folin-Ciocalteu's phenol reagent, Trolox [(S)-(-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], ABTS [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)], fluorescein disodium, AAPH [2,2′-azobis (2-methyl-propionamide)], ascorbic acid, *meta*-phosphoric acid, DTT (dithiothreitol), BHT (butylated hydroxytoluene) as well as acetonitrile, ethanol, methanol, acetone, methyl tert-butyl ether, formic acid and acetic acid (all HPLC grade) (Sigma-Aldrich, St. Louis, MO, USA). In all experiments Milli-Q (Merck Millipore, Darmstadt, Germany) water was used.

Sample Extraction

Fruits were collected from the two lines (WT and SB) at three developmental stages: mature green (MG, fruit fully developed with surface of the tomato completely green), breaker (BR, about 50% of the fruit with color changed to red) and red ripe (RR, fruit completely ripe with red color). Developmental stages in purple fruits were inferred by inspecting the blossom end that is usually lacking anthocyanin accumulation.

Tomato fruits (at least three fruits for each developmental stage) were collected from three plants of both lines, immediately cooled at 4°C, washed, cut into pieces and reduced to a fine powder with a Waring blender, in presence of liquid N_2. The powder was then freeze-dried using a Freezone® 2.5 model 76530 lyophiliser (Labconco Corp., Kansas City, MO, USA) for 48 and stored in polyethylene tubes at −20°C (−80°C for carotenoids), until analysis. For ascorbic acid determination, aliquots of fresh fruit were stored at −80°C until analysis.

Anthocyanin extraction (for structure elucidation) was done from the peel of at least 1 kg (fresh weight, FW) of SB tomato at MG and RR stages. Freeze-dried peel (25 g) was extracted with Methanol:Water:Trifluoracetic acid (70:29.5:0.5 v/v/v), with a ratio 1/20 (W/V), o.n., in static at room temperature. After centrifugation at 3,500 g for 10 min., the extraction was repeated for 1 h in the same condition, the supernatants were combined and solvent evaporated at 32°C to 1/3 of the initial volume, then freeze-dried.

Polyphenols (including anthocyanins, for quantitative purposes) were extracted in triplicate from 100 mg freeze dried material (whole fruit), macerated overnight at 4°C in 10 mL of extraction solvent [Methanol:Ethanol:Water:Formic acid (35:35:28:2 v/v/v/v)]. After centrifugation at 3,500 g for 10 min., the extraction was repeated on a rotary shaker at room temperature, the supernatants were combined and organic solvent evaporated at 32°C, then brought to a known volume with acidified water (0.1% formic acid). Extracts were filtered through a 0.45 μm nylon membrane (PTFE) (Millipore, Bedford, MA), stored at −20°C and analyzed in triplicates within 1 month.

Carotenoids were extracted from triplicate aliquots of freeze-dried tomato powder (50 mg, whole fruit) by the method of Sadler et al. (31) modified by Perkins-Veazie et al. (32). Carotenoids were extracted with 20 mL mixture of hexane/ethanol/acetone (2/1/1 v/v/v) containing 0.05% of BHT. Samples were shaked on an orbital shaker at 180 rpm for 15 min. Then, 3 mL of distilled water was added and the suspension was centrifuged at 4,500 g for 10 min. The organic phase was dried under nitrogen, resuspended in 1 mL of ethyl acetate and analyzed by HPLC.

Ascorbic acid extraction was done from 1 g fresh tomato fruit (from freshly-grinded fruits in Waring blender with liquid N_2) in 10 mL 5% meta-phosphoric acid, centrifuging at 10,000 g for 20 min at 4°C.

Analysis of Anthocyanins, Other Polyphenols, Carotenoids, and Vitamin C

The concentrated filtered freeze-dried anthocyanin extract was purified using partition against ethyl acetate and Amberlite XAD-7 column chromatography. Isolation of individual compounds was performed using Sephadex LH-20 column chromatography and preparative HPLC. Structural elucidations of the main anthocyanins were mainly based on the following NMR experiments: One-dimensional ^{1}H, 2D heteronuclear single quantum coherence (^{1}H−^{13}C HSQC), heteronuclear multiple bond correlation (^{1}H−^{13}C HMBC), double quantum filtered correlation (^{1}H−^{1}H DQF-COSY), and total correlation (^{1}H−^{1}H TOCSY) spectroscopy [experimental procedure according to Skaar et al. (33)]. See **Table 1** for assignments of individual ^{1}H and ^{13}C chemical shifts for anthocyanin **1** and **2**, and **Figures S1, S2** for the HSQC and HMBC spectra of anthocyanin **1**.

Sample extracts were analyzed for phenolic compounds by an HPLC apparatus with photodiode array (PDA) detection

TABLE 1 | ^{1}H and ^{13}C NMR spectral data for the anthocyanins petunidin 3-*O*-[6″-*O*-(4‴-*O*-*E*-*p*-coumaroyl-α-rhamnopyranosyl)-β-glucopyranoside]-5-*O*-β-glucopyranoside (petanin, **1**) and malvidin 3-*O*-[6″-*O*-(4‴-*O*-*E*-*p*-coumaroyl-α-rhamnopyranosyl)-β-glucopyranoside]-5-*O*-β-glucopyranoside (negretein, **2**) isolated from the peel of "Sun Black" tomato recorded in CF_3COOD-CD_3OD (5:95, v/v) at 25°C.

	1 (^{1}H)	2 (^{1}H)	1 (^{13}C)	2 (^{13}C)
ANTHOCYANIDIN				
2			164.34	164.66
3			146.03	146.32
4	9.06	9.02	133.92	135.41
5			156.56	156.86
6	7.11	7.04	105.63	105.90
7			169.40	169.95
8	7.16	7.14	97.24	97.76
9			156.94	157.45
10			112.82	113.31
1′			119.71	119.75
2′	8.11	8.04	109.48	111.16
3′			149.65	149.84
4′			146.35	147.35
5′			147.56	149.84
6′	7.94	8.04	113.95	111.16
OMe	4.10	4.01	57.04	57.44
3-*O*-GLUCOSIDE				
1″	5.59	5.50	102.26	106.14
2″	3.82	3.70	74.47	75.00
3″	3.67	3.59	78.29	78.58
4″	3.56	3.47	71.51	71.76
5″	3.91	3.81	77.81	78.05
6A″	4.11	4.01	67.30	67.59
6B″	3.82	3.73	67.30	67.59
6″-*O*-RHAMNOSYL				
1‴	4.80	4.71	101.63	102.13
2‴	3.88	3.80	72.34	72.50
3‴	3.92	3.82	70.34	70.61
4‴	5.01	4.91	75.27	75.52
5‴	3.83	3.71	67.17	67.71
6‴	1.09	0.98	17.73	18.21
5-*O*-GLUCOSIDE				
1⁗	5.28	5.20	102.36	102.90
2⁗	3.78	3.69	74.64	75.02
3⁗	3.66	3.59	77.97	78.51
4⁗	3.63	3.54	70.90	71.31
5⁗	3.73	3.69	76.81	78.29
6A⁗	4.03	3.94	62.05	62.37
6B⁗	3.87	3.79	62.05	62.37
4‴-*E*-*p*-COUMAROYL				
1			127.06	127.29
2	7.53	7.43	130.64	131.27
3	6.90	6.81	116.39	117.03
4			161.17	161.39
5	6.90	6.81	116.39	117.03
6	7.53	7.43	130.66	131.27
α	6.35	6.27	114.38	115.07
β	7.67	7.57	146.58	147.40
COO			168.80	169.06

*See **Figure 2B** for structures.*

(Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA). Instrument control, data acquisition and processing were provided by the ChemStation software (Agilent Technologies). Separations were performed at 30°C on a Luna RP-C18 column (250 × 4.6 mm, 5 μm internal diameter) (Phenomenex, Torrance, CA, USA) equipped with a guard cartridge column. The samples were eluted following the multi-segment linear gradient employed in Gerardi et al. (14), using 5% (v/v) formic acid both in water (mobile phase A) and in acetonitrile (mobile phase B). UV-visible spectra were recorded in the 200–800 nm range and chromatograms were acquired at 280 and 520 nm. Individual polyphenolic compounds were identified by comparing their peak retention times and UV-visible spectra with those of commercial standards, and by co-chromatography of samples spiked with the standards.

The identified phenolic compounds were quantified by the external standard method using calibration graphs obtained with solutions of available authentic standards at six different concentration within the linearity range of concentration.

Anthocyanins were quantified by using a calibration graph (six concentration levels from 0.001 to 0.5 μg/L, analyzed in triplicate) constructed with petanin standard purified in house.

Carotenoids analyses were carried out using an Agilent 1100 Series HPLC system as described by Durante et al. (34).

Determination of vitamin C (Ascorbic acid, AA, plus Dehydroascorbic acid, DHA) was done according to Sánchez-Moreno et al. (35), with some modifications. Samples were kept in the dark and on ice all the time. The total AA + DHA was obtained after reduction of DHA to AA by DTT. Sample extract (0.2 mL) and DTT solution (50 mg/mL) (0.2 mL) were mixed and diluted to 1 mL with 5% meta-phosphoric acid and let to react at room temperature in darkness for 2 h, then injected onto the HPLC system, following the same conditions as in Gerardi et al. (14). DHA content was obtained by subtracting the initial AA content to the final total AA content after reduction.

Antioxidant Capacity Analysis

The Folin-Ciocalteu (F-C) reducing capacity assay, the TEAC and the ORAC assays were evaluated in WT and SB hydrophilic extracts, as described in Gerardi et al. (14). A rapid microplate methodology, using a microplate reader (Infinite M200, Tecan Trading AG, Switzerland) and 96-well plates (Costar, 96-well black round bottom plate, Corning) were used. All experiments were performed in triplicate, and two independent assays were performed for each sample.

Statistical Analysis

Assays were carried out in triplicate and the results were expressed as mean values ± standard deviation (SD). Differences between samples were analyzed by one-way analysis of variance (ANOVA) with Tukey's HSD *post hoc* test. A p-value lower than 0.05 was considered statistically significant. Statistical analysis was performed using SigmaPlot version 13.0 software (SyStat Software Inc., Chicago, IL).

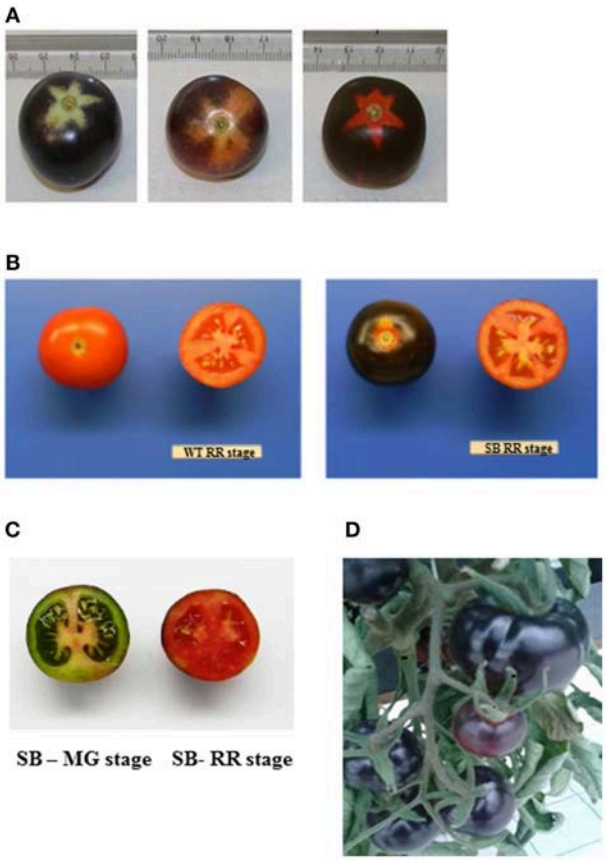


FIGURE 1 | Sun Black (SB) tomato fruit. (**A**) SB at three developmental stages—mature green (MG), breaker (BR), and red ripe (RR) (from left to right). (**B**) Whole and cross section of wild type (WT) (left) and SB (right) both at RR stage. (**C**) Cross section of SB (MG, left, RR right); (D): SB tomato fruits on the vine, at RR stage.

RESULTS

Occurrence and Profile of Polyphenolic Compounds: Comparison of "Sun Black" and Wild Type Tomatoes

Anthocyanins

The presence of anthocyanin pigment in SB tomato fruit was visually detected already at the immature (MG) stage, making difficult the inspection of developmental stages, unless to focus on the calix imprint or shaded area where anthocyanin biosynthesis was not activated by the light (**Figure 1**).

The only but very significant difference between WT and SB tomato fruits was the color of the peel (**Figure 1A**), instead the flesh was regularly red, both at immature (MG) and mature (RR) stage (**Figures 1B,C**).

The HPLC profile of SB fruit extracts, regardless of the ripening stage, showed two major peaks (corresponding to pigments **1** and **2**), in addition to small amounts of other anthocyanins (**Figure 2A**).

The downfield part of the ^{1}H NMR spectrum of anthocyanin **1** showed a 1H singlet at δ 9.06 (H-4), a 2H AX system at δ 8.11 (H-2′) and δ 7.94 (H-6′), and a 2H AX system at δ 7.16 (H-8) and δ 7.11 (H-6) (**Table 1**). A singlet at δ 4.10 integrating for 3H, corresponding to a methoxy group, identified the aglycone as petunidin. The acyl moiety was identified as *p*-coumaric acid by the 4H AA'XX' spin system at δ 7.53 (H-2,6) and δ 6.90 (H-3,5) and the AX system at δ 7.67 (H-β) and δ 6.35 (H-α). A coupling constant of 15.8 Hz between H-α and H-β showed the *E*-configuration. The chemical shifts of the carbons of the aglycone and acyl moiety assigned by the HMBC and HSQC NMR experiments were also in agreement with the presence of petunidin and one p-coumaroyl acid moiety (**Table 1**). In the ^{1}H NMR spectrum three anomeric proton signals were detected. The proton and carbon resonances belonging to the respective sugar moieties were assigned by DQF-COSY, TOCSY, and HSQC experiments to be in accordance with two glucopyranosyl and one rhamnopyranosyl units (**Table 1**).

The connection sites of the sugars of **1** on the aglycone were derived from the HMBC experiment. Cross-peaks between the anomeric proton at δ 5.59 and the carbon at δ 146.03 (C-3), and the anomeric proton at δ 5.28 and the carbon at δ 156.56 (C-5), revealed that the aglycone 3- and 5-positions were both connected to a glucopyranosyl with β-linkages showing coupling constants of 7.8 Hz. A cross-peak between the anomeric proton at δ 4.80 and the carbon at δ 67.30 revealed that the rhamnopyranosyl was connected to the 6″-position on the 3-glucopyranoside. The downfield shift (5.25 ppm) of the 6″-carbon resonance compared to the analogous signal of the 5-glucopyranosyl (**Table 1**) also confirmed this connection site. A downfield shift of H-4‴ (δ 5.01) together with a cross-peak in the HMBC spectrum between this resonance and the carbon signal at δ 168.80, revealed that the p-coumaroyl moiety was connected to the rhamnosyl 4‴-position. Thus, **1** was identified as petunidin 3-*O*-[6″-*O*-(4‴-*O*-*E*-*p*-coumaroyl-α-rhamnopyranosyl)-β-glucopyranoside]-5-*O*-β-glucopyranoside called petanin [**Figure 2B**; (36)].

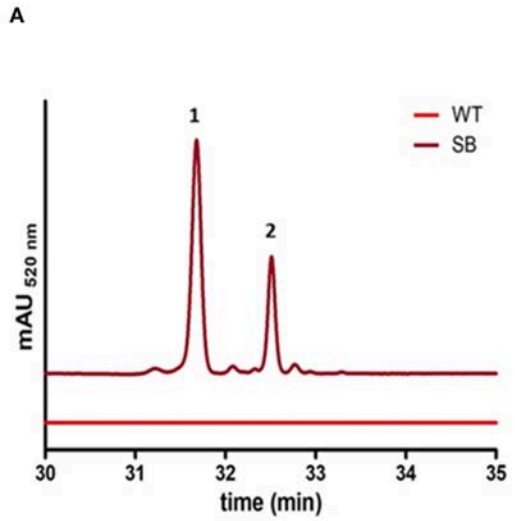
The NMR spectra of anthocyanin **2** showed many similarities with the corresponding spectra of **1** (**Table 1**). After assignments of the proton and carbon resonances, pigment **2** revealed an anthocyanidin B-ring with one hydroxyl group replaced with an methoxy group compared to that of **1**, in accordance with malvidin. Thus, **2** was identified as malvidin 3-*O*-[6″-*O*-(4‴-*O*-*E*-*p*-coumaroyl-α-rhamnopyranosyl)-β-glucopyranoside]-5-*O*-β-glucopyranoside called negretein [**Figure 2B**; (37)].

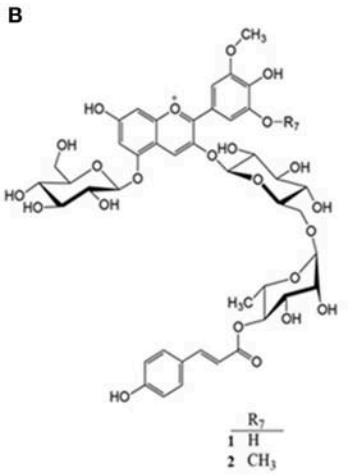
Petanin and negretein represented 56.6 and 21.4% of the total anthocyanins in SB peel, respectively.

No anthocyanin compound was detected in WT tomato fruit (flesh or peel) nor in the flesh of SB fruit (data not shown). The quantification of anthocyanin in SB (whole fruit) was done using standard curves based on petanin, the standard purified in house. This has made the quantification more reliable than in other reports, where aglycone standard curves were used. **Figure 2C** reports the anthocyanin quantification in whole berry of SB line, both as total or individual petanin.

Phenolic Acids and Flavonols

For the analysis of polyphenols we used a reproducible extraction protocol using aqueous acidified methanol/ethanol





C

	Petanin mg/g DW	Total Anthocyanin mg/g DW	Total Anthocyanin mg/100 g FW
MG stage	0.69 ± 0.01^a	1.06 ± 0.03^b	7.01 ± 0.18^a
BR stage	0.41 ± 0.03^b	0.67 ± 0.04^c	4.35 ± 0.29^b
RR stage	0.75 ± 0.04^a	1.16 ± 0.03^a	7.09 ± 0.19^a

FIGURE 2 | Anthocyanin characterization of SB tomato fruit extract. (**A**) Chromatografic profile of SB tomato fruit RR stage (at MG and BR stages the chromatographic profiles were similar) (**B**) structures of the main anthocyanins (petanin, **1**, and negretein, **2**) found in SB tomato peel. **(C)** Anthocyanin quantification in whole berry of SB tomato line, both as total or individual petanin.

as extraction solvent. This solvent was able to extract polar and semi-polar compounds, and to stabilize anthocyanins on their flavylium forms due to the acid content. Each sample was extracted in triplicate and two technical replicates were adopted to assure reliable results as recovery and repeatability.

We decided to conduct the study with the whole fruit in order to simulate the conditions while eating the fresh fruit.

With the chromatographic method already established in our laboratory (14) we were able to identify several peaks corresponding to phenolic compounds commonly found in traditional tomatoes (38) (**Figure 3A**).

The chromatographic profile of SB tomato extract at 280 and 320 nm appeared much more complex than other purple tomato genotype (ex. V118 from Ontario) (39) for the more polar compounds eluting earlier (hydroxycinnamic acids). SB extract showed a group of predominant peaks, eluting before chlorogenic acid, which could be assigned, on the basis of similarity to V118 purple tomato, to the unknown peak 1, tentatively identified as caffeic acid hexoside by the authors (39).

The quantification of polyphenolic compounds is reported in **Figure 3B** and **Table S1**. In addition to anthocyanins, not present in WT, also phenolic acids and flavonols were found at higher level in SB tomato.

As a quantity, chlorogenic acid was the most abundant phenolic compound quantified in SB tomato extract (from 0.5 to 1.3 mg/g DW, depending on the developmental stage).

Among flavonols, only two peaks were present with maximum absorbance at $\lambda = 350$ nm. Rutin (RT = 29 min) was identified and quantified (**Table S1**). The peak with RT = 25.9 min showed an absorption spectrum similar to rutin, and from literature data on tomato, particularly in tomato bearing *high pigment* (*hp*) gene, it has been tentatively assigned to rutin pentoside (24, 38). Total flavonols were quantified as Rutin Equivalent (RE) based on the area of rutin plus rutin pentoside (**Table S1**).

A more detailed characterization of unknown phenolic compounds present in SB extract will be addressed in a future study; here we report a general characterization of nutraceutical components of the new bred purple tomato SB, in respect to the wild type fruit.

Carotenoids Profile and Their Occurrence

The identification and quantification of carotenoids by HPLC is shown in **Table 2**.

On a dry weight basis, at the MG stage both in WT and SB the main carotenoid was lutein, followed by β- and α-carotene, respectively, in both genotypes, while lycopene was not detected. When compared, the various carotenoids occurred at the MG

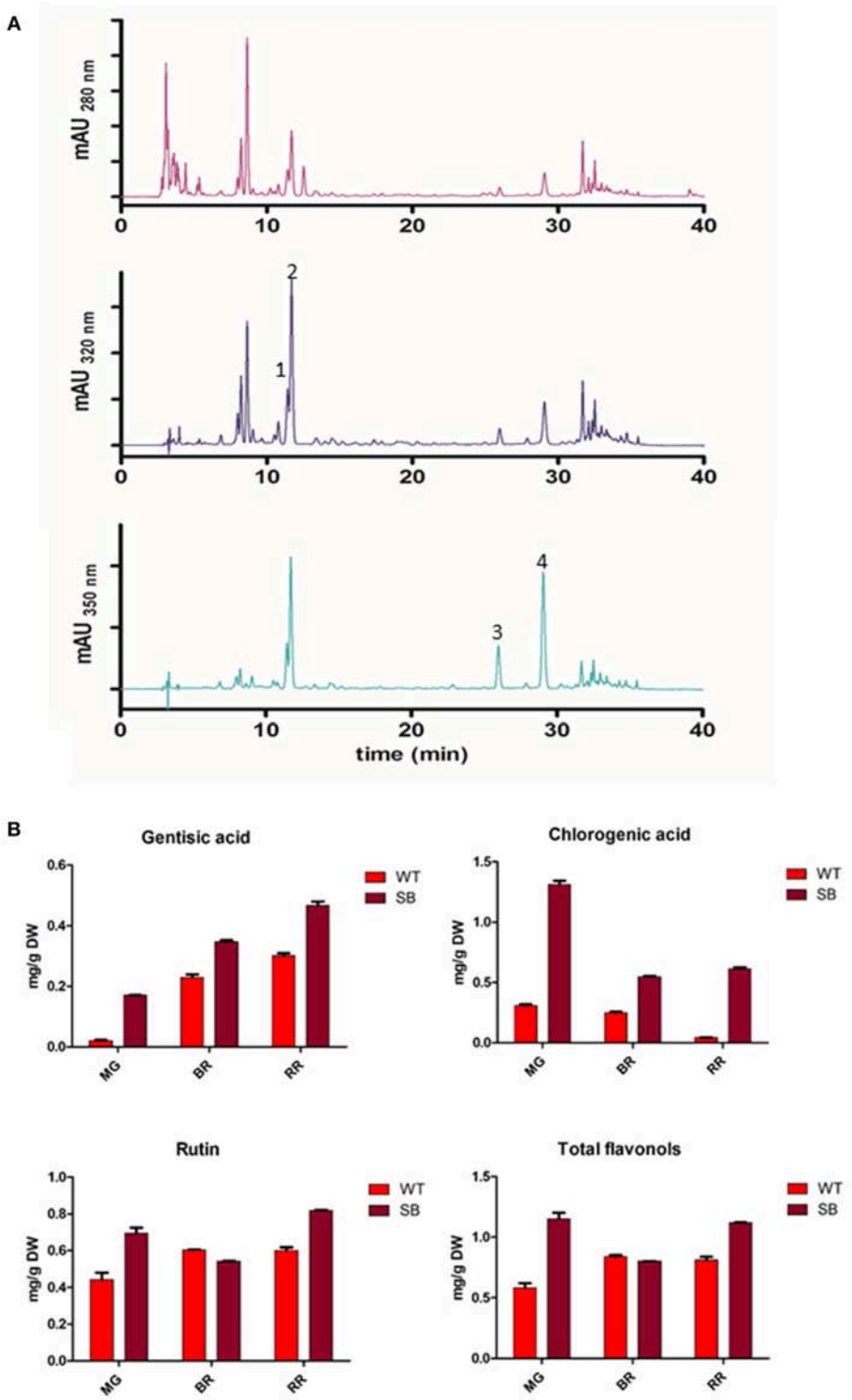


FIGURE 3 | Phenolic acids and flavonols in SB tomato extract. (**A**) Chromatographic profile of SB tomato fruit extract at λ = 280-320-350 nm. (**B**) Quantification of some phenolic acids and flavonols. Peak 1, gentisic acid; peak 2, chlorogenic acid; peak 3, rutin pentoside; peak 4, rutin.

stage in similar amounts in both genotypes. At the BR stage, differences could be found between WT and SB: The content of lutein and α-carotene was higher in SB than in WT. At the RR stage, the total carotenoids content was statistically similar in the SB and WT lines. However, the β-carotene content was significant higher in the SB sample, while the lycopene content was lower.

TABLE 2 | Carotenoid contents (μg/g dry weight, DW, and μg/g fresh weight, FW) in wild type (WT) and Sun Black (SB) tomato at different stages of ripening, mature green (MG), breaker (BR), and red ripe (RR).

	Lutein		β-carotene		α-carotene		Lycopene		*Total carotenoids*	
Sample	**μg/g DW**	**μg /100 g FW**	**μg/g DW**	**μg /100 g FW**	**μg/g DW**	**μg /100 g FW**	**μg/g DW**	**μg /100 g FW**	**μg/g DW**	**μg/100 g FW**
WT-MG	$39.75 \pm 3.29^{a,b}$	278.25 ± 23.03^{a}	21.33 ± 2.61^{b}	149.31 ± 18.27^{b}	3.49 ± 0.53^{b}	24.43 ± 3.71^{b}	ND	ND	64.58 ± 6.44^{c}	452.06 ± 45.08^{d}
WT-BR	29.70 ± 0.58^{c}	187.11 ± 4.06^{b}	37.20 ± 0.86^{b}	234.36 ± 5.41^{b}	4.07 ± 0.61^{b}	25.64 ± 3.84^{b}	24.67 ± 0.84^{c}	155.42 ± 5.29^{c}	$95.65 \pm 2.9^{b,c}$	$602.59 \pm 18.27^{b,d}$
WT-RR	$35.58 \pm 1.20^{b,d}$	273.96 ± 9.24^{a}	46.33 ± 0.82^{b}	356.74 ± 6.31^{b}	4.11 ± 0.12^{b}	31.64 ± 0.92^{b}	75.42 ± 2.58^{a}	580.73 ± 19.86^{a}	161.46 ± 4.73^{a}	$1243.24 \pm 36.42^{a,c}$
SB-MG	41.92 ± 2.55^{a}	276.67 ± 16.83^{a}	24.20 ± 3.88^{b}	159.72 ± 25.60^{b}	4.58 ± 0.15^{b}	30.22 ± 0.99^{b}	ND	ND	$70.71 \pm 6.69^{b,c}$	$466.68 \pm 44.15^{b,d}$
SB-BR	44.31 ± 1.79^{a}	288.01 ± 11.63^{a}	49.23 ± 5.34^{b}	319.99 ± 34.71^{b}	9.82 ± 1.49^{a}	63.83 ± 9.68^{a}	22.26 ± 3.58^{c}	144.69 ± 23.27^{c}	125.64 ± 12.22^{b}	$816.66 \pm 79.43^{b,c}$
SB-RR	$31.75 \pm 2.28^{c,d}$	190.5 ± 13.68^{b}	112.79 ± 43.11^{a}	676.74 ± 258.66^{a}	4.71 ± 0.25^{b}	28.26 ± 1.5^{b}	62.07 ± 6.0^{b}	372.42 ± 36.0^{b}	211.33 ± 51.65^{a}	1267.98 ± 309.9^{a}

The same letters in the same column indicate that mean values (n = 3) are not significantly different ($p < 0.05$).

Phenolic Content and Antioxidant Capacity

The antioxidant capacity of SB was evaluated by considering only the hydrophilic extract, which is the main contributor to the total antioxidant capacity in fruits and vegetables, particularly in the purple tomato (39).

The total phenols content was measured by the Folin-Ciocalteu (F-C) assay, as reported in Gerardi et al. (14), and it increased during ripening, in both tomato genotypes.

In case of SB tomato at MG stage, the phenolic content (5.8 mg GAE/g DW) was higher than WT by 152%, and at RR stage (8.6 mg GAE/g DW) by 134%. On a fresh weight base, at MG stage, SB had more phenols than WT by 137%, at RR stage SB had 85% more phenols than WT (**Table 3**).

The TEAC value for SB at RR stage (31.6 μmol TE/g DW) was 200% more than WT (10.3 μmol TE/g DW); instead the ORAC value for SB, at the same stage of ripening (140.3 μmol TE/g DW) was 86% higher than WT (75.5 μmol TE/g DW) (**Table 3**).

The antioxidant capacity of SB assessed by TEAC and ORAC at MG stage (22.9 and 104.1 μmol/TE g DW, respectively) was much lower than at RR stage, probably because of the great increase in polyphenols accumulation during ripening (from 5.8 to 8.6 mg GAE/g DW, in MG and RR, respectively).

The HPLC ascorbate determination was done on WT and SB extracts only at RR stage, revealing a much higher total ascorbic acid (AA + DHA) content in SB than WT (37.3 ± 1.4 vs. 27.1 ± 1.1 mg/100 g FW, respectively). The ascorbic acid (AA, reduced form) was 82% of total AA (30.8 ± 1.1 mg/100 g FW) in SB, while in WT was much lower (around 50%, 13.2 ± 0.79 mg/100 g FW) (data not shown).

DISCUSSION

Despite the remarkable success in increasing flavonoid content in tomato fruits by transgenic approaches (8, 21, 23), in the last 15 years there has been a growing interest in breeding a high flavonoid GM-free tomato (40). This interest is motivated by customers' reluctance to consume transgenic food and by restriction in EU Countries to cultivate genetically modified plants (Directive EU 2015/412).

The exploitation of the biodiversity available in the wild germplasm can allow a remarkable success for specifically exploring metabolic pathways to produce healthier food. This approach has been followed by the Soressi's group in order to combine different alleles from tomato-related wild species into cultivated tomato, promoting anthocyanin production (25, 41).

The characterization of phytochemicals in naturally bred purple tomatoes has already been reported in different genotypes, from the US accession LA1996 (9) and the Canadian purple tomato line V118 (39) to the Brasilian (42) and the Japanese ones (43). Here we report the characterization of the anthocyanin content and other nutraceuticals of "Sun Black" (SB), an Italian tomato line with purple skin color, both in green and in red fruit stages. The latter is caused by the biosynthesis of anthocyanins in the peel, whereas the flesh is red color, similar to the wild type.

TABLE 3 | Total phenols and antioxidant capacity (by TEAC and ORAC assays) of wild type (WT) and "Sun Black" (SB) tomato at different stages of ripening, mature green (MG), breaker (BR), and red ripe (RR).

Sample	Total phenolics[§]		TEAC°		ORAC°	
	mg GAE/g DW	mg GAE/100 g FW	μmol TE/g DW	μmol TE/100 g FW	μmol TE/g DW	μmol TE/100 g FW
WT- MG	2.28 ± 0.26^{d}	15.96 ± 1.82^{d}	6.93 ± 1.21^{d}	48.51 ± 8.47^{d}	60.04 ± 3.24^{c}	420.28 ± 23.32^{c}
WT- BR	4.34 ± 0.34^{c}	27.34 ± 2.14^{c}	12.11 ± 0.92^{c}	76.29 ± 5.79^{c}	64.48 ± 5.80^{c}	406.27 ± 36.54^{c}
WT- RR	3.66 ± 0.37^{c}	28.18 ± 2.84^{c}	10.35 ± 0.82^{c}	79.69 ± 6.31^{c}	75.49 ± 5.93^{c}	581.32 ± 45.66^{b}
SB- MG	5.76 ± 0.35^{b}	38.01 ± 2.31^{b}	22.95 ± 2.53^{b}	151.47 ± 16.69^{b}	104.13 ± 3.05^{b}	687.25 ± 20.13^{b}
SB- BR	6.03 ± 0.41^{b}	39.19 ± 2.66^{b}	19.9 ± 2.08^{b}	129.35 ± 13.52^{b}	129.44 ± 9.28^{a}	841.36 ± 60.32^{a}
SB- RR	8.56 ± 0.08^{a}	52.21 ± 0.48^{a}	31.64 ± 3.91^{a}	193 ± 23.85^{a}	140.30 ± 9.18^{a}	855.83 ± 55.98^{a}

[§]*Total Phenols as GAE = Gallic Acid Equivalent;* °*Antioxidant Capacity as TE = Trolox Equivalent. The same letters in the same column indicate that mean values (n = 3) are not significantly different ($p < 0.05$).*

In SB tomato, the anthocyanidin (aglycone) composition has previously been reported as detected mass (*m/z*) of delphinidin, petunidin and malvidin (44). In the present study, the structure of the major anthocyanins has been elucidated for the first time.

The anthocyanin composition of SB peel is similar to the one previously reported in other purple breeding lines (9, 39). Probably all these genotypes share a common genetic base. In the V118 line (39), petunidin-derivatives accounted for approximately 91.9% of the total anthocyanins, more than in SB (which was 56.6%). In the triple mutant genotype *Aft*/*atv*/*hp2*, mostly petunidin and lower amount of delphinidin, in acylated form, were found (42).

The GM purple tomato from C. Martin's Lab. contains (both in the peel and in the flesh) acylated derivatives of delphinidin and petunidin, and at lower extent, malvidin (45). Moreover, in a recent study on a transgenic *Del/Ros1* tomato, two malvidin-based anthocyanins were reported (46).

The similarity of the anthocyanin structures (delphinidin-based) in cross-bred and transgenic tomatoes (overexpressing the *SlANT* gene) demonstrated that the biosynthetic machinery for anthocyanin biosynthesis is under control of the same *MYB* transcription factors (47), although two candidates remain to underlie the *Aft* variant (*SlANT* and *SlAN2*) (28). In addition, the first anthocyanidin product (delphinidin) is promptly methylated by the action of methyltransferases (48) in the two systems.

The anthocyanin content of SB tomato fruit (1.2 mg/g DW or 7.1 mg/100 FW) is comparable to some anthocyanin-rich vegetables (eggplant or red lettuce) or fruits (light-colored strawberries or cherries).

The not linear anthocyanin accumulation in SB tomato at different ripening stages (**Figure 2C**) could be explained by the different developmental behavior of the fruit: while at immature stage the biosynthesis of anthocyanin is already promptly activated (by the light), at the breaker stage the fruit undergoes cells division and expansion, with fruit enlargement and metabolite biosynthesis slowdown. At ripe stage the full development of the fruit and the anthocyanin biosynthesis (as well all the other typical metabolites of the ripe fruit) is completed.

The anthocyanin content in the peel of SB tomato is controlled by environmental factors, light in particular. It is known that the anthocyanin biosynthesis is subjected to the environmental activation on regulatory genes (*MYB* transcription factors), by light or cold (26, 49). In the environmental condition described here (unheated tunnel, Central Italy), the anthocyanin content of SB fruits was 1.16 mg/g DW. When SB tomatoes were hydroponically grown in South Italy (where light irradiation is generally higher), preliminary data suggest that at the RR stage both anthocyanin content and ORAC antioxidant capacity were 20% higher than the values reported here (data not reported). In purple tomato V118, the authors reported a total anthocyanin content of nearly 0.25 mg/g DW (39). SB tomato contains four times more anthocyanin as determined more precisely using a real standard (petanin) purified in house.

In transgenic anthocyanin-rich tomatoes a much higher anthocyanin content was reported, as expected, because of the presence of anthocyanins in the flesh (21, 46).

For colorless phenolic compounds, the HPLC chromatographic profile of the SB tomato extract was not different (for major peaks) from the WT. However, the peaks area was greater, revealing a higher content of hydrophilic polyphenolic compounds. Indeed the phenolic content assessed by Folin- Ciocalteu was higher by 100% and more in SB extract than in WT.

Gentisic and chlorogenic acids were present in SB tomato extracts at higher level in respect to WT, for each stages of ripening. Rutin was more abundant in SB, except for breaker stage. Total flavonols were not different in SB and WT at RR stage. It is interesting to note that, while during ripening the content of gentisic and chlorogenic acids followed a linear trend (increasing for gentisic, decreasing for chlorogenic), for rutin, total flavonols, and total anthocyanins the trend was not linear. As already discussed above in the anthocyanin section, during ripening and particularly at the breaker stage, the fruit undergoes many physiological and metabolic changes, not consistent with the other stages. While analyzing three stages of ripening, we focused our interest to the ripe stage, which is important for consumers.

Gentisic acid content was present in SB tomato at higher level than reported in the V118 purple tomato genotype, while chlorogenic acid was at the same level (39).

The total carotenoids content in SB (RR stage) was similar to the average amount reported for tomatoes (50), particularly for greenhouse-grown tomatoes (1), and even similar to the reported values in other purple genotypes, as the breeding line V118 from Ontario, although the carotenoid profile was different (39). Borghesi et al. (44) reported a higher content of carotenoids in SB tomatoes, and a very different pattern in lycopene, β-carotene and lutein than reported in our study on the same tomato line. In that study, tomatoes were hydroponically grown, that probably influenced the carotenoid biosynthesis. Moreover, difference in carotenoid analysis methodology can greatly influence the final metabolite quantification.

As far as the cross-talk between lipophilic carotenoids and hydrophilic flavonoids was concerned, interestingly, when transgenic lines with altered carotenoid contents were analyzed, no significant differences were found in phenolic or flavonoid content (51). Breeding or transgenic lines for increased phenylpropanoids did not show an increase of flavonoids at expenses of carotenoids (7, 21). In a transgenic tomato cultivar, total carotenoid and lycopene levels did not vary, in spite of a higher anthocyanin content of 13 mg/100 g FW (22). It has been supposed that both classes of compounds have different role and subcellular localization (plastids for carotenoids vs. vacuoles for anthocyanins), and do not compete for precursors (42).

In tomatoes bearing *high pigment* (*hp*) gene, with phenotypic effects similar to those due to the *Aft* locus, a slight increased level of carotenoids and polyphenols in respect to the control line was reported (24).

Recently, the potential health benefits of anthocyanins have been related to not yet identified chemical properties beyond the antioxidant capacity of the molecules (12). However, the antioxidant properties of a food extract, assessed *in vitro*, is an inceptive parameter to be tested when studying the nutraceutical properties of a food. Our results on antioxidant capacity of SB hydrophilic extract show a significantly higher value in respect to WT.

The phenolic content (by F-C assay) of SB tomato, at all stage of ripening, was significantly higher than that one found in WT, and in other selected tomato lines (24). At RR stage of ripening phenolic content of SB tomato was higher than the reported value for the Canadian purple tomato V118 (39).

Two different chemical assays (TEAC and ORAC) have been adopted for evaluating the antioxidant capacity of SB extract, as it has been recommended in order to take into account the different mechanisms of action (52). In both assays, the antioxidant capacity (hydrophilic) of SB tomato, at the three stages of ripening, was significantly higher than WT at the same stage, due to the presence of anthocyanin compounds in the peel, and to an increased content of other polyphenolic compounds.

The TEAC of WT tomato was in agreement with the reported value for commercial tomato cultivar (1, 53). The TEAC value for SB tomato was 200% more than WT, thanks to the increased polyphenolic content. The ABTS assay has been previously applied to SB tomato samples, on skin area with different level of pigmentation, revealing increased antioxidant capacity with increasing anthocyanin content, although not comparable to our results which are on whole fruit (54). The TEAC antioxidant capacity of our SB tomato was lower, as expected, than the value reported for transgenic tomatoes, because of the anthocyanin presence at whole berry fruit (21).

The antioxidant capacity (as ORAC assay) in SB tomato, at all stage of ripening, was statistically higher than that one found in WT. At RR stage of ripening, ORAC was lower in SB than in the Canadian purple tomato V118, in spite of a higher phenolic content in SB (39).

Tomato fruit is considered a good source of vitamin C. The vitamin C content was quite high both in WT and SB, with reference to commercial cultivar or purple tomato (1, 42). SB extracts at RR stage had a higher vitamin C content than WT. The ascorbic acid (AA, reduced form) was much higher in SB than WT. One possible explanation is the protective function of the increased polyphenol content in SB, which protects AA from oxidation. Anyway, the contribution of the ascorbic acid to the antioxidant capacity of plant extract is usually low. As it has been reported, the main contributor to the antioxidant capacity of hydrophilic extracts is not ascorbate but rather polyphenols (55).

It has been reported that purple tomatoes both obtained by transgenesis or conventional breeding showed improved shelf life compared to wild-type, with a delayed ripening and a reduced pathogen susceptibility, due to anthocyanin accumulation and increased level of natural antioxidant (54, 56). Postharvest losses by pathogenic infection can reduce the yield of vegetable crops, therefore the harvest of tomato when green and firm, and successive ethylene exposition, is a usual technique to induce color and ripeness, without developing flavor and aroma. This is likely the main reason why the marketed tomatoes are often characterized by extremely low flavor regardless of the cultivar. Preliminary panel tests on SB at commercial stage of ripeness showed a good performance for flavor, aroma and texture (data not reported). Moreover, ripening SB tomatoes showed that the ethylene climacteric peak was delayed, resulting in a higher firmness at commercial stage of ripening (57). Since SB has been bred to promote fresh consumption of a tomato rich in bioactive compounds, all this information taken together make SB tomato valuable both for nutraceutical and market qualities.

CONCLUSION

From the Kuopio study which demonstrated that the consumption of large amount of anthocyanin-rich food significantly lowered the risk of CVD (58) to the recent advancement in anthocyanin health benefits (12), it can be concluded that consuming anthocyanin-rich fruits and vegetables is a recommended style of eating.

Transgenesis has been always seen in a suspicious way, due to the consumer's concerns over the consumption of GM foods. Moreover, EU rules discourage the application of transgenic cultivations. Therefore, the naturally bred SB tomato can be a valuable alternative to transgenic purple tomato, despite its lower

concentration of anthocyanins and antioxidant phytochemicals. On the other hand, the anthocyanin (and phenolic) content of SB is comparable to some anthocyanin-rich vegetables (eggplant or red lettuce) or fruits (light-colored strawberries or cherries), with the advantage that tomatoes are highly consumed in many countries, and are available all year-round. In conclusion, consumption of SB tomatoes can contribute to ameliorate human health, thanks to the increased content of nutraceutical compounds.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

FB, HB, GMa, MD, IN, and CG performed the experiments. AM and MP produced the plant material. FB, IN, GMi, HB, and ØA designed the experiments. FB, HB, GMa, MD, IN, GMi, and ØA analyzed and interpreted the data. FB wrote the manuscript with the input from all the authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2019.00133/full#supplementary-material

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Conflict of Interest Statement: AM and MP are participating as inventors in owing the trademark "Sun Black" and the rights on the registered variety "Solenero." The ownership is the University of Tuscia, Viterbo, Italy.

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Variation of Anthocyanin Content and Profile Throughout Fruit Development and Ripening of Highbush Blueberry Cultivars Grown at Two Different Altitudes

Anna Spinardi[1], Gabriele Cola[1], Claudio Sebastiano Gardana[2] and Ilaria Mignani[1]*

[1] Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy, Università degli Studi di Milano, Milan, Italy, [2] Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Milan, Italy

Blueberry (*Vaccinium corymbosum L.*) is a widely consumed fruit and a rich source of bioactive compounds, namely, the polyphenol class of anthocyanins. Little information is available about the influence of internal (genetic and developmental) and external (environmental) factors on the levels of phenolic metabolites in blueberry fruit. In light of this consideration, total polyphenolic and flavonoid content, anthocyanin accumulation and composition were evaluated in cv. "Duke" and "Brigitta" grown at two different altitudes in Valtellina, a valley of the Alps in Northern Italy. During berry ripening, there is a developmentally coordinated shift from cyanidin-type, di-substituted anthocyanins toward delphinidin-based, tri-substituted pigments. At the lower altitude location, higher temperatures, not exceeding optimum, resulted in a more quickly berry developmental pattern and in higher anthocyanin concentrations in the early phases of ripening. At later stages of ripening, berries of both cultivars at higher altitude compensate for these initial temperature effects, and no differences were recorded in ripe fruit grown in the two locations. We conclude that anthocyanin accumulation is strongly regulated by development and genotype, and the environmental factors, associated to the altitude gradient, exert in the trial conditions only a fine-tuning influence. Fruits reach the full-ripening stage simultaneously at both sites because the initial gap in pigment levels is counterbalanced at the higher altitude by a faster rate of accumulation at later phases of the ripening process.

Keywords: blueberry, bioactive compounds, ripening class, polyphenols, anthocyanins, altitude, temperature

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****Correspondence:***
Anna Spinardi
anna.spinardi@unimi.it

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INTRODUCTION

The consumer's concern for fruit quality is, at the present, becoming greater due to increasing interest in food aspects related to health care and better quality of life. For these reasons, the modern concept of quality combines sensorial features and nutritional contents (Knee, 2002; Tromp, 2005). According to this trend, small fruit industry is increasing because their high contents in bioactive compounds meet the consumer expectations of healthy food (Gosch, 2003; Kähkäonen et al., 2003).

Blueberry is an interesting fruit for its potential health benefits attributed to antioxidant properties of bioactive compounds (polyphenols, in particular anthocyanins, and ascorbic acid);

thus, the worldwide production of blueberry has been growing in the last decades. In particular, blueberry consumption has been reported to induce improvements in memory and cognitive performance (Williams et al., 2008), to prevent oxidative stress and damage, to inhibit inflammation (Youdim et al., 2002), and to improve cardiovascular health (Erlund et al., 2008; Beccaro et al., 2004). Moreover, small fruit cultivation is proper for hill field because of its attitude to mountain climate, aside from lands, and generally in small-scale farms giving an extra income to family businesses and the opportunity to improve the economy of marginal areas. Finally, blueberry production is rapidly increasing because of its excellent productivity, adaptability to different environments, and pest resistance. Moreover, in the recent years, the small fruit production was often oriented to environmental friendly agricultural methods as integrated pest management or organic agriculture, endowing the growing areas with an added value of environmental respect.

In order to better understand the drivers of nutraceutical traits in blueberry, it will be challenging to study the different growing conditions and techniques (Häkkinen and Törrönen, 2000) to assess fruit quality. The content of nutraceutical substances is influenced by ripening stage, genotype, cultivation techniques, pre-harvest climatic conditions, and the operations carried out during the post-harvest storage. The environmental factors play a crucial role in qualitative and quantitative accumulations of antioxidant compounds in many types of fruits: temperature, solar radiation, water stress, and soil are considered the major elements affecting anthocyanin content in fruit (Connor et al., 2002; Castellarin et al., 2007b; Stevenson and Scalzo, 2012). Acidity and phenolic compounds vary markedly from southern to northern latitudes (Åkerström et al., 2010; Thomas et al., 2013, Lätti et al., 2008, Lätti et al., 2010), while the altitudinal gradient induces higher accumulation of anthocyanins in bilberry (Jovančević et al., 2011; Wang et al., 2014). Among the climatic parameters affected by altitude, as sunlight spectra, visible light, and UV radiation, the lower daily temperatures at higher altitudes seem to increase anthocyanin accumulation in bilberry and blueberry (Karppinen et al., 2016; Zoratti et al., 2015a, Zoratti et al., 2015b). The direct sun exposure enhances the expression of flavonoid pathway genes and the concentrations of anthocyanins, catechins, flavonols, and hydroxycinnamic acids in bilberry leaves (Jaakola et al., 2004) and fruit (Jovančević et al., 2011), giving support for the protective role of flavonoids and hydroxycinnamic acids against high solar radiation in plants (Routray and Orsat, 2011). In Finnish bilberry, the proportions of anthocyanins vary in the berries from South compared to those in the Central and Northern regions (Åkerström et al., 2010; Lätti et al., 2008).

According to these considerations, the present work studies the effect of the different altitude on quality attributes of two cultivars of blueberry grown in Valtellina, a Northern Italy valley. Particular attention was focused on quantitative and qualitative changes in anthocyanin accumulation during berry development, as anthocyanins are key factors of quality in various fruits. Fruit colors depending on anthocyanin types, and their contents, have both commercial and aesthetic values.

MATERIALS AND METHODS

Fruit Sampling and Quality Trait Measurement

Highbush blueberry (*Vaccinium corymbosum* L.) samples of two cultivars ("Brigitta" and "Duke") were harvested in the Valtellina area (Northern Italy) during 2015 and 2016 from two commercial orchards located in Postalesio (46,175° N; 9,776° E) and Gaggio di Berbenno (46,168° N; 9,746° E) at two different altitudes (450 and 650 m ASL, respectively). An East–West exposure characterizes Valtellina, a valley in the Italian Alps. Potential Yearly Photosynthetically Active Radiation (PPAR) was calculated by means of the topographic tools of SAGA GIS (Conrad et al., 2015), based on a 20-m resolution digital elevation model.

Both the orchards were endowed with micro-irrigation systems and monitoring of water status, to avoid summer water stress and grant the correct water supply.

Postalesio soil was characterized by sandy texture class (sand> 61%), sub acidic pH (6.04), and high organic matter content (4.66%). The soil of Gaggio was characterized by sandy texture class (sand >71%), acidic pH (4.98), and high organic matter content (3.15%). Both the soils were optimal for blueberry growth, without differences for nutrient content. Conventional farming practices were carried out in the two fields.

Samples were collected from 4- to 5-year-old plants. "Duke" is an early ripening variety, harvested in Northern Italy at middle June—first week of July, whereas "Brigitta," a mid-season variety, ripens at middle July—first week of August. "Duke" berries were harvested on the third week of June, and "Brigitta" berries were sampled on the third week of July, in two consecutive growing seasons (2015 and 2016).

The trial considered six plants of each cultivar. All fruits of each bush were picked and divided visually into four homogenous classes corresponding to different stages of ripening: mature green, fully expanded (class I), less than 50% pigmented (class II), 50% to completely pigmented except the stem-end (class III), and fully ripe (class IV) (**Figure 1**). Immature fruits not fully enlarged, i.e., that had not reached at harvest the typical final shape and size due to a complete cell expansion, were discarded. Fruits of each of the four different ripening stages of a single plant represented one individual sample. Consequently, the pool of berries harvested from the same plant at the assigned stage of ripening represented one biological replicate for each cultivar, and the total number of samples was 48 (4 ripening stages per 6 individual plants per 2 cultivars) for each of the growing sites, located at 450 and 650 m ASL, respectively. Total soluble solid content (TSS) and titratable acidity (TA) were also evaluated to better characterize the different ripening stages (**Supplementary Figure 1**). TSS content, expressed as percent of soluble solids, was determined by a hand refractometer (Atago mod., N1, Tokyo, Japan) on juice obtained by squeezing the berries. TA was measured in an aliquot of the juice (about 1 g) through titration to pH 8.1 by 0.01 N NaOH and a semi-automated Titrator Compact D (Crison Instruments SA, Alella, Spain). Acidity was

FIGURE 1 | Different ripening stages of blueberry fruit (from left, stage 1: mature green, stage 2: less than 50% pigmented, stage 3: 50% to completely pigmented except the stem-end, stage 4: fully ripe).

calculated and reported as meq/L. Aliquots of fruits from each sample were used to perform all the analyses. Distance between individual plants allowed optimal illumination of all sides of the canopy. Immediately after harvest and sorting, samples were cooled to below 8°C and placed within the day at −80°C until chemical analyses.

Temperature Determination

The air temperature of the two sites was directly monitored by two ONSET HOBOware (HOBO, Onset Computer Corporation, Pocasset, MA, USA) experimental weather station equipped with air temperature sensors and solar radiation shields. Daily maximum and minimum temperature and thermal excursion are presented.

Fruit Skin Color Measurement

Fruit skin color was measured only in 2015 by a compact tristimulus colorimeter (Minolta CR-300, Ramsey, NJ, USA) with an 8-mm diameter viewing aperture and described by the CIE L*, a* and b* color space coordinates. (Dussi et al., 1995). L* represents the lightness of colors on a scale of 0 to 100 and is low on the scale for dark colors and high for bright colors. The parameter a* is negative for bluish-green and positive for red-purple. The parameter b* is negative for blue and positive for yellow. The L*, a* and b* values were used to calculate the hue angle ($H° = \arctan [b^*/a^*]$, reported as degrees) and the chroma index ($C^* = [a^{*2} + b^{*2}]^{1/2}$) (McGuire, 1992). Hue angle, denoting visual color appearance, was expressed on a 360° color wheel where 0° and 360° represent red-purple, 90° the yellow, 180° the bluish-green, and 270° the blue. Chroma is the degree of deviation from gray towards pure chromatic color and thus indicates color intensity or saturation (high values are more vivid). Color measurements were assessed on 30 freshly harvested fruit per cultivar and maturity stage at each growing location, amounting to 480 determinations.

Total Phenolics, Total Flavonoids, and Total Anthocyanin Analysis

For total phenolics, total flavonoids, and total anthocyanin determinations, 25 g of berries were ground in 25 mL of acidified ethanol (EtOH:H_2O:HCl conc.; 70:29:1; v/v/v) and placed 14 h on a rotary shaker, then centrifuged at 10,000 *g* for 10 min. The supernatant was analyzed after proper dilution with acidified ethanol. Six different samples per cultivar (2)/ripening stage (4)/ altitude (2) were extracted, accounting for 96 samples.

Total phenolic contents were determined following the Folin–Ciocalteu procedure. One milliliter of Folin–Ciocalteau reagent, 0.5 ml of distilled water, and 2 ml of 20% Na_2CO_3 were added to 0.1 mL of the extract. The solution was immediately diluted to a final volume of 20 mL with distilled water and then agitated. The optical density was measured after 90 min at 700 nm on a UV–vis spectrophotometer (Jasko model 7800, Tokyo, Japan). Results were expressed as mg of catechin per g of fresh weight (FW).

Total flavonoids were evaluated spectrophotometrically at 280 nm. A catechin standard curve was set, and results were expressed as milligrams of catechin per g of FW (Beghi et al., 2013).

Total anthocyanin contents of blueberry extracts were estimated spectrophotometrically according to Sinelli et al. (2008) as malvidin 3-glucoside at 520 nm using a molar absorptivity coefficient of 28,000 and reported as mg per g of FW.

Anthocyanin Identification and Quantification

Approximately 10 g of frozen berries were mixed with 30 mL of a solution methanol/TFA 2% in water (10:90, v/v) and homogenized by an Ultra-Turrax (IKA-Werke, Staufen, D) for 1 min. The homogenate was extracted for 30 min under agitation in the dark at room temperature. The suspension was centrifuged at 1,000 × *g* for 10 min at 4°C, and the supernatant recovered. The residue was extracted again until disappearance of the red

color (4 × 20 mL) with a solution of methanol/TFA 2% in water (10:90, v/v), and treated as described above. The supernatants were combined, and the volume was adjusted to 200 mL by solution of 2% TFA in water. All extracts were stored at −20°C and centrifuged at 3,000 × *g* for 1 min before the LC analysis.

Anthocyanin identification was performed using an ACQUITY UHPLC system (Waters, Milford, MA) equipped with a model E-Lambda photodiode array detector (Waters) and an Exactive high-resolution mass spectrometer (Thermo Scientific, Rodano, Italy) equipped with an HESI-II probe for ESI and a collision cell (HCD). A C18 Kinetex column (150 × 4.6 mm, 2.6 µm, Phenomenex, Torrence, CA) protected with guard column, carried out the separation at 1.7 mL/min, and flow-rate split 5:1 before electrospray ionization (ESI) source. The column and sample were maintained at 45° and 20°C, respectively. The eluents were (A) 0.2% TFA in water and (B) acetonitrile: 0.2% TFA in water (35:65, v/v). The linear gradient was as follows: 0–15 min 14% B; 15–25 min from 14% to 20% B; 25–35 min from 20 to 32% B; 35–45 min from 32% to 50% B; 45–48 min 50% to 90% B; and 90% for 3 min. The MS operative conditions were as follows: spray voltage +4.0 kV, sheath gas flow rate 60 (arbitrary units), auxiliary gas flow rate 20 (arbitrary units), capillary temperature 350°C, capillary voltage +30 V, tube lens +80 V, skimmer +25 V, and heater temperature 130°C.

The acquisition was assessed in the full-scan mode in the range (m/z) + 200–2,000 u, using an isolation window of ±2 ppm. The AGC target, injection time, mass resolution, energy, and gas in the collision cell were 1 × 106, 100 ms, 50 K, 20 V, and N_2, respectively. The MS data were processed using Xcalibur Software (Thermo Scientific). Peaks were identified by evaluating the accurate mass, the fragments obtained in the collision cell, and the on-line UV spectra (220–700 nm). Working solutions (n = 5) were prepared in the range of 2–50 µg/mL, and 20 µL was injected into the chromatographic system. Chromatographic data were integrated at 520 nm, and each analysis was carried out in triplicate (three technical replicates).

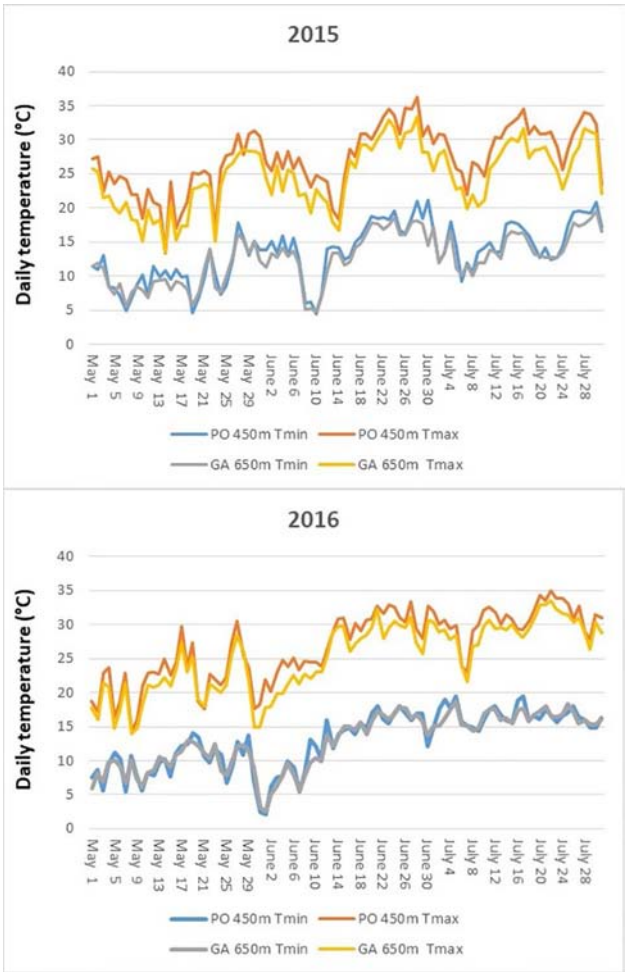

FIGURE 2 | Maximum daily temperatures and minimum daily temperatures recorded along in the two growing locations of Postalesio (450 m) and Gaggio (650 m) during berry development and ripening (May–July) in 2015 and 2016.

Statistical Analysis

Analysis of variance was performed by IBM SPSS Statistics software, version 25 (SPSS Inc., Chicago, IL), using general linear model univariate analysis, with growing year, altitude, or cultivar as fixed factors. Significant differences among different ripening classes were calculated by Tukey's mean test. Differences at $p \leq 0.05$ were considered as significant. Additional information is reported in the figure legends.

RESULTS

Environmental Factors

The course of daily maximum and minimum temperatures for the period May–July of both seasons for each site is reported in **Figure 2**. Minimum temperatures were very close in the two sites with an average difference of 0.8°C in 2015 and 0.2°C in 2016. Maximum temperatures were constantly higher in Postalesio, with +2.6°C on average in 2015 and +1.6°C in 2016 over the period May–July. As a consequence, daily thermal excursion was higher in Postalesio, with an average excursion in the May–July of 13.8°C in 2015 and 13.9°C in 2016, while in Gaggio, it was 12.1°C and 12.4°C, respectively.

Considering thermal resources useful for blueberry growth, growing degree days with 0°C base (White et al., 2012) were calculated for the May–July period: in agreement with temperature courses, Postalesio reached higher values of 1877 in 2015 and 1833 in 2016 GDD, while Gaggio 1718 and 1749 GDD.

Regarding solar radiation, PPAR, calculated by means of the topographic tools of SAGA GIS, reached in Gaggio 3,544 MJ/m^2, while it reached 3,109 MJ/m^2 in Postalesio.

Since the two sites are very close from one to the other and on the same side of the valley, we can assume similar patterns in the cloudiness and argue that the proportion of real PAR between the two sites is the same as for Potential PAR.

The monthly cumulated Potential PAR showed constantly higher availability of radiation (+17% on average) in Gaggio

(421, 466, 498 MJ m^{-2} in May, July, and June, respectively) over Postalesio (362, 398, 423 MJ m^{-2} in the same months).

Similar results were obtained simulating real PAR on the base of maximum and minimum daily temperatures by means of the Hargreaves model (Allen et al., 1998) (**Supplementary Figure S2**); considering the experimental time span May–July, Gaggio showed on average +15% of PAR in 2015 and +14% in 2016 compared to Postalesio.

Total Phenolics, Total Flavonoids, and Total Anthocyanins

"Duke" exhibited in both years higher amounts of total phenols than "Brigitta" throughout development and ripening (**Figure 3**). In both cultivars, the levels did not change markedly in the first two classes and then slightly increased due to the accumulation of anthocyanins starting from ripening class II. During the two growing seasons, ripe berries of "Duke," compared to berries of class II, presented an average increase in total phenol concentrations of 66% at the lower altitude and an average increase of 46% at the higher growing site. Conversely, in "Brigitta," total phenol accumulation, comparing class II and class IV fruits, increased averagely by 35% at the lower site and by 47% at the higher site. There was no clear effect of different altitudes on this parameter, although in 2015, "Duke" class I and "Brigitta" class III and in 2016 "Brigitta" class IV polyphenol contents were greater at higher altitude.

Remarkably, the pattern of accumulation of polyphenols did not change between years, even if the amounts were significantly higher in 2015 respect to 2016, both for "Duke" and for "Brigitta."

Similar trends were observed for total flavonoids and total anthocyanins, analyzed in berries starting from class II (turning stage) (**Figure 3**). The accumulation of these two groups of phenols increased progressively throughout ripening, determining significant differences among the three classes of both blueberry cultivars. During the two subsequent years, anthocyanin concentrations in "Duke" berries increased during ripening averagely by 432% and by 1,192% at the lower and at the higher growing sites, respectively. In "Brigitta" berries, anthocyanin accumulation was more pronounced than in "Duke," as fruit of "Brigitta" at stage II presented lower pigment levels than fruit of "Duke." In "Brigitta" fruits, the anthocyanin concentrations augmented 25-fold during maturation at lower and 34-fold at higher altitudes. As shown in **Figure 3**, total flavonoids and total anthocyanins were always higher in "Duke" compared to "Brigitta." As for polyphenol content, altitude did not affect total flavonoid and total anthocyanin contents and did not alter their pattern of accumulation.

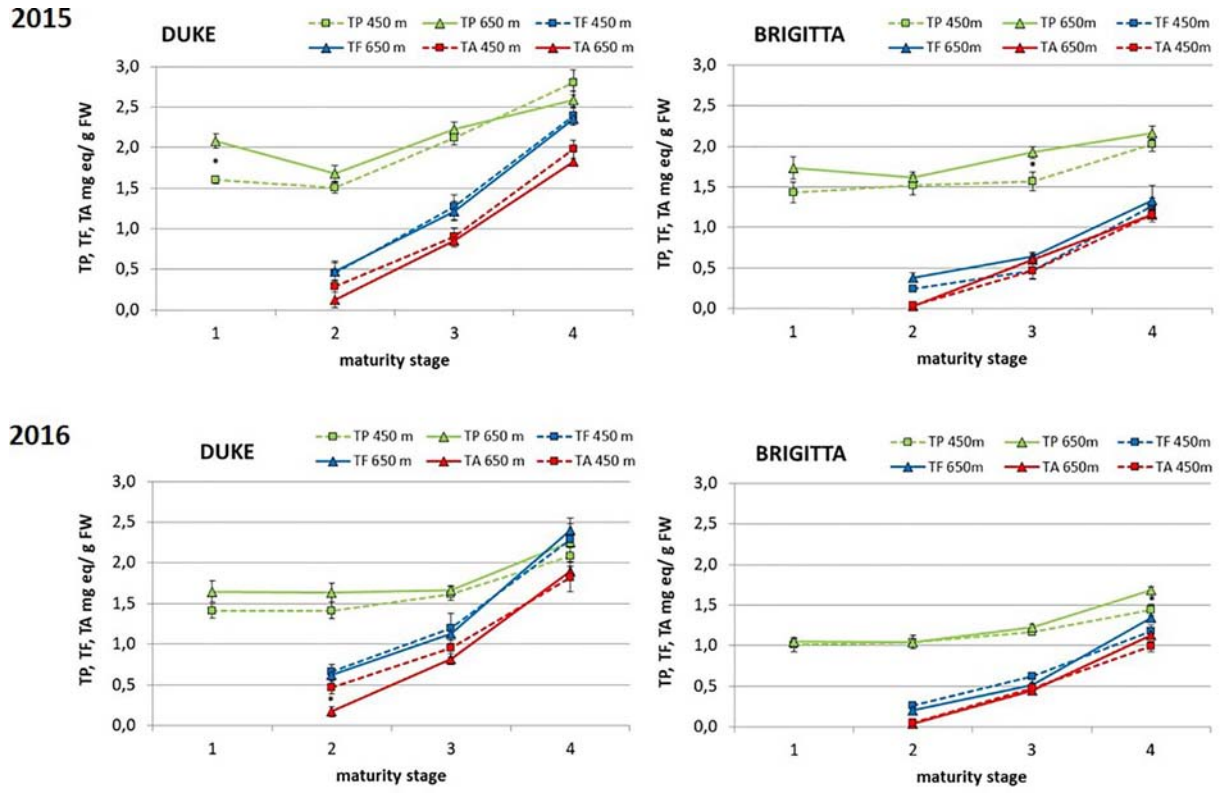

FIGURE 3 | Total phenols (expressed as mg catechin equivalents/g FW), total flavonoid (expressed as mg catechin equivalents/g FW) and total anthocyanins (expressed as mg malvidin 3-glucoside equivalents/g FW) (mg/100 g FW) in blueberries cv. "Duke" and "Brigitta" in 2015 and 2016. Values are means of six replicates. Asterisks indicate statistical differences between altitudes ($P \leq 0.05$).

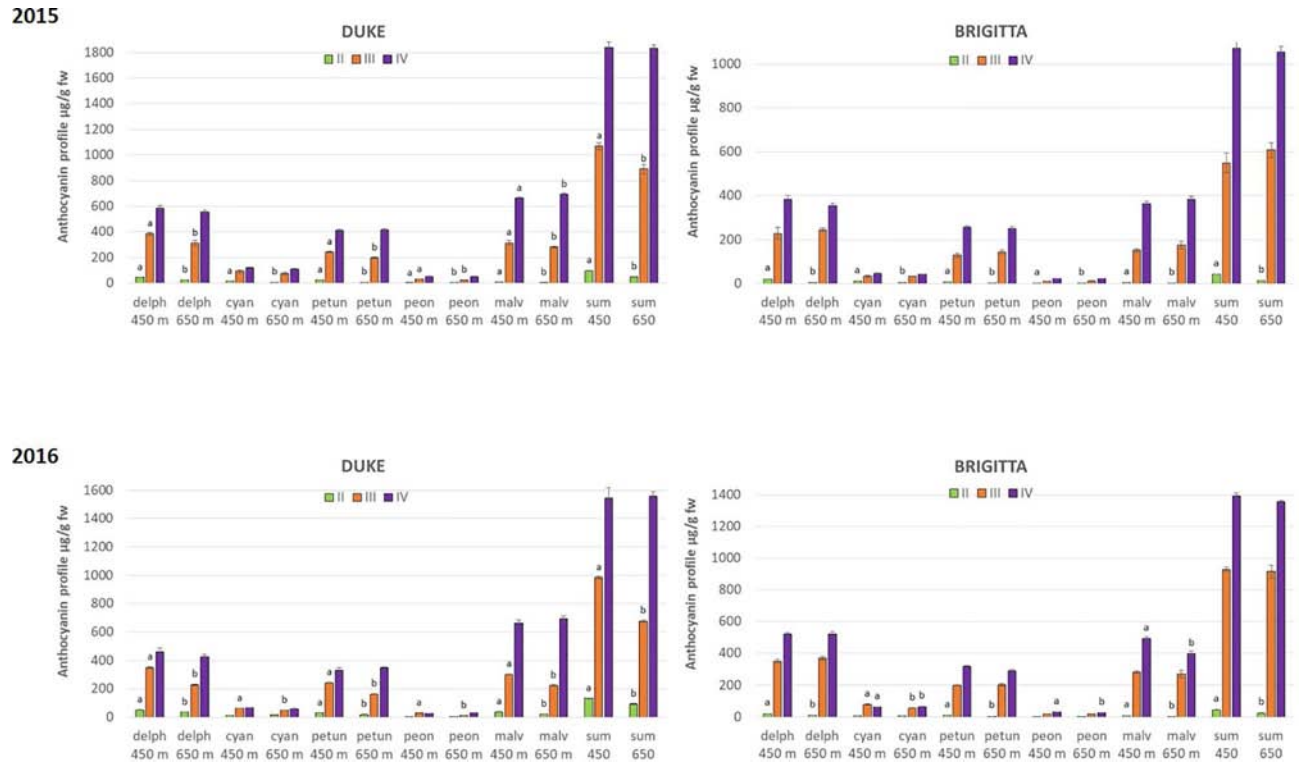

FIGURE 4 | Anthocyanin accumulation (µg/g FW) in blueberries cv. "Duke" and "Brigitta" in 2015 and 2016. Values are means of six replicates. Different letters indicate statistical differences between altitudes for each ripening class ($P \leq 0.05$).

Anthocyanin Profile

Anthocyanin profiles of "Duke" and "Brigitta" were systematically analyzed over the ripening process starting from the beginning of fruit pigment accumulation at stage II. The anthocyanins identified in both cultivars were monoarabinosides (ara), monogalactosides (gal) and monoglucosides (glu) of delphinidins (Dp), cyanidins (Cy), petunidins (Pt), peonidins (Pn), and malvidins (Mv) (**Supplementary Figure S3**; **Supplementary Table S1**). The acetylated form Pt-acetyl-galactoside was also detected in both varieties. In "Brigitta" berries, the predominant anthocyanidins were Dp and Mv. In ripe fruits of "Duke," the main anthocyanidin was Mv followed by Dp and Pt. As in "Brigitta," Cy and Pn were in minor proportion. The anthocyanidins identified were in both varieties conjugated prevalently with gal and secondarily with ara. Only Mv was detected as glu.

The total amount of anthocyanins, calculated as the sum of all the individual anthocyanins resulting by the chromatographic profiles, showed significant differences among all the classes of ripening, confirming the results obtained by the spectrophotometrical analysis (**Figure 4**).

Importantly, the chromatographic analysis revealed differences also between growth locations at particular berry developmental stages. During berry development, anthocyanin amounts were higher in berries grown at lower altitudes. "Duke" berries of classes II and III accumulated more anthocyanins at 400 m in both years, whereas in "Brigitta," higher levels of pigments were observed at 400 m in class II in both years. Berries of "Duke" and "Brigitta" class IV (full-ripening stage), however, did not differ in total anthocyanin amounts in any growing season. Remarkably, Dp and Pt glycosides reached significant higher amounts in fruits of class II at the lower growing location, regardless of the cultivar and growing season.

In addition to absolute concentrations, it is useful to express anthocyanin composition in relative terms (**Figure 5**). Considering the relative proportion of the individual anthocyanins during blueberry ripening, a shift of anthocyanin biosynthesis from cyanidin-type, di-substituted molecular structures (Cy, Pn) toward delphinidin-based, tri-substituted pigments (Dp, Pt, Mv) was evident. At color appearance, di-substituted anthocyanin relative proportions were highest. During ripening, however, a progressive increase in the proportion of tri-substituted and methoxylated (Pn, Pt, Mv) pigments was observed (**Figure 6**). The proportions of tri-substituted and methoxylated anthocyanins were always lower at higher altitude at the early stage of ripening (class II). Conversely, Cy and Pn glycosides reached higher proportions in berries belonging to class II grown at the higher sites, in both cultivars and years.

In the first growing season, fruit skin color was determined (**Figure 7**). No distinction was perceived between unripe fruits (class I) of both cultivars, which displayed a green color. Berries belonging to class II, associated to the initial appearance of

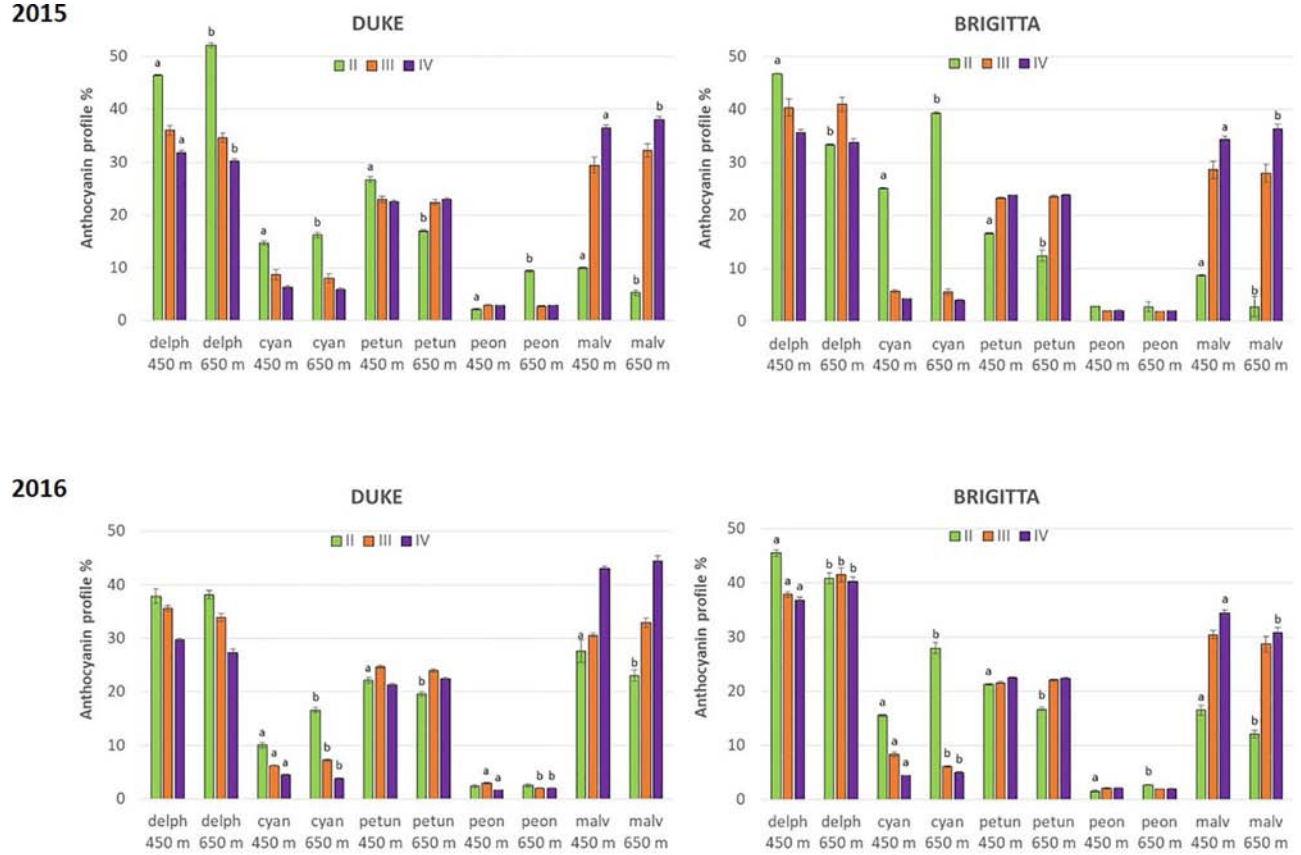

FIGURE 5 | Relative proportion of individual anthocyanins (%) in blueberries cv. "Duke" and "Brigitta" in 2015 and 2016. Values are means of six replicates. Different letters indicate statistical differences between altitudes for each ripening class ($P \leq 0.05$).

pigment accumulation, showed genotype-specific differences. "Brigitta" skin color shifted toward a reddish hue (average hue value moving toward lower values) whereas "Duke" fruit tended to a bluish coloring (average hue value moving toward higher values). This is in line with the ratio of red-/cyanidin- and blue-/delphinidin-based anthocyanins found in the two cultivars at the breaker stage. Moreover, fruit skin hue of blueberries grown at the lower altitude location showed more pigmentation with decreased values for "Brigitta" and increased values for "Duke." Fruit belonging to classes III and IV of both cultivars showed a shift from a violet (290°) to a blue color (210°). During blueberry ripening, chroma values progressively decreased as berry skin colors became duller (**Figure 8**). On the other hand, L* values also decreased at the same time (**Figure 8**), due to the peel becoming increasingly pigmented and darker in the course of maturation.

DISCUSSION

Environmental Factors

The availability of environmental resources strongly affects quality and quantity of plant production (Larcher, 1995; van Keulen and Wolf, 1986), with a primary role played by air temperature, solar radiation, and precipitation. Air temperature is the direct result of the energy balance driven by solar radiation and is influenced by topography, soil physical and chemical characteristics, soil management and water content, canopy shape, and management.

Similar topographic features, soil characters and same management (training system, cultural practices, and water supply) characterize the two sites object of this study. Consequently, we can assume that most of the environmental variability can be associated with the different availability of solar radiation by the specific courses of air temperature, both strongly related to altitude.

The current study focuses on the thermal course of the two sites, where higher availability in thermal resources determined quicker phenological development and affected ripening processes and anthocyanin accumulation. A complementary approach focuses on the relevant role played by solar radiation, taking into consideration other flavonoids such as flavonols. In fact, flavonol synthesis is upregulated by solar radiation, which shapes the flavonol profile, and especially the proportion of kaempferol, over time (Martínez-Lüscher et al., 2019). Additionally, the quality of solar radiation and its relationship with elevation and other geographic factors (Brillante et al., 2017; Martínez-Lüscher et al., 2019; Zoratti et al., 2015a) should be

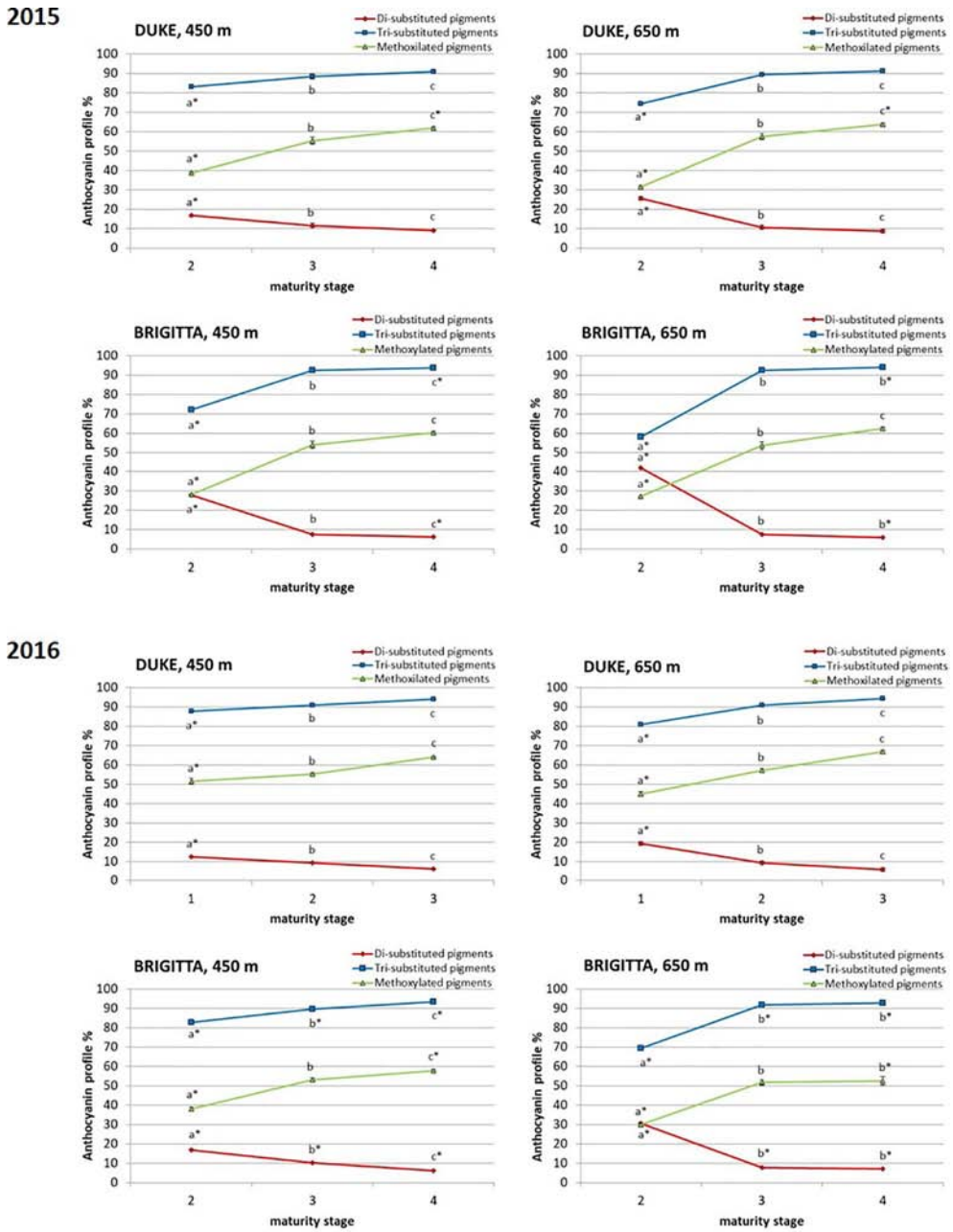

FIGURE 6 | Relative proportion of di-substituted anthocyanins, tri-substituted anthocyanins, and methoxilated anthocyanins (%) in blueberries cv. "Duke" and "Brigitta" in 2015 and 2016. Values are means of six replicates. Asterisks indicate statistical differences between altitudes ($P \leq 0.05$). Different letters indicate statistical differences among ripening classes of each cultivar ($P \leq 0.05$).

also investigated in order to assess the effects on the dynamics of metabolites—for instance, the relationship between variations of UV with elevation (Blumthaler et al., 1997, Diffey, 1991).

In the same environment of the present study and on grapevine, Failla et al. (2004) analyzed the spatial distribution of solar radiation and the effects on vine phenology and grape ripening. They concluded that different factors cooperated in the phenological timing of plants and in their ripening dynamics, with temperature (decreasing with altitude) and PPAR (increasing with altitude in the viticultural belt) as predictors for the considered variables.

Total Phenolics, Total Flavonoids, and Total Anthocyanins

Phenolic compounds including flavonoids (i.e., flavanols, anthocyanins and flavonols) and phenolic acids are considered

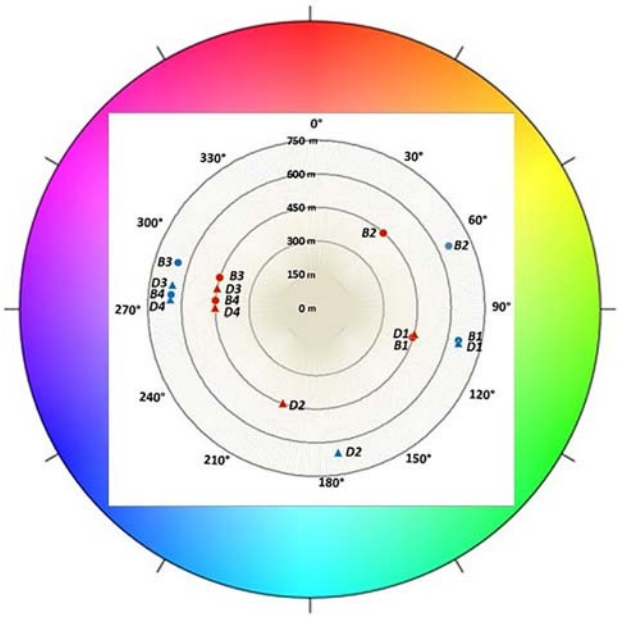

FIGURE 7 | Color expressed as hue angle (degrees) recorded in different classes of ripening (1, 2, 3, 4) of blueberries cv. "Duke" (D) and "Brigitta" (B) grown at two different altitudes. Polar plot: 0° and 360° indicate red, 90° indicates yellow, 180° indicates green, and 270° indicates blue. Values are means of 30 replicates recorded in 2015.

as nonnutrient biologically active compounds and concur in determining the antioxidant capacity of fruits. In blueberry, antioxidant activity correlates well with total phenolic content and anthocyanin content (Prior et al., 1998; Ehlenfeldt and Prior, 2001).

The increasing trends of "Duke" and "Brigitta" total phenols in 2015 and 2016 were partially different from the accumulation patterns reported for other varieties in Germany, which showed a decrease or steady level during color break and ripening (Castrejón et al., 2008). In Nova Scotia, blueberries displayed no changes or increasing levels during ripening and a decrease at full ripeness (Kalt et al., 2003). Differences of our results in comparison to those obtained in these studies may arise due to genotypic variation, different cultivation, and climate conditions and to different extraction solvents and analytical methods used. Moreover, contribution to blueberry total phenolic content is mainly associated to the phenolic acid group of hydroxycinnamic acids derivatives and to the flavonoid subclasses of flavanols (and their polymers proanthocyanidins), flavonols and anthocyanins. Flavonols, proanthocyanidins, and hydroxycinnamic acids and the corresponding transcripts are most concentrated in young fruits. By contrast, the levels of these compounds markedly decrease at later stages of berry development, the accumulation of anthocyanins begins at the onset of ripening, and they are the major flavonoids in the ripe berry (Jaakola et al., 2002; Castrejón et al., 2008; Zifkin et al., 2012; Vvedenskaya and Vorsa, 2004). Polyphenolic content therefore integrates all the different

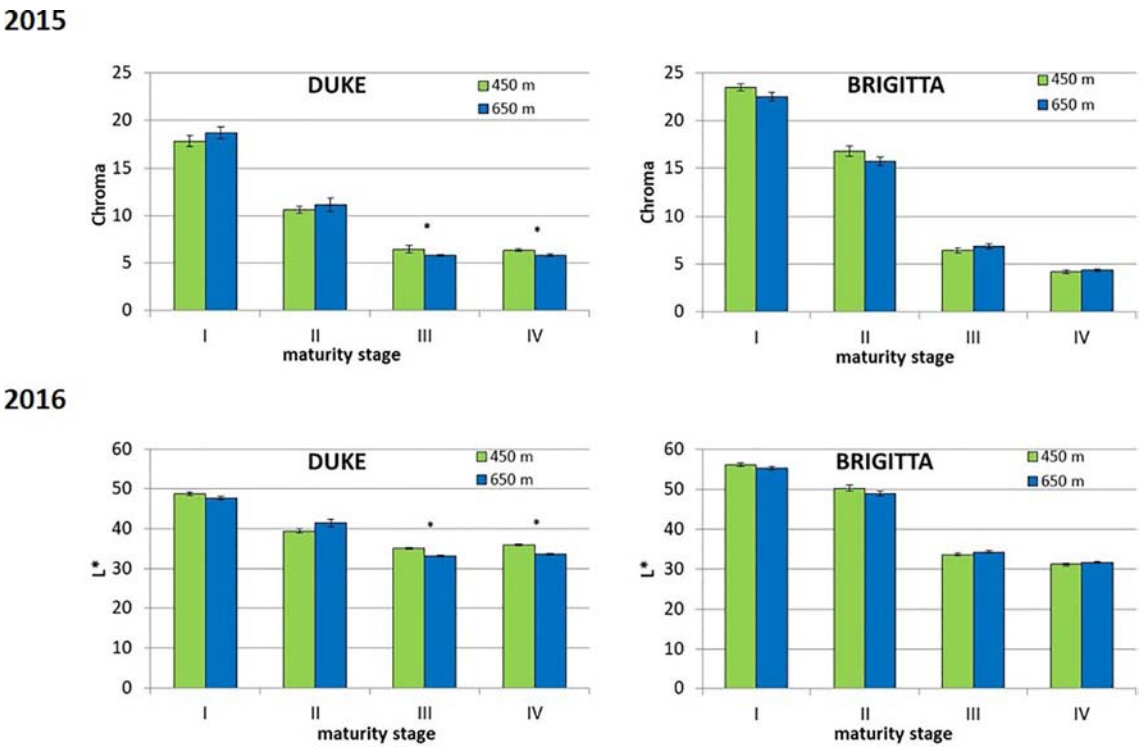

FIGURE 8 | Chroma (top) and L* (bottom) recorded in different classes of ripening of blueberries cv. "Duke" and "Brigitta" grown at two different altitudes. Asterisks indicate statistical differences between altitudes ($P \leq 0.05$). Values are means of 30 replicates recorded in 2015.

accumulation patterns of these classes of compounds that may vary among cultivars (Ribera et al., 2010; Guofang et al., 2019). Comparison of six highbush blueberry cultivars and *Vaccinium angustifolium* showed relative low amounts of chlorogenic acid and proanthocyanidins and high contents of anthocyanidins in "Brigitta" berries, suggesting a relative high contribution of the pigment class of phenols to the total phenolic content. Starting from turning stage (class II), "Duke" and "Brigitta" steadily accumulated flavonoids and anthocyanins. These results are in line with those of Ribera et al. (2010) who reported total anthocyanin concentrations for "Brigitta" ranging from 3.79 to 190 mg cyanidin-3-glucoside 100 g^{-1} FW and with comparable trends in rabbiteye blueberry (Guofang et al., 2019), in lowbush blueberry (Gibson et al., 2013), and bilberry (Jaakola et al., 2002).

Beside the strongly consistent developmental pattern and the genotypic effect, growth altitude did not apparently affect the total amounts of flavonoids and anthocyanins, nor did it the harvest season.

Anthocyanin Profile

The anthocyanin profile of ripe berries of "Brigitta" was similar to the profiles reported in other studies (Rodriguez-Mateos et al., 2012; Zoratti et al., 2015b); Dp and Mv were the dominant forms, followed by Pt and, in lowest proportion, Cy and Pn. In ripe fruits of "Duke," the main anthocyanidin was Mv; Cy and Pn were present in minor proportion. Similar results were reported in a study on blueberries grown in China (Li et al., 2016).

Anthocyanin accumulation begins at the onset of maturation and proceeds until full ripening. The flavonoid pathway has been intensively studied in cultivated and wild berry species, such as blueberries and bilberries, and the main structural genes have been isolated from highbush blueberry (Zifkin et al., 2012) and bilberry (Jaakola et al., 2002). The researches have indicated the increase in transcription levels particularly of chalcone synthase (*CHS*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*), and UDP-glucose flavonoid 3-*O*-glucosyltransferase (*UFGT*) at the ripening stage leading to anthocyanin accumulation. In addition, several transcription factors that play a role as key regulators of the flavonoid pathway have been identified (Falcone Ferreyra et al., 2012).

In our study, anthocyanin concentrations during berry development were higher in berries grown at lower altitudes. Remarkably, berries at turning stage accumulated more anthocyanins at the lower altitudes in both years. Berries of "Duke" and "Brigitta" class IV (full-ripening stage), however, did not differ in total anthocyanin amount in any growing season. The results are partially in accordance with the data on ripe bilberry grown at different altitudes in the eastern Alps of Austria (Rieger et al., 2008). The influence of altitudinal variation on the anthocyanin content of bilberry fruits was evident in both years, with a decrease moving from 800 to 1,200 m; in particular, all the individual anthocyanin levels were lower at higher altitude. Conversely, fruits grown at the highest altitude of 1,500 m showed only few differences compared to those at 1,200 m. Different results were reported by Zoratti et al. (2015b) on blueberry cv. "Brigitta" and bilberry grown in the eastern Alps of Italy. Bilberry revealed a progressive increase in anthocyanin accumulation along the altitudinal gradient of about 650 m. On the other hand, the effect of altitude increase on "Brigitta" was not clear, and contradictory data were obtained in the two growing years. These seasonal differences in anthocyanin pattern of accumulation were considered linked to environmental effects and to the markedly different temperatures recorded in some locations during the development and ripening of the fruits in the two growing years. In our study, temperatures at the lower altitude appeared to affect positively anthocyanin accumulation, mainly in the early ripening cv. "Duke." Differences in maximum daily temperatures between the two locations were about 2.6°C in the first half of July 2015 (average maximum temperatures of 24.7°C in Berbenno *vs*. 22.1°C in Gaggio) and about 2.2°C in the second half (average maximum temperatures of 32.0°C in Berbenno *vs*. 29.8°C in Gaggio). In the subsequent year, the temperature flow recorded in the same period did not differ substantially (the data recorded were 25.1°C in Berbenno *vs*. 23.0°C in Gaggio in the first half of July 2016 and 30.9°C and 28.9°C, respectively, in the second half). Optimum temperatures for blueberry fruit set and ripening are 20–26°C during the day and 16°C at night, although genetic variation has been documented (Lobos and Hancock, 2015). Therefore, "Duke" appeared to take advantage of the higher nighttime and daytime temperatures during color break and ripening by accumulating more anthocyanins within the different developmental stages. In "Brigitta," which ripens later, the positive effect of the higher temperature at the lower location was visible only at the onset of ripening, when the berry started pigmentation (class II). After this stage, the patterns of pigment accumulation were similar at both altitudes, suggesting that the environmental conditions allowed the kinetic of pigment deposition coordinated to the ripening process to proceed faster in berries grown at the higher location. Pattern of phenol accumulation and related gene expression observed in blueberry, bilberry, strawberry, and grape (Zifkin et al., 2012; Jaakola et al., 2002; Carbone et al., 2009; Castellarin et al., 2007a) highlights the coordination of phenol biosynthesis with the developmental stages of the fruit. Therefore, environmental factors that alter the rate of berry development may indirectly affect metabolite, e.g., anthocyanin accumulation, and environmental effects impacting particular metabolites would be time-dependent.

Zoratti et al. (2015b) reported as optimal temperature conditions for "Brigitta," high maximum daily temperatures (as high as 33.5°C) during the pink stage, followed by lower temperatures fluctuating around an average of 26°C until ripening, relatively higher than optimum temperatures of literature. Ideal conditions for anthocyanin accumulation are nowadays not known, and it has not yet been established whether anthocyanin accumulation is due to an integrated response to temperature as expressed by the concept of thermal time, to specific threshold temperatures, or to a duration of exposure to critical temperatures. Likewise, the critical stage of development for a response to temperature has not

been characterized. In grape, *in vitro* study indicated that a key time point might be the 2 weeks following the first skin color appearance (Yamane et al., 2006).

In our study, the temperature conditions at different altitudes had significant but temporary effects on anthocyanin accumulation. In both blueberry cultivars, berries appeared to compensate for these initial temperature effects. Higher temperatures during day and night led to an initial difference in anthocyanin concentration, but subsequently, berries at the final stage of ripening counterbalanced the gap through accumulating pigments at a more rapid rate, resulting in equal levels recorded at stage IV. Similar compensation mechanisms could be associated to observations reported in grape by Gouthu et al. (2014) which showed that, in a cluster, during the last phase of fruit maturation, the ripening rate of under-ripe berries is higher than that determined in the ripest berries to reach a synchronized development. Also, Corso et al. (2016) reported that grapevine rootstocks, characterized by a different effect on the rate of ripening of "Cabernet Sauvignon" berries, although determining different timings at the beginning of fruit development, (e.g., an earlier onset of ripening in the graft combination CS/M4 compared to CS/1103P), did not affect skin colorimetric parameters of berries at full-ripening stage. These results suggest an acceleration of ripening induced by 1103P rootstock at last stages of maturation and the need of the ripening transcriptional program to be completed in a genetically defined temporal window, independently by exogenous factors affecting the early phases of berry ripening initiation.

Climate differences associated to different altitudes affected accumulation of individual anthocyanins in "Duke" and "Brigitta" (**Figure 4**). In both years, the tri-substituted anthocyanins Dp, Pt, and Mv reached higher levels at the lower location, characterized by higher temperatures, during the early stages of fruit ripening (stages II and III of "Duke" and stage II of "Brigitta"). An opposite trend has been reported in bilberry, which increased accumulation of tri-substituted anthocyanins with increasing altitude (Zoratti et al., 2015b) and in grape "Pinot noir" grown at high daytime temperatures, which reduced the levels of Dp 3-glucoside, Pt 3-glucoside, and Mv 3-glucoside in the berry skin (Mori et al., 2007). Tarara et al. (2008) showed in a complex study on "Merlot" grapes, in which temperature-control regimens were dynamic paralleling the diurnal temperature fluctuation in the vineyard that, with increasing berry temperature, total concentrations of Dp, Cy, Pt, and Pn-based anthocyanins decreased, whereas the total concentration of Mv-based anthocyanins was unaffected. The temperatures, particularly daytime temperatures during ripening, appeared to be an environmental determinant of anthocyanin accumulation in blueberry under the field conditions encountered in this study. Moreover, the higher temperatures encountered at the lower altitude location may not have exceeded optimum for maximum anthocyanin accumulation, resulting in a quicker berry developmental pattern and in higher anthocyanin concentrations in the early phases of ripening, compared to the higher location.

Anthocyanin production is controlled by several transcription factors that affect the ratio of di-/trihydroxilated anthocyanins through trans-regulation of flavonoid 3′-hydroxylase (*F3'H*) and flavonoid 3'5'-hydroxylase (*F3'5'H*) gene expression. The biosynthesis of di-substituted anthocyanins is promoted by the enzyme F3'H activity, which is responsible for the hydroxylation of the precursor dihydrokaempferol at position 3′ of the B-ring and promotes Cy and Pn accumulations. F3'5'H activity drives the synthesis of tri-substituted anthocyanins, being responsible for the hydroxylation at the 3',5'-positions of the B-ring, that results in the production of Dp and the methylated derivatives Mv and Pt. Increase in flavonoid 3'5'-hydroxylase activity also diverts the biosynthesis from the Cy and Pn branch toward the branch resulting in Dp, Mv, and Pt as final anthocyanin structures. In blueberry, *F3'5'H* is weakly expressed during the earliest ripening stages and increases during the late ripening stages, closely paralleling the accumulation of tri-substituted anthocyanins (Zifkin et al., 2012).

Early in the ripening process of "Duke" and "Brigitta" blueberries, Cy-type anthocyanins were abundant, likely accounting for the red blush to parts of the green berry. As the fruit ripened and the exocarp color changed from mostly green and reddish to partially pink, blue-purple Dp-type anthocyanins began to accumulate. The appearance of the trihydroxylated anthocyanidin Dp and its derivatives Mv and Pt is coordinated with the abundance of *VcF3'5'H* transcripts at developmental stage coincident with the onset of ripening (Zifkin et al., 2012). Similar results were observed in grape, where the correlation between transcript profiles and the kinetics of accumulation of red-/cyanidin- and blue-/delphinidin-based anthocyanins indicated that *VvF3'H* and *VvF3'5'H* expressions were consistent with the chromatic evolution of ripening bunches (Castellarin et al., 2006). In the present study, developmental regulation based on genotypic information appeared to be more relevant and environmental factors related to different altitudes had only fine-tuning influence. The present results are comparable with studies conducted on bilberry (Jaakola et al., 2004) and on strawberry (Carbone et al., 2009), in which anthocyanin accumulation showed to be under strong developmental control. Moreover, light seems to play a secondary, fine-tuning role on the accumulation of flavonoids in different *Vaccinium* species. Many of the wild *Vaccinium* berries, like bilberries, grow in shaded habitats and do not require high light for induction of anthocyanin biogenesis. Blueberries are also shade-adapted species, although they seem to require higher solar exposure for normal ripening and anthocyanin accumulation (Zoratti et al., 2015a). In fact, regarding anthocyanin production, fruits can be classified into those that have anthocyanins in both their skin and flesh, those that have anthocyanins only in their skin, and those that accumulate anthocyanins in their skin only as response to a light stimulus. In the first two classes, developmental regulation has a major role in anthocyanin biosynthesis, whereas in the third, anthocyanin biosynthesis is more under environmental control (Jaakola, 2013). There is strong evidence that blueberry and bilberry belong to the first group. Findings reported by Karagiannis et al. (2016) on the regulation of peach skin quality traits by altitude, conversely, suggested that, in this case, environmental factors had a more important role in skin anthocyanin accumulation and higher altitude favored peach pigment biosynthesis, as already proposed in apple (Lin-Wang et al., 2011).

Our results indicate that, during bilberry ripening, when the relative proportion of individual anthocyanins is considered, the shift of biosynthesis from cyanidin-type, di-substituted molecular structures toward delphinidin-based, tri-substituted pigments were evident. De Lorenzis et al. (2016) reported similar trends in "Corvina" grape. Grapes subjected to different water stress conditions showed an analogous behavior, with an altered ripening process and subsequent anthocyanin profile (Brillante et al., 2017). In fact, skin total amount of anthocyanins was negatively affected by severe water stress; in particular, the dihydroxylated forms were more affected than trihydroxylated. At the same time, a pronounced water stress reduced net carbon assimilation, but not TSS, which, on the contrary, increased. Since the berry mass was not significantly affected, the authors suggested that higher water stress accelerated ripening. Moreover, the observed reduction of TA could be related to water stress, but could also be linked to a more advanced ripening in the severely stressed condition. Therefore, the increase in the tri-/dihydroxylated ratio could be related to a stressful factor, as the anthocyanin biosynthesis appeared to be shifted toward the production of more complex and stable molecules, and to an acceleration of the ripening process.

In the present study, blueberry plants at both altitudes were well watered throughout the developmental stages, and no stresses due to water deficit or other soil properties were expected. Moreover, tri-substituted and methoxilated anthocyanin proportions were always lower at higher altitude at the early stage of berry ripening and were likely associated with a slower progression of the ripening process. The developmentally regulated proportion changes between di- and tri-substituted anthocyanins occurred earlier at the lower altitude location characterized by higher temperatures, where blueberries initially ripened more quickly. These differences however diminished along the ripening process and suggest that berries grown at higher altitude compensated for this initial disparity through accumulating tri-substituted pigments at a much more rapid rate as the temperature became higher, closer to the optimum. In "Brigitta," this was more evident compared to "Duke," and the increase in the proportion of tri-substituted anthocyanins between class II fruit and class III fruit was more steep. This compensatory mechanism suggests a feedback response resulting in part from the coordinated regulation of flavonoid pathway genes, mainly driven by developmental and genetic cues and by specific environmental effects (Cohen et al., 2012). Moreover, a different response in anthocyanin accumulation between "Brigitta" and "Duke" was also reported following methyl jasmonate treatment (Cocetta et al., 2015).

Fruit skin color evaluated in the first growing season was in line with the ratio of red-/cyanidin- and blue-/delphinidin-based anthocyanins found in the two cultivars at the breaker stage. Fruit skin hue of blueberries grown at the lower altitude location showed more pigmentation with decreased values for "Brigitta" and increased values for "Duke." Fruit belonging to classes III and IV of both cultivars showed a shift from a violet (290°) to a blue color (210°), a trend associated with the progression in the ripening process.

The observed progressive decline in the chroma values may be an effect of epicuticular waxes deposition during berry development (Saftner et al., 2008; Konarska, 2015). Indeed, cuticular wax load (fruit bloom) increased in blueberry during the fruit development, leading to a thick cuticle at maturity (Chu et al., 2018; Trivedi et al., 2019) and resulting in less vivid skin color. On the other hand, L* values also decreased at the same time and reflected anthocyanin accumulation rather than fruit bloom, in accordance with the data reported in other blueberry cultivars (Chung et al., 2016; Matiacevich et al., 2013).

CONCLUSIONS

The present study suggests that anthocyanin accumulation is strongly regulated by development and genotype. The environmental effect exerted by temperature, when not far exceeding optimum, on anthocyanin concentration and composition in blueberry fruit is mainly associated to the pattern of ripening progression. Furthermore, this fine-tuning influence may only be temporary, and with increasing temperatures, differences in anthocyanin levels may disappear due to compensatory mechanisms in anthocyanin accumulation.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and the supplementary files.

AUTHOR CONTRIBUTIONS

AS and IM developed the concept of the paper. AS wrote the paper, performed spectrophotometric analyses and colorimetric determinations, and together with CG performed the analysis of anthocyanin profile by HPLC and MS. GC provided the environmental data. All authors discussed and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01045/full#supplementary-material

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Salinity in Autumn-Winter Season and Fruit Quality of Tomato Landraces

Tommaso Michele Moles[1,2], Rita de Brito Francisco[3]*, Lorenzo Mariotti[1]*, Antonio Pompeiano[4,5], Antonio Lupini[6], Luca Incrocci[1], Giulia Carmassi[1], Andrea Scartazza[7], Laura Pistelli[1], Lorenzo Guglielminetti[1], Alberto Pardossi[1], Francesco Sunseri[6], Stefan Hörtensteiner[3] and Diana Santelia[2]*

[1] Department of Agriculture, Food and Environment, University of Pisa, Pisa, Italy, [2] Institute of Integrative Biology, ETH Zürich, Zürich, Switzerland, [3] Department of Plant and Microbial Biology, University of Zürich, Zürich, Switzerland, [4] International Clinical Research Centre, St. Anne's University Hospital, Brno, Czechia, [5] Central European Institute of Technology, Brno University of Technology, Brno, Czechia, [6] Department of Agraria, University Mediterranea of Reggio Calabria, Reggio Calabria, Italy, [7] Institute of Research on Terrestrial Ecosystems, National Research Council, Pisa, Italy

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Edited by:
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Alexios A. Alexopoulos,
Technological Educational Institute of Peloponnese, Greece

***Correspondence:**
Tommaso Michele Moles
tommaso.moles@env.ethz.ch
Rita de Brito Francisco
rfrancisco@botinst.uzh.ch
Lorenzo Mariotti
lorenzo.mariotti@unipi.it

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Tomato landraces, originated by adaptive responses to local habitats, are considered a valuable resource for many traits of agronomic interest, including fruit nutritional quality. Primary and secondary metabolites are essential determinants of fruit organoleptic quality, and some of them, such as carotenoids and phenolics, have been associated with beneficial proprieties for human health. Landraces' fruit taste and flavour are often preferred by consumers compared to the commercial varieties' ones. In an autumn-winter greenhouse hydroponic experiment, the response of three Southern-Italy tomato landraces (Ciettaicale, Linosa and Corleone) and one commercial cultivar (UC-82B) to different concentrations of sodium chloride (0 mM, 60 mM or 120 mM NaCl) were evaluated. At harvest, no losses in marketable yield were noticed in any of the tested genotypes. However, under salt stress, fresh fruit yield as well as fruit calcium concentration were higher affected in the commercial cultivar than in the landraces. Furthermore, UC-82B showed a trend of decreasing lycopene and total antioxidant capacity with increasing salt concentration, whereas no changes in these parameters were observed in the landraces under 60 mM NaCl. Landraces under 120 mM NaCl accumulated more fructose and glucose in the fruits, while salt did not affect hexoses levels in UC-82B. Ultra-performance liquid chromatography–tandem mass spectrometry analysis revealed differential accumulation of glycoalkaloids, phenolic acids, flavonoids and their derivatives in the fruits of all genotypes under stress. Overall, the investigated Italian landraces showed a different behaviour compared to the commercial variety UC-82B under moderate salinity stress, showing a tolerable compromise between yield and quality attributes. Our results point to the feasible use of tomato landraces as a target to select interesting genetic traits to improve fruit quality under stress conditions.

Keywords: tomato, landraces, off-season, salinity, fruit quality, metabolites

INTRODUCTION

Tomato is the most consumed berry fruit worldwide as well as one of the most important constituents of the Mediterranean diet representing a key source of minerals, vitamins and antioxidants (Canene-Adams et al., 2005). Fruit quality is affected by environmental conditions, such as seasonal changes, the occurrence of biotic/abiotic stress and agronomic practices (water management and fertilizer supply), as well as genetic factors (Poiroux-Gonord et al., 2010), but their mechanism of action is not completely clear. To enhance health-related compounds, different agronomic strategies have been applied, namely grafting (Sánchez-Rodríguez et al., 2012; Casals et al., 2018) or controlled water management techniques (Barbagallo et al., 2008).

Salinity induces changes in physiology and metabolism that affect the final crop yield (Pompeiano et al., 2016). Tomato is generally considered a moderately salt-tolerant crop, often cultivated in areas polluted by salinization of aquifers and consequent use of saline water for irrigation (Santa-Cruz et al., 2002). Salinity can positively modulate tomato fruit metabolism and improves the sensorial/nutritional value of the production (D'Amico et al., 2003). Salinity can increase the total soluble content (°Brix) and the titratable acidity, two important parameters influencing the quality of tomato fruits. Moreover, a high salt concentration in irrigation water generally stimulates the defence system of the plant, thereby leading to accumulation of secondary metabolites in different tissues. One common feature of plant secondary compound classes, such as carotenoids, polyphenols and terpenoids, is reactive oxygen species (ROS) scavenging activity (Ndhlala et al., 2010). Due to their strong antioxidant activity, these bioactive metabolites have been recognized as beneficial players against human cardiovascular and chronic degenerative diseases (Borguini and da Silva Torres, 2009) and tumours (Barone et al., 2018). High salinity can accelerate lycopene biosynthesis in hydroponically-grown tomato plants (Wu and Kubota, 2008). Several water management techniques applying controlled and moderate drought/salt stress in the pre-harvesting period of tomato fruits have been implemented to maintain a sufficient yield and also to produce fruits with improved nutritional level (Kubota et al., 2012).

The diurnal and seasonal changes in light intensity, vapour pressure and temperature can also explain the differences observed between seasonal experiments (spring-summer vs autumn-winter) and cultivation systems (open-field vs greenhouse) (Incerti et al., 2009; Asensio et al., 2019). Yield and quality of tomato fruits from off-season greenhouse cultivation are often reduced compared to open-field production (Hu et al., 2006). This effect depends on the association of the different climatic conditions with the covering materials used in the protected environment—that can deplete the intensity and the quality of the light spectrum inside the greenhouse (Toor et al., 2006; Gent, 2007; Mariz-Ponte et al., 2019). Light quality and intensity are indeed major constraints influencing quality parameters in tomato fruit (Slimestad and Verheul, 2005). Generally, °Brix and titratable acidity are positively correlated with increasing light intensity (Claussen et al., 2006) and temperature (Adams et al., 2001). High light intensity and the modulation of UV-B in the light spectra enhance flavonoid accumulation in tomato fruit tissues. Low night temperature, which often occurs in non-heated greenhouses during winter, drastically affects plant growth and crop yield (Jing et al., 2016).

The genetic background is a critical factor that can significantly influence fruit quality (Steward et al., 2000; Vallverdú-Queralt et al., 2011). In the present work the response to salt stress of long-storage tomato landraces (or traditional varieties) were assessed. In particular, the effects of moderate and high concentrations of sodium chloride (60 and 120 mM NaCl) on yield and quality-related fruit metabolites were evaluated on three Italian tomato landraces from different geographic origin (Ciettaicale, Corleone and Linosa). These landraces are traditionally used to prepare fresh sauce or dried fruits stored in olive oil. A tomato ancient variety (UC-82B) was included in the experiment as commercial control. According to our preliminary screening of a tomato landrace collection, the local accessions selected for this study represented promising candidates for traits related to abiotic stress tolerance, which are notably found in other Mediterranean tomato landraces (Galmes et al., 2013; Patanè et al., 2016). Indeed, compared with other processing tomato genotypes, Ciettaicale previously showed an interesting tolerance profile to salt and drought stress at vegetative stage (Moles et al., 2016; Moles et al., 2018), whereas Linosa exhibited a high nitrogen use efficiency (Abenavoli et al., 2016; Lupini et al., 2017). These promising findings prompted to carry out experiments in autumn-winter off-season climatic conditions, which allowed us to adopt a hydroponic irrigation system with different salt concentrations (60 and 120 mM NaCl can be considered as 10% and 20% seawater dilutions, respectively) that normally are considered high for a moderate salt-sensitive crop such as tomato (Cuartero et al., 2006). This information is essential for selecting potential metabolic traits to be used as biomarkers on which to focus for new breeding strategies.

MATERIALS AND METHODS

Plant Material and Growth Condition

Three Southern Italy tomato landraces and a standard tomato variety were used as genetic material in this study. Among the landraces, Ciettaicale (from Basilicata region) and Linosa (from Pelagic Islands, Sicily) belong to the category of tomatoes with indeterminate growth habit and with pear/globose fruits, whereas Corleone (from Sicily region) is an indeterminate tomato type with flattened/ribbed fruits. The commercial variety UC-82B (supplied by the Tomato Genetics Resource Center, Department of Plant Sciences, University of California-Davis, CA, USA) belongs to the category of determinate tomatoes with pear/globose fruits. Plants were grown in rockwool cube (Rockwool B.V., The Netherlands), in an open nutrient solution system at a plant density of approximately 3 m^{-2} in a glasshouse

at the University of Pisa (Italy) from September 2016 to February 2017. The plants (16 individuals for each of the 4 genotypes for each of 3 experimental conditions, divided in eight pairs which were arranged in a randomized block design on the glasshouse benches, for a total of 192 plants) were grown vertically trimming the plant below the fourth fruit truss. Climatic parameters were continuously monitored by means of a weather station located inside the glasshouse. The mean air temperature and relative humidity were 17.3°C and 74.6%, respectively (T_{min} = 11.4°C and T_{max} = 27.7°C; RH_{min} = 50.2% and RH_{max} = 96.7%). Mean values of daily inside global radiation was 3.44 MJm^{-2} (GR_{min} = 1.02 MJm^{-2} and GR_{max} = 10.30 MJm^{-2}). Two salinity levels of nutrient solution were used with electrical conductivities (EC) of 8.3 and 14.6 mS cm^{-1}, which corresponded roughly to 60 and 120 mM NaCl, respectively. The concentration of nutrient solution was as reported by Incerti et al. (2007). Salt stress was applied 3 weeks after planting; the process was stepped up in roughly 2.1 mS cm^{-1} (20 mM NaCl) daily increments to avoid osmotic shock. Irrigation was controlled by a timer that opened the irrigation lines for 1 min up to 12 times per day, depending on growing stage and environmental conditions.

Biometrical Measurements, Fruit Yield and Sampling

Fresh fruit yield (FW) was determined based on total fruits picked two times per week in February from all the three trusses of each plant and genotype under different experimental treatments. The red-ripe fruits from the second truss (which consistence was evaluated by penetrometer) were separately collected early in the morning, weighted and randomly grouped in six biological replicates (15-20 whole fruits composed a single biological replicate) to be ground altogether at 4°C (homogenate) and then used for laboratory determinations. An aliquot of the homogenate was added to the remaining harvested material and stored at 80°C for 2 weeks to record fruit dry weight (DW). Aliquots of the homogenate were separately stored and placed at 80°C for 1 week for cation quantifications; additional aliquots were used to determine °Brix and titratable acidity (Beckles, 2012); finally, other samples were collected in tubes and stored at -80°C for further metabolic analyses (per biological replicate, four technical replicates were analysed). At the end of experiment, leaf area was estimated using a digital planimeter; then, shoot organs (leaves and stems) were weighted (shoot FW) and put in 80°C for 2 weeks to record the dry weight (shoot DW).

Total Soluble Sugar Measurements

Glucose, fructose and sucrose were extracted from tomato fruit homogenate aliquots according to the protocol described in Hostettler et al. (2011), and then quantified enzymatically according to Thalmann et al. (2016).

Cation Determination

Fruit dried samples were powdered and mineralized (60 min at 220°C) using a solution of HNO_3:$HClO_4$ (2.5:1, v/v). Sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) were determined using an atomic absorption spectrometer (Varian AA 24FS, Australia).

Lycopene Determination

Lycopene content was assayed according to Giovannetti et al. (2012). Briefly, an aliquot of tomato fruit homogenate was extracted in a solution of acetone:ethanol:hexane (1:2:1, v/v/v) and agitated on an orbital shaker for 15 min. Then one volume of distilled water was added, followed by 5 min agitation. After centrifugation, the hexane phase was measured at 503 nm, blanked with pure hexane.

Total Flavonoids, Total Phenols and Total Antioxidant Activity Contents

Tomato fruit homogenates were mixed in 70% (v/v) methanol and agitated overnight at 4°C in the dark. After incubation, the extracts were centrifuged at 12000 x g for 15 min at 4°C and the supernatants were utilized for the analyses indicated below. Total soluble phenols content (TPHE) and total flavonoids content (TFL) were assayed using the respective protocols reported in Caser et al. (2016). Total antioxidant capacity (TAC) was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as previously reported in Moles et al. (2016), with some modifications. Briefly, an aliquot of the methanolic fruit extract was added to 0.1 mM methanolic DPPH solution. After 30 min of incubation at room temperature in the dark, absorbance was measured at 515 nm, and the results were expressed as µmol of Trolox per gram of plant material on dry basis.

Metabolite Profiling

Tomato fruit homogenate (100 mg) was extracted with 100% methanol. The samples were ground using a mixer mill with 1.25–1.65 mm glass beads for 1.5 min at 30 Hz, centrifuged at 15,000 × g at 4°C and the supernatants collected. All samples were de-salinized over a silica-based classic cartridge (WAT051910, Waters) according to the manufacturer instructions. The eluted samples were concentrated using a Savant SpeedVac concentrator (Thermo Fisher Scientific) at 42°C. Prior to ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) analysis the samples were re-suspended in 80% methanol, 0.1% formic acid. After sonication for 5 min, the samples were centrifuged at 15,000 × g, at 4°C for 5 min, and transferred to liquid chromatography vials. Samples were analysed on a UPLC (Dionex UltiMate 3000, Thermo Fisher Scientific) coupled to a Bruker compact electrospray ionisation (ESI)-quadrupole-time-of-flight tandem-mass spectrometer (Bruker Daltonics). The UPLC separation was performed at 28°C with a C18 reverse-phase column (ACQUITY UPLC™ BEH C18, 1.7 µm, 2.1 × 150 mm, Waters) using the following gradient of solvent B [acetonitrile with 0.1% (v/v) formic acid] and solvent A [water with 0.1% (v/v) formic acid]: 0–0.5 min, 5% B; 0.5–12 min, 5–100% B; 12–14 min, 100% B; 14–16 min, 100–5% B. The flow rate was set to 0.3 mL min^{-1} and 5 µl of each sample was injected. The ESI source was operated in positive mode and parameters were set as follows: gas temperature, 220°C; drying gas, 9 L min^{-1}; nebuliser, 2.2 bar; capillary voltage, 4,500 V; end plate offset, 500 V. The instrument was set to acquire an m/z range of 50–1,300. Conditions for MS/MS were set as described by Christ et al. (2016). All data were recalibrated

internally using pre-run injection of 10 mM sodium hydroxide in 0.2% formic acid, 49.8% water, 50% isopropanol (v/v/v). DataAnalysis v.4.2 and TargetAnalysis v.1.3 softwares (Bruker Daltonics) were used to analyse the data. Metabolites were identified and annotated by comparison with MS and MS/MS data spectra either generated by authentic reference standards or deposited in the literature and databases, such as PubChem and MoTo (Moço et al., 2006).

Statistical Analysis

A randomized block design as previously described was performed. Data were subjected to two-way analysis of variance (ANOVA) and the mean values were compared using Duncan's test ($P < 0.05$) to check the significant differences. For the metabolomics data, in order to examine the differences between the treatments, the percentage contribution of each compound to the average dissimilarity between the aforementioned factors was calculated using similarity percentage analysis (SIMPER) (Vaníčková et al., 2015). A cut-off was imposed where $\sum\delta i\%$ reached 70%. Also, the differences in the chemical composition and extra characteristics of the samples from the study were analysed by principal component analysis (PCA). Prior to PCA, peak areas were subjected to logarithmic transformation; intraspecific scaling was performed by dividing each treatment/experimental condition score by its standard deviation; the data were centred by treatment scores. In PCA analyses, hierarchical clustering based on Pearson correlation showed that treatments with similar chemical profiles cluster together. All computations were performed with R 3.5.3 language and environment (R Core Team, 2019) and the R packages FactoMineR (Le et al., 2008) and vegan (Oksanen et al., 2019).

RESULTS

We assessed the effects of moderate and high salt treatments (60 and 120 mM NaCl) applied during an off-season greenhouse experiment, thereby evaluating yield and quality-related fruit metabolites in three Italian tomato landraces (Ciettaicale, Corleone and Linosa) and in a commercial variety (UC-82B). Analysis of all the biometric and metabolic traits revealed a significant ($P < 0.05$) genotype × treatment interaction. Following that, subsequent data were presented in treatment combinations.

Biometrical Measurements and Fruit Yield

To investigate whether (or how) salinity stress impacted on leaf growth, we measured leaf area and epigeal biomass at the end of the experiment. In the landraces leaf area did not decrease upon treatment with 60 mM NaCl (**Supplementary Figure 1A**), while a significant reduction in this parameter was recorded under 120 mM NaCl, particularly in Ciettaicale (approximately 46% smaller than its control). By contrast, the commercial variety UC-82B decreased leaf area about 30% and 60% under 60 and 120 mM NaCl, respectively. However, a common reduction in the vegetative biomass (shoot FW and DW) was recorded in all the tomato genotypes comparing to their respective controls (**Supplementary Figures 1B**, **C**).

Although salinity treatments differently affected fruit size (**Figure 1** and **Table 1**), the number of fruits was unaffected (**Table 1**). Ciettaicale and UC-82B already reduced fruit yield FW under 60 mM NaCl (**Figure 2A**). By contrast, Corleone and Linosa decreased fruit yield FW only under 120 mM NaCl by 47% and 20%, respectively. Moreover, Ciettaicale, Linosa and UC-82B did not exhibit significant differences when considering fruit yield DW (**Figure 2B**), except Corleone which significantly increased fruit yield DW under 60 mM NaCl (about 14%) and decreased under 120 mM Na Cl by 27% compared to the control.

Soluble Sugars Content

In all genotypes, 120 mM NaCl treatment caused significant increases in both fruit °Brix and titratable acidity (**Table 1**). We did not detect sucrose in fruit samples, but different trends in glucose and fructose contents among tomato genotypes were observed (**Figure 3**). Under 120 mM NaCl, landraces had increased glucose and fructose contents, while no significant

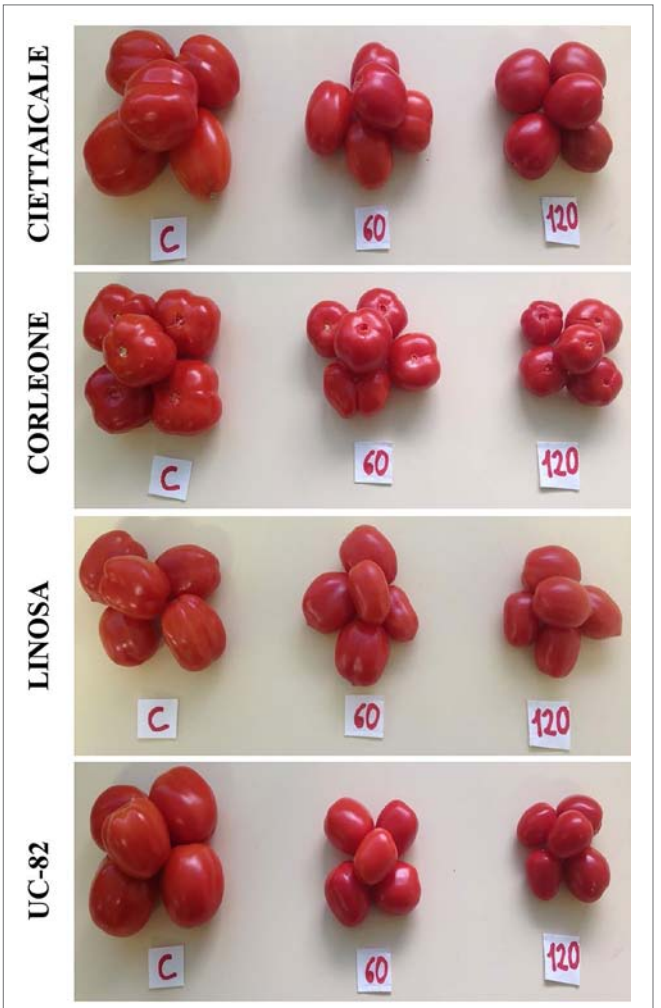

FIGURE 1 | Effect of different salt concentrations (60 mM and 120 mM NaCl) on fruit size of three tomato landraces (Ciettaicale, Corleone and Linosa) and a commercial variety (UC-82B) compared to respective control condition (C).

TABLE 1 | Effect of different salt concentrations (60 mM and 120 mM NaCl) on number of fruits, single fruit fresh weight (FW), fruit total soluble solids (°Brix) and titratable acidity (TA) of three tomato landraces (Ciettaicale, Corleone and Linosa) and a commercial variety (UC-82B).

Genotype	[NaCl]	n° fruits plant^{-1}	Single fruit FW (g plant^{-1})	°Brix	TA
Ciettaicale	Control	18.25 ± 0.74 b	51.12 ± 3.66 a	3,84 ± 0.25 e	0,26 ± 0.01 d
	60 mM	17.94 ± 0.87 b	41.29 ± 1.69 cd	4,94 ± 0.15 cd	0,29 ± 0.01 c
	120 mM	18,25 ± 0.63 b	32.47 ± 1.44 e	6,28 ± 0.13 b	0,33 ± 0.01 b
Corleone	Control	14.81 ± 0.86 cd	48.57 ± 0.84 ab	4,10 ± 0.10 e	0,29 ± 0.01 ce
	60 mM	16.81 ± 0.78 bc	39.24 ± 0.97 d	4,60 ± 0.18 d	0,30 ± 0.01 c
	120 mM	14.38 ± 0.64 d	25.83 ± 1.04 fg	6,44 ± 0.13 b	0,39 ± 0.01 a
Linosa	Control	24.44 ± 0.89 a	29.32 ± 0.49 eg	5,04 ± 0.11 c	0,27 ± 0.01 cd
	60 mM	23.63 ± 0.69 a	29.99 ± 0.71 ef	6,02 ± 0.12 b	0,33 ± 0.01 b
	120 mM	23.13 ± 0.64 a	24.72 ± 0.71 gh	7,14 ± 0.12 a	0,29 ± 0.01 c
UC-82B	Control	13.00 ± 0.82 d	44.43 ± 2.32 bc	2,50 ± 0.16 f	0,17 ± 0.01 e
	60 mM	13.50 ± 0.64 d	26.91 ± 1.10 fg	4,14 ± 0.12 e	0,25 ± 0.01 d
	120 mM	13.06 ± 0.61 d	21.09 ± 1.03 h	4,70 ± 0.07 cd	0,36 ± 0.02 a

Data are means ± SE of 16 replicates for the biometrical parameters and of 6 replicates for fruit quality estimations, respectively. Means within a column followed by the same letter are not significantly different based on Duncan's test (P < 0.05).

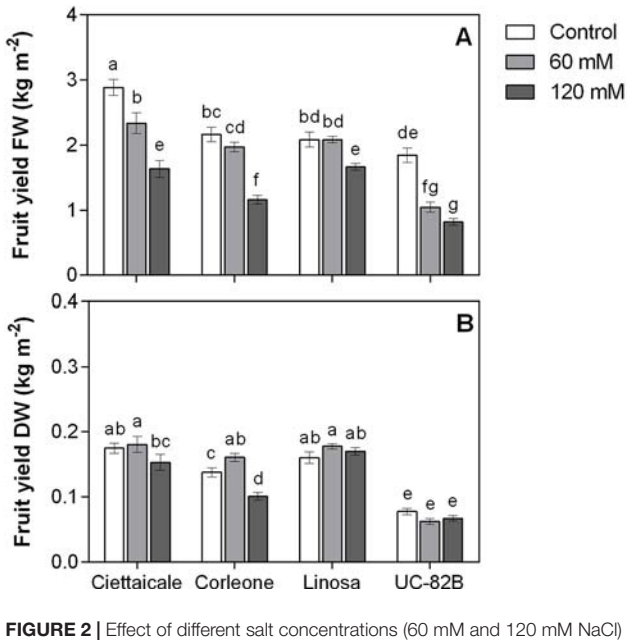

FIGURE 2 | Effect of different salt concentrations (60 mM and 120 mM NaCl) on fruit yield of three tomato landraces (Ciettaicale, Corleone and Linosa) and a commercial variety (UC-82B). **(A)** Yield fresh weight (FW) and **(B)** yield dry weight (DW). Error bars represent the standard error of the mean (n = 16). Bars with same letters are not statistically different from one another according to Duncan's test (P < 0.05).

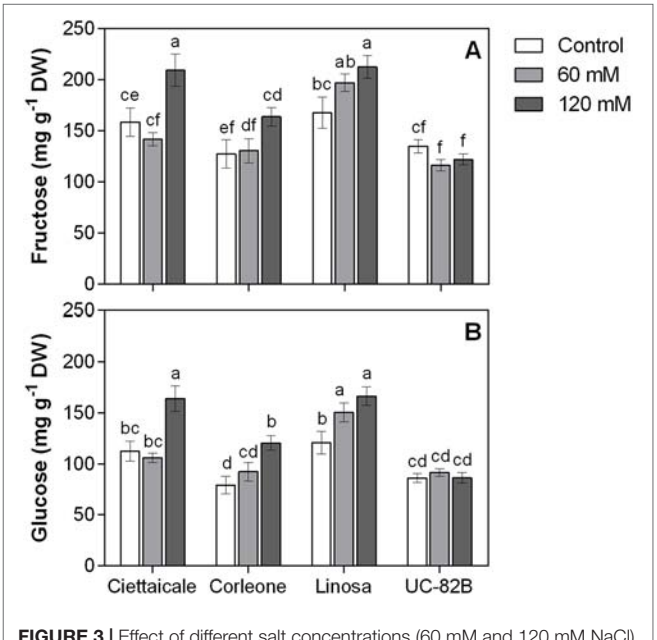

FIGURE 3 | Effect of different salt concentrations (60 mM and 120 mM NaCl) on fruit hexose contents of three tomato landraces (Ciettaicale, Corleone and Linosa) and a commercial variety (UC-82B). **(A)** Fructose content and **(B)** glucose content. Error bars represent the standard error of the mean (n = 6). Bars with same letters are not statistically different from one another according to Duncan's test (P < 0.05).

changes in glucose and fructose levels were observed in the commercial variety under the salt treatment.

Cation Content

Salt treatments differently affected the fruit contents of Na^+, K^+ and Ca^{2+}, depending on the genotype (**Table 2**). Ciettaicale showed the highest fold increase, reaching 3.4 fold more under 60 mM NaCl and 5.3 fold more under 120 mM NaCl compared to the control. Under 120 mM NaCl, the lowest Na^+ concentration was found in Linosa fruits (2.5 fold more than the control), while the highest Na^+ level was recorded in UC-82B (4 fold more than the control). Notably, Ciettaicale accumulated more K^+ under both salinity treatments than in control, and more K^+ was also found in Linosa under 60 mM NaCl. However, 120 mM salt induced a decrease of K^+ concentration in Corleone and UC-82B. Commercial variety fruits showed reduced Ca^{2+} content already at 60 mM NaCl, while Linosa had less Ca^{2+} only under 120 mM compared to respective controls. Overall, Linosa maintained higher K^+/Na^+ and Ca^{2+}/Na^+ ratios at 60 mM and 120 mM NaCl compared to the other genotypes, while Ciettaicale and UC-82B more markedly decreased Ca^{2+}/Na^+ ratio with increasing salinity level.

TABLE 2 | Effect of different salt concentrations (60 and 120 mM NaCl) on fruit cation contents of three tomato landraces (Ciettaicale, Corleone and Linosa) and a commercial variety (UC-82B). Data are means ± SE of six replicates.

Genotype	[NaCl]	Na^+ (g kg^{-1} DW)	K^+ (g kg^{-1} DW)	Ca^{2+} (g kg^{-1} DW)	K^+/Na^+	Ca^{2+}/Na^+
Ciettaicale	Control	0.62 ± 0.03 f	30.03 ± 0.94 f	1.70 ± 0.09 cd	48.96 ± 2.92 a	2.76 ± 0.20 a
	60 mM	2.12 ± 0.22 cd	36.47 ± 2.26 bd	1.61 ± 0.13 cd	17.38 ± 1.03 d	0.77 ± 0.06 e
	120 mM	3.31 ± 0.31 b	36.29 ± 1.22 bd	1.71 ± 0.09 bd	11.18 ± 1.27 ef	0.52 ± 0.04 eg
Corleone	Control	0.95 ± 0.09 f	39.21 ± 0.81 ab	1.57 ± 0.14 cd	41.94 ± 3.35 b	1.65 ± 0.04 e
	60 mM	1.99 ± 0.02 de	38.22 ± 0.89 ac	1.56 ± 0.12 cd	19.20 ± 0.30 d	0.79 ± 0.06 c
	120 mM	3.48 ± 0.11 b	34.38 ± 0.65 de	1.45 ± 0.04 d	9.90 ± 0.43 ef	0.42 ± 0.01 fg
Linosa	Control	0.89 ± 0.07 f	36.93 ± 1.39 bd	1.78 ± 0.13 ac	42.10 ± 3.68 b	2.02 ± 0.20 b
	60 mM	1.67 ± 0.06 e	41.29 ± 1.04 a	2.02 ± 0.03 a	24.79 ± 1.23 c	1.21 ± 0.05 d
	120 mM	2.26 ± 0.61 cd	35.38 ± 0.47 ce	1.49 ± 0.03 cd	15.73 ± 0.53 de	0.66 ± 0.02 ef
UC-82B	Control	1.00 ± 0.02 f	37.46 ± 0.70 bd	1.99 ± 0.10 b	37.50 ± 1.21 b	2.00 ± 0.12 b
	60 mM	2.50 ± 0.20 c	35.83 ± 1.06 bd	1.16 ± 0.06 e	14.61 ± 1.69 de	0.47 ± 0.03 fg
	120 mM	3.98 ± 0.14 a	32.00 ± 0.94 ef	1.10 ± 0.06 e	8.05 ± 0.04 f	0.28 ± 0.01 g

Means within a column followed by the same letter are not significantly different based on Duncan's test (P < 0.05).

Lycopene

Landraces treated with 60 mM NaCl did not show changes in lycopene content (**Figure 4**). However, Corleone fruits under 120 mM NaCl contained about 40% less lycopene compared to controls. Notably, UC-82B in control conditions showed the highest values of fruit lycopene among the tomato genotypes, but exhibited a progressive decrease in lycopene content already at 60 mM NaCl (-38%) and more marked at 120 mM NaCl (-55%) compared to the control.

Total Flavonoids, Total Phenols, and Total Antioxidant Activity

Salt conditions did not affect TFL in Ciettaicale and Linosa (**Figure 5A**). An increase (+ 21%) in TFL was recorded in Corleone fruits under 120 mM NaCl compared to controls. Conversely, salinity negatively affected TFL in UC-82B. No differences in TPHE in the landraces under 60 mM NaCl were recorded (**Figure 5B**). Under the same stress condition, UC-82B reduced TPHE by around 29% compared to its control. The highest salt concentration caused a decrease in fruit TPHE with a similar magnitude in Corleone, Linosa and UC-82B compared to respective controls (-26%, -28% and -33%, respectively). At harvesting point, fruits of Ciettaicale and Linosa maintained roughly control TAC values under 60 mM NaCl (**Figure 5C**). Corleone and UC-82B had decreased TAC under 60 mM (-17%

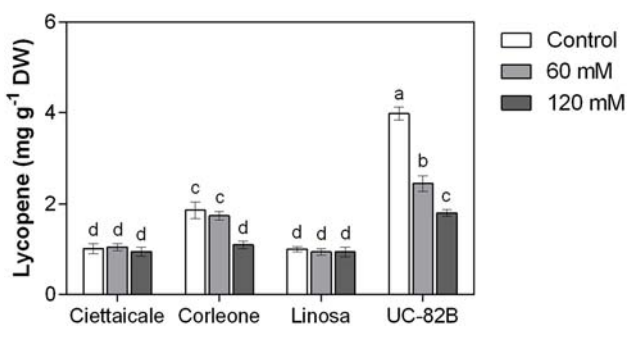

FIGURE 4 | Effect of different salt concentrations (60 mM and 120 mM NaCl) on fruit lycopene content of three tomato landraces (Ciettaicale, Corleone, and Linosa) and a commercial variety (UC-82B). Error bars represent the standard error of the mean (n = 6). Bars with same letters are not statistically different from one another according to Duncan's test (P < 0.05).

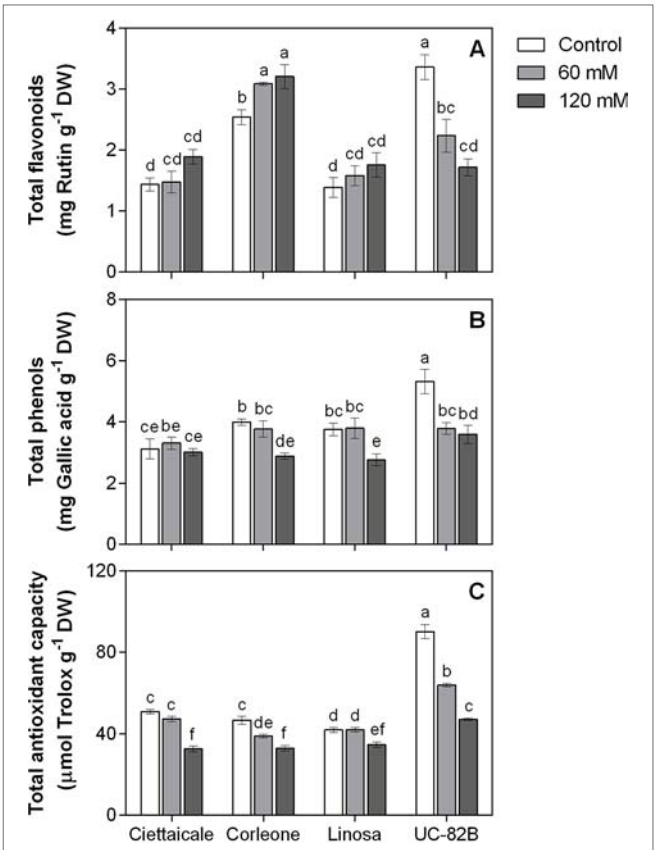

FIGURE 5 | Effect of different salt concentrations (60 mM and 120 mM NaCl) on fruit phenolic content and antioxidant capacity of three tomato landraces (Ciettaicale, Corleone and Linosa) and a commercial variety (UC-82B). **(A)** Total flavonoids, **(B)** total phenols and **(C)** total antioxidant capacity. Error bars represent the standard error of the mean (n = 6). Bars with same letters are not statistically different from one another according to Duncan's test (P < 0.05).

and -29% compared to their respective controls). However, a common reduction in TAC was observed in all genotypes under 120 mM compared to controls, i.e. from -17% in Linosa to -48% in UC-82B.

Metabolite Profiling

An untargeted UPLC-MS/MS analysis profiled the same sample sets as described above. We were able to identify 32 metabolites (**Table 3**) based on accurate mass measurements and MS/MS spectra from biological standards or publicly available data, namely literature and/or databases such as PubChem and Moto. ANOVA results are reported in **Supplementary Table 1** and graphically represented in **Figure 6**. Most metabolites detected were phenylpropanoids (10 hydroxycinnamic acids and 7 flavonoids) and glycoalkaloids (8). The remainder metabolites were assigned as phenylamides (4), amino acids (2) and vitamins (1).

Among the hydroxycinnamic acids (**Figure 6A**), coumaric acid (detected as two isomers) was the compound that presented the most intense mass signal (**Supplementary Table 1**). We could observe only a significant decrease of coumaric acid II under 120 mM NaCl in UC-82B. Interestingly, a similar trend was observed for coumaric acid-hexose accumulation in Linosa. Two ferulic acid isomers were detected: ferulic acid I significantly increased in all genotypes under salt stress compared to control conditions, whereas ferulic acid II did not significantly change in the case of Ciettaicale and Corleone upon salt stress. Linosa and UC-82B displayed a similar trend of reduction of the intensity of ferulic acid II mass signal upon 120 mM NaCl. The 1,3-O-dicaffeoylquinic acid I showed a remarkably low mass signal intensity in UC-82B fruits as compared to the landraces. At 120 mM NaCl, Corleone fruits showed a 3 fold increase of 1,3-O-dicaffeoylquinic acid I mass signal intensity. Also, 3,4,5-tricaffeoylquinic acid showed a significant increase in mass signal intensity in Corleone fruits upon 120 mM NaCl.

Apart from hydroxycinnamic acids, the phenylamides conjugated with caffeic acid (caffeoylputrescine I and II isomers) and ferulic acid (feruloylputrescine I and II isomers) were also identified (**Figure 6C** and **Supplementary Table 1**). Caffeoylputrescine I was significantly reduced in Ciettaicale and Linosa upon 120 mM NaCl, whereas the same salt concentration induced an increase of this compound in UC-82B fruits. Feruloylputrescine I was significantly reduced under 120 mM NaCl for the three landraces. Feruloylputrescine II increased in Corleone and UC-82B fruits upon salt stress, but in Linosa fruits the opposite trend was observed.

Regarding the flavonoids identified, rutin was the compound that exhibited the most intense mass signal (**Figure 6B** and **Supplementary Table 1**). Interestingly, UC-82B fruits showed the highest signal for all the flavonoids detected. Nevertheless, rutin and rutin-O-pentoside did not significantly change upon salt stress in all landraces, whereas UC-82B fruits showed a significant decrease of rutin upon 60 and 120 mM NaCl.

The list of identified compounds also includes 8 glycoalkaloids that presented a variable accumulation pattern in the studied conditions (**Figure 6D** and **Supplementary Table 1**). Esculeoside A was the glycoalkaloid that showed the highest mass signal intensity. Under control conditions, Ciettaicale accumulated significantly higher amounts of esculeoside A than all the other genotypes, while 60 mM NaCl promoted its accumulation in Corleone and UC-82B fruits. Under 120 mM NaCl all genotypes accumulated similar levels of esculeoside A. Tomatidine content was significantly higher in the commercial variety and Corleone under 120 mM NaCl, when compared to Ciettaicale and Linosa. Tomatidine+3hexoase showed the highest mass intensity signal in Corleone fruits upon 60 mM NaCl treatment. Tomatine, delta-tomatine and lycosperoside A were significantly high in the commercial variety in all conditions and in Corleone upon 120 mM NaCl. On the other hand, lycoperoside H was significantly higher in all landraces compared to UC-82B variety, and its content was not modulated by salt treatment.

We also identified the amino acids phenylalanine and tryptophan, and the vitamin derivative, pantothenic acid–hexose (**Supplementary Table 1**). Phenylalanine showed a marked increase in mass signal intensity upon 60 mM NaCl treatment in Ciettaicale fruits, whereas for the other genotypes the levels remained unchanged. The lowest mass signal intensity of tryptophan was observed in Corleone at 120 mM NaCl treatment, whereas the other genotypes showed the same mass signal intensity for this compound. Finally, the levels of pantothenic acid-hexose were high upon 60 mM NaCl in all genotypes except for Linosa, which showed unaltered levels of this metabolite in all treatments.

Moreover, the UPLC-MS/MS data were used in a further analysis known as SIMPER (**Supplementary Table 2**). The objective of this analysis was to find key compounds that allow for differentiation of one experimental condition from another when compared in a pair-wise analysis. The results display the contribution of each compound to the average overall dissimilarity of the two compared samples. A cut-off is imposed when $\Sigma\delta i\%$ reaches 70%. The metabolomic profiles of the experimental treatments differed qualitatively. In the first analyses, we identified the compounds that were consistently present in all the three salinity comparisons (control vs 60, control vs 120, and 60 vs 120), regardless of the genotypes. Among them, comparing conspecific different salinity treatments among all the genotypes, we identified the persistence of seven compounds: feruloylputrescine II (PA4), caffeoylputrescine I (PA1), naringenin (FL2), rutin-O-pentoside (FL7), naringenin chalcone (FL1), esculeoside A (GA8) and tomatidine+3hexoase (GA3).

The PCA (**Figures 7A**, **B**) performed on metabolomics data showed that the first two dimensions (PC1 and PC2) account for 47.5% of the total variance (total inertia). The first axis (PC1) explains 30.9% of the total variance and the second axis (PC2) 16.6%. The contribution of individual compounds to sample differentiation is displayed as a correlation circle (**Figure 7A**) where normalized vectors graphically represent the quantitative variables. The length and the direction of the vectors directly correlate with their significance within each treatment. A positive correlation between compounds is greater when the angle between their directions is smaller (close to 0 degree), whereas the correlation is negative if the angle reaches 180 degrees. No linear dependence exists if the angle is exactly 90 degrees. Overall, in our dataset we observed strong positive correlation

TABLE 3 | UPLC-MS/MS analysis of plant specialized metabolites responsive to salinity stress found in this work. For the identification level: A, literature; B, database, S, standard.

Peak #	Ret time (min)	Metabolite name	Metabolite class	Code	Molecular formula	$[M+H]_{ob}$	$[M+H]_{theo}$	MS/MS fragments	Identificaton level	Ref
1	3.33	Phenylalanine	Aminoacids	AA1	$C_9H_{11}NO_2$	166.0869	166.0863	120, 103	B	PubChem
2	4.52	Tryptophan		AA2	$C_{11}H_{12}N_2O_2$	205.0976	205.0971	188, 170, 159, 146, 118	B	PubChem
3	7.90	Naringenin chalcone	Flavonoids	FL1	$C_{15}H_{12}O_5$	273.0763	273.0757	153, 147, 119	S, A, B	Moço et al., 2006; MoTo
4	8.05	Naringenin		FL2	$C_{15}H_{12}O_5$	273.0764	273.0757	153, 147, 119	S, A, B	Moço et al., 2006; MoTo
5	6.43	Naringenin 7-O- glucoside		FL3	$C_{21}H_{22}O_{10}$	435.1304	435.1285	273, 153	A, B	Moço et al., 2006; MoTo
6	6.80	Naringenin chalcone 7-O- glucoside		FL4	$C_{21}H_{22}O_{10}$	435.1298	435.1285	273, 153	A, B	Moço et al., 2006; MoTo
7	5.81	Phloretin 3',5'-di-C-glucoside		FL5	$C_{27}H_{34}O_{15}$	599.1970	599.1990	497, 479, 461, 449, 431, 419, 413, 407, 395, 383, 377, 365, 353, 341, 329, 301, 259, 247, 235, 107	A	Slimestad et al., 2008; Beelders et al., 2014
8	5.69	Rutin		FL6	$C_{27}H_{30}O_{16}$	611.1616	611.1607	303	S, A, B	Moço et al., 2006; MoTo
9	5.39	Rutin-O-pentoside		FL7	$C_{32}H_{38}O_{20}$	743.2041	743.2029	465, 303	A, B	Moço et al., 2006; MoTo
10	6.52	Tomatidine	Glycoalkaloids	GA1	$C_{27}H_{45}NO_2$	416.3524	416.3523	398, 273, 255, 161	A, B	Caprioli et al., 2015; MoTo
11	6.50	Delta-tomatine		GA2	$C_{33}H_{55}NO_7$	578.4057	578.4051	417, 273, 255, 161	A, B	Cataldi et al., 2005; PubChem
12	5.95	Tomatidine+3hexoase		GA3	$C_{45}H_{75}NO_{18}$	918.5081	918.5057	432, 245, 162	A	Iijima et al., 2008
13	6.50	Tomatine		GA4	$C_{50}H_{83}NO_{21}$	1034.5538	1034.5530		A, B	Moço et al., 2006; MoTo
14	5.72	Lycoperoside H		GA5	$C_{50}H_{83}NO_{22}$	1050.5475	1050.5480	1032, 594, 432, 325, 273, 255, 163, 145, 127	A	Adato et al., 2009
15	6.45	Lycoperoside A		GA6	$C_{52}H_{85}NO_{23}$	1092.5577	1092.5585		A	Moço et al., 2006
16	5.93	Acetoxy-Hydroxytomatine		GA7	$C_{52}H_{85}NO_{24}$	1108.5517	1108.5534		A	Iijima et al., 2008
17	5.55	Esculeoside A		GA8	$C_{58}H_{95}NO_{29}$	1270.6011	1270.6063	1210, 1090, 1048, 1030, 652, 592	A	Iijima et al., 2008
18	4.33	Coumaric acid I	Hydroxycinnamic acids	HC1	$C_9H_8O_3$	165.0550	165.0546	147, 119, 91	A, B	Moço et al., 2006; MoTo
19	4.99	Coumaric acid II		HC2	$C_9H_8O_3$	165.0552	165.0546	147, 119, 91	A, B	Moço et al., 2006; MoTo
20	4.59	Ferulic acid I		HC3	$C_{10}H_{10}O_4$	195.0659	195.0651	177, 145, 117	A, B	Moço et al., 2006; MoTo
21	6.25	Ferulic acid II		HC4	$C_{10}H_{10}O_4$	195.0663	195.0651	177, 145, 117	A, B	Moço et al., 2006; MoTo
22	4.33	Coumaric acid-hexose		HC5	$C_{15}H_{18}O_8$	327.1082	327.1074	165, 147, 119	A, B	Moço et al., 2006; MoTo
23	4.28	Caffeic acid glucoside I		HC6	$C_{15}H_{18}O_9$	343.1035	343.1023	181, 163	A, B	Moço et al., 2006; MoTo
24	4.80	Caffeic acid glucoside II		HC7	$C_{15}H_{18}O_9$	343.1033	343.1023		A, B	Moço et al., 2006; MoTo
25	6.40	1,3-O-Dicaffeoylquinic acid I		HC8	$C_{25}H_{24}O_{12}$	517.1351	517.1341	163	B	PubChem
26	6.77	1,3-O-Dicaffeoylquinic acid II		HC9	$C_{25}H_{24}O_{12}$	517.1354	517.1341	323, 295, 273, 163	B	PubChem
27	7.15	3,4,5-Tricaffeoylquinic acid		HC10	$C_{34}H_{30}O_{15}$	679.1679	679.1657	499, 163		
28	3.24	Caffeoylputrescine I	Phenylamides	PA1	$C_{13}H_{18}N_2O_3$	251.1399	251.1390	234, 163, 145, 135,117	A	Gaquerel et al., 2010
29	3.96	Caffeoylputrescine II		PA2	$C_{13}H_{18}N_2O_3$	251.1398	251.1390	234, 163, 145, 135,117	A	Gaquerel et al., 2010
30	4.36	Feruloylputrescine I		PA3	$C_{14}H_{20}N_2O_3$	265.1539	265.1546	177, 145, 117	B	PubChem
31	4.60	Feruloylputrescine II		PA4	$C_{14}H_{20}N_2O_3$	265.1532	265.1546	177, 145, 117	B	PubChem
32	3.92	Pantothenic acid-hexose	Vitamins	VA1	$C_{15}H_{27}NO_{10}$	382.1722	382.1707	252, 220, 202, 184, 116, 90	A	Mintz-Oron et al., 2008

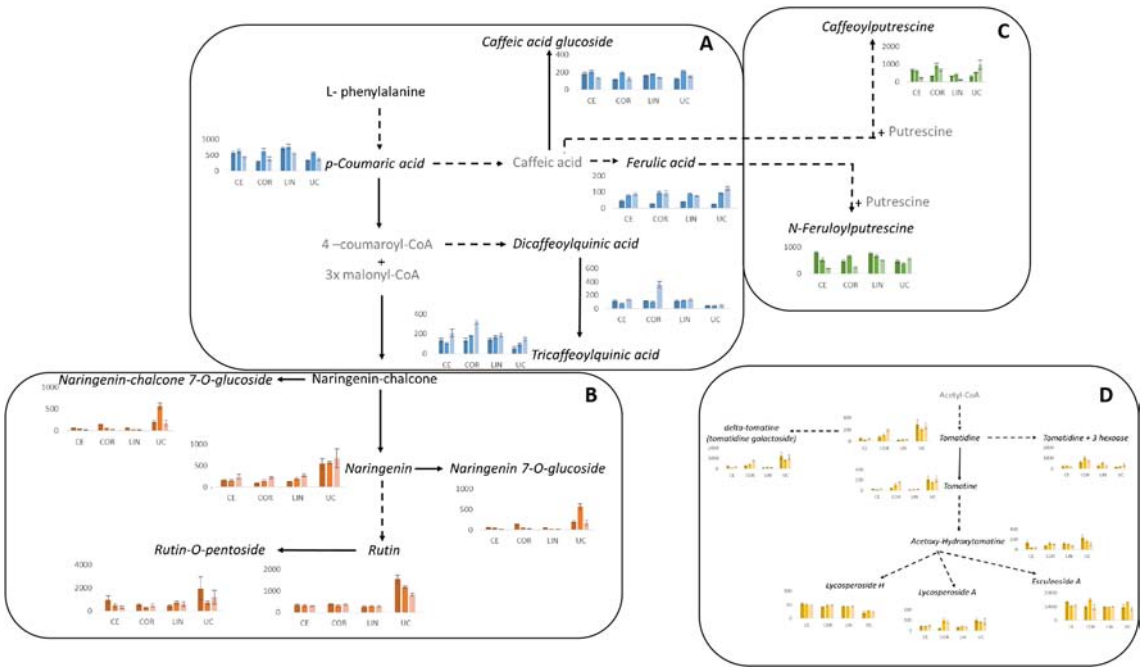

FIGURE 6 | Pathways overview of the plant specialized metabolites responsive to salinity stress. **(A)** Hydroxycinnamic acids, **(B)** flavonoids, **(C)** phenylamides and **(D)** glycoalkaloids. Metabolites that were identified in the present study are represented in black, whereas non identified metabolites are in grey. Metabolites that showed statistically significant changes (genotype x salinity treatment) are represented in italic and graphical representations are presented. The graphs represent the mean of relative ion intensity/dry weight of six biological replicates ± SE. Dark, middle and light colours (blue, orange, green and yellow) represent control, 60 and 120 mM NaCl, respectively; CE, Ciettaicale; LIN, Linosa; COR, Corleone; UC, commercial variety UC-82B.

between caffeic acid glucoside II (HC7) and coumaric acid II (HC2), caffeoylputrescine I (PA1) and caffeoylputrescine II (PA2), and 1,3-O-dicaffeoylquinic acid II (HC9), 3,4,5-tricaffeoylquinic acid (HC10) and phenylalanine (AA1). From the spatial distribution of the treatments (**Figure 7B**), we observed that Corleone distinguished itself the most. At 60 mM NaCl, Corleone showed a distinct metabolomic profile, while it shared more similarities under control conditions and at 120 mM NaCl. Ciettaicale and Linosa clustered separately but formed one cluster regardless of the salinity level. Furthermore, the commercial variety UC-82B under 120 mM NaCl showed a distinct profile compared to the control and mild salinity level. In the PCA analysis performed on the biochemical and biometric characteristics (**Figures 7C**, **D**), the landraces segregated into two main groups mainly according to the salinity level, whereas the standard variety clustered separately. In particular, untreated UC-82B was uniquely distinct, while the salt-treated ones grouped together forming one cluster.

DISCUSSION

Tomato landraces are a valuable resource for many traits of agronomic interest. This is mostly due to their resilience against abiotic stresses, which contributes to yield stability and adaptation to low input and/or adverse growth conditions. Landraces are also associated with distinctive organoleptic and nutritional quality traits and could exhibit peculiar and often contrasting metabolic profiles (Baldina et al., 2016; Gascuel et al., 2017; Patanè et al., 2017; Siracusa et al., 2018). High contents in functional compounds are frequent traits found in Mediterranean traditional tomato varieties (Pinela et al., 2012; Berni et al., 2018), but often no differences in sensory profile have been identified between commercial varieties and landraces (Ruiz et al., 2005; Sinesio et al., 2007; Casals et al., 2011). Moreover, the promoting effect on fruit quality metabolites is frequently ascribed to the concentration effect and not to the absolute accumulation (Zushi and Matsuzoe, 2015). However, different studies consistently concluded that the interaction between genotype and environment is the key component able to modulate the expression of specific metabolic patterns (Berni et al., 2019).

In this study we compared the effects of moderate and high concentrations of sodium chloride on yield and quality-related ions and metabolites in three tomato landraces (Ciettaicale, Corleone and Linosa) and a commercial variety (UC-82B). We showed that salinity promoted the anticipation of fruit ripening in all genotypes, but differentially caused fresh fruit yield losses. Notably, at 60 mM NaCl all landraces showed better performance in terms of yield FW compared to the commercial variety. The most interesting results were the absence of yield loss in Corleone and Linosa at the aforementioned salinity level and the observation that Linosa reduced only around 20% fresh fruit production under 120 mM NaCl. The capacity to

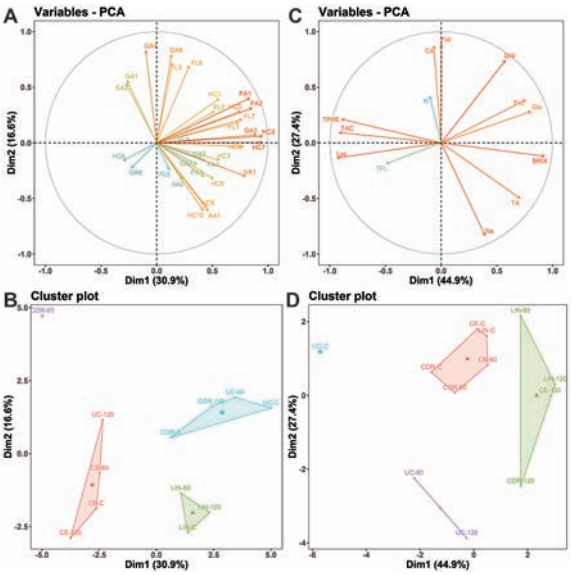

FIGURE 7 | Graphical representations of Principal Component Analysis (PCA) results. **(A** and **C)** Vector representation of the contribution of each metabolite identified by UPLC-MS/MS **(A)** and each laboratory determination/assay **(C)** to the distinction of experimental treatments. The coordinates of each variable are the correlation coefficients with the two-first principal components. Between vectors and between a vector and an axis, there is positive correlation if the angle is less than 90 degrees whereas the correlation is negative if the angle reaches 180 degrees. There is no linear dependence if the angle is 90 degrees. **(B** and **D)** Cluster map of PCA results of UPLC-MS/MS data **(B)** and laboratory determinations/assays **(D)** obtained from experimental treatments. For UPLC-MS/MS metabolite codes see **Table 3**; FW, yield fresh weight; DW, yield dry weight; Na, sodium; K, potassium; Ca, calcium; Fru, fructose; Glu, glucose; BRIX, total soluble solids; TA, titratable acidity; LYC, lycopene; TPHE, total phenols; TFL, total flavonoids; TAC, total antioxidant capacity; CE, Ciettaicale; COR, Corleone; LIN, Linosa; UC, commercial variety UC-82B; C, control condition; 60 and 120 mM NaCl used as experimental treatments, respectively.

maintain an adequate yield has also been found in a Kenyan tomato landrace grown in site soil polluted by high salty water irrigation (Agong et al., 1997).

Yield and functional quality traits can be influenced by salinity mainly due to sodium competition for other cations, such as K^+ and Ca^{2+} (Adams, 1991; Petersen et al., 1998, Pompeiano et al., 2017). Among the genotypes, Linosa maintained higher K^+/Na^+ and Ca^{2+}/Na^+ ratios along the salt gradient. Calcium participates both in the alleviation of sodium toxicity and in the fruit size development (Plieth, 2005; Manaa et al., 2013). This observation could support the ability of Linosa to maintain an adequate yield under salt stress. Additionally, fresh tomato fruits with high Ca^{2+} content represent a natural mineral supply indispensable in human dietary (Soetan et al., 2010). On the contrary, in the commercial variety the Ca^{2+}/Na^+ ratio was more affected by the salinity gradient, leading to a more marked reduction of fruit size and weight. Several studies reported that calcium deficiency affects tomato fruit development (Park et al., 2005), often resulting with the appearance of the blossom-end rot (Taylor and Locascio, 2004). However, in the present study, as well as in the winter greenhouse experiment conducted by Zushi and Matsuzoe (2009), the fruits of all genotypes were not affected by this marketable injury.

The increases in soluble solids and titratable acidity are common responses of tomato fruits under salt stress (Balibrea et al., 2003; Zushi and Matsuzoe, 2015). High values of these parameters have been found in Spanish tomato landraces under salt stress (Massaretto et al., 2018). Soluble solids content (°Brix) mainly estimated the sugar amounts in tomato fruit pulp, but also organic acids, amino acids, soluble pectins, phenolic compounds and minerals (Beckles, 2012). Nevertheless, the sugar/acid ratio generally increases during summer and decreases during winter. Primary metabolism is more affected when light and temperature changes occur during early fruit development than when environmental conditions mutate during the ripening phase (Gautier et al., 2008). Under 120 mM NaCl all genotypes showed higher °Brix content compared to the respective controls. High °Brix improves the taste of tomato fruits and is a desirable trait for the processing of tomato products (De Pascale et al., 2001). Also 120 mM NaCl promoted the accumulation of fructose and glucose in the landraces, but not in the commercial variety. Zushi and Matsuzoe (2015) reported that the tomato cultivar Mini Carol accumulated more fruit glucose and fructose under 50 mM NaCl, while the tomato cultivar House Momotaro increased total soluble sugars only under 100 mM NaCl. The authors concluded that the salt effect on sugar levels depends essentially on the genotype. Interestingly, sucrose was detectable only in traces in the fruits of any of the studied tomato accessions, suggesting that salt stress promotes invertase activity and consequently the release of hexoses during tomato ripening (Balibrea et al., 2003). Also, low solar radiation conditions, such as the ones experienced in our study, could affect sugar concentration in sink tissues due to a limitation in carbon fixation/transport in/from source leaves (Hu et al., 2006).

The content of lycopene, the main carotenoid that confers the red pigmentation to the tomato fruit, is a genotype-dependent trait. Lycopene metabolism can be modulated by water deficit (Atkinson et al., 2011; Coyago-Cruz et al., 2017), low light radiation as well as low temperature (Dumas et al., 2003; Jarquín-Enríquez et al., 2013). Even though several tomato landraces have been identified with constitutive high carotenoid content (Pinela et al., 2012; Figàs et al., 2015; Zushi and Matsuzoe, 2015), the studied landraces showed lower fruit lycopene content under control conditions compared to the commercial variety. Upon 60 mM NaCl treatment, the landraces roughly maintained control values of lycopene, while the commercial variety had a significantly reduced content. Ciettaicale and Linosa displayed no changes in lycopene amount under 120 mM NaCl. The decrease in K^+ content, as we observed in Corleone and UC-82B under 120 mM NaCl, could affect lycopene production. Indeed, K^+ plays a role as cofactor of several enzymes involved in the biosynthesis of isopentenyl diphosphate, the first precursor of carotenoids in the mevalonate pathway (Trudel and Ozbun, 1971). Li and Yuan (2013) and Coyago-Cruz et al. (2019) suggested that the fruit ripening-related accumulation of lycopene can also be influenced due to limited amount of sucrose. Overall, our results were in agreement with those found by Petersen et al. (1998), which reported that salinity did not promote an increase of lycopene levels per dry weight in tomato

fruits. Nevertheless, moderate salinity stress has previously been used to improve lycopene content in tomato (De Pascale et al., 2001; Kubota et al., 2012).

Phenolic acids and flavonoids represent a complex class of compounds with specific biological activity. Their profile in tomato fruits have been widely investigated and often used as taxonomical markers to discriminate tomato varieties (Minoggio et al., 2003; Vallverdú-Queralt et al., 2011). Flavonoids content has been positively correlated with environmental radiation, resulting in seasonal changes (Incerti et al., 2009). For example, one light-dependent effect is the up-regulation of the gene expression of chalcone synthase, the first committed enzyme in flavonoid biosynthesis (Feinbaum and Ausubel, 1988). Flavonoids have been found highly concentrated in epidermal and placental tissues of tomato fruits, acting as chemical defences against pathogens and UV radiation (Slimestad et al., 2008). Flavonoids, also known as vitamin P, have recently been targeted as important functional compounds with benefits for human health (Perez-Vizcaino and Fraga, 2018). New emerging studies showed that flavonoids may interfere with the signalling of several kinases that in turn modulate cellular functions by altering the phosphorylation state of target molecules and by modulating gene expression (Williams et al., 2004; Van Der Rest et al., 2006).

Most of the flavonoids found in tomato are mainly present as O-glycosides, but also as non-conjugated forms (aglycones). The main flavonoid reported in tomato fruits is rutin (quercetin 3-rutinoside) (Le Gall et al., 2003). However, naringenin chalcone and its more stable cyclized form naringenin, as well as naringenin-7-O-glucoside, have been also detected as additional frequent flavonoids in fresh tomato skins (Moço et al., 2006) and have been investigated *in vitro* as potential anti-allergic compounds (Yamamoto et al., 2004). In addition, the presence of the aglycones kaempferol, quercetin and naringenin is also noticed, but with a discrepancy in the amount among experimental studies (Stewart et al., 2000). Overall, the content of flavonoids in terms of quantity and quality greatly varies depending on genotype, growth conditions, stage of ripeness and tissue, as well as on the detection method (Slimestad and Verheul, 2005; Slimestad et al., 2008). In this study, most of the abovementioned flavonoids were identified with the exception of the aglycones, possibly due to the absence of acid hydrolysis of the analysed sample extracts. The commercial variety clearly differed from the landraces for rutin and rutin-O-pentoside contents both under control and salt. Overall, the TFL content, as well as rutin content, in the commercial variety decreased according to the salt gradient. In Ciettaicale and Linosa, TFL content was not affected by salinity, while 120 mM NaCl induced an accumulation of TFL in Corleone fruits.

Tomato fruits also contain hydroxycinnamic acids and respective quinic acid ester derivatives. During ripening, an increase of these esters generally occurs, especially in the pulp (Whitaker and Stommel, 2003). Caffeic acid and its quinic acid ester (chlorogenic acid), as well as ferulic acid and coumaric acid, are detected in quite high levels in tomato fruits. In particular, chlorogenic acid and its derivatives reduce the incidence of fungal disease in tomato (Ruelas et al., 2006; Wojciechowska et al., 2014). Moreover, hydroxycinnamic acids can modulate auxin and ethylene metabolism, which are both involved in fruit size development and ripening (Fleuriet and Macheix, 1981). The highest salt stress condition promoted di- and tricaffeoylquinic acid accumulation in Corleone compared to the commercial variety. Coumaric acid (I+II) showed a similar profile of accumulation in all genotypes under 60 mM NaCl, while a decreasing trend was observed in Linosa and in the commercial variety under 120 mM NaCl compared to the respective controls. Cinnamate 3-hydroxylase, a key limiting enzyme for hydroxycinnamic acid biosynthesis (Ferrer et al., 2008), was shown to be up-regulated at transcriptional and protein levels by salinity (Martinez et al., 2016). Our data suggest that cinnamate 3-hydroxylase may be differentially regulated in the landraces compared to the commercial variety, but further investigations need to be conducted to validate this hypothesis.

The observed decrease in TPHE in salt-treated plants may be the result of the reallocation of phenolic compounds to lignin polymers as a protective mechanism (Humphreys and Chapple, 2002). However, decreased values of phenolic contents are often observed under low temperature (Rivero et al., 2001; Gautier et al., 2008).

Tomato, a fully-fledged member of the *Solanaceae*, produces steroidal glycoalkaloids, which belong to the terpenoid family. Tomatine represents the main glycoalkaloid in tomato fruit. From a pharmaceutical point of view, dietary tomatine leads to a reduction of plasma cholesterol content thanks to the capacity of this terpenoid to form insoluble complexes with cholesterol, which are poorly absorbed from the intestinal tract (Friedman, 2013). During fruit ripening, tomatine is normally converted in esculeoside, while concomitantly lycopene contents increases (Katsumata et al., 2011). However, as for many other metabolites, this relationship is deeply affected by agronomic practices, genotype and environmental conditions (Koh et al., 2013). The pattern of accumulation of glycoalkaloids was variable in the landraces and in the commercial variety upon salt stress. The levels of esculeoside A were almost similar in all genotypes. Although salinity was shown to induce accumulation of these compounds in tomato leaves (Han et al., 2016), this trend was only confirmed for Corleone.

The class of polyamines, which includes putrescine, spermidine and spermine, is required for tomato fruit development (Cohen et al., 1982). These metabolites can donate amino groups to different other plant compounds, such as hydroxycinnamic acids. Hydroxycinnamic amides seem to play a key role in the reproductive tissues since their catabolism provides nitrogenous and phenolic carbon skeletons for reproductive development (Balint et al., 1987). Caffeoylputrescine and feruloylputrescine isomer contents showed different trends in the genotypes along the salt gradient. Since polyamines compete with ethylene for the biosynthetic precursor S-adenosylmethionine, high content of hydroxycinnamic amides may delay the fruit softening-inhibiting production of ethylene (Liu et al., 2006).

Overall, despite the salinity-induced rearrangement in the stoichiometry of the antioxidant metabolites identified by UPLC-MS/MS, UC-82B and Corleone progressively decreased fruit TAC with increasing salt concentrations, while in Ciettaicale and Linosa TAC only declined under 120 mM NaCl.

CONCLUSION

The combination of moderate/high salt concentrations with low light irradiance differently affected the yield and the metabolism of the studied tomato genotypes. Despite these non-optimal environmental conditions for tomato cultivation, the Italian landraces showed a different behaviour as compared to the commercial variety UC-82B under moderate salinity stress, showing a tolerable compromise between yield and quality attributes. Salt stress markedly reduced yield and functional metabolite contents in the commercial variety. Among the landraces investigated, Linosa showed better performance in terms of yield/quality parameters under 60 mM NaCl. However, off-season high salinity stress (120 mM NaCl) significantly reduced the antioxidant activity both in UC-82B and in the landraces. In conclusion, these data point to the use of tomato landrace germplasm as a suitable strategy to counteract detrimental environmental factors, such as salinity and off-season cropping, and also as resource of metabolic biomarkers which can be used to improve commercial varieties.

DATA AVAILABILITY

All datasets for this study are included in the manuscript and the **Supplementary files**.

AUTHOR CONTRIBUTIONS

TM, RBF, LM, LI, and FS conceived and designed the experiments with the help of all authors. TM, RBF, LM, GC, AS, and LP performed the experiments. TM, RBF, AnP, AL, and LI analyzed the data. TM, RBF, and AnP drafted the manuscript. All authors led the critical review of the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01078/full#supplementary-material

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REVIEW
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Effect of Preharvest Abiotic Stresses on the Accumulation of Bioactive Compounds in Horticultural Produce

Stefania Toscano[1], Alice Trivellini[2], Giacomo Cocetta[3], Roberta Bulgari[3], Alessandra Francini[2], Daniela Romano[1] and Antonio Ferrante[3]*

[1] Department of Agriculture, Food and Environment, Università degli Studi di Catania, Catania, Italy, [2] Institute of Life Sciences, Scuola Superiore Sant'Anna Pisa, Pisa, Italy, [3] Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy, Università degli Studi di Milano, Milan, Italy

The quality of horticultural products is the result of the interaction of different factors, including grower's crop management ability, genotype, and environment. Sub-optimal environmental conditions during plant growth can induce abiotic stresses and reduce the crop performance with yield reduction and quality losses. However, abiotic stresses can induce several physiological, biochemical, and molecular responses in plants, aiming to cope with the stressful conditions. It is well known that these abiotic stresses are also elicitors of the biosynthesis of many metabolites in plants, including a wide range of bioactive compounds, which firstly serve as functional molecules for crop adaptation, but they have also a great interest for their beneficial effects on human health. Nowadays, the consumer is oriented to low-energy foods with low fat content, but at the same time, growing attention is paid to the presence of bioactive molecules, which are recognized as health-related compounds and concur to the nutraceutical value of plant-derived foods. In this context, fruit and vegetables play an important role as sources of bioactive ingredients in the diet. At the cultivation level, the understanding of crop responses to abiotic stresses and how they act in the biosynthesis/accumulation of these bioactive compounds is crucial. In fact, controlled abiotic stresses can be used as tools for improving the nutraceutical value of fruit and vegetables. This review focuses on the quality of vegetables and fruits as affected by preharvest abiotic stressors, with particular attention to the effect on the nutraceutical aspects.

Keywords: cold, water stress, light stress, salinity, UV, wounding

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Edited by:
Cai-Zhong Jiang,
Crops Pathology and Genetics Research Unit, USDA-ARS, United States

Reviewed by:
Daqi Fu,
China Agricultural University (CAU), China
David Obenland,
San Joaquin Valley Agricultural Sciences Center, United States

****Correspondence:***
Giacomo Cocetta
giacomo.cocetta@unimi.it

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BRIEF INTRODUCTION ON ABIOTIC STRESS AND CROP RESPONSES

Bioactive phytochemical compounds represent non-nutrient plant molecules such as pigments or secondary metabolites (Ismail et al., 2010), influencing the functional and nutritional values of commonly consumed fruit and vegetables commodities due to their established role related to human health and well-being as health-promoting compounds (Liu, 2013).

Abiotic stresses are potent elicitors of bioactive compound biosynthesis, and they should be wisely used for growing crops that are naturally enriched and with high nutraceutical value.

Plant Metabolism

The main biosynthetic pathway that leads to bioactive molecules accumulation in plants is the phenylpropanoids. These chemical compounds are accumulated in plants with defense or signaling functions. The phenylpropanoids are especially accumulated under stressful conditions, and the different chemical compounds can be associated with specific stresses (Dixon and Paiva, 1995). The shikimate pathway is considered the core of phenylpropanoids biosynthesis. These molecules are classified as secondary metabolites, because they were considered as molecules that do not contribute to the vital processes of plants (Vogt, 2010). The importance of these compounds has been completely revised in plant biology because many molecules of secondary metabolism have a crucial function in plant growth and development such as lignin biosynthesis and its role in plant defense, water, and nutrient transportation. Nevertheless, secondary metabolism is often wrongly considered less important than the primary metabolism. The phenylpropanoids can be absent in different tissues during development, and for this reason, they were considered not essential for plant development. In particular environments, the biosynthesis of phenylpropanoids and related compounds is essential for plant survival. The concentration of phenylpropanoid compounds can vary during plant growth and adaptation to sub-optimal growing conditions. Plants are not able to escape the adverse environmental conditions; therefore, their survival is related to the adaptation ability to different stresses. The plasticity of the plants is associated with the accumulation of bioactive molecules that increase the tolerance to stresses by modulating the main physiological and biochemical processes (Oh et al., 2009a and Oh et al., 2009b). In agricultural systems, the discovery of the traits associated with the crop adaptation strategies can be exploited for reducing yield and quality losses. The tight association of the specific tolerant traits will be used in breeding programs for enhancing the crop performance under abiotic stressful conditions. The yield losses of crops under abiotic stresses have been estimated to be 69% in average (Boyer, 1982; Mariani and Ferrante, 2017).

Primary metabolism of plants involves photosynthesis and related processes, respiration, sugars (starch and sucrose), and amino acids metabolism (**Figure 1**). Abiotic stresses usually reduce the plant growth by slowing down photosynthesis. Crops invest their energy in defense mechanisms by the activation of specific biosynthetic pathways (Caretto et al., 2015).

Photosynthesis provides the carbohydrates that can supply carbon skeletons for nitrogen assimilation, which leads to amino acids biosynthesis and related bioactive compounds. The amino acids can be used in plants as monomeric molecules for the biosynthesis of proteins or other functional molecules such as nucleic acids, glucosinolates (GSLs), plant hormones, and other nitrogen-containing compounds. Primary metabolism is linked to the secondary metabolism, since several substrates can serve to activate the phenylpropanoids pathway, enabling the biosynthesis of secondary metabolites, including polyphenols. Phenolic compound biosynthesis has erythrose 4-phosphate and phosphoenolpyruvate as first substrates (**Figure 1**). These two metabolites are intermediates of the pentose phosphate pathway and the glycolysis (Vogt, 2010).

The phenylpropanoids biosynthetic pathway is the source of secondary metabolites in plants. These compounds are involved in defense mechanisms and can exert antioxidant functions. Moreover, fruits and vegetables are an important source of these molecules, which in the diet provide beneficial effects on human health.

THE EFFECT OF SALINITY ON THE NUTRACEUTICAL PROPERTIES OF FRUITS AND VEGETABLES

High salt concentration in soil and water is a stressful condition that severely affects crop quality and yield. This phenomenon

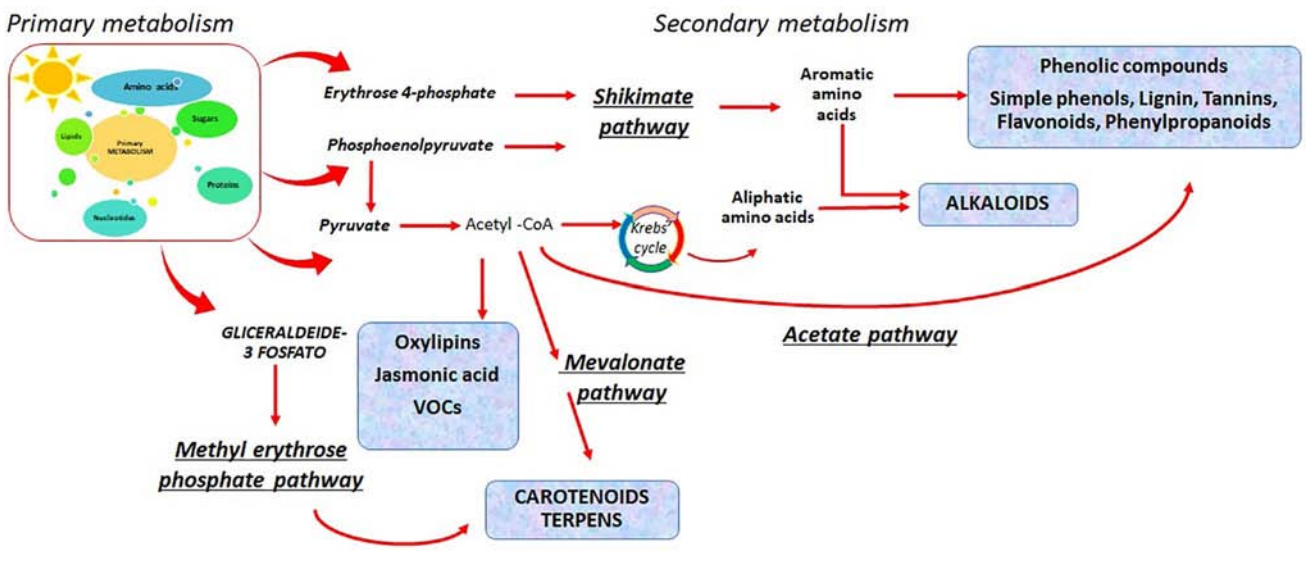

FIGURE 1 | Primary and secondary metabolisms in plants and the different pathways that lead to the bioactive compounds biosynthesis.

can occur in arid or semi-arid regions as well as in coastal areas, in which the proximity to the sea strongly affects soil and water quality. Moreover, in case of soilless cultivations (such as hydroponically grown leafy greens), the bad quality of the water or the sub-optimal management of the nutrient solutions used can cause a stressful condition for crops. Because of salinity, plants must face a reduction in the water potential of soil and nutritional imbalance, and this turns finally into a decrement in yield and a loss in quality. Plants have developed several strategies to counteract the increment in salt concentration, including the accumulation of osmotically active metabolites, antioxidant compounds, and specific secondary metabolites (Parvaiz and Satyawati, 2008). These strategies aim to reduce the oxidative stress, which could derive from the altered ion and water flux and help to re-establish the water balance within the cell. As a side effect, the changes in the metabolites accumulated in the plant's edible parts can positively affect the nutraceutical value of the crops, as several stress-related plant metabolites are also appreciated as health-related compounds in human nutrition. For this reason, salt stress, among others, has been recently suggested as a potential eustressor to be used for enhancing the quality of vegetables (Rouphael et al., 2018).

Salinity Stress and Bioactive Compound Accumulation in Vegetable Crops

Among the leafy vegetables, lettuce is one of the most relevant in terms of economic value and cultivated area; it is also highly appreciated by consumers as fresh-cut commodity. Lettuce is moderately sensitive to salinity; for this reason, the effects of salinity on the nutritional and nutraceutical properties of this crop are widely studied. Salinity affects the mineral composition of leaves and the biosynthesis of health-related phenolic compounds as shown in green and red baby lettuces (Neocleous et al., 2014). An increase in phenols is a common response to salinity in lettuce; in fact, it was also observed in two differently pigmented lettuces subjected to high salinity or CO_2 levels. In a red-pigmented cultivar, salinity (200 mM) maintained higher phenolic amounts and antioxidant activity. Moreover, in combination with elevated CO_2 (700 ppm), salinity caused a lower reduction in yield and a higher accumulation of luteolin than does salinity alone (Sgherri et al., 2017). The effect of salinity can vary depending on the species or varieties studies as well as on the salt concentration or the duration of the stress application. For example, the phenolic content of the romaine lettuce (*Lactuca sativa* L. var. *longifolia*) declined as a response to short-term salt irrigation. In the same study, with long-term irrigation with 5 mM of NaCl-enriched water, the total carotenoid (particularly the lutein and β-carotene) content increased without color change (Kim et al., 2008).

Rocket is another leafy vegetable particularly appreciated as fresh-cut salad, rich in phytochemicals such as phenols and GSLs. In wild rocket (*Diplotaxis tenuifolia* L.) plants subjected to a moderate salt stress (200 mM of NaCl for 48 h), the levels of GSLs were reduced, and a key role in the response to salinity has been hypothesized for the gene encoding for the enzyme thio-methyl transferase (Cocetta et al., 2018). Further analyses should be performed to estimate the effect of salinity on the accumulation of GSL-derived products such as isothiocyanates, which have also a proven health-promoting action.

The nutritional quality of broccoli florets was improved under moderate saline stress (40 or 80 mM of NaCl). Salinity caused an increment in phenolic compounds and GSLs, while the mineral composition remained within the range of recommended values (Lopez-Berenguer et al., 2009).

The role of certain GSLs in salt stress response in broccoli has been recently shown by Di Gioia et al. (2018). The authors reported that the use of saline water improved broccoli dry matter and soluble solid content, while it had no impact on total GSL concentration. However, salinity induced an increase of indolic GSLs (glucobrassicin and neoglucobrassicin) potentially affecting nutritional properties and flavor (**Figure 2**).

The quality and shelf-life of fresh-cut cauliflowers grown under high salinity was improved; in fact, preharvest salt stress (20 mM of NaCl) increased the concentration of GSLs in a genotype-dependent way, improving also the concentration of total polyphenols and ascorbic acid, hence enhancing the antioxidant activity of florets (Giuffrida et al., 2018).

Colla et al. (2013) studied the effect of increasing salinity (0 and 30 mM of NaCl) applied on artichoke and cultivated cardoon grown in a floating system. Salinity decreased the leaf dry biomass, leaf number, and macroelement and microelement accumulation. On the other hand, antioxidant activity, total polyphenols, chlorogenic acid, cynarine, and luteolin levels were improved in response to salinity.

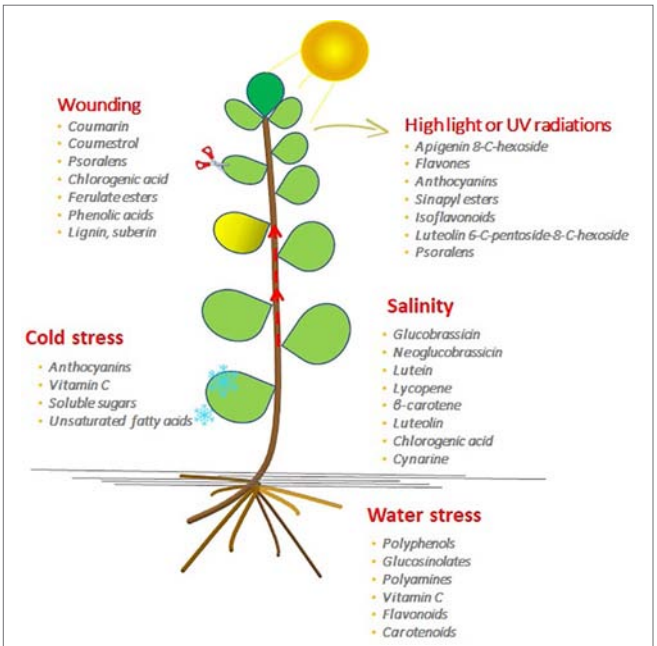

FIGURE 2 | The bioactive molecule accumulation in plants induced by the different abiotic stresses.

Salinity Stress and Bioactive Compound Accumulation in Fruits

Salinity has been reported to affect the nutritional and nutraceutical properties of fruits by inducing metabolic changes in response to the stressful condition. The effect of salinity has been largely studied in tomato fruit and in other important fruit crops (Rouphael et al., 2018). For example, tomato plants grown in a greenhouse were treated with a nutrient solution with electrical conductivities (ECs) of 2, 4, or 6 dS m^{-1}, and yield per plant and fruit size were reduced. The antioxidant capacity [oxygen radical absorbance capacity (ORAC)], β-carotene, lycopene, and vitamin C concentrations increased with EC, while lutein was only partially affected by salinity. The same study also showed that salinity does not influence the expression of several key genes involved in antioxidant production in ripe fruit (Ehret et al., 2013). Similarly, Fanasca et al. (2007) showed that the quality of tomato fruits was improved by high EC (2.5 or 8 dS m^{-1} in the root zone). A high EC increased the dry matter content, total soluble solids content, titratable acidity, and glucose, fructose and citric acid contents. Significantly higher lycopene and β-carotene contents were also observed with a high EC.

Salinity stress led in some cases to a two- to three-fold increase in the lycopene content in different tomato genotypes. At the same time, salinity differentially affected the accumulation of total anthocyanins in two anthocyanin-rich tomato genotypes. In fact, anthocyanin content was enhanced in fruits of the genotype 'Sun Black' (two-fold increase) and reduced in fruits of 'Anthocyanin fruit type' (10-fold decrease) (Borghesi et al., 2011).

Pepper is a good source of nutraceuticals such as ascorbic acid, carotenoids, and phenolics. The effect of salinity depended on the maturity state of peppers, showing a more relevant effect on red fruits. Salinity had no effect on antioxidant activity of the hydrophilic fraction, β-carotene, or sugars, and it decreased ascorbic acid and total phenolic compounds and increased lipophilic antioxidant activity and lycopene (Navarro et al., 2006). A positive effect of salinity on pepper fruit quality was also observed by Giuffrida et al. (2014); the authors showed that total phenol content was slightly increased (+10%) by NaCl salinity and that the concentration of carotenoids was enhanced by 40% to control.

Strawberry fruits obtained from plants subjected to high salinity showed, in some cases, lower total acidity and higher values for soluble solids content; fruit taste was, therefore, enhanced. Moreover, salinity improved the accumulation of antioxidant compounds (Cardeñosa et al., 2015).

Field salinity stress induced an increase of carotenoids and sugars in melon fruits, but at the same time, it has a negative effect in quality and yield, affecting several physiological stress indexes such as malondialdehyde, oxygen peroxide, chlorophylls, and proline (Akrami and Arzani, 2018).

WATER STRESS AND PRODUCE QUALITY

Climate change influences changes in rainfall patterns, causing increasing severity of droughts and floods (Sheffield and Wood, 2008). One of the consequences of climate changing is an increase of extreme events, such as drought and waterlogging events. Water stress is one of the most common and dramatic environmental stress in many cultivated areas. It influences plant growth and reduces crop productivity (González-Chavira et al., 2018). The effect of drought stress on plant growth and crop yield depends on the genotype sensitivity, the phenological stage, the organs (leaves and fruits) of the plants, and the intensity and duration of the stress (Marjanović et al., 2012; Mirás-Avalos and Intrigliolo, 2017). Despite the negative effects of water deficit, some studies also reported positive effects on produce quality, such as activating the biosynthesis of secondary metabolites (Sangwan et al., 2001). It has been demonstrated that drought stress can stimulate the metabolism of phytochemicals with health-promoting properties (González-Chavira et al., 2018). Plants stressed by water shortage could represent potential sources of antioxidants such as polyphenols. Consequently, it can be hypothesized that an increase in polyphenols could be obtained with the use of stress-tolerant species (de Abreu and Mazzafera, 2005). The ability of plants to tolerate water scarcity is determined by multiple biochemical mechanisms that improve water retention or uptake, chloroplast functionality, and cell ion homeostasis. One of the main adaptation strategies is the biosynthesis of osmotically active molecules that control the flow of ions and water, eliminating oxygen radicals that can function as chaperones (Munns, 2002; Kapoor et al., 2015). Beyond the type of vegetables (leaf or fruit), the increase of bioactive compounds is connected to the reduction of weight increase of the different organs, for which the percentage incidence of phytochemicals increases, but not the content total of bioactive compounds. In any case, the differences found, beyond the level of water stress imposed, are a function of the characteristics of the organs used and of the various vegetable species.

Bioactive Compounds in Leafy Vegetable Quality

Among the leafy vegetables, particular attention has been paid to Brassicaceae vegetables for the relevance of their health products. The cultivation under stress conditions can stimulate the biosynthesis of bioactive compounds (Oh et al., 2009b), often associated with antioxidant systems linked to plant defense mechanisms (Mittler, 2002).

In broccoli, the most effective abiotic stress that can affect the content of bioactive phytochemicals is not yet clear. This could be related to the fact that stress affects specific phenolic compounds (i.e., phenolic acids), although the magnitude of the effect depends on the cultivar and the plant organ considered (Domínguez-Perles et al., 2010). Thus, Fortier et al. (2010) observed a higher content of phenolic compounds in broccoli subjected to water and nitrogen stress (Khan et al., 2011). The increase in the phenolic content could be an indirect effect of drought stress, due to the higher temperatures of plants under reduced evapotranspiration (Krumbein et al., 2007). An increase of phenolic content was observed in rapeseed (*Brassica napus* L.) during flowering and pod fill under drought stress (Jeffery et al., 2003). In broccoli, besides phenolic compounds, water deficit also induced anthocyanin accumulation (Chalker-Scott, 1999; Rodríguez-Hernández et al., 2012).

In a study focused on the possible role of six different *Brassica* vegetables (*Brassica oleracea* L. and *Brassica rapa* L.) as a natural source of antioxidant compounds, an increase in total flavonoids and L-ascorbic acid was found few days before harvest when plants were subjected to high water deficit (Aires et al., 2011). An increase in vitamin C content with moderate water deficits was found in broccoli (Toivonen et al., 1994) and leeks (*Allium porrum* L.) (Ahmed et al., 2014), which led to the hypothesis that a high content of vitamin C acts as a protective strategy against drought damage (Bozhenko, 1965).

On the contrary, also, the excess of water can induce the activation of the biosynthesis of bioactive molecules. In onion (*Allium cepa* L.), waterlogging reduced bulb quality traits such as phenol, pyruvic acid, flavonoids, antioxidant activity, and total soluble solids content (Ghodke et al., 2018).

In broccoli during drought conditions, higher kaempferol levels in well-watered plants were found, while stressed plants showed a decreased biomass production. The higher kaempferol content in drought and water-logged plants suggests that plants produced kaempferol as a biochemical adaptation towards water stress (**Figure 2**). Decreased plant growth might be related to higher kaempferol content, as plants need to invest photosynthates as resources of carbon required for the kaempferol biosynthesis; defensive flavonoids are expensive for the plants, and their accumulation causes a reduction of the plant's growth (Gayler et al., 2004). Robbins et al. (2005) observed that water stress decreased GSL content relative to unstressed broccoli (Pék et al., 2012). In broccoli, a higher level of water supply determined an increase of GSL level, making plants more tolerant of pest and insects (Khan et al., 2011).

Schreiner et al. (2009) analyzed the water stress response of two lines of Ethiopian mustard (*Brassica carinata*), in order to evaluate the GSL metabolism response (**Figure 2**). In both lines, 2-propenyl glucosinolate and 3-indolyl methyl glucosinolate were the most representative GSLs with an increase of 80% and 120%, respectively, in both lines. The increase was inversely correlated to the soil water content with severe yield losses (Nora et al., 2012).

An accumulation of GSLs in rapeseed leaves (*B. napus*), grown in low water potential, was found (Jensen et al., 1996). Unfortunately, it is not always possible to distinguish whether increases in GSL concentrations are related to a real increase in GSL biosynthesis or are due to lower biomass production, while the quantity of GSLs produced is not affected (Bloem et al., 2014). However, the answer is complex and depends on the plant phenophase. If the water stress, in fact, was determined in the branching phase, the concentration of glucotropaeoline (GT) was higher, while in the subsequent phenophases, the increases were smaller (Bloem et al., 2014). In rapeseed, there was a 50% increase in GSLs if water unavailability occurred after flowering (Mailer and Cornish, 1987). This could explain, at least in part, the contradictory conclusions published. While Robbins et al. (2005) found a reduction in the concentration of GSLs in drought-stressed broccoli, Schreiner et al. (2009) found an increase in GSL concentration in *B. carinata* with a soil water content of less than 80%, and Radovich et al. (2005) found the increase in GSL in cabbage (*B. oleracea* L. Capitata Group).

In *B. napus*, waterlogging affected the oil quality by increasing erucic acid (C22:1) and GSL content. Waterlogging also caused an increase in linolenic acid (C18:3) and a decrease in linoleic acid (C18:2), indicating that this kind of stress might affect metabolic pathways involving lipid biosynthesis (Xu et al., 2015).

In nasturtium (*Tropaeolum majus* L.), moderate drought stress (65–70% of the amount of irrigation water that was applied to the control plants) and the application of methyl jasmonate (MeJA) were considered as suitable tools to increase the GT content, representing the sole GSL measured as target compound (Bloem et al., 2014).

In lettuce (*Lactuca sativa* L.), interesting bioactive compounds for the human diet are, among others phenolic substances, carotenoids and dietary fiber (Schreiner and Huyskens-Keil, 2006). Oh et al. (2010) found increasing levels of phenolics during drought stress in lettuce. In American lettuce, grown in a greenhouse and subjected to four levels (25%, 50%, 75%, and 100%) of evaporation restitution, it was noted that the content of each amine, with the exception of agmatine, increased with stress water (Coelho et al., 2005). Polyamines can eliminate free radicals, protecting the membranes from lipid peroxidation and oxidative stresses. Lettuce contains two main classes of phenols and polyphenols: caffeic acid derivatives (Ke and Saltveit, 1988) and flavonols (Hermann, 1976). The amount of these antioxidants and micronutrients in the leaves is modified by the cultivar (Llorach et al., 2008), growth conditions, and environmental stresses (Galieni et al., 2015).

In two cultivars of green leaf lettuce ('Lollo Bionda' and 'Vera'), subjected to different levels of water deficit (25%, 50%, and 75% management allowable depletion [MAD] levels), it was noted that 50% of MAD caused an increase in chicoric acid, caftaric acid, chlorogenic acid, and caffeic acid, while 75% of MAD increased levels of kaempferol, quercetin, and myricetin (Malejane et al., 2018). The content of ascorbic acid, on the other hand, decreased with increasing levels of MAD. The water deficit, therefore, stimulates the biosynthesis of phytochemicals in plants and improves the quality of crops. Water stress also induces gene expression and the enzymatic activity of phenylalanine ammonia lyase (PAL; E.C. 4.3.1.5), the primary enzyme of the phenylpropanoid pathway (**Figure 1**), responsible for the biosynthesis of phenolic compounds (Oh et al., 2010). In this study, also the antioxidant capacity [ferric reducing antioxidant power (FRAP) method] appeared higher with MAD at 50%, probably due to the increase in hydroxycinnamates (Malejane et al., 2018). Among the lettuce cultivars, 'Vera' was the most suitable cultivar for deficit irrigation (DI), due to its increase in phytochemicals and the quality of the crop without compromising the fresh biomass. In iceberg lettuce, DI has led to a reduction in chlorogenic acid and an increase in chicoric acid (Luna et al., 2012). In contrast, Oh et al. (2010) found, also in lettuce, an increase in total phenolic concentration and antioxidant capacity in the presence of water stress.

Sucrose, glucose, and fructose are soluble sugars in plants. The first two sugars participate as substrates for cellular respiration or as osmolytes to maintain cell homeostasis (Gupta and Kaur, 2005). An increase in fructose, instead, is connected to the phenolic compound biosynthesis (Hilal et al., 2004) rather

than provides osmoprotection (Rosa et al., 2009). Like drought, oxidative stress in photosynthetic tissue might result in the decomposition of carotenoids, which was found in lettuce in the water-logged treatment (Eichholz et al., 2014). The phenol content, in particular the chicoric acid, is increased under water stress (Oh et al., 2010).

'Teodore' lettuce showed significantly lower contents of β-carotene under waterlogged conditions compared with the well-watered treatment (−27%). Under waterlogged conditions, neither phenolic compounds nor dietary fiber were influenced in their contents (Eichholz et al., 2014).

Secondary compounds, essential oils, and aromatic components of leaves often increase due to environmental stress. An increase in antioxidant compounds was observed in Oman basil plants (*Ocimum basilicum*) subjected to various water regimens [from 65 (12.5%) to 500 ml/day (100%)]. The maximum amount of total phenols and total flavonoids was observed with an irrigation intensity of 25%. Also, the DPPH scavenging activity and the reduction of the antioxidant capacity of the basil leaf extract were also higher with 25% water regimen. A further reduction in the availability of water up to 12% has instead caused a reduction in antioxidant compounds and antioxidant activities (Khan et al., 2012).

Short-term exposure to moderate water stress in thyme (*Thymus vulgaris*), nipplewort (*Chelidonium majus*), and parsley (*Petroselinum crispum*) increased concentrations and overall content of the related secondary metabolites in *T. vulgaris* and *C. majus*. However, longer periods of drought have led to a clear reduction in the overall content of the metabolites also due to greater growth reductions. *P. crispum*, very sensitive to drought, even with short-term stress, has experienced significant reductions in growth (Kleinwächter et al., 2015).

Bioactive Compounds in Fruit Vegetable Quality

Tomato (*Solanum lycopersicum* L.) is a crop in which the relationship between phytochemical production and water stress has been deeply analyzed for the relevance of health effects of its fruit and the interest of cultivation with reduced quantity of water (i.e., DI).

DI, in fact, can be used both to save water and also to improve the quality of some products; nevertheless, special attention must be given when stress was induced, because the water deficit in sensitive phenological phases (such as flowering) can reduce the content of sugars, acids, and carotenoids (Ripoll et al., 2016). Different authors observed how DI increased the content of lycopene, vitamin C, and β-carotene (Favati et al., 2009; Patanè and Cosentino, 2010; Patanè et al., 2011; Chen et al., 2013), regardless of dependence on period and the degree of water stress (Pulupol et al., 1996; Nuruddin et al., 2003; Marouelli et al., 2004; Favati et al., 2009). DI could, therefore, be considered a useful tool to increase in tomato fruits the content of these nutrients (Juroszek et al., 2009), although it reduces the yield (Nuruddin et al., 2003; Marouelli et al., 2004).

Regulated DI (RDI) increased the content of several secondary metabolites (carotenoids and phenolics) in different cultivars of tomato ('Tigerella', 'Palamós', 'Byelsa', 'Lazarino', and 'Summerbrix'). The response depended on the cultivar and linked to cultivar resistance to water deficit; for example, 'Palamós' did not change total carotenoid, while 'Summerbrix', 'Tigerella', and 'Palamós' did not modify the total phenolic compounds (Coyago-Cruz et al., 2017).

Riso et al. (2004) observed that the phytochemicals in tomatoes, in particular lycopene, reduces reactive oxygen species (ROS), thus avoiding cellular damage; however, several other mechanisms of healthy carotenoid action have been suggested (Krinsky and Johnson, 2005). With lower water availability, a higher ascorbic acid content was found during fruit ripening (Kumar et al., 2012). In a study conducted by Helyes et al. (2014), the β-carotene level was positively influenced by water stress. In particular, in the first ripening stages, β-carotene/lycopene ratio was also influenced by drought stress, and results suggested that this stress preferably induces the carotenoid biosynthetic pathway of β-carotene (Riggi et al., 2008). Also, Favati et al. (2009) found that the lycopene and β-carotene levels were higher during drought stress conditions.

Four tomato cultivars were grown in a greenhouse under drought conditions compared with well-watered conditions. At the end of the experiment, the drought stress caused significant differences in antioxidant compositions (lycopene, total phenolics, and flavonoids) and antioxidant activities (DPPH and ABTS) (Klunklin and Savage, 2017).

Drought stress, imposed on five tomato cultivars ('Kosaco', 'Josefina', 'Katalina', 'Salomé', and 'Zarina'), caused an increase in phenolic compounds, especially in flavonoids, only in 'Zarina'. This increase was correlated to DAHPS activity (+33%) (Sánchez-Rodríguez et al. (2011).

Phenolic compounds (phenolic acids and derivatives) and polyphenols (flavonoids and polymeric compounds) play an important role in detoxification of free radicals (Ksouri et al., 2007). Water stress, like numerous other environmental stresses, which determine the accumulation of ROS in plants, can increase these scavenger molecules.

The activity of the key enzymes of the phenylpropanoid pathway also intensifies in response to environmental stresses (Weisshaar and Jenkins, 1998), whereby PAL has been associated with greater resistance to drought stress (Keleş and Oncel, 2002), as was demonstrated by the response of PAL-deficient mutants (Gitz III et al., 2004).

Water-deficit conditions generally determine the production of fruits with a higher antioxidant activity, due to a decrease in enzymatic activity and an increase in vitamin C and total phenolic content (Helyes et al., 2012; Barbagallo et al., 2013; Pék et al., 2014).

In tomato, waterlogging reduced the total sugar content (Singh et al., 2017).

Hot pepper subjected to deficit of water (100%, 85%, 70%, and 55% of water-holding capacity) showed a reduction of vitamin C content in DI treatments (Ahmed et al., 2014). Other studies (Mahendran and Bandara, 2000) on chili cultivars demonstrated the negative effect of drought stress on vitamin C content. Marín et al. (2009), indeed, have showed an increase of 23% of this vitamin in peppers subjected to water deficit.

Sweet peppers (*Capsicum annuum*) subjected to drought stress increased the phytochemical contents (phenols and

anthocyanins) (Dixon and Paiva, 1995). In this sense, as reported by Sánchez-Rodríguez et al. (2012), water stress could represent a suitable tool for managing plant growth and enhancing fruit quality. In fact, López-Marín et al. (2017) showed that the water deficit generally increases the chemical parameters of product quality. As previously reported, the stress response is cultivar dependent and is related to the harvest stage. In a study conducted by Marín et al. (2009), green peppers showed an increased in vitamin C content by 23%, whereas red fruits showed an increase in total carotenoids and provitamin A (30% and 15% respectively) as a consequence of DI. Furthermore, an increase of 30% in total carotenoids was observed in pepper red fruits subjected to water stress.

In pepper, waterlogging significantly reduced soluble proteins, soluble sugars, free amino acids, P, Fe, vitamin C, and vitamin E contents of the fruit (Ou et al., 2017).

Since the strawberry is sensitive to water scarcity, especially during flowering and ripening phase, cultivation is carried out under irrigated conditions (Krüger et al., 1999). Strawberry fruits treated by DI had increased concentration of some compounds linked to taste and health. In strawberry subjected to DI, higher concentrations of anthocyanins and antioxidant (Terry et al., 2007; Giné-Bordonaba and Terry, 2016) and ascorbic acid (Bordonaba and Terry, 2010) were found.

Watermelon is a natural source of lycopene, vitamin C, and L-citrulline (Fish and Bruton, 2014). In drought stress conditions, L-citrulline protects plants from oxidative stress (Akashi et al., 2001; Yokota et al., 2002). In fact, during drought stress, there is an accumulation of L-citrulline, which limits oxidative stress (Akashi et al., 2001) and the development of osmotic pressure and reduces the mechanical characteristics of the pulp (Soteriou and Kyriacou, 2015). Full irrigation treatment in watermelon reduced the vitamin C content, while the different irrigation treatments did not influence the lycopene content (Kuşçu et al., 2015); Leskovar et al. (2004), instead, found that watermelon cultivated in DI showed an increase of quality characteristics (lycopene and vitamin C content).

Drought stress induced an increase of total soluble solids content (23%) and β-carotene content (25%) in 'Mission' (muskmelon; reticulatus) and 'Da Vinci' (Tuscan; reticulatus), respectively (Sharma et al., 2014).

In a study by Balakumar et al. (1993), drought stress in cowpea plants (*Vigna unguiculata*) increased anthocyanin levels. Drought stress involves the water migration from cells, causing dehydration and plasmolysis. Given the induction from osmotic stress, it is not surprising to find that plants that are resistant to drought stress contain elevated contents in anthocyanins (Chalker-Scott, 1999).

COLD AND HEAT STRESS EFFECTS ON PRODUCE QUALITY

Cold Stress and Accumulation of Bioactive Compounds in Crops

The exposure to low but non-freezing temperatures, called cold stress (Raison and Lyons, 1986), can cause severe crop losses. Among the numerous phenotypic symptoms observable after cold stress exposure, we can list poor seed germination, stunted seedlings, leaf senescence, reduction in the leaf expansion, increase of wilted leaves, and finally the death of tissues. Severe membrane damage may also occur in case of cold stress (Galindo et al., 2007; Yadav, 2010). However, moderate stress conditions could induce in plants the accumulation of antioxidants and secondary metabolites as a defense mechanism (Rivero et al., 2001). The use of controlled abiotic stresses may represent an alternative strategy to increase the presence of healthy plant compounds in many vegetables and fruits (Rajashekar et al., 2009), and this issue is of interest to both producers and consumers. In fact, various crops exposed to cold stress have been shown to have higher nutritional values (Yoon et al., 2017, and references therein). Usually, in conditions of low growth temperatures, plants tend to increase the concentration of soluble sugars to promote osmotic adjustment, enhancing freezing tolerance. These findings are reported in a work conducted on spinach (*Spinacia oleracea* L.), cultivated in greenhouse and exposed to cold stress (Yoon et al., 2017). For humans, sugars are compounds with nutritional value, and they provide energy to the human body. The accumulation of soluble sugars could also affect taste, by increasing sweetness, and leaf tenderness (Decoteau, 2000; Watanabe and Ayugase, 2015). In kale leaves, it was observed that low temperatures increased soluble sugar content (Hagen et al., 2009), and an increment in the content of health-promoting phenolic compounds, in particular flavonols, was also reported (Neugart et al., 2012). Ascorbic acid accumulation is favored at lower temperatures. Cold stress exposure enhanced by almost double the vitamin C levels in spinach leaves cultivated in greenhouse as observed by Yoon et al. (2017); also, Watanabe and Ayugase (2015) found that the nutritional quality of winter sweet spinach (*S. oleracea* L.) was higher if the crop was subjected to low temperatures, considering the abundance in ascorbic acid resulting from chilling stress. Proietti et al. (2009) observed that the exposure at 10°C increased the total ascorbic acid concentration in spinach leaves by 41% compared with the levels registered in plants grown at 25°C/20°C day/night temperature regimen. Pak Choi plants (*Brassica rapa* ssp. *chinensis* L.) grown in greenhouse with three mean temperature settings (18°C, 21°C, and 25°C) showed increased ascorbic acid content at sub-optimal growing temperature exposure (Mahmud et al., 1999). As reported by Oh et al. (2009a), the total phenolic content increased in response to chilling (4°C for 1 day) in lettuce plants (*Lactuca sativa* L.) grown in a growth chamber. A significant increment was observed within 1 h of chilling, and the highest phenolic compound level occurred after 3 days of plant recovery. Similarly, in lettuce plants, the antioxidant capacity showed an increment when stress conditions occurred. In three cultivars of Japanese parsley (*Oenanthe stolonifera* D.C.) (Hasegawa et al., 2001) and in strawberry cell suspension cultures (Zhang et al., 1997), it was found that low temperatures caused an accumulation of anthocyanins. Phenolic compounds are important for the appearance, taste, flavor, and aroma of food products, as well as for their health-promoting aspects (Tomás-Barberán and Espín, 2001).

Watermelon plants (*Citrullus lanatus* [Thomb.] Mansf. cv. Dulce Maravilla), in a condition of sub-optimal growth

temperature (15°C), showed increased levels of phenolic compounds, as an adaptation strategy to cold stress. At this temperature, also, the highest PAL activity was registered (Rivero et al., 2001). Also, changes in the composition of membrane fatty acids are plants' strategy to enhance membrane stability, integrity, and function by increasing the proportion of the unsaturated fatty acids. These cold-enhanced polyunsaturated fatty acids and their higher intake are also linked to several health-promoting benefits for humans, in particular related to a reduced risk of both cardiovascular diseases and cancers (De Lorgeril and Salen, 2012).

In apple fruits, the low temperatures or high carbon dioxide during storage induced the accumulation of 4-aminobutyrate and 4-hydroxybutyrate (Brikis et al., 2018). These metabolites seem to be correlated to multiple abiotic stresses and play an important role in the regulation of transcriptional and biochemical mechanisms, which lead to the accumulation of bioactive compounds.

Low temperatures can also activate specific genes that can enhance the tolerance of plants to the freezing temperature. In grapevine, it has been observed that in chilling temperature compared with freezing conditions at the transcriptomic level, the plants showed different patterns of transcript profiles and enriched pathway responses relative to bioactive compounds. The most differentially expressed genes were those that belonged to the ethylene signaling; ABA signaling; the AP2/ERF, WRKY, and NAC transcription factor families; and the sugar pathways (Londo et al., 2018).

Heat Stress and Bioactive Compounds in Crops

Heat stress is a common abiotic stress in Mediterranean areas and in crops grown in greenhouse or plastic tunnels during spring–summer. High temperatures directly affect plant metabolism, acting on the enzyme activities. Therefore, many physiological processes are slowed down or impaired. Photosynthesis and phenylpropanoid pathways are primarily affected by heat stress. In particular, high temperatures can induce the accumulation of antioxidant in order to protect the cell membrane from breakdown and peroxidation. Heat stress usually induces the accumulation of ROS and the activation of detoxification systems. Tomato plants exposed to 35°C showed an increase of ascorbic acid (vitamin C) and improved the activity of the ascorbate/glutathione-related enzymes (Rivero et al., 2004). Heat-stressed plants undergo changes in carbon metabolism including the increase of soluble sugars. The stressed plants show also an increase in bioactive compounds such as proline, glycine betaine, and sugar alcohols (Wahid et al., 2007). The main function of these molecules is to stabilize proteins/enzymes and the membrane bilayer structure of plant tissues under heat stress conditions.

The phenolic concentrations in heat-stressed plants can have different behaviors depending on the species and their tolerance or sensitivity to high temperatures. Tomato plants exposed to 35°C showed a significant increase of total phenols, while watermelon showed a reduction of these compounds. In both species, the increase or decrease of total phenols was correlated to the higher or lower PAL enzyme activity (Sánchez-Rodríguez et al., 2010), confirming a key role for this enzyme in regulating plant stress responses.

LIGHT SUB-OPTIMAL STRESS AND PRODUCE QUALITY

The light is the most important energy source for plant growth, influencing morphogenesis and yield, and its signaling pathway plays a key role in the modulation of phytochemical profile through a light-mediated metabolic reprogramming (Ghasemzadeh et al., 2010; Karimi et al., 2013; Bian et al., 2015). In horticulture products, the bioactive phytochemical profile is directly or indirectly influenced by light intensity, which can be either a limiting or promoting factor for bioactive compounds accumulation (**Figure 2**).

Generally, the light excess can induce severe damage to the photosynthetic apparatus and can compromise the quality of plants, but it depends on the time and intensity. A prolonged exposure of plants to excessive radiation results in light-induced inactivation of photosystem II (PSII), which may cause the death of the organism when the rate of damage exceeds the rate of repair process, leading to complete disintegration of chlorophyll protein complexes (Kataria et al., 2014). The partial or complete photo-destruction of these pigments (chlorophyll photo-bleaching) are strictly related to a reduction of carotenoids content because of their failure to provide, in this condition, protection from photooxidative degradation (Kataria et al., 2014). Under this condition, the photosynthesis is inhibited (Jordan et al., 1994; Sztatelman et al., 2015), resulting in an increase in ROS production and finally death of the photosynthetic organism (Apel and Hirt, 2004). However, plants develop different photoprotection strategies to dissipate the excess of light energy when exposed to high light levels favorable to photoinhibition (i.e., light absorbed exceeds the capacity of photosynthesis), for example, through the xanthophyll cycle. The constituents of photosynthetic protein pigment LHCII complexes (the xanthophyll pool) increase as a photoprotective mechanism to prevent the light-induced damage of the photosynthetic machinery due to the formation of ROS under excess light (Yoo et al., 2003), resulting in an increment of carotenoids to reinforce preservation against photodamage (Yoo et al., 2003; Fanciullino et al., 2014). Generally, along with antioxidant activity of carotenoids, other lipophilic antioxidants are involved as showed in pea (*Pisum sativum*) grown under high irradiance (Ariz et al., 2010) as well as in spinach and lettuce (Oyama et al., 1999). Nonetheless, light exposure appears to have little or no effect on carotenoid content of the majority of the edible portion of fruits and vegetables (Kalt, 2005), whereas activation of secondary metabolism, and in particular, the phenylpropanoid pathway (**Figure 3**), can induce the biosynthesis of a wide range of bioactive compounds by exposure to elevated light in horticultural products. Polyphenols contribute to the increase in quality of fruit, vegetable, and their processed products (Heim et al., 2002; Cheynier et al., 2006); and it is widely accepted that high light intensity acts as stress inducer driving their biosynthesis (Ramakrishna and Ravishankar, 2011).

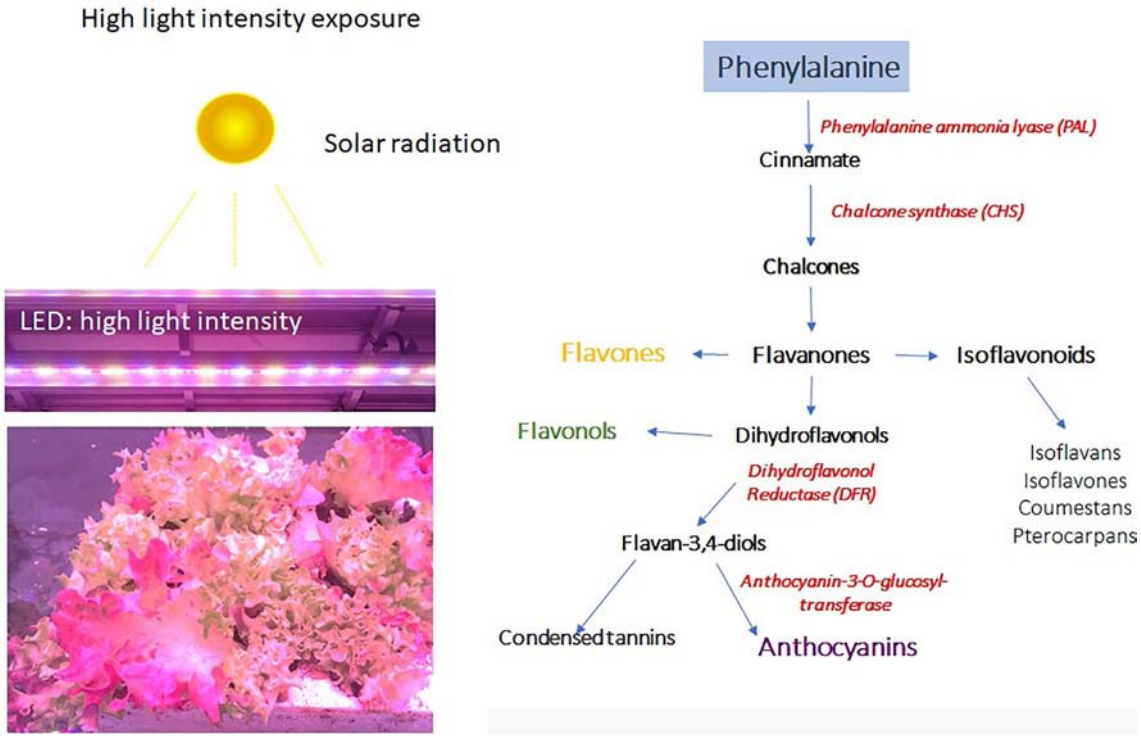

FIGURE 3 | High solar radiation or artificial lighting can induce the biosynthesis of anthocyanins and other phenylpropanoid compounds.

These bioactive compounds in fact may function as a scavenger and as a protective screen layer against harmful high-energy radiation (Agati and Tattini, 2010).

In lettuce plants, the exposure to mild to high light (800 μmol m^2 s^{-1} for 1 day) stresses induced the highest accumulation of all the phenolic compounds examined (caffeic acid derivatives, chicoric acid, and chlorogenic acid), showing a phenolic compound level six-fold higher in treated plants than in the control plants (Oh et al., 2009a). The influence of fertilization, light, and temperature on flavonoids and caffeoyl derivatives and related gene expression was investigated in tomato (*Solanum lycopersicum*, 'Suzanne'). In this study, PAL activity, and expression levels of chalcone synthase (CHS2) and flavanone 3-hydroxylase (F3H) genes were significantly enhanced at a higher light intensity, in agreement with a corresponding increase in flavonoid and caffeoyl content (Løvdal et al., 2010). Grape berry (*Vitis vinifera* L.) development and its phenolic compound accumulation are influenced by light environment and are considered to have a significant impact on wine quality (Downey et al., 2006). The light-induced phenolic metabolism and transcriptome changes were recently analyzed in Cabernet Sauvignon grape berries under sunlight exposure treatments at different phenological stages (Sun et al., 2017). In this study, the accumulation and compositional changes of hydroxycinnamic acids and flavonoids drastically increased by sunlight in exposed grape berries, and this metabolic reprogramming correlated well with transcriptional network changes of genes coding PAL, 4-coumarate:CoA ligase (4CL), flavanone 3-hydroxylase (F3H), and flavonol synthase (FLS) family members and their regulatory TF genes, highlighting the importance of theoretical foundations for cultural practice and environmental impact in wine production.

On the other hand, low light availability is the limiting factor for plant production due to adverse environmental conditions (cloudy days) or light-block horticulture facilities (greenhouses). Physiological and morphological traits such as photosynthesis, carbon and nitrogen fixation, leaf morphology and anatomy, gas exchange, and water relations (water use efficiency, stomatal conductance, and thus photosynthesis) are substantially affected in horticulture crops by ambient low light stress (Bjorkman, 1981; Atanasova et al., 2003; Heuvel et al., 2004; Gregoriu et al., 2007). Thus, horticulture products that were grown under low irradiance showed a decrease in flowering, fruit set, and fruit size (Passam and Khah, 1992; Pearson, 1992; Hampson et al., 1996).

In addition, low light intensities determine a metabolic reprogramming, leading an adjustment in the antioxidant profiles and pigment contents (Gruda, 2005). Vitamin C, including ascorbic acid and dehydroascorbic acid, is one of the most important and powerful antioxidants provided by fruits and vegetables having many biological activities in the human body (Lee and Kader, 2000). It is well known that the lower the light intensity, the lower the content of ascorbic acid in many fruits and vegetables (Weston and Barth, 1997; Lee and Kader, 2000). Leaves of lettuce grown with increasing levels of shading manifested a narrow shape and a reduced fresh weight and leaf number, and their sugar and ascorbic acid contents significantly

decreased (Shinohara and Suzuki,1981). A similar relationship between the light conditions and the ascorbic acid content was observed for different fruit and green leafy vegetables (Shinohara, 1987; Oyama et al., 1999; Weerakkody, 2003). For example, harvested strawberry fruits in spring showed a higher content of ascorbic acid together with other nutrients than did those harvested in autumn (Caruso et al., 2003), highlighting a seasonal influence on the amount of vitamin C formed.

The color of fruits and vegetables is a key and critical factor to consumer acceptance and the marketing success of these products, and it is dependent on light irradiation intensity (Gironi and Testoni, 1990; Kays, 1999; Schreiner et al., 2002). Since the primary pigments conferring colorful features to these agri-food commodities are chlorophylls (green), carotenoids (yellow, orange, and red), anthocyanins (red, blue), and flavonoids (yellow), there is an intrinsic association between the color, texture, and nutritional composition of fruits and vegetables (Barrett et al., 2010). In tomato plants grown under low light intensity, the carotenoids and, in particular, lycopene biosynthesis were reduced (Dorais et al., 2001). When lettuce plants were exposed to shading conditions, the biosynthesis of anthocyanins was generally reduced in almost all the examined cultivars (Kleinhenz et al., 2003). Similarly, in red radish, plants cultivated in protected environment were compared with plants grown in an open field, and a positive correlation was reported between the development of pigment and the light intensity (Schreiner et al., 2002)

In green asparagus, the chlorophyll, ascorbic acid, and rutin (the main flavonoid with strong antioxidant properties, in asparagus, quercetin-3-*O*-beta-rutinoside), and in purple asparagus, the anthocyanin content, were shown to be positively dependent on light intensity (Guillén et al., 2008; Kohmura et al., 2008; Maeda et al., 2008, Maeda et al., 2010; Wambrauw et al., 2016). When light is under optimal level, the overall color and nutritional quality were negatively affected, the concentration of rutin decreased, and the spear color was pale (Wambrauw et al., 2016).

Moreover, radiation intensity was reported to affect GSL biosynthesis in different *Brassica* species (Ciska et al., 2000). The concentration of GSL in rape leaves (*B. rapa*) was reduced by low light, and this effect was linked to a significant decrease in the flavin-containing monooxygenases, which catalyze a key regulatory step in GSL biosynthesis (Wallsgrove and Bennett,1995). A similar low light-induced effect was reported also for broccoli and *B. oleracea* varieties (Charron et al., 2005; Schonhof et al., 2007; Cartea et al., 2011).

Finally, preharvest environmental conditions, particularly light intensity (both high and low stress), cause responses at the physiological and biochemical levels, which may impact the postharvest life of many fruits and vegetables influencing their susceptibility to postharvest disorders or causing damages directly during postharvest (Ferguson et al., 1999; Gruda, 2005). The lower level of vitamin C reported under low light environment was linked to the worst performance of fruits and vegetable to sub-optimal environmental condition during the postharvest chain (Lin and Jolliffe, 1996; Noctor and Foyer, 1998; Gruda, 2005; Witkowska and Woltering, 2010).

In the next paragraph are reported some studies on the possibility of being able to improve the quality of fruit and vegetables through moderate levels of preharvest light stresses.

MODULATION OF ABIOTIC STRESSES FOR IMPROVING THE QUALITY OF AGRICULTURAL CROPS

Abiotic stresses in the cropping systems negatively affect produce quality and reduce the yield of crops. Genetic improvement has been providing new cultivars with enhanced tolerance against abiotic stresses, but in certain periods of the year or in specific geographical areas, the cultivation can be carried out only under greenhouses. As described above, abiotic stresses activate the biosynthesis of bioactive compounds and mild stresses can even enhance the quality of the products. In modern and technologically equipped greenhouses, abiotic stresses can be applied at low intensity, and if opportunely managed, they can represent an innovative strategy for enhancing the product quality. The accumulation of bioactive compounds with antioxidant properties in fruit and vegetables represents an interesting research topic in terms of human nutrition and the beneficial effect of these functional molecules in the diet. The bioactive compounds are essential for plants, which can enhance the tolerance to abiotic stresses and can be also interesting as source of these molecules (**Figure 2**). Each abiotic stress can be a trigger for specific biosynthetic pathway leading to the accumulation of specific metabolites. Understanding crop responses to abiotic stresses and the mechanisms of accumulation of bioactive compounds can be used for the development of agronomic strategies for producing high-quality fruits and vegetables.

Salinity Stress Application and Produce Quality

Hydroponic growing systems are widely used for the cultivation of fruit and vegetables. Optimized nutrient solutions can be used in these systems for improving yield and quality. During cultivation, the nutrient solution can be modified and enriched in salts.

The salinity of nutrient solution can be enriched by adding sodium chloride (NaCl) or using naturally salty water, commonly available along coastal areas in the Mediterranean regions.

Crops have specific tolerance threshold to salinity, which is usually expressed as EC of the nutrient solution or the substrate extract. The increase of salinity reduces the growth of crops, but if applied few days before harvest, it can induce positive quality variations of the products such as the reduction of nitrate accumulation in leaves. In leafy vegetables, for the commercial sales, they must have the nitrate concentration below the limits imposed by the EU regulation 1258/2011 (Cavaiuolo and Ferrante, 2014). The nitrate that is not assimilated is stored in

the vacuole. Since nitrate has also an osmoregulation function, under water stress or low light intensity, it can be accumulated.

The increase of salinity in the nutrient solution reduces the nitrate accumulation (Zanin et al., 2009), because the excess of sodium is stored in the vacuole, avoiding its concentration in the cytoplasm. The high concentration of sodium in the vacuole in saline conditions avoids the accumulation of the nitrate. Moreover, the Cl^- is also a competitor in the uptake of nitrate ion (NO_3^-); therefore, the high concentration of Cl^- reduces the nitrate uptake at the root level and the accumulation in the tissues (Abdelgadir et al., 2005). This crop response can be exploited as a strategy for reducing the nitrate concentration in leafy vegetables such as lettuce, spinach, and rocket by increasing the salinity of nutrient solution 2–3 days before harvest.

The same strategy can be used for improving the sensory quality of tomato. It has been reported that the quality of tomato fruits with higher sodium concentration has higher sensorial quality (Restuccia et al., 2002).

The application of salty water can be only exploited in greenhouse cultivations using hydroponic systems, because the beneficial effects can only be obtained if the stress application is limited to few days before the harvest.

Low Temperatures

In different species, low temperatures can induce anthocyanins and phenol biosynthesis. This strategy can be exploited for increasing the accumulation of these antioxidant compounds in the edible parts of different vegetables. Low temperatures are required, for example, in certain varieties of lettuce or radicchio plants for reaching the commercial color, which depends on the anthocyanins concentration.

A practical application can be exploited in protected cultivations by lowering the environmental temperature just by reducing the heating of the greenhouse few days or weeks before the harvesting. The critical step is the identification of the functional low temperatures specific for the crop of interest. Cold stress can be easily applied on crops grown in greenhouses during winter. The temperature reduction can be obtained by opening the windows and reducing the heating. This strategy is also applied for controlling the growth/height of many ornamental species. This technique is named "morning drop," because the windows opening early in the morning induces a rapid heat loss with a drastic temperature decline. The temperature drops to about 6–7°C and can reduce or inhibit plant growth. This strategy works for different species, but it requires appropriate adjustments to avoid chilling injury and quality losses, as well.

Wounding or Slicing

Harvesting procedures can induce wounds on the products due to cutting operations or mechanical damage derived from handling and transportation. These wounds can lead to quality losses in color alteration or tissue degradation. However, wounding induced by the slicing operations for postharvest products preparation, such as fresh-cut industries, can induce phenolic compound accumulation and enhance the produce antioxidant capacity. The crops showing highest wounding stress during harvest are baby leaf vegetables such as lettuce, spinach, and rocket. The wounds activate PAL, the key enzyme of the phenylpropanoid pathway, leading to an accumulation of secondary metabolites.

Wounding induced by slicing or chopping the fruit and leafy vegetables can be also used as postharvest strategy for improving anthocyanins or phenolic compounds concentration (Cisneros-Zevallos, 2003). Purple-flesh potatoes after slicing showed an increase of PAL enzyme activity with an increase of 60% of phenolic compounds and subsequently also an increase of the antioxidant capacity (Reyes and Cisneros-Zevallos, 2003). Analogous results have been observed in sliced lotus root (Hu et al., 2014). These evidences can be applied in the fresh-cut industries for increasing the antioxidant compounds of the products.

High Light Intensity and UV Light Treatments

High light intensity applied as supplementary lighting in a greenhouse can induce the biosynthesis of bioactive compounds such as vitamin C and carotenoids. Ascorbic acid is an important antioxidant with well-known beneficial effects on the human health. Ascorbic acid biosynthesis comes from the primary metabolism and from fructose-6P (Akram et al., 2017). Therefore, during the fruit ripening or before the vegetables are harvested, the application of additional light intensity, such as supplemental lighting, can increase the ascorbic acid content. At the practical level, the high light treatments can be applied in the greenhouse or other indoor cultivation. It is important to balance the light intensity and environmental temperature; otherwise, the high lighting can induce an excess of light with photodamage problems. Therefore, the highest effect of light treatments can be obtained when the temperature inside the greenhouse is in the range of the optimal range for the photosynthetic activity of the crop. It means that during winter the supplemental lighting should be provided during the daylight around noon, when the temperature inside the greenhouse is the highest possible (optimal range), especially in cold countries.

High light intensity can also increase the carotenoids concentration. Carotenoids are molecules with protective function against photobleaching of chlorophylls (Biswall, 1995); hence, high light intensity can have a positive effect on leaf carotenoids concentration. The high light intensity has also a positive effect on fruit carotenoids. Tomato plants grown under high light intensity (traditional greenhouse) and low light conditions (photovoltaic greenhouse) showed different lycopene and β-carotene concentrations. The tomato fruits obtained from plants grown under high light conditions showed double the lycopene and β-carotene concentrations (Bulgari et al., 2015).

UV radiations can induce the accumulation of phenolic compounds and increase the antioxidant capacity of products. During cultivation or few days before harvesting, the UV radiation

applied as pulse treatments can induce the phenylpropanoid pathway and increase the phenolic compounds.

The high light intensity can also activate the biosynthesis of phenylpropanoids (**Figure 3**). Lettuce plants exposed to high light show the accumulation of anthocyanins in leaves, which change color from green to blue. The color change is due to the activation of the phenylpropanoids pathway and in particular of the PAL enzyme and the correlated enzymes, which can lead to the accumulation of a wide range of metabolites, including anthocyanins (**Figure 3**).

The application of UV-B radiations to sweet basil with doses of 0.5, 34, 68, and 102 kJ m^{-2} day^{-1} delivered in 6 days (sub-chronic exposure) significantly enhanced the phenolic concentrations without compromising the efficiency of the PSII and the leaf functionality (Mosadegh et al., 2018).

Chili pepper (*Capsicum annuum* L.) exposed to 1.14 kJ m^{-2} day^{-1} UV-B radiation increased phenolic compounds in particular: chlorogenic acid, luteolin 8-C-hexoside, apigenin 6-C-pentoside-8-C-hexoside, and apigenin 8-C-hexoside (Rodríguez-Calzada et al., 2019). The combination of UV-B light exposure and drought can have a synergistic effect as it was observed for luteolin 6-C-pentoside-8-C-hexoside with an increase of more than 50% in chili pepper or an antagonist effect as found for apigenin 6-C-pentoside-8-C-hexoside, which showed lower levels in combined stresses (Rodríguez-Calzada et al., 2019). A practical application of UV lights can be exploited using light-emitting diode (LED) lamps, which can be set with the optimal UV spectrum (nm) and intensity for the species of interest. The highest performance and light use efficiency (LUE) can be also obtained by placing the lamps in the right positions and distance from the canopy of the crops (Cocetta et al., 2017). The LED lamps can be also placed between rows of crops such as tomato (interlighting). Since UV light can be also dangerous for the growers or operators who work in the greenhouse, it is advisable to apply the UV treatments during the night, when the energy cost is also lower.

Moderate Water Stress Induced by DI

Water stress as described above can have a negative effect on growth and, hence, on yield and quality. However, moderate water stress applied as DI can be exploited for inducing the accumulation of bioactive compounds (Ahmed et al., 2014; Bogale et al., 2016). The most important issue is the identification of the tolerance threshold of each species and the response time after the water stress application. The crops should be in the primary response stage of the water stress. It means that the plant metabolism is partly shifted in the biosynthesis of bioactive compounds such as osmolytes, antioxidants, and plant hormones, without showing any external symptom of stress.

It is important to constantly control the soil or substrate moisture content using tensiometers or other water content sensors and contemporarily the crop stress status. The most useful non-destructive measurement of plant stress is represented by chlorophyll *a* fluorescence and derived parameters. The application of DI can be carried out using appropriate mathematical models and software that can manage the water availability in order to induce bioactive molecules biosynthesis without reducing the crop performance.

Since the aim of the DI is the accumulation of the bioactive compounds, the most important issue is to define when the water reduction must be applied and how long the treatment should be carried out before harvesting to obtain the highest bioactive compounds accumulation.

CONCLUSION AND FURTHER PROSPECTIVE

Abiotic stresses can be used as tools to enhance the nutraceutical quality of crops and fruits. However, the effects of practical applications can vary depending on genetic diversity, agronomical practices, environmental conditions, and the combination of all these factors. The improvement of health-related properties of products in response to abiotic stresses should be obtained without affecting the yield. For these reasons, understanding the mechanisms adopted by plants to counteract these stresses (involving both the primary and secondary metabolisms) is the key step to control the abiotic stresses and use them as tools to improve the nutraceutical properties of crops. At the same time, the individuation of physiological, biochemical, and molecular markers related to stress tolerance and linked to improved nutraceutical quality may serve in breeding and crop selection program.

The practical application of abiotic stresses for bioactive compound accumulation requires the identification of the optimal application time, the crop sensitivity threshold, the intensity of the stress to apply, and the best methods for stress control during the application.

Controlled abiotic stress can be a new frontier of the applied sciences and can lead to the production of produce with a higher nutraceutical value. At the same time, this practice can help in reducing the use of natural resources, such as water, and in enhancing usage of saline or sub-optimal cultivation environments.

AUTHOR CONTRIBUTIONS

The manuscript was prepared with the following contributions: AlF wrote the primary and secondary metabolism sections; GC wrote the section related to effect of salinity on nutraceutical properties; ST and DR wrote the water stress and produce quality section; RB wrote the cold stress and produce quality section; AT wrote the sub-optimal light and quality section; and AnF wrote the section related to the modulation of abiotic stresses. All authors revised and approved the final version of the manuscript.

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