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The role of temperature in mediating postharvest polyamine homeostasis in tomato fruit

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ABSTRACT

Polyamines are actively involved in diverse processes, including fruit ripening and stress responses. The aim of this study was to investigate the role of storage temperature on polyamine metabolism of tomato fruit, which were either harvested at the turning stage or left to mature on-plant. The applied temperatures (5, 10 and 25 °C) and storage duration (7 d) are regularly employed in real-world scenarios. The metabolic profile of polyamines (putrescine, spermidine and spermine), gene transcription of the enzymes mediating polyamine biosynthesis and catabolism, protein accumulation of the putrescine synthesis enzyme and the putrescine-produced H_2O_2 were evaluated. Putrescine was the major polyamine in all cases, and its content generally increased during ripening, as well as in chilled fruit (stored at 5 °C). Increases in arginine decarboxylase protein content and in arginine decarboxylase transcription of both attached fruit and detached ones stored at either 10 or 25 °C showed that putrescine accumulation was mainly driven via the arginine decarboxylase biosynthetic pathway. Interestingly, putrescine catabolism by copper-containing amine oxidase was favored in parallel with increases in arginine decarboxylase and ornithine decarboxylase transcripts in fruit stored at 5 °C. However, the arginine decarboxylase protein accumulation suggests that ornithine decarboxylase is mainly responsible for putrescine accumulation at 5 °C. This study indicates that storage temperature modifies the homeostasis of polyamines in tomato fruit, which in turn orchestrates ripening-associated physiological processes.

1. Introduction

Market demand for high quality tomato fruit is currently rising (de Oliveira Silva et al., 2018). An increasing trend of consumption is evident due to antioxidants, contained by tomato, which promote consumer health by reducing the risk of various diseases, including cardiovascular ones (Cheng et al., 2017). However, tomato is a rather perishable commodity (Tsaniklidis et al., 2014, 2016). Thus, the prolongation of storage and shelf-life of tomato fruit is critical for its commercial value, since it increases the product marketability period and reduces the associated losses. Improving the nutritional value and extending the postharvest life of tomato fruit, appears to be a rather difficult undertaking, which requires a deeper insight into the underlying processes.

Abbreviations: ADC, arginine decarboxylase; CM, commercial maturity; CuAO, copper-containing amine oxidase; dw, dry weight; ODC, ornithine decarboxylase; PA, polyamine; PAO, polyamine oxidase; Put, putrescine; ROS, reactive oxygen species; RR, red ripe; SAM, S-adenomethionine; SAMDC, S-adenosylmethionine decarboxylase; Spd, triamine spermidine; SPDS, spermidine synthase; Spm, tetramine spermine.

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Fruit ripening is one of the most delicate and complex physiological procedures, involving multiple signaling and biosynthetic pathways (Pesaresi et al., 2014). These underlying processes are programmed with unique precision, and greatly influence both the quality properties and postharvest life. As tomatoes, similarly to other agricultural commodities, are generally excised from the mother plant prior to full ripening for larger expected longevity (Moneruzzaman et al., 2009), the delivery of water and nutrients is irreversibly interrupted, triggering an altered physiological equilibrium (Sorrequieta et al., 2013). On top of that, storage at low temperatures (≈ 10 °C), typically employed for prolongation of postharvest life, decelerates the ripening process and causes additional physiological reprogramming affecting major metabolites, which in turn may adversely influence fruit colour, flavor and nutritional value (Cantwell et al., 2009). Chilling temperatures (< 10 °C) in tomatoes, often used in households for short-term storage, may cause injuries, adversely affecting texture (Rugkong et al., 2010), colour, carotenoid content (Rugkong et al., 2011), and aroma volatiles similarly to other tropical and subtropical fruits (Bai et al., 2011), and may stimulate additional metabolic changes (Guillén et al., 2006; Petric et al., 2018; Tsaniklidis et al., 2014). Inversely, the ripening process is typically accelerated by exposure of detached fruit to higher holding temperatures (≈ 25 °C) (Tsaniklidis et al., 2014).

Polyamines (PAs) are simple-structured aliphatic polycations, including diamine putrescine (Put), which is rather abundant *in planta*. Put serves as a precursor for more complex forms, which are classified as higher PAs. Higher PAs include the triamine spermidine (Spd) and the tetramine spermine (Spm) (Tsaniklidis et al., 2016). Among other physiological roles, PAs are implicated in the process of fruit ripening, exhibit anti-senescence properties and hold a protective role against abiotic stress (Mo et al., 2020; Paschalidis et al., 2019; Serrano and Valero, 2018). Additionally, PA content is linked to fruit quality traits and shelf-life, suggesting a role of these molecules in early fruit development and ripening *per se* (Fortes and Agudelo-Romero, 2018).

Accumulated data from several species suggests that PAs act pleiotropically in a plethora of crucial physiological and developmental processes. They regulate a great number of metabolic pathways, mainly via transcriptional/post-transcriptional regulation of various genes, including protein kinases. They also contribute to protein stability and functionality especially under stress conditions, and are thus considered as stress indicators *in planta* (Bigeard and Hirt, 2018; Moschou and Roubelakis-Angelakis, 2014; Paschalidis et al., 2019, 2009; Paschalidis and Roubelakis-Angelakis, 2005). Under adverse conditions, PA accumulation plays a pivotal role in plant ability to protect biomolecules and membrane systems (Hussain et al., 2011). In addition, exogenous Put application has been linked to both enhanced abiotic stress tolerance and improved postharvest quality of fruit and vegetables (Gill and Tuteja, 2010; Sharma et al., 2017). By employing transgenic plants, PAs have also been related to postharvest life prolongation (Lasanajak et al., 2014).

Studies employing either exogenous PA application or transgenic lines overexpressing PA biosynthesis genes suggest that PA accumulation not only confers a shelf-life prolongation, but also significantly promotes carotenoid content and consequently enhances the produce nutritional value (Law et al., 1991; Mehta et al., 2002; Nambeesan et al., 2012). Although Put and higher PAs do not fall within the most prominent food bioactive compounds, they exhibit valuable pro-health properties for humans, mainly supporting the metabolic processes and regulating the immune system functionality (Ali et al., 2011). Among ordinary foodstuff, tomatoes are a rather rich source of Put dietary intake, considering the significant amount of *per capita* consumption (Okamoto et al., 1997).

Although alterations in the PA pool under the common temperature management play a considerable role in tomato fruit postharvest life prolongation, they have received relatively limited attention. Notably, recent work indicates that the cold stress-induced expression of several genes orchestrating PA metabolism, was mitigated by a preceding viral infection in tomato plants (Tsaniklidis et al., 2020). These findings not only denote a possible priming-like mechanism, but also point to crosstalk between biotic and abiotic stress signalling (Tsaniklidis et al., 2020). Since post-harvest treatments of tomato fruit are mainly associated with low-temperature storage conditions, it is crucial to examine the PA metabolism under these environments. Therefore, for the first time in the present study, the effect of temperature on the PA metabolism was addressed during tomato fruit ripening. The chosen temperature regimes are frequently applied in the respective supply chain, such as domestic refrigerators (5 °C), storage facilities and transport trucks (10 °C), as well as ripening at household room temperature (25 °C). It is well known, however, that storage at 5 °C results in cold-chilled fruit, whereas exposure to 25 °C accelerates the ripening process. The intermediate storage temperature (10 °C) comprises the lower recommended limit, and does not usually result in chilling-injured tomatoes, depending on the genotype and length of storage (Cantwell et al., 2009; Watkins et al., 1990). In this study, the transcript accumulation of all annotated genes implicated in tomato PA metabolism was evaluated together with the activity of enzymes involved in PA catabolism, and the protein concentration of enzymes involved in PA biogenesis. Additionally, Put, Spd, Spm and total PA concentrations were estimated during ripening under different storage temperature regimes. The obtained results suggest a direct effect of both developmental stage and storage temperature on PA metabolism.

2. Materials and methods

2.1. Plant material and growing conditions

Tomato (*Solanum lycopersicum* L. cv. Chondrokatsari) was employed in this study, which is a Greek locally-cultivated variety with excellent organoleptic properties (Darras et al., 2017). Plants were cultivated in a greenhouse compartment (9.9 × 7.6 m) located at the south part of Peloponnese (Kalamata, Greece; Lat 37° N). Growth period ranged between February and May. Throughout this period, plants were maintained under naturally fluctuating conditions of light intensity, temperature, and relative air humidity. During cultivation, min and max air temperature averaged 16.5 ± 2.0 and 27.1 ± 4.0 °C, accordingly. Mean solar radiation was 15.5 MJ m^{-2} d^{-1}. No artificial illumination was employed during cultivation. Plants were automatically watered with a nutrient solution. Electrical conductivity and pH were constantly monitored and adjusted to 2 mS cm^{-1} and 5.8, respectively.

Fruit at commercial maturity (CM), being physiologically at turning ripe stage (Supplementary Fig. S1), were either tagged and left intact to mature on the plants, or harvested.

The attached (tagged) fruit were harvested after an additional 7 d period. Following this period, they were at the Red Ripe (RR) stage. The ripening stage evaluation was based on both colour (assessed visually) and firmness. The latter was assessed with finger pressing and by using a Chatillon DFIS-10 penetrometer (John Chatillon, Greensboro, NC, USA). The latter was mounted on a frame (Chatillon TCM 201-M). A 6.3 mm probe was employed to induce a 0.6 cm penetration with a constant speed of 20 cm min^{-1} (Supplementary Table S1).

The CM harvested fruit were placed in plastic cases, allowing gas exchange, and were then stored at 80 % relative air humidity and 5, 10 and 25 °C for 7 d (hereafter named 7d5 °C, 7d10 °C, and 7d25 °C, respectively). Following this period, skin (epidermis) colour was yellowish in fruit at 5 °C, yellow-orange in those at 10 °C and deep red in fruit at 25 °C. Namely, the colour of RR fruit (ones that left to mature on the plants) was in between those of 7d10 °C and 7d25 °C (Supplementary Fig. S1).

Border plants (adjacent to greenhouse walls) were not sampled. Plants with uniform appearance were selected for measurements. Replicate fruit were sampled from separate plants. Each harvest was carried out at 11:00 am (22–24 °C during harvest). Samples were replicated three times (10 fruit per replicate). Samples were sliced,

immediately frozen in liquid nitrogen, homogenized (by using a pestle and mortar), and eventually stored at -80 °C for further analyses. Harvesting, as well as placing in either cases or storage chambers were carried out randomly.

2.2. Determination of total PA content

Total PAs were extracted from (0.1 g) freeze-dried tissue, at 2–4 °C for 1 h using 1 mL cold 5 % (v/v) $HClO_4$ water solution and 1.6 hexanediamine, as an internal standard. The samples were centrifuged (4000*g* at 4 °C) for 15 min, and 0.2 mL of the supernatant was used with dansyl chloride for PA derivatization. HPLC analysis was performed by HP-1050 isocratic pump system using a fluorescence detector and Inertsil ODS-3 (250 × 4.6 mm) reverse phase column for PA quantification. Mobile phase included 82 % acetonitrile and deionized water, while flow rate was 1.0 mL min^{-1} (0–7 min), 1.5 mL min^{-1} (7.8–11 min), and 1.8 mL min^{-1} (> 11.5 min) at 30 °C. Detector λex and λem were 360 and 510 nm, respectively. For every sample, each assay was performed twice. Three biological replications were made. PA content was expressed as mg g^{-1} dry weight (dw).

2.3. CuAO activity

CuAO activity was estimated by the method of Lin and Kao (2001), with modification as described below. Liquid nitrogen grounded tissue (0.4 g) was extracted by 1 mL extraction buffer containing 50 mM phosphate buffer (pH 7.8), 1 mM EDTA, 5 mL dithiothreitol and 10 % (v/v) glycerol. Next, centrifugation (12,000*g* at 4 °C) was performed for 20 min. Then, 200 μL of the supernatant were used for the CuAO activity assay along with 500 mL reaction buffer containing 50 mM phosphate buffer (pH 7.8), 10 mM Put and 0.1 mM pyridoxal phosphate. The mixture was incubated at 30 °C for 60 min, and afterwards the reaction ended by the addition of 250 μL 20 % (w/v) trichloroacetic acid. The mixture was rested for 30 min at room temperature (25 °C), and was subsequently centrifuged (5000*g*) for 15 min. Finally, 200 mL of ninhydrin solution (0.25 g ninhydrin in 10 mL of 3:2 acetic acid/-phosphoric acid) was added, and the mixture was left at 100 °C for 30 min to develop colour. Before absorbance measurement at 510 nm by using a UV–vis spectrophotometer (UV-1800, Shimadzu, Japan), 200 mL acetic acid was added to the mixture. The CuAO enzyme unit represented one photometric unit increase at 510 nm h^{-1}. Results were expressed as units mg^{-1} protein.

2.4. qPCR expression analysis

Total RNA from each sample was extracted as previously described by (Tsaniklidis et al., 2016). The extraction was performed using a lysis buffer containing 8 M GuHCl, 1 % Sarcosyl, 2 % Triton X-100, 25 mM sodium citrate, 25 mM EDTA and 0.2 M sodium acetate (pH 5.2). The samples were incubated at 65 °C for 10 min and then centrifuged (16, 000 *g*) for 5 min. Afterwards, 625 μL of absolute ethanol was added, and each sample was then filtered using FT-2.0 Filter-Tube Spin-Column System according to manufacturer's instructions (G. Kisker GbR, Steinfurt, Germany). The genomic DNA was eliminated by DNAse I treatment according to manufacturer's instructions (ThermoFisher, Waltham, USA). The successful genomic DNA elimination was verified by qPCR prior to cDNA synthesis using primers for *UBQ* (Supplementary Table S2) and genomic tomato DNA as a positive control. Gene expression was estimated using PowerUpTM SYBR® Green Master Mix (ThermoFisher, Waltham, USA), in a QuantStudio 3 Real-Time System (ThermoFisher, Waltham, USA). All qPCR assays were performed three times.

2.5. Western blot for ADC and PAO detection

ADC and PAO protein levels were estimated as previously described by Tsaniklidis et al. (2020). In brief, 0.1 g tissue was grounded using sterile mortar and pestle. Then, 0.3 mL extraction buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10 % glycerol, 0.1 % NP-40, 1 mM EDTA, was added in each sample. The extraction buffer was supplied with 1 × protease inhibitor cocktail for plants (Sigma–Aldrich Inc., MO, USA). The powder mixtures were vigorously vortexed at 4 °C for 20 min, and then centrifuged (14,000 *g* at 4 °C) for 20 min. Afterwards, 200 μL of the extract were transferred to new tubes and the total protein content was estimated with the method of Bradford, with a BSA standard curve. Subsequently, 15 μg from each normalized for protein level sample were used for analysis on a 10 % SDS-polyacrylamide gel at 200 V. The gel wet transferring was performed at 40 °C for 1 h at 100 V constant voltage using a methanol-activated PVDF membrane. The blot blocking performed with 1 × PBS containing 0.05 % Tween-20 (1 × PBST) and 5 % skimmed milk for 60 min at room temperature (25 °C). The blot was initially washed with 1 × PBST, followed by the primary antibody application, which was diluted (1:2000) in 1 × PBST, 3 % BSA and incubated at 25 °C. The blot was then washed using 1 × PBST and incubated for 2 h at room temperature (25 °C) in diluted (1:1500) anti-rabbit Hrp conjugate in (1 × PBST) and 5 % skimmed milk. A final washing of the blot with 1 × PBST was made. Eventually, ECL Clarity (Promega, Wisconsin, USA) was used for signal development, and photographed with a Sapphire Biomolecular Imager (Azure Biosystems, CA, USA) (Paschalidis and Roubelakis-Angelakis, 2005).

2.6. Hydrogen peroxide assay

Hydrogen peroxide (H_2O_2) content was measured as descripted by Tsaniklidis et al. (2020).

2.7. Statistical analysis

Experiments were analysed by one-way analysis of variance by using Statgraphics Centurion (Statpoint Technologies Inc., Warrenton, USA).

3. Results

3.1. Free Put and higher PA concentrations of tomato fruit

Put content fluctuated between 0.055 and 0.11 mg g^{-1} dw (Fig. 1A), Spd content between 0.011 and 0.017 mg g^{-1} dw (Fig. 1B) and Spm content ranged from 0.0015 to 0.0065 mg g^{-1} dw (Fig. 1C). The lowest Put content was observed in CM stage, which was nearly half than the Put content of the remaining treatments. A pairwise test between CM and RR stages confirmed that this difference was statistically significant. The highest Put content was noted in fruit stored at 10 °C (7d10 °C), intermediate values in fruit stored at 5 °C (7d5 °C), and the lowest content was observed in fruit of the remaining treatments (Fig. 1A). However, these differences in Put content were not significantly different. In contrast, Spd concentration was similar in attached fruit and in detached fruit stored at the two higher temperatures (10 and 25 °C), whereas it was lower in those stored at 5 °C (Fig. 1B). Thus, the contributions of Put and Spd concentrations to the total PA homeostasis were somehow complementary. Spm was highly accumulated in RR fruit as compared to all other treatments, and was followed by the levels in 7d25 °C and CM stage. Spm content was the lowest under the two lower applied temperatures (5 and 10 °C). Thus, Spm content was favorably concentrated in mature fruit, while it was down-regulated by low temperature (Fig. 1C).

Total PA levels exhibited remarkable homeostasis and were of similar magnitude (≈ 0.12 mg g^{-1} dw) among examined treatments, with the exception of the attached fruit at CM stage, where lower levels were detected (Fig.1D). CM fruit, which represent a less mature stage, had approximately 50 % lower total PA accumulation, as compared to the rest of the treatments (Fig. 1). It is worth noticing that Put was the most abundant metabolite, representing approximately 75 % of the total

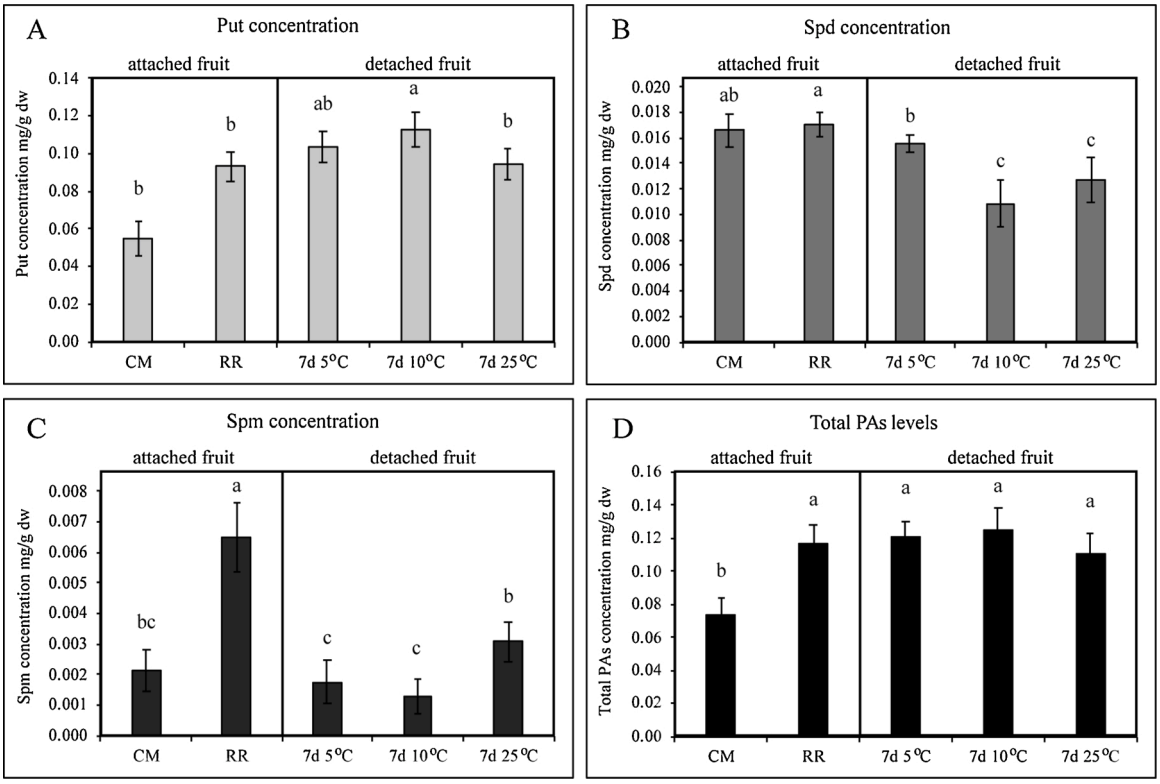

Fig. 1. Concentration of (A) putrescine (Put), (B) triamine spermidine (Spd), (C) tetramine spermine (Spm), and (D) total polyamines (PAs) in tomato fruit. Attached fruit were assessed at Commercial Maturity (CM) and Red Ripe (RR) stages. Detached fruit were sampled at CM stage, and then stored for 7 d at 5, 10 and 25 °C (abbreviated as 7d5 °C, 7d10 °C, and 7d25 °C, respectively). Significant differences between treatments were determined by one-way ANOVA. Values with different letters show statistically significant differences ($\alpha = 0.05$) according to Duncan's test. Bars represent means (±SE) of three replications.

free PAs in all treatments under study, apart from the attached fruit at CM stage, where this fraction was 50 % (Fig. 1D).

3.2. *Analysis of tomato genome for genes involved in PA metabolism and expression analysis of genes involved in PA biosynthesis coding for decarboxylases*

In silico analysis of the tomato genome was carried out as previously described by Tsaniklidis et al. (2020). However, no additional genes implicated in the PA metabolism were identified (see also https://solgenomics.net). Thus, the 23 previously-identified genes (Tsaniklidis et al., 2020), involved in PA metabolism were further subjected to expression analysis. All genes implicated in Put biosynthesis were ubiquitously expressed in both attached and detached fruit exposed to different temperature regimes. Put is biosynthesized directly via ornithine decarboxylase (ODC) enzyme activity or indirectly via the arginine decarboxylase (ADC) enzyme activity. So far, two genes have been identified in tomato plants coding for each of the ADC and ODC enzymes (Tsaniklidis et al., 2016, 2020). In fruit stored at 5 °C (Fig. 2A–D), the transcripts' accumulation of all four examined genes of ADC and ODC enzymes was high. Particularly, *ADC1* transcript levels in 7d5 °C were higher than in the advanced mature fruit (RR) and 7d25 °C, while transcript levels were the lowest in CM stage. Similarly, in fruit stored at 10 °C, the detected gene expression was also high, being at similar levels with those at 5 °C for *ADC1* and *ADC2,* but at intermediate levels for *ODC1,2* genes among all samples. On the contrary, in fruit stored at 25 °C, the transcription of *ADC2* and *ODC1* was remarkably down-regulated (Fig. 2B, C). Minor differences were found in attached fruit at either developmental stage (i.e., CM and RR), with their levels being similar to 7d5 °C for *ADC2* (Fig. 2B), but lower than the remaining transcripts (Fig. 2A, C, D).

In the tomato genome, three genes have been so far identified coding for SAM decarboxylase (SAMDC). The transcription patterns for *SAMDC1* were similar to those for *SADC3* (Fig. 2E, G), where both were up-regulated in fruit exposed to low temperatures (5 and 10 °C) and especially to the lowest one. By contrast, the lowest *SAMDC1,3* transcript levels were detected in fruit at 25 °C, followed by the attached fruit at either maturity stage (CM and RR; Fig. 2E, G). However, *SAMDC2* transcription peaked in fruit stored at 25 °C, whereas it showed low transcription level in attached fruit (Fig. 2F). Transcript accumulation for *SAMDC2* in fruit at 10 °C (7d10 °C) was in between the attached fruit and those at 5 °C.

3.3. *Expression analysis of genes involved in higher PA biosynthesis*

Although transcripts for all the genes coding for SMDS enzyme were ubiquitously detected in fruit under study, their expression pattern exhibited significant differences. In general, however, the expression of the genes was induced under low-temperature conditions (7d5 °C and 7d10 °C) (Fig. 3A–E). The highly expressed genes *SPDS1,2,5* were induced in fruit at 5 °C at higher levels than at 10 °C (Fig. 3A, B, E). By contrast, the *SPDS4* gene was similarly expressed in fruit at both 5 and 10 °C (Fig. 3D). At 25 °C, *SPDS5* was also highly expressed and similarly to fruit at 5 °C (Fig. 3E). However, *SPDS1* and *SPDS2* were expressed at lower levels than 5 °C (Fig. 3A, B). The expression of *SPDS3* gene was rather stable and exhibited no significant differences among the treatments under study. In RR, the transcript levels for *SPDS1* gene were lower than in CM stage, but similar to fruit at 25 °C. In RR stage, the *SPDS1* transcript level was in between those at 5 and 10 °C, whereas the levels for *SPDS2* gene were the highest observed. The levels for *SPDS5* gene in RR stage were similar to CM stage and to 7d10 °C, and lower than those exposed to two extreme temperatures under study (5 and 25 °C).

The expression of Spermine synthase 1 (*SPMS1*) was relatively at

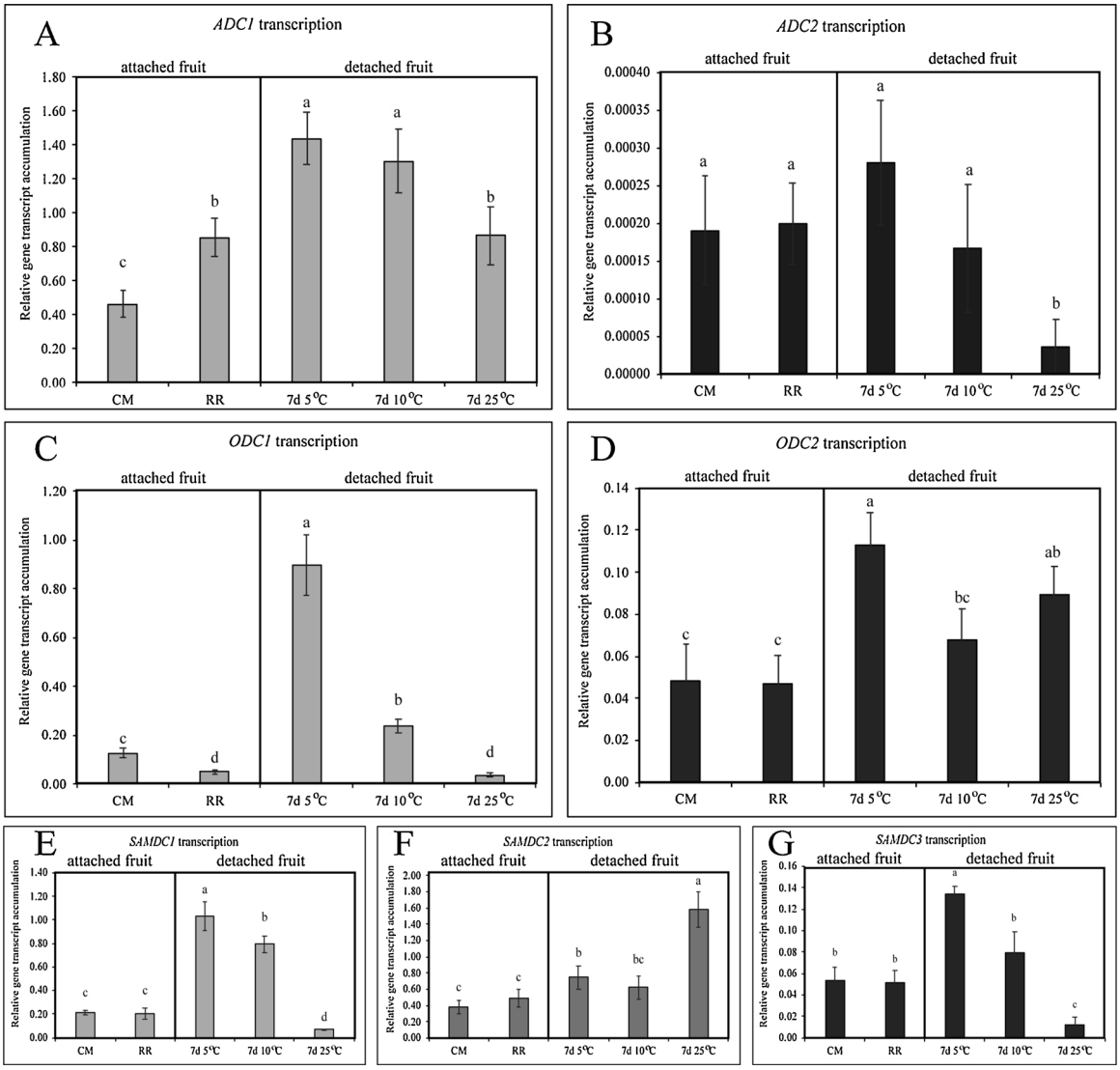

Fig. 2. Expression levels of genes coding for enzymes (A, B) arginine decarboxylase (ADC; *ADC1,2*), (C, D) ornithine decarboxylase (ODC; *ODC1,2*) and (E, F, G) S-adenosylmethionine decarboxylase (SAMDC; *SAMDC1,2,3*) in tomato fruit. Attached fruit were assessed at Commercial Maturity (CM) and Red Ripe (RR) stages. Detached fruit were sampled at CM stage, and then stored for 7 d at 5, 10 and 25 °C (abbreviated as 7d5 °C, 7d10 °C, and 7d25 °C, respectively). Significant differences between treatments were determined by one-way ANOVA. Values with different letters showed statistically significant differences ($\alpha = 0.05$) according to Duncan's test. Bars represent means (±SE) of three biological replications.

high levels in the attached fruit and decreased at 10 °C, while being at rather intermediate levels at 5 and 25 °C (extreme employed temperatures). By contrast, *SPMS2* was clearly induced by the lowest temperature (5 °C), while being at similarly lower levels in the remaining samples (Fig. 3G). Levels of *SPMS3* expression lowered in RR stage in comparison to CM stage, but increased similarly in all stored fruit (Fig. 3H).

3.4. Expression analysis of genes coding for CuAO and PAO isoenzymes

Unlike other taxa, where the CuAO enzyme is coded by a gene family (i.e., twelve *CuAO* genes in *Arabidopsis thaliana* (Wimalasekera et al., 2011), only two genes have been so far annotated as copper-binding amine oxidases in tomato (Tsaniklidis et al., 2020). Similarly, to genes involved in Put biosynthesis, *CuAO1* transcription was the highest in fruit stored at 5 °C, followed by the similar values in attached fruit at either developmental stage (i.e., CM and RR) and in fruit at 10 °C. *CuAO1* transcription was lowest in fruit stored at 25 °C (Fig. 4A). For the much lower expressed *CuAO2*, no specific pattern was recognized (Fig. 4B). The *CuAO2* transcription clearly peaked in RR fruit, followed by the similarly lower values in CM stage, 7d5 °C and 7d10 °C. *CuAO2* transcription was the lowest in fruit stored at 25 °C, as observed for *CuAO1* (Fig. 4A, B).

PAO is implicated in both spermidine and spermine catabolism, and is coded by at least six genes in tomato plants. Among the highly expressed genes, transcript levels in *PAO2, 6, 7* increased in detached fruit in comparison to the attached ones (Fig. 4E, H, I). Thus, these genes exhibited a rather temperature independent regulation, while their expression was induced upon detachment. On the contrary, *PAO1, 4* genes' expression was clearly increased by both lower employed temperatures (5 and 10 °C; Fig. 4D, F). In RR attached fruit, there was an upregulation of *PAO5* gene and downregulation of *PAO7*, while no difference was observed in the other genes, as compared to CM stage.

3.5. Activity of CuAO and accumulation of H_2O_2

CuAO activity pattern seems to be formed by the synergistic expression of the two genes coordinated in CuAO transcripts'

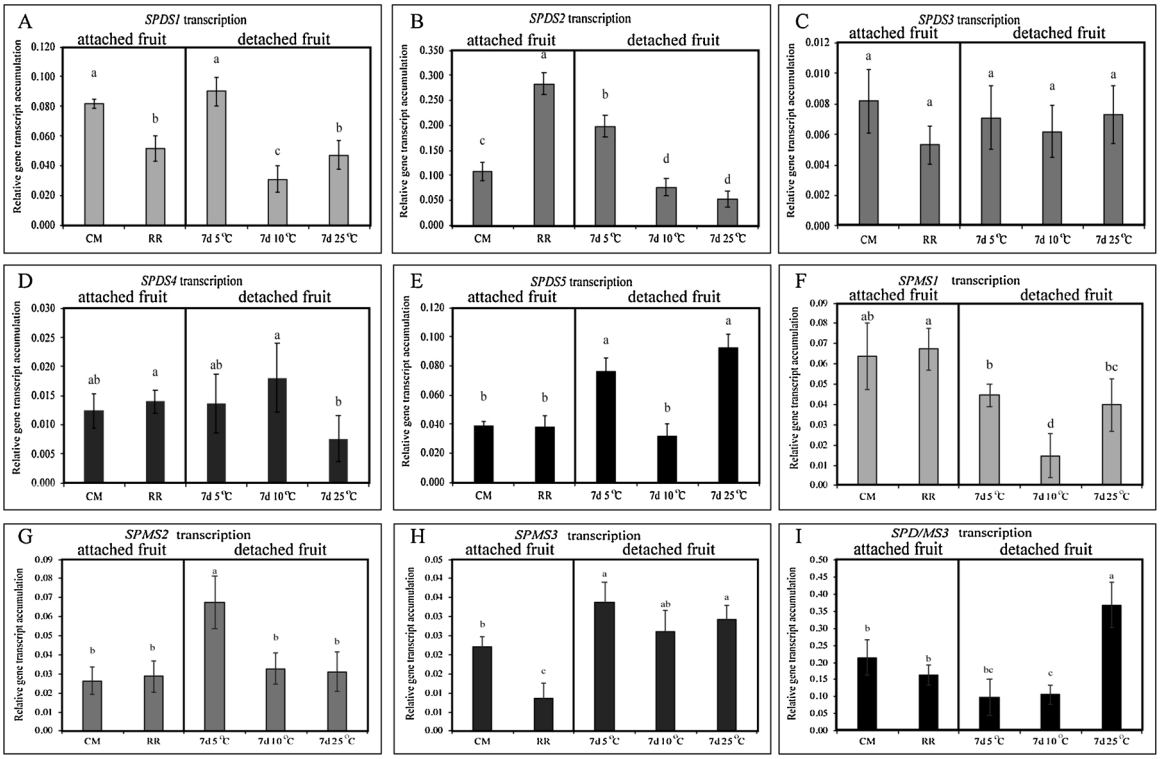

Fig. 3. Expression levels of genes coding for enzymes (A, B, C, D, E) spermidine synthase (SPDS; *SPDS 1,2,3,4,5*), (F, G, H) spermine synthase (SPMS; *SPMS1,2, 3*) and (I) spermidine/spermine synthase (*SPD/MS3*) in tomato fruit. Attached fruit were assessed at Commercial Maturity (CM) and Red Ripe (RR) stages. Detached fruit were sampled at CM stage, and then stored for 7 d at 5, 10 and 25 °C (abbreviated as 7d5 °C, 7d10 °C, and 7d25 °C, respectively). Significant differences between treatments were determined by one-way ANOVA. Values with different letters showed statistically significant differences ($\alpha = 0.05$) according to Duncan's test. Bars represent means (±SE) of three biological replications.

accumulation. Indeed, CuAO activity was induced in attached fruit at RR stage and in detached cold-treated ones (7d5 °C) (Fig. 4C), where *CuAO1,2* picked (Fig. 4A, B).

Concerning the levels of H_2O_2, values ranged between 0.13 and 0.27 μmol g^{-1} fresh weight. No remarkable differences in H_2O_2 content were noted between RR attached fruit and those stored at 5 or 10 °C, although the H_2O_2 content at 10 °C was lower than the one at 5 °C (Fig. 5B). However, the accumulation of H_2O_2 was considerably elevated and significantly the highest one in fruit at 25 °C (Fig. 5B).

3.6. ADC and PAO protein quantification

The anti-ADC antibody recognized one protein with a molecular weight of approximately 74 kD, corresponding to ADC, which had been previously characterized by Paschalidis and Roubelakis-Angelakis (2005). The highest accumulation of the ADC protein was detected in tomatoes stored at 10 °C. Similarly, the ADC protein was also high in attached fruit at RR stage and in fruit stored at 25 °C for 7 d, with the former slightly exceeding the content of the latter (Fig. 5A). The accumulation of ADC protein was considerably lower in attached fruit at CM stage and in detached fruit stored at 5 °C (Fig. 5A). Although two PAO isoenzymes with molecular weights of 53 and 54 kD have been earlier successfully detected in tomato leaves by anti-PAO antibody (Tsaniklidis et al., 2020), in this study PAO isoenzymes were not immunologically detected in fruit.

4. Discussion

In planta, a plethora of PA roles has been elucidated, underpinning their function as a major player in stress responses (Paschalidis et al., 2019) PAs act as stress-relievers and their catabolism is tightly connected with fruit ripening and senescence (reviewed in Wang et al. (2019)). Thus, PAs hold a pivotal role in fruit development and ripening (Fortes et al., 2019; Mellidou et al., 2016; Rastogi and Davies, 1991; Tavladoraki et al., 2016; Tsaniklidis et al., 2016, 2020) and have been directly associated to major quality attributes, as well as to postharvest life prolongation (Mehta et al., 2002). Their mediating role extends to postharvest acclimation processes, including stress responses, which, in several cases, are part of the post-harvest practice in real-world situations.

4.1. PA biosynthesis and catabolism during late development of attached fruit

The developmental stage of tomato fruit is critical for the free PA accumulation. In our previously published work, PAs accumulated in mature as compared to immature fruit (Tsaniklidis et al., 2016) and this accumulation has been directly connected to ripening and carotenoid biosynthesis (Mehta et al., 2002). Similarly, in the present study, both Put and higher PAs accumulated in mature fruit, as compared to the CM stage (Fig. 1). In mature fruit, higher ADC protein content (Fig. 5A), increased CuAO activity (Fig. 4C) and enhanced transcripts' accumulation of both *ADC1* (Fig. 2A) and *CuAO2* (Fig. 4B) were also recorded. These results suggest that both Put biosynthesis and catabolism processes are simultaneously highly active, while Put is notably accumulated at high levels in RR fruit. Increased transcription of the *CuAO2* gene was prominent at RR stage, as compared to the CM stage (Fig. 4B). Put is mainly catabolized by the CuAO enzyme, while this oxidation produces H_2O_2 and ammonia as by-products. H_2O_2 functions as a signaling molecule, influencing the ripening process (Guo et al., 2019; Steelheart et al., 2019). Moreover, the produced gamma-aminobutyric acid *via* CuAO and pyrroline dehydrogenase activities can be subsequently transaminated and oxidized to succinic acid, which is further incorporated into the tricarboxylic acid cycle, serving as a link between

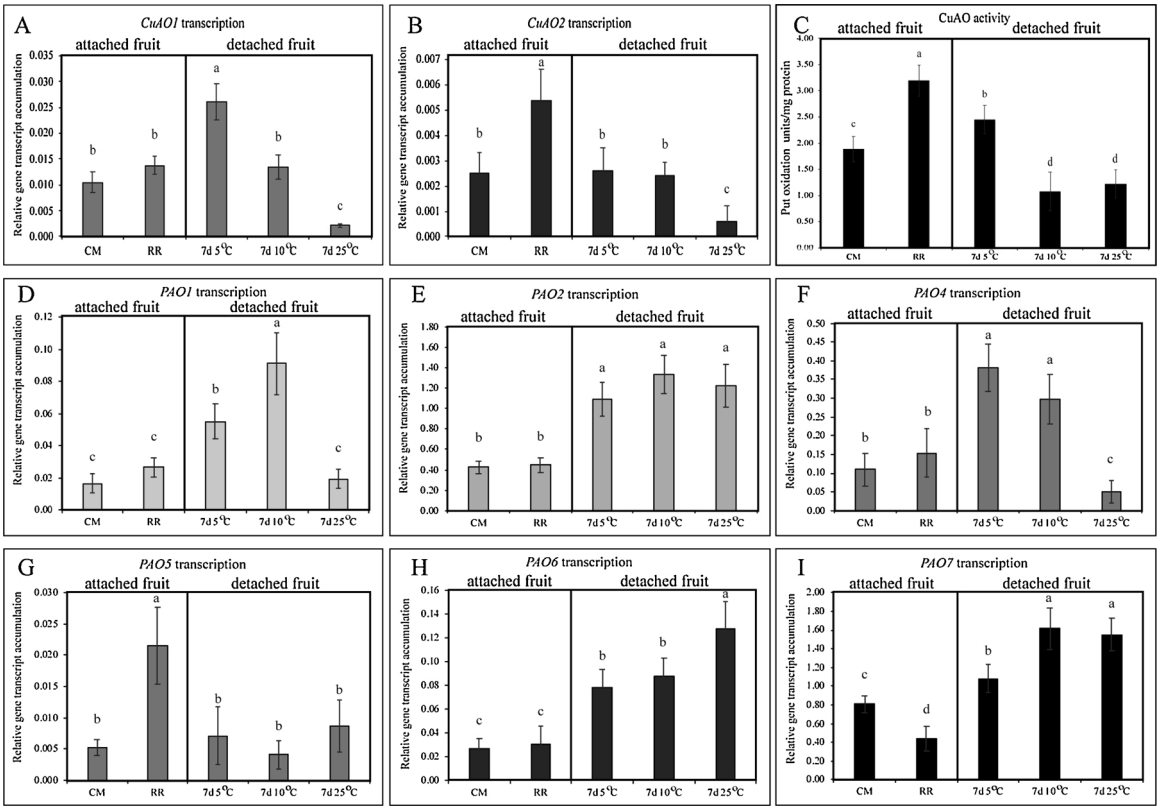

Fig. 4. Expression levels of genes coding for enzymes (A, B) copper-containing amine oxidase (CuAO; *CuAO1,2*) and (D, E, F, G, H, I) polyamine oxidase (PAO; PAO*1,2,4,5,6,7*) in tomato fruit. The activity of CuAO enzyme involved in Put catabolism is indicated in panel C. The attached fruit were assessed at Commercial Maturity (CM) and Red Ripe (RR) stages. Detached fruit were sampled at CM stage and then stored for 7 d at 5, 10 and 25 °C (abbreviated as 7d5 °C, 7d10 °C, and 7d25 °C, respectively). Significant differences between treatments were determined by one-way ANOVA. Values with different letters showed statistically significant differences (α = 0.05) according to Duncan's test. Bars represent means (±SE) of three biological replications.

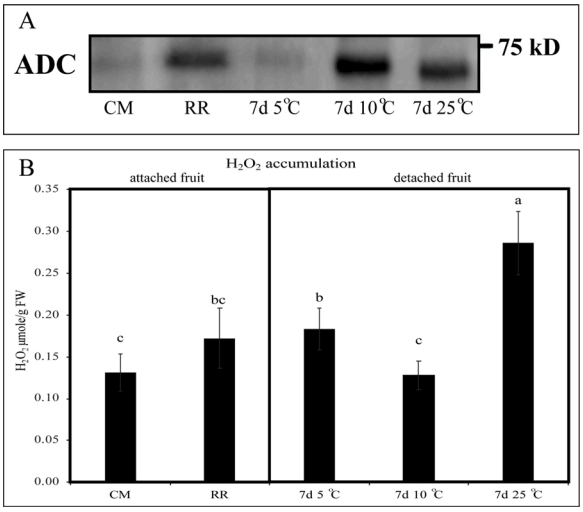

Fig. 5. (A) Western blot analysis of ADC protein, and (B) H_2O_2 accumulation in tomato fruit. Attached fruit were assessed at Commercial Maturity (CM) and Red Ripe (RR) stages. Detached fruit were sampled at CM stage, and then stored for 7 d at 5, 10 and 25 °C (abbreviated as 7d5 °C, 7d10 °C, and 7d25 °C, respectively). Significant differences between treatments were determined by one-way ANOVA. Values with different letters showed statistically significant differences (α = 0.05) according to Duncan's test. Bars represent means (±SE) of three biological replications.

amino acid biosynthesis and carbon metabolism (Agudelo-Romero et al., 2013; Tavladoraki et al., 2016).

PA levels are generally elevated during the initial fruit developmental stages, and subsequently rapidly diminish (Fortes et al., 2015; Tsaniklidis et al., 2016). In some cases, however, PA levels may rebound during late ripening under specific genotype by environment combinations (Dibble et al., 1988; Saftner and Baldi, 1990; Van de Poel et al., 2012). Since PAs have been associated with the prolongation of postharvest life, this late PA accumulation may be extremely important (Dibble et al., 1988; Nambeesan et al., 2008). Furthermore, indirect evidence supporting this notion comes from the fact that both the above-mentioned cultivars ('Chiou' and 'Chondrokatsari') exhibit a rather long shelf life.

ADC protein content was substantially increased during fruit ripening (RR versus CM stage; Fig. 5A). Increased transcription of *ADC1* gene was also noted at RR stage, as compared to CM stage, whereas marginal differences in the transcription of *ODC* genes were found between these two stages (Fig. 2). These results clearly indicate that the increased Put biosynthesis during fruit ripening (RR *versus* CM stage) mainly originates through the ADC biosynthetic pathway, whereas the contribution of the ODC biosynthetic pathway in this process appears to be less prominent. Put biosynthesis during late tomato ripening mainly though the ADC activity has also been earlier suggested (Lasanajak et al., 2014; Rastogi and Davies, 1991).

The variation in the transcription of the five genes coding for *SPDS* owing to fruit ripening (RR *versus* CM stage) did not follow a common pattern. *SPDS1* transcription probably is favored under recommended storage conditions (10 °C), while *SPDS5* appears to be cold stress-induced. By contrast, *SPDS3* and *SPDS4* were mostly unchanged during storage. Finally, chilling favored the transcription of *SPDS2* (Fig. 3).

Different transcription patterns of the two studied *SPDS* genes have also been earlier noted during fruit development and ripening in another tomato cultivar ('Chiou'), which may indicate a more complex and possibly individual regulation (Tsaniklidis et al., 2016).

No substantial difference in *SAMDC* transcription was noted between RR and CM stages (Fig. 2). In previously published reports, transgenic lines over-expressing a *SAMDC* gene showed a tendency to accumulate more Spd and Spm (higher PAs), though their concentration also depended on additional factors, such as substrate availability (Lasanajak et al., 2014).

In the cultivar under study ('Chondrokatsari'), the fruit content in higher PAs (Spd and Spm; Fig. 1) was similar to 'Alcobaca' (Dibble et al., 1988), while it was considerably lower than 'Chiou' (Tsaniklidis et al., 2016). Therefore, the ratio of Put to higher PA content is clearly genotype-dependent. This genotype-dependency adds another layer of complexity in the study of PA metabolism.

4.2. PA metabolism of detached tomato fruit after cold (5 or 10 °C) or 25 °C storage

Cold storage affected the tomato fruit PA metabolism in a temperature-dependent manner (5, 10 or 25 °C). The lowest temperature (5 °C), typically applied in domestic refrigerators, generally induced the transcription of genes involved in Put metabolism (Figs. 2 and 3). Several previously published reports have also shown an increase in the transcriptional levels of genes involved in biosynthetic pathways contributing to the cellular antioxidant protection of chilled-treated tomato fruit and leaves (Kevers et al., 2007; Mellidou et al., 2016; Tsaniklidis et al., 2016, 2020). Moreover, exogenous Put application has been shown to enhance antioxidant activity and to protect membrane lipids from chilling injuries in kiwifruit under cold storage at 5 °C (Yang et al., 2016). The exposure of fruit to 10 °C elicited different PA homeostasis, as compared to the chilling temperature (5 °C). Although total PA levels did not significantly differ between fruit exposed to either 5 or 10 °C, the latter temperature led to enhanced Put content, whereas Spd levels were reduced (Fig. 1). This antithetical trend in terms of direction (i.e., an increase versus decrease; Fig. 1) highlights the differential regulation between Put and higher PA (i.e., Spd) biosynthesis. This diverse regulation of PA metabolism and catabolism was also documented by Majumdar et al. (2016).

In line with the findings of this study (Fig. 1), a minor increase in Put accumulation following storage at the recommended temperature range (≈0 °C) has also been noticed in sweet cherry (Karagiannis et al., 2018). One-week fruit exposure to 25 °C led to a slight de-escalation of Put and Spd (Fig. 1). In accordance to our results, diminishing Put and Spd levels were noted in overripe Lorena hybrid tomato fruit (Casas et al., 1990). A similar tendency in Put levels has also been noted in overripe fruit of other taxa, such as orange (Tassoni et al., 2004), banana (Adão and Glória, 2005), and peach (Liu et al., 2006). Since PAs are generally correlated to the retardation of fruit senescence, the reduced PA levels in fruit stored at 25 °C suggest that irreversible abscission-related events were initiated, eventually leading to the disorganization of fruit structure (Gómez et al., 2014; Perrakis et al., 2019). In tomato, the enhanced Put content, as a result of storage at 10 °C, is expected to contribute to both the prolonged postharvest life and the absence of cold injury symptoms (Luengwilai et al., 2012).

Although the transcription of *ADC* genes, which is a rate limiting enzyme for Put biosynthesis, was up-regulated (Fig. 2), the ADC protein levels in tomato fruit exposed to 5 °C were significantly lower than fruit stored at higher temperatures (10 or 25 °C) or remained attached at RR stage (Fig. 5B). Interestingly, the transcription of both *ODC* genes was remarkably enhanced at 5 °C (Fig. 2). Similar expression patterns of *ADC* and *ODC* genes have been reported in cherry tomatoes following a short period of cold storage at 5 °C (Zhang et al., 2012) suggesting an enhanced role for ODC under these conditions. In contrast to our results, Fortes et al. (2019) concluded that the ODC route is stimulated by biotic stress, whereas the ADC route is elicited by abiotic stress (such as cold stress applied here) based on the reduced arginase activity. However, Zhang et al. (2012) raised additional factors to be considered. In that study, significantly reduced arginine levels were found in cherry tomatoes after a period of 5 d cold storage at 5 °C, while ornithine levels initially fluctuated and afterward increased. This reduced availability of arginine (i.e., the ADC substrate) may be the cause of diminishing the ADC protein accumulation in fruit retained at 5 °C, and the concomitant shift in the ODC pathway for Put production. Similarly, cold storage has been also shown to drastically reduce arginine levels in sweet cherries (Karagiannis et al., 2018).

As compared to the rest of the treatments, one-week exposure to 10 °C was also characterized by enhanced ADC protein accumulation (Fig. 5A), relatively low CuAO activity (Fig. 4C), as well as low H_2O_2 levels (Fig. 5B). These findings provide additional evidence that this temperature level generally favors the flux towards Put. Put serves as a substrate to higher PA biosynthesis, which as discussed plays a pivotal role in ripening. Moreover, although Put and total PA contents are not substantially different between storage at 5 and 10 °C, both the ADC protein accumulation and the *ADC/ODC* transcription results suggest a rather complex regulation of Put biosynthesis in tomato fruit. Storage of detached fruit at elevated temperature (25 °C) has been related to accelerated ripening (Tsaniklidis et al., 2016), which was also noted in the current study (Supplementary Table S2).

Detached fruit stored at 25 °C had an *ADC1* transcript level (Fig. 2) and a corresponding protein content (Fig. 5A) similar to those of the attached fruit at the RR stage, but lower than in fruits stored at 10 °C. Similarly, *ADC* and *ODC*, as well as *SAMDC* and *SPDS* transcription levels were rather unchanged at the respective stage in peach (Liu et al., 2006). Given the unaltered levels of these genes, the quality deterioration (assessed by loss of firmness) at this stage was rather related to the reduced Put levels (Liu et al., 2006). The CuAO enzymatic activity was significantly induced in fruit stored at 5 °C for 7 d, as compared to CM fruit (Fig. 4C). It is well documented that the PA oxidizing enzymatic activity is generally up-regulated in response to cold stress, as well as to other abiotic stresses. In addition, under adverse conditions the transcription of apoplastic PA catabolism enzymes is also increased. Thus, the PA catabolism-induced increase in H_2O_2 content acts as a long distant signal, stimulating the antioxidant mechanism and orchestrating the physiological responses (Gupta et al., 2016; Moschou et al., 2008; Velikova et al., 2000). In fruit stored at 25 °C, the *CuAO* transcription levels and the CuAO activity were low (Fig. 4A–C), whereas H_2O_2 levels were elevated (Fig. 5B). These results clearly indicate that the contribution of PA oxidation to H_2O_2 synthesis was rather limited. One possible reason can be the limited PAO accumulation in tomato fruit, since no immunoreactive PAO protein was detected. PAO is critical for the control of $O_2^{\bullet-}$ to H_2O_2 ratio *via* their biosynthesis and scavenging, which regulates the respiratory chain in mitochondria (Andronis et al., 2014). Moreover, the reactive oxygen species' (ROS; e.g., H_2O_2) accumulation is indeed increased in some cases upon senescence or during storage at near-ambient temperatures, mostly owing to the balance disruption between ROS production and the antioxidant machinery capacity (Chen et al., 2020; Dong et al., 2015; Tian et al., 2013). Although PA oxidation-produced H_2O_2 contributes to the signaling processes that govern ripening (Fortes and Agudelo-Romero, 2018), the H_2O_2 level generated by this process is rather minimal in comparison to other physiological processes (Mondal et al., 2004).

5. Conclusion

In conclusion, our results further suggest that storage at 10 °C favors Put accumulation *via* both stimulating its biosynthesis (Figs. 2 and 5A) and reducing its catabolism (Fig. 4C). In tomato fruit, Put derives from the *ADC1*, *ADC2*, *ODC1* and *ODC2* genes and is catabolized by the *CuAO1* and *CuAO2* genes during chilling. However, during ripening, the *ADC1* and *CuAO2* genes are mostly responsible for Put synthesis and

catabolism, respectively. The reduced content of higher PAs (i.e., Spd; Fig. 1) and the generally lower transcription of both *SPDS* (Fig. 3) and *SAMDC* (Fig. 2) at 10 °C storage, as compared to 5 °C, hint to the lower requirements for the production of protective metabolites under this temperature regime. Higher PAs (Spd and Spm) have been shown to be critical for the cold stress tolerance of several species (Liu et al., 2015), while SAMDC has been associated with stress response (Mellidou et al., 2016). On the other hand, the declining PA levels in fruits stored at 25 °C, may be an indication of the initiation of abscission-related events. The differential PA responses encountered in this study may assist to further unravel the cellular and physiological mechanisms associated with the postharvest tomato fruit quality.

Authors' contribution

GT and CD, designed the research, GT, QPCR experiments, H_2O_2 quantification. SC, western blot analysis. AT and PR, polyamine quantification. KP, enzyme activity assays, data interpretation. DF, NN, EG, ET and IS, assisted the data interpretation, and provided valuable insights. GT, CD, DF, NN and ET, contributed to the writing of the manuscript.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2021.111586.

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