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Proceeding Paper

Tocopherol Biosynthesis Dynamics in Almond Kernel Development †

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† Presented at the 2nd International Electronic Conference on Plant Sciences—10th Anniversary of Journal Plants, 1–15 December 2021; Available online: https://iecps2021.sciforum.net/).

Abstract: Almond is an important nut tree with seeds rich in tocopherol (Vitamin E). We studied tocopherol accumulation during kernel maturation (March to September) in the cultivar Soleta, and characterized a candidate *tocopherol cyclase* gene (*PdVTE1*), putatively involved in tocopherol biosynthesis. The tocopherol profile was analyzed and quantified by HPLC. An increase in α-tocopherol content was observed along kernel development, with a higher increment between T2 and T3 stages. Sequence of *PdVTE1* was identified and high similarity with Texas and *P. persica* sequences were confirmed. In the future, the differential expression level of candidate genes will be characterized using RT-qPCR.

Keywords: *Prunus dulcis*; almond kernel; fruit development; tocopherol

Citation: Almeida, G.; Faustino, A.; Pires, R.C.; Soldado, D.; Cachucho, L.; Oliveira, M.M.; Jerónimo, E.; Marum, L. Tocopherol Biosynthesis Dynamics in Almond Kernel Development. **2021**, *1*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Feibo Wu

Published: 29 November 2021

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1. Introduction

Prunus dulcis (Miller) D.A. Webb, known as almond, is one of the most important tree nut crops in terms of commercial production in Mediterranean climate areas [1]. As most cultivars are self-incompatible, seed production depends on cross-pollination, which provides genetic variability and adaptability to different environments [2]. This crop is widely cultivated for its edible seed, named kernel, which has a high nutritional value as it contains high concentration of lipids, mainly unsaturated fatty acids, and tocopherols.

Tocopherols, also named Vitamin E, are lipid soluble natural antioxidants. There are four homologues of tocopherol (α-, β-, γ-, δ-tocopherol) which differ in the number and position of the methyl groups on the ring structure, being the α-tocopherol the most abundant. All isomers are derived from 6-chromanol and have a saturated 16-carbon phytol side chain [1]. Tocopherols protect unsaturated fatty acids from peroxidation, increasing almond storage-life. Moreover, vitamin E has a key role in preventing cardiovascular diseases, because of its antioxidant activity, its inhibition of platelet aggregation and its immune-enhancing activity [3]. Due to all these benefits, almond quality is highly associated with tocopherol concentration in kernel.

In the last years, the production of almond in Portugal have significantly increased, being ranked 18th worldwide (FAOSTAT, 2019) [4]. Most almond cultivars currently produced in Portugal have foreign origin, coming from European countries where almond breeding programs are being conducted. The improved cultivars have higher yields than

traditional ones and also incorporated the late flowering trait, which reduces chances of flowering when frost events may still arise and bees are still unactive.

The aim of this work was to study tocopherol accumulation during almond kernel maturation, and to characterize a candidate *tocopherol cyclase* gene (*PdVTE1*), putatively involved in tocopherol biosynthesis.

2. Material and Methods

2.1. Plant Material and Fruit Characterization

Almonds at different developmental stages were collected, from March to September, from four-year-old 'Soleta' trees. The almond orchard was installed with irrigation in Penedo Gordo, Beja, Portugal (37°58′32.4″ N 7°55′46.2″ W). Fruits were collected from 3 different almond trees at 6 developmental stages: T0 = 26 days post-anthesis (DPA) (1st march), T1 = 56 DPA, T2 = 86 DPA, T3 = 116 DPA, T4 = 156 DPA, T5 = 164 DPA, and T6 = 171 DPA (corresponding to the commercial harvest time). The developmental stages were defined by visual morphological assessment and by measuring kernel and shell length in five selected almonds from each developmental stage (n = 5). At the mature stage, twenty five almonds with shell and kernels were weighed and the yield was calculated. Kernels were isolated, immediately frozen in liquid nitrogen and stored at 80 °C. Samples were later grinded in liquid nitrogen for chemical and molecular analysis.

2.2. α-Tocopherol Analysis

The α-tocopherol levels in the samples were quantified using a method adapted from Zhu et al. [5]. Almond powder (0.25 g) was mixed with ascorbic acid (0.025 g), ethanol (2.5 mL) and 80% potassium hydroxide solution (0.25 mL). Samples were vortexed for 30 s, incubated in a water-bath at 70 °C and periodically vortexed every 10 min. After a 30 min incubation period, the tubes were cooled in ice water for 5 min prior to addition of water (1.5 mL) and n-hexane (2.5 mL). After vortexing for 30 s, samples were centrifugated (1000× *g* at 20 °C) for 10 min. The n-Hexane layer was collected to vials and the tubes were centrifugated again. n-Hexane was evaporated in a nitrogen stream and n-hexane for HPLC (2 mL) was added to the vials for HPLC analysis [5]. The analysis was performed using a Dionex Ultimate 3000 uHPLC (Thermo Fisher Scientific, Waltham, MA, USA) and a normal-phase silica column (Zorbax RX-Sil, with the corresponding 12.5 mm analytical guard column, 4.6 mm ID, 250 mm, 5 µm particle size, Agilent Technologies Inc., Palo Alto, CA, USA). The α-tocopherol was identified using fluorescence detection (excitation wavelength of 295 nm and emission wavelength of 325 nm). α-Tocopherol standard (Calbiochem, USA) was used to prepare external calibration curve in order to calculate α-tocopherol content. Mobile phase was n-hexane (with 1% isopropanol) and the flow rate was 1.5 mL/min. The run last for 15 min with a column temperature of 20 °C. Data were subjected to analysis of variance (ANOVA), considering developmental stages as a single fixed factor, and using the GLM procedure from SAS software (SAS Institute Inc., Cary, NC, USA). Tree as the experimental unit (n = 3). Least square means were estimated and when a significant ($p < 0.05$) developmental stages effect was detected, the differences between means were determined using the LSD test.

2.3. Sequence Analysis

Sequences of *VTE1* annotated as putative *tocopherol cyclase*, involved in tocopherol biosynthesis, were obtained in Genome Database for Rosaceae (https://www.rosaceae.org (accessed on)) from *P. persica*. Using the BLASTn tool between this sequence against *Prunus dulcis* cv. Texas genome (Genome Database for Rosaceae—GDR) it was possible to identify the putative *VTE1* in almond, named *PdVTE1*. The Primer3Plus software (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi (accessed on)) was used for primer design, and primer quality was verified using PCR Primer Stats software (https://www.bioinformatics.org/sms2/pcr_primer_stats.html (accessed on)).

Total RNA was isolated from leaves and nuts of a 'Soleta' almond cultivar using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with minor modifications. After the addition of RLC buffer, 45µL of Plant RNA Isolation Aid (Invitrogen, Waltham, MA, USA) were added to each sample, followed by vortex and 5 min incubation at room temperature. Centrifuge force was increased from 8000× *g* to 12,000× *g* (in steps 5–8) and to 15,000 (in step 9). RNA was eluted in 30µL of water in the last step. A treatment with RNase-free DNASE I (Qiagen) was included to avoid contamination with DNA. For first-strand cDNA synthesis, total RNA was converted in cDNA using QuantiTect Reverse Transcription Kit (Qiagen), following its protocol. The coding sequence was amplified from cDNA using specific primers (Forward: 5′—CCTCCATTTCCTCTTCCACA—3′; Reverse: 5′—GCGTCAGCTTCTTACGGAAC—3′) and the amplified fragments were sequenced through Sanger platform (STABVIDA). The sequence obtained was aligned with *Arabidopsis thaliana* NP_567906.1, *Prunus dulcis* cv. Texas XP_034201471.1, *Prunus persica* XP_007222342.1, *Prunus mume* XP_008220061.1 and *Prunus avium* XP_021819281.1 (http://multalin.toulouse.inra.fr/multalin (accessed on)) for phylogenetic analysis (http://www.phylogeny.fr/simple_phylogeny.cgi (accessed on)). For the phylogenetic tree the "One-Click mode" (Standard) was used, which aligned the sequences through MUSCLE and curated through Gblocks. Phylogeny was made through PhyML and ultimately tree was rendered through TreeDyn (http://www.phylogeny.fr/simple_phylogeny.cgi (accessed on)).

3. Results and Discussion

3.1. Morphological Analysis

During almond kernel development and maturation, it was possible to observe several morphological changes (Figure 1). At the T0 and T1 stages, the epicarp and mesocarp were green, while the kernel tegument was whitish. Inside the almond kernel, a clear endosperm was found. As kernels developed, the embryo began to develop, and the endosperm reduced in size, while the tegument remained whitish (T2). At T3 and T4 stages, the tegument began to darken and to get a more brownish color. Cotyledons were fully developed and the kernel became firm. The mesocarp acquired a brownish color, started to open and became dry (T4). At the T5 stage the mesocarp withered and separated from the endocarp. Kernels were fully developed and comparatively dry. Tegument was almost fully developed. At commercial stage (T6), seed coat acquired a brown color as a whole. The growth is similar to other cultivars of almond. The biggest growth occurs between March and April, growing slightly over the remaining months. The decrease between T4 and T5 is due to the fact that measurements were done in fruits without mesocarp. An effect of the growth period on the size of fruits and kernels is described in Figure 1A. In Sánchez-Pérez et al. [6] the Ramillete and Marcona cultivars had a similar growth pattern, however fruits reached mature stage in early August. Soleta fruits grown in Beja, Portugal, achieved the commercial maturity in mid-September.

In the mature stage (T6), weight of almond with shell and kernel, from Penedo Gordo, Portugal, was measured and the results obtained (Table 1) were identical to those obtained in Aragón, Spain [6]. It is possible to conclude that the evolution of almond growth did not vary from what was described and the almond weight and yield remained within the values already reported for this cultivar.

Table 1. Average weights of almond with shell and kernels, in the commercial stage (T6).

Morphological Characteristics	Soleta (Beja, Portugal)	Soleta [1] (Aragón, Spain)
Average weight of almond with shell	3.64 g	3.63 g
Average weight of kernels	1.24 g	1.27 g
Yield	34.80%	27–35%

[1] Socias I Company, R.; Felipe, A. 'Belona' and 'Soleta' Almonds. HortScience **2007**, 42(3), 704–706.

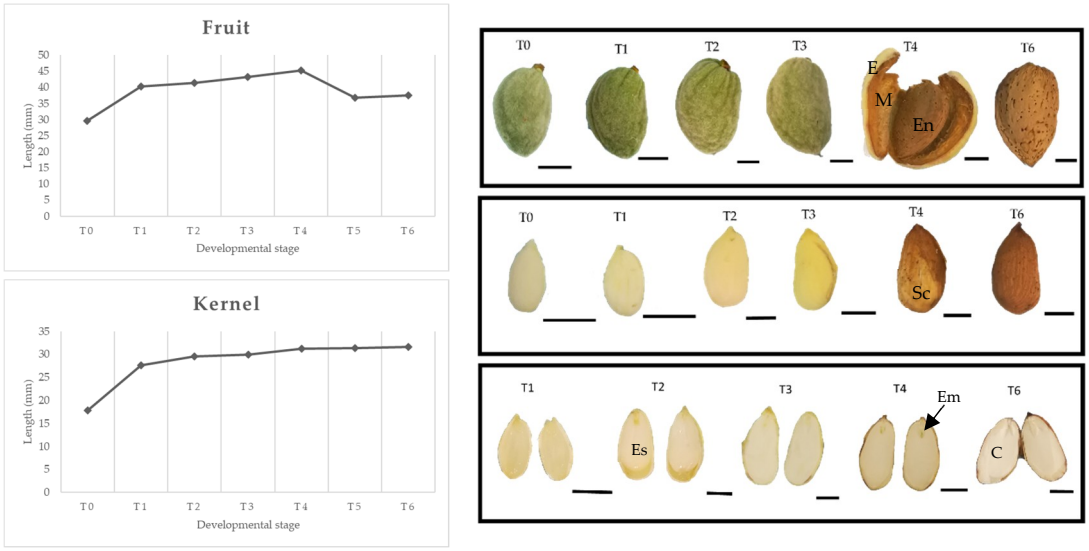

Figure 1. (**A**)—Evolution of fruits and kernels size during developmental stages; n = 5. (**B**)—Fruit developmental stages. Fruit with shell (on top); Almond kernel (on middle); Kernel cut lengthwise (on bottom) Scale bars = 1 cm. E—Exocarp, M—Mesocarp, En—Endocarp, Sc—Seed coat/ Tegument, Es—Endosperm, Em—Embryo, C—Cotyledons.

3.2. α-Tocopherol Content

α-Tocopherol content varied across developmental stages (Figure 2). In the early stages (T0, T1 and T2), the amount of tocopherol present was quite residual (2.91 µg/g). In stage T3, the highest content was reached (281 µg/g). In the mature fruit stage (T6), the concentration remained constant, which indicates that the production of tocopherol was greatly reduced from T3 to T6. This variation of the α-tocopherol content is within the expected results. As described in Zhu et al. [7], the greatest increase in α-tocopherol concentration occurs around 95 days post-anthesis. The α-tocopherol concentration in the mature stage is higher than it was described for Soleta in Spain [6]. It is possible to conclude that the soil and climatic conditions of Alentejo region may have influenced a greater accumulation of tocopherol in the mature fruit.

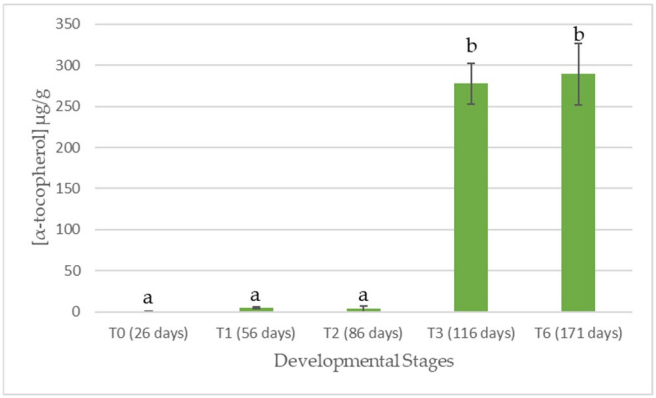

Figure 2. α-Tocopherol contents along almond kernel development (n = 3). Bars show ± S.D.

3.3. Sequence Analysis

The new PdVTE1 aminoacid sequence from Soleta was aligned with VTE1 protein sequences from Texas, other Prunus sps and Arabidopsis thaliana (Figure 3A). The sequences present high similarity among Prunus species and slightly vary compared to A. thaliana. In terms of analysis phylogeny of the genus Prunus, a greater proximity between *Prunus persica* and Prunus dulcis has been already described [8,9], which was confirmed in our study (Figure 3B), by the closer relationship between the two cultivars, Soleta and

Texas, and *Prunus persica*. Otherwise, *Prunus avium* and *Prunus mume* are more distant phylogenetically from other *Prunus* spp analysed. The VTE1 sequences of the different species of Prunus evolved over time and underwent changes. However, the variations between species, may not influence protein activity, keeping its cyclase activity.

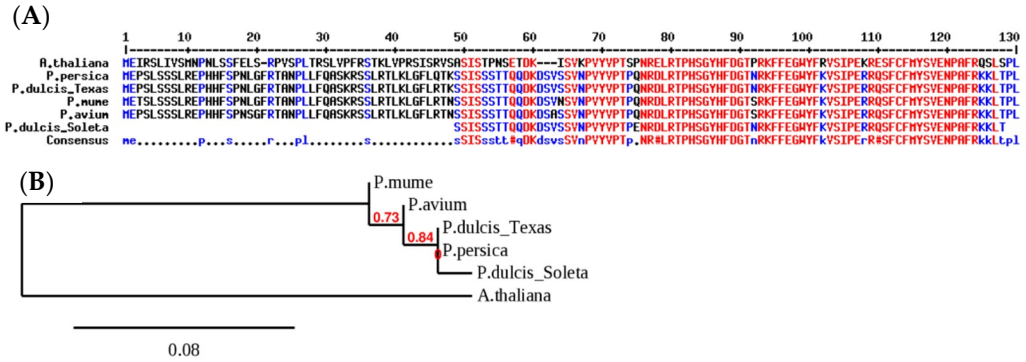

Figure 3. (**A**)—Protein alignment of VTE1 in different Prunus species and in *A. thaliana*. (**B**)—Phylogenetic tree of VTE1 proteins. *Prunus dulcis* cv. Soleta, *Prunus dulcis* cv. Texas (XP_034201471.1), *Prunus persica* (XP_007222342.1), *Prunus avium* (XP_021819281.1), *Prunus mume* (XP_008220061.1) and *Arabidopsis thaliana* (NP_567906.1).

4. Conclusions and Future Work

This study allowed the determination of the changes in tocopherol content throughout kernel development of almonds from 'Soleta' cultivar grown in Beja, Portugal. It is possible to conclude that the period of time where α-tocopherol has the biggest accumulation is between 86 and 116 DPA. Soil and climatic conditions of Alentejo may have influenced a higher accumulation of α-tocopherol in the commercial stage. For characterization of the candidate tocopherol cyclase gene, involved in tocopherol synthesis, the sequence of PdVTE1 transcript was identified in Soleta and a high similarity with other Prunus sequences was confirmed by sequences alignment and phylogenetic study. For better understanding tocopherol biosynthesis pathway, the differential expression level of candidate PtVTE1 by RT-qPCR is ongoing. This study expects to discover new insight into tocopherol biosynthetic pathway, which is revealed to be an important compound for determining the quality of the almond.

Funding: This work was funded by Inov-Amendo-AL: Microenxertia in vitro de amendoeiras selecionadas para a promoção do amendoal no Alentejo (ALT20-03-0246-FEDER-000068) supported by Program Alentejo 2020, through the European Fund for Regional Development (ERDF), within the scope of the Collective Action Support System—Transfer of scientific and technological knowledge. Domain of Competitiveness and Internationalization. Authors also acknowledge FCT for Contrato—Programa to L. Marum (CEECINST/00131/2018) and FCT for UIDB/05183/2020 and UIDB/04551/2020 (UIDP/04551/2020) projects.

References

1. Kodad, O.; Rafel Socias i Company; Alonso, J. Genotypic and Environmental Effects on Tocopherol Content in Almond. *Antioxidants* **2018**, *7*, 6, doi:10.3390/antiox7010006.
2. Socias i Company, R.; Felipe, A.J. Almond: A diverse germplasm. *HortScience* **1992**, *27*, 717–718, doi:10.21273/HORTSCI.27.7.718.
3. Rizvi, S.; Raza, S.T.; Ahmed, F.; Ahmad, A.; Abbas, S.; Mahdi, F. The role of vitamin E in human health and some diseases. *Sultan Qaboos Univ. Med J.* **2014**, *14*, e157–e165.
4. Oliveira, I.; Meyer, A.; Afonso, S.; Aires, A.; Goufo, P.; Trindade, H.; Gonçalves, B. Phenolic and fatty acid profiles, α-tocopherol and sucrose contents, and antioxidant capacities of understudied Portuguese almond cultivars. *J. Food Biochem.* **2019**, 43, e12887, doi:10.1111/jfbc.12887.
5. Zhu, Y.; Taylor, C.; Sommer, K.; Wilkinson, K.; Wirthensohn, M. Influence of deficit irrigation strategies on fatty acid and tocopherol concentration of almond (*Prunus dulcis*). *Food Chem.* **2015**, *173*, 821–826, doi:10.1016/j.foodchem.2014.10.108.

6. Sánchez-Pérez, R.; Jørgensen, K.; Olsen, C.; Dicenta, F.; Møller, B. Bitterness in Almonds. *Plant Physiol.* **2008**, *146*, 1040–1052, doi:10.1104/pp.107.112979.
7. Zhu, Y.; Wilkinson, K.; Wirthensohn, M. Changes in fatty acid and tocopherol content during almond (*Prunus dulcis*, cv. Nonpareil) kernel development. *Sci. Hortic.* **2017**, *225*, 150–155, doi:10.1016/j.scienta.2017.07.008.
8. Socias i Company, R.; Felipe, A. 'Belona' and 'Soleta' Almonds. *HortScience* **2007**, *42*, 704–706.
9. Hodel, R.G.; Zimmer, E.; Wen, J. A phylogenomic approach resolves the backbone of Prunus (Rosaceae) and identifies signals of hybridization and allopolyploidy. *Mol. Phylogenet. Evol.* **2021**, *160*, 107118.