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Pomegranate (*Punica granatum* L.) Fruits: Characterization of the Main Enzymatic Antioxidants (Peroxisomal Catalase and SOD Isozymes) and the NADPH-Regenerating System

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Abstract: Pomegranate (*Punica granatum* L.) is a common edible fruit. Its juice can be used as a source of antioxidative compounds, primarily polyphenols and vitamin C, in addition to other vitamins and minerals. Nevertheless, little is still known about how the enzymatic machinery, mainly that related to oxidative metabolism, is influenced by the genotype and the environmental and climate conditions where pomegranate plants grow. In this work, seeds and juices from two pomegranate varieties (Valenciana and Mollar) grown in two different Spanish locations were assayed. Both varieties showed clear differences in their respective polypeptide profiles. The analysis of the isoenzymatic superoxide dismutase (SOD) activity pattern displayed one Mn-SOD and five CuZn-SODs (I–V) whose abundances depended on the variety. Furthermore, by immunoblot assays, at least one additional Fe-SOD with a subunit size of about 23 kDa was also detected in both varieties. Besides this, the presence of the H_2O_2-scavenging peroxisomal catalase in seeds and juice indicates that an active metabolism of reactive oxygen species (ROS) takes place in this fruit, but the two pomegranate varieties showed opposite activity profiles. The activities of the main NADPH-regenerating enzymes, including glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphlogluconate dehydrogenase (6PGDH), NADP-dependent isocitrate dehydrogenase (NADP-ICDH), and NADP-dependent malic enzyme (NADP-ME), were studied in the same plant materials, and they behaved differently depending on the genotype. Finally, our data demonstrate the presence of two specific enzymes of the peroxisomal glyoxylate cycle, malate synthase (MS) and isocitrate lyase (ICL). These enzymes participate in oilseeds by channeling the lipid catabolism to the carbohydrate synthesis for further use in seed germination and early seedling growth. The results obtained in this work indicate that a similar mechanism to that reported in oilseeds may also operate in pomegranate.

Keywords: catalase; glyoxylate cycle; isozymes; juice; NADP-dehydrogenases; peroxisomes; SDS-PAGE; seeds; superoxide dismutase; varieties Valenciana and Mollar

1. Introduction

Pomegranate (*Punica granatum* L.) is a tree/shrub whose edible fruits have been used for centuries in human nutrition. Pomegranate has also been used as a symbol of life, fertility, immortality, and human-related activities, and has idiosyncratic features [1,2]. Pomegranate is predominantly cultivated in the Mediterranean area; southeastern Spain, which cultivates more than 2500 ha (mainly Alicante)

of pomegranate [3], is one of the main European producers [4], with production reaching close to 20,000 tm/year. Anatomically, pomegranate fruits are mainly composed of arils where the seeds are immersed, and all arils are integrated within the full fruit which is surrounded by a moderately lignified peel. The selection practices of this crop species have allowed for a number of varieties/genotypes able to adapt to and colonize diverse environments, thus covering a spectrum of organoleptic features [5–7].

The nutritional value given to pomegranate fruits resides in their high content of compounds with antioxidant properties, such as polyphenols (ellagic acid), flavonoids (particularly anthocyanins), vitamin C, and hydrolysable tannins, as well as fatty acids and proline, among others. This fruit also contains important levels of certain minerals such as potassium, calcium, phosphorous, magnesium, and sodium [1–4,7–12]. Accordingly, it is not rare that, since their properties were first discovered by humans, pomegranate fruits have been used as health sources due to their many medical applications [1,2,11–13]. Thus, extracts from pomegranate have been useful as potential treatments for inflammation, bacterial infections, healing wounds, neurodegenerative disorders, obesity, and diabetes mellitus, and also to protect from cancer and as effective chemotherapeutic agents with no toxic side effects [12–18].

On the other hand, besides these non-enzymatic antioxidants, a battery of enzymatic systems with antioxidative ability has been reported in pomegranate tissues under different conditions. Thus, the activity of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and other peroxidases (POX), as well as glutathione reductase (GR) enzymes were studied as indexes of cultivar and ripening stage [19], and also as partners involved in the response against chilling, salinity, ozone, irrigation stress, and aril senescence [20–24]. The activity detection of other redox enzymes, including phenylalanine ammonia-lyase (PAL) polyphenol oxidase (PPO), lipoxygenase (LOX), and phospholipase D (PLD) has been recently reported [20,21]. Those studies, however, did not use an analysis of the respective isoenzyme patterns as a way to regulate the expression of those enzyme systems depending on the prevailing growth conditions and/or the genotype, as reported elsewhere [25–29].

New high throughput technologies, such as proteomics by either bimensional electrophoresis (2-D) plus (Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight-Mass Spectrometry) MALDI-TOF-TOFMS or Isobaric Tag for Relative and Absolute Quantitation (iTRAQ) [30–33] or gene expression and metabolite profiling, have also allowed for widening the metabolic picture of pomegranate [33–35]. These approaches have revealed that the majority of the identified proteins participate in global processes such as carbohydrate metabolism and transport, energy production and conversion, posttranslational modifications, protein turnover, protein folding/unfolding, translation, ribosomal structure, anthocyanin biosynthesis, photosynthesis, flavonoid biosynthesis, phenylalanine and α-linolenic acid metabolism, peroxisome metabolism, phenylpropanoid biosynthesis, and biosynthesis of secondary metabolites [32,33]. However, those technologies have provided few data on proteins related to the oxidative metabolism promoted by reactive oxygen species (ROS).

Likewise, little information is available on enzymes which generate NADPH as part of the secondary antioxidative barrier. Thus, the coenzyme NADPH, besides being very important for cell growth and development and useful for multiple metabolic routes, is also a source of reducing power for the antioxidant response under diverse situations [36,37]. However, no data on NADPH metabolism in pomegranate have been reported thus far.

The expression and activity of enzymes given above, mainly those related to oxidative metabolism (SOD, CAT, APX, POX, and GR) and the NADPH-generating dehydrogenases depend on the genotype/variety/cultivar, and they are also susceptible to regulation by growth and developmental conditions. In fact, their activity and isoenzyme profiles are commonly used as markers of those situations. An investigation including all these marker enzymes would help to discriminate among the diverse pomegranate genotypes and also to understand how this species modulates its metabolism depending on the environmental conditions.

Accordingly, in this work, the isozyme pattern of SOD in pomegranate arils has been investigated by non-denaturing polyacrylamide gel electrophoresis (PAGE) and the activity profiles of catalase,

peroxidases, and the NADPH-generating dehydrogenases have been studied using two pomegranate cultivars grown in two Spanish locations with different climate conditions. Likewise, the activity of malate dehydrogenase, malate synthase, and isocitrate lyase has been determined, thus providing evidence for the involvement of the glyoxylate cycle in the metabolism of pomegranate seeds.

2. Materials and Methods

2.1. Plant Material

Pomegranate (*Punica granatum* L.) samples belonging to two varieties were obtained from two different locations in southern Spain which differ in their altitude and climate conditions. The first is the Valenciana variety from El Fargue, Granada (F-GR; latitude: 37.2079; longitude: −3.558597; altitude: 960 m; average temperatures: 16–23 °C (min.–max.); average precipitation: 0.2–26.9 mm (min.–max.)). The second is the Mollar variety from Elche, Alicante (E-A; latitude: 38.265318; longitude: –0.698849; altitude: 81 m; average temperatures: 11–26 °C (min.–max.); average precipitation: 4.8–56.3 mm (min.–max.)). Arils from 3–5 fruits of each variety/location were taken and their respective seeds and juices were processed independently for further analyses.

2.2. Determination of Brix and pH from Juices

The Brix degree was measured at 20 °C using a refractometer Atago (Atago CO, Ltd., Tokyo, Japan) and was expressed as sucrose percentage. The pH was determined in juices using wide range pH-test strips.

2.3. Preparation of Crude Extracts

Arils were deposited on two nylon-cloth layers and squeezed manually by twisting the tissue over a beaker placed on ice. The juice was then centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was taken and, without any addition of buffering solutions, it was frozen under liquid nitrogen and stored at −80 °C until further use. For processing of seeds, aril membranes were removed after squeezing, and seeds were thoroughly washed under tap water and then with distilled water. Afterwards, seeds were pulverized under liquid nitrogen using a grinder IKA®A11 Basic (IKA®, Staufen, Germany). The obtained power was homogenized in 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 10% (v/v) glycerol, 5 mM DTT (ratio 1:4, plant material:buffer). Homogenates were filtered through two layers of nylon-cloth, centrifuged at 15,000 g for 15 min (4 °C) and stored at –80 °C until use.

2.4. Enzyme Activity Assays

Catalase (CAT; EC 1.11.1.6) activity was recorded by following the H_2O_2 break-down at 240 nm according to Aebi [38] and using a molar extinction coefficient, ε = 39.58 $M^{-1}{\cdot}cm^{-1}$. For the determination of the ascorbate peroxidase (APX, EC 1.11.1.11) activity, the method based on the ascorbic acid oxidation at 290 nm was assayed, with ε = 26.6 $mM^{-1}{\cdot}cm^{-1}$ as the molar extinction coefficient [39]. Peroxidase (POD; EC 1.11.1.7) activity was determined at 470 nm by plotting the guaiacol oxidation and using ε = 26.6 M^{-1} cm^{-1} [40]. The activity of the NADPH-generating enzymes glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44), NADP-isocitrate dehydrogenase (NADP-ICDH; EC 1.1.1.42), and NADP-malic enzyme (NADP-ME; EC 1.1.1.40) was spectrophotometrically determined following the reduction of NADP at 340 nm, with $\varepsilon = 6.22 \times 10^3$ M^{-1} cm^{-1}. All assays were carried out in 50 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid HEPES, pH 7.6, using glucose-6-phosphate, 6-phosphogluconate, 2R,3S-isocitrate, and L-malate as substrates, respectively, as reported earlier [41–43]. Likewise, the NAD-dependent malate dehydrogenase (MDH; EC 1.1.1.37) activity was assayed by recording the formation of NADH at 340 nm ($\varepsilon = 6.22 \times 10^3$ $M^{-1}{\cdot}cm^{-1}$) with L- malate as substrate, but using NAD as cofactor [41].

The peroxisomal glyoxylate cycle enzymes isocitrate lyase (ICL; 4.1.3.1) and malate synthase (MS; EC 2.3.3.9) were also analyzed in pomegranate samples. For ICL, the method consisting of the detection of the glyoxylate-phenylhydrazine hydrochloride complex formation at 324 nm (ε = 1.74×10^4 $M^{-1} \cdot cm^{-1}$) was followed [44,45]. MS activity was assayed by the method which allows for combining coenzyme A (CoA) released from acetyl-CoA, due to the action of the enzyme, with dithiobis nitrobenzoic acid (DTNB) to form the colored species TNB, which absorbs at 412 nm (ε = 13.6 $mM^{-1} \cdot cm^{-1}$) [45,46].

Finally, the activity of the acyl-CoA oxidase (ACOX; EC 1.3.3.6), the first enzyme of the fatty acid β-oxidation, was achieved by following the oxidation of antipyrine at 500 nm ($\varepsilon = 6.4 \times 10^{-3}$ $M^{-1} \cdot cm^{-1}$) in the presence of FAD and *p*-hydroxymercuribenzoate (*p*HMB), and using palmitoyl-CoA as substrate [45,47].

Protein concentration was determined by the method of Bradford [48], with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) solution and bovine serum albumin as standard.

2.5. Non-Denaturing Electrophoresis and SOD Detection in Gels

For separation of SOD (EC 1.15.1.1) isozymes non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed in 10% acrylamide gels. After proteins were separated, isozymes were visualized in gels by the photochemical nitroblue tetrazolium (NBT) reduction method [45,49]. To identify the different isozymes, before staining the pre-incubation of gels in the presence of specific inhibitors was carried out, with either 5 mM KCN of 5 mM H_2O_2. Cyanide inhibits copper, zinc-containing SODs (CuZn-SODs), and H_2O_2 is able to inhibit CuZn-SODs and iron-containing SODs (Fe-SODs), whereas Mn-SODs are resistant to both chemicals.

2.6. SDS-PAGE and Western Blotting

Polypeptides from pomegranate samples were separated by SDS-PAGE on 12% acrylamide [50] and afterwards detected in gels by a silver staining method [51]. Prestained Precision Plus Protein Standards (Dual Color, Bio-Rad) ranging from 7 to 250 kDa were used as molecular size markers.

For immunoblots, proteins were separated by SDS-PAGE as above and then transferred onto PVDF membranes using a Trans-Blot SD (Bio-Rad) equipment and 10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), pH 11.0, as transfer buffer [52]. An antibody against pepper Fe-SOD (dilution 1:1000), obtained and characterized in our laboratory [53–55], was used and the detection of the antibody-recognizing proteins was achieved using the Clarity^{TM} Western ECL Substrate (Bio-Rad) kit following the manufacturer's instructions.

3. Results

In this work, the antioxidative enzyme profiles of two pomegranate varieties that are grown in two Spanish cultivation regions with different climate conditions have been studied. The two pomegranate varieties had almost the same Brix values of about 15–15.8%, whereas the pH of their juices was 3–4 in both F-GR (Valenciana) and E-A (Mollar de Elche).

As shown in Figure 1, the analysis of the polypeptide pattern in pomegranate seeds from both populations apparently showed similar profiles, although some bands displayed differential expression. Thus, whereas polypeptides P1, P9, and P10 were more abundant in seeds from F-GR, P2–P8 and P11 were present in higher amounts in seeds from the population E-A which is closer to sea level. This electrophoretic analysis was also done with their respective juices directly obtained after squeezing. As also depicted in Figure 1, juice from F-GR contained much lower protein content than that from E-A pomegranates. In spite of that, polypeptide P12 was more abundant in F-GR juice. The figure also shows that, although the patterns in seeds and juice from E-A samples were qualitative close, the polypeptide content varied, with P3, P9, and P11 being considerably prominent in juice. In Table 1, the differential expression of the polypeptides detected in the seeds from the two pomegranate populations together with their respective molecular sizes are given.

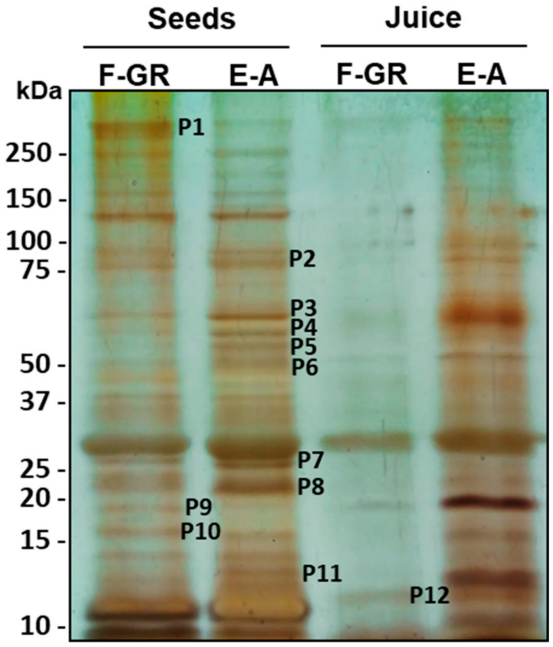

Figure 1. Polypeptides detected in seeds and juice from two pomegrante Spanish populations. SDS-PAGE was run on 12% acrylamide gels, and polypeptides were detected by silver staining. Two pomegranate populations from Spain were studied: from El Fargue, Granada (F-GR) and Elche, Alicante (E-A). Differentially abundant polypeptides are labelled (P1-P12). For seed samples, 13.8 of µg protein was loaded. For juice samples, 25 µl were directly used after incubating with sample buffer. Molecular markers are displayed on the left.

Table 1. Polypeptides detected by SDS-PAGE showing differential abundance in seeds from two pomegranate Spanish populations. Pomegranate fruits were collected from El Fargue, Granada (F-GR) and Elche, Alicante (E-A). The polypeptide molecular weights are displayed in parentheses. Marks show where polypeptides were more abundant.

Polypeptide (Molecular Weight)	F-GR	E-A
P1 (> 250 kDa)	√	
P2 (87)		√
P3 (67)		√
P4 (64)		√
P5 (60)		√
P6 (50)		√
P7 (25)		√
P8 (22)		√
P9 (18)	√	
P10 (16)	√	
P11 (13)		√

Regarding the SOD activity detected in non-denaturing gels, 6 different isozymes were observed in seeds from both F-GR and E-A locations (Figure 2A). The incubation with specific inhibitors showed that all isozymes excepting the slower one was inhibited by both KCN (Figure 2B) and H_2O_2 (Figure 2C) and, therefore were assigned to the CuZn-SOD type. Thus, according to their increasing electrophoretic mobility, the SOD isozymes were designated as Mn-SOD and CuZn-SOD I–V (Figure 2A). No isozymes were detected in juice from either F-GR or E-A samples (results not shown). As depicted in the figure, Mn-SOD and CuZn-SODs I and II were more abundant in seeds from the E-A location. Most plant tissues contain at least one Fe-SOD, although in this work, we were not able to detect any Fe-SOD activity in pomegranate. So, in order to confirm the absence of this isozyme, western blotting assays

using an antibody against Fe-SOD from pepper (*Capsicum annuum*) fruits [54], (also characterized in other plant species like *Olea europaea* [53] and *Cakile maritima* [55]), were carried out. Interestingly, one cross-reacting band of about 23 kDa was detected in all seeds and juices from F-GR and E-A samples. Besides, in seeds from E-A a smaller band of about 20 kDa was found (Figure 2D).

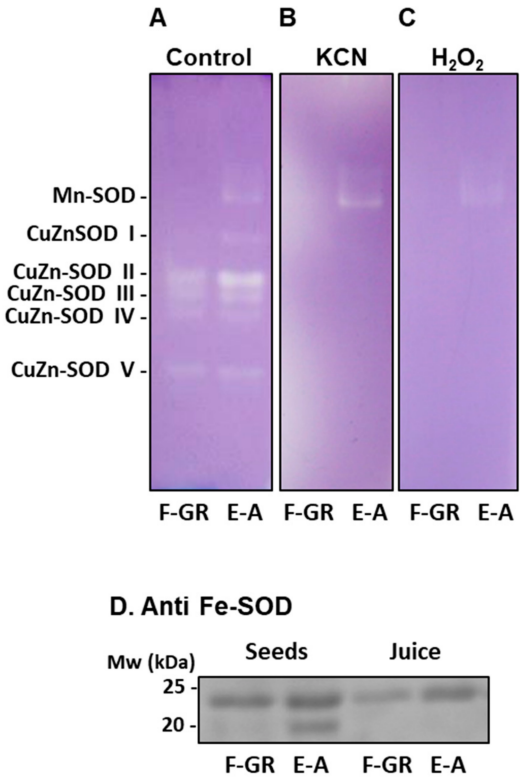

Figure 2. Superoxide dismutase (SOD) isozymes from seeds of two Spanish pomegranate populations. SOD activity was analyzed by non-denaturing PAGE on 10% acrylamide gels. After electrophoresis, enzyme activity was detected in gels by the NBT photochemical method under control conditions (**A**) and under the presence of specific inhibitors, either 5 mM KCN (**B**) or 5 mM H_2O_2 (**C**). One Mn-SOD and five CuZn-SODs (I–V) were identified. (**D**) Western blotting was carried out using an antibody against Fe-SOD from pepper fruits (dilution 1:1000). Molecular weights are indicated on the left. Pomegranate fruits from El Fargue, Granada (F-GR) and Elche, Alicante (E-A) were analyzed.

Three enzymes involved in H_2O_2 scavenging were investigated in this work: catalase, ascorbate peroxidase, and peroxidase. CAT displayed opposite behavior in the two aril parts analyzed. Thus, whereas in seeds the activity was significantly higher in samples from E-A with respect to F-GR seeds, in juice, the activity in pomegranate samples E-A was lower (Figure 3). Both seeds and juice showed activities in the same order of magnitude. Neither APX nor POD activity could be detected by either spectrophotometric assay or by native PAGE in any on the samples. Regarding NADP-dehydrogenases (G6PDH, 6PGDH, NADP-ICDH, and NADP-ME), it was found that all activities were mostly higher in E-A samples than in F-GR fruits, with juice being the tissue which displayed the greatest values (Figure 4). This was especially visible for G6PDH which showed much higher activity in E-A juice than in the other samples analyzed (Figure 4A). The NADP-ME activity was almost undetectable in seeds from F-GR (Figure 4C).

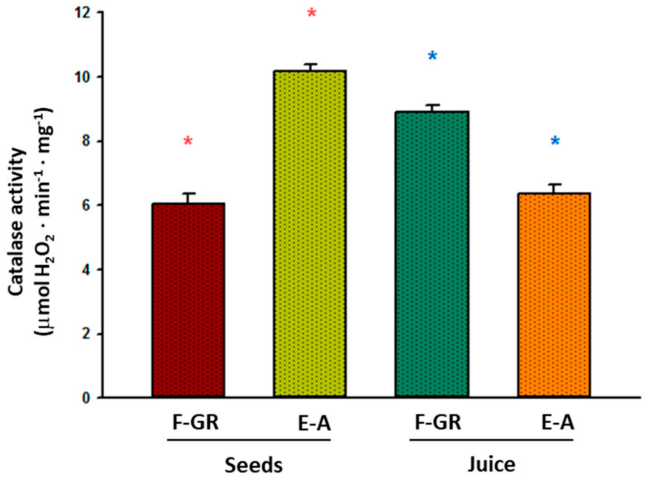

Figure 3. Catalase activity in seeds and juice from two Spanish pomegranate populations. Pomegranate fruits from El Fargue, Granada (F-GR) and Elche, Alicante (E-A) were analyzed. Colored asterisks depict significant differences ($p < 0.05$) between either seed (red) or juice (blue) samples according to the Student's *t*-test.

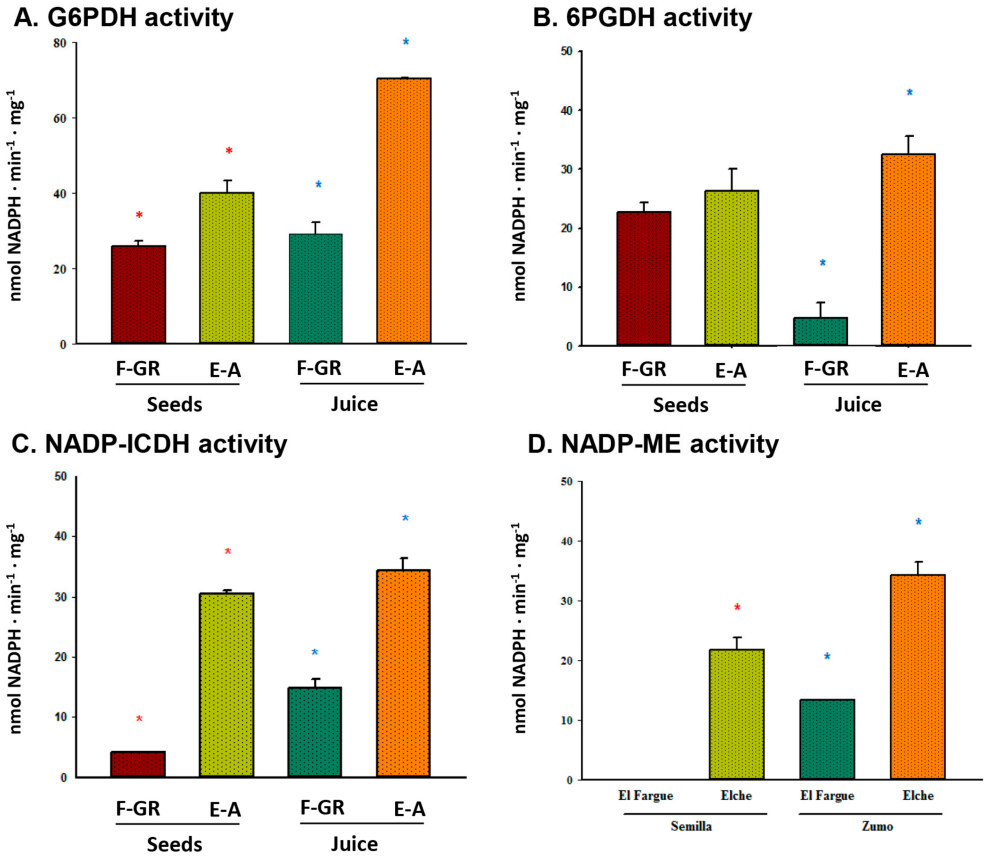

Figure 4. Activity of NADP-generating dehydrogenases in seeds and juice from two Spanish pomegranate populations. Pomegranate fruits from El Fargue, Granada (F-GR) and Elche, Alicante (E-A) were analyzed. (**A**) glucose-6-phosphate dehydrogenase (G6PDH); (**B**) 6-phosphlogluconate dehydrogenase (6PGDH); (**C**) NADP-dependent isocitrate dehydrogenase (NADP-ICDH); (**D**) NADP-dependent malic enzyme (NADP-ME). Colored asterisks depict significant differences ($p < 0.05$) between either seed (red) or juice (blue) samples according to the Student's *t*-test.

The activity profile of two peroxisomal glyoxylate cycle enzymes ICL and MS was also investigated. They showed opposite behavior. Thus, while ICL was higher in pomegranate from both locations, MS activity was almost negligible in that material (Figure 5). The highest ICL activity was determined in juice from E-A fruits (Figure 5A). Conversely, the highest MS activity was detected in seeds, mainly in those from F-GR (Figure 5B). Interestingly, another malate-metabolizing enzyme, the MDH, whose role involves both the glyoxylate and the tricarboxylic acid cycles, displayed similar activity pattern than the ICL reported above, with the greatest value being obtained in juice from E-A samples and very little activity in seeds from both locations (Figure 5C). Attempts to measure the β-oxidation ACOX enzyme were made, but no activity was detected.

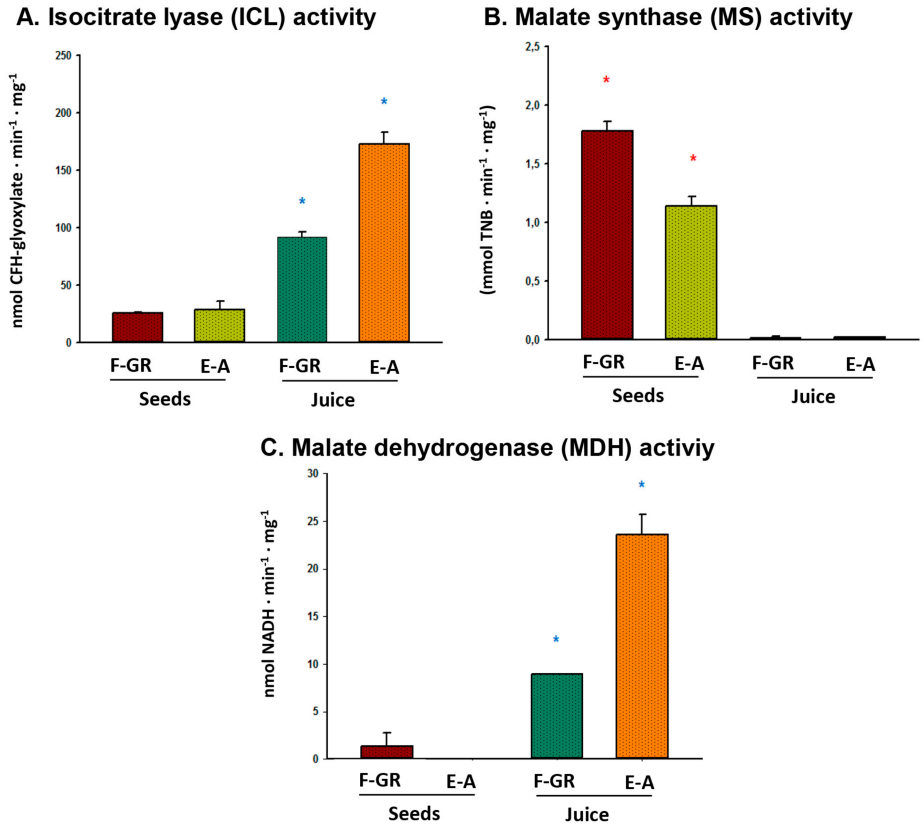

Figure 5. Activity of the peroxisomal glyoxylate cycle enzymes and malate dehydrogenase in seeds and juice from two Spanish pomegranate populations. Pomegranate fruits from El Fargue, Granada (F-GR) and Elche, Alicante (E-A) were analyzed. (**A**) Isocitrate lyase (ICL); (**B**) malate synthase (MS); (**C**) malate dehydrogenase (MDH). Colored asterisks depict significant differences ($p < 0.05$) between either seed (red) or juice (blue) samples according to the Student's *t*-test.

4. Discussion

This work has been focused to determining the antioxidative enzyme profiles which could be used as differential traits of pomegranate varieties that, due to their genetic and acclimation backgrounds, grow and develop in distinct topographical and climate conditions. The present research provides data on the protein patterns as well as on the activity of diverse antioxidative enzymes in two pomegranate varieties grown in two different environments. Early studies carried out on the protein content of pomegranate revealed that the major identified proteins corresponded to storage proteins, mainly albumins, globulins, prolamines, and glutelins [56]. Contemporary works that applied 2-D/LC/MS (2-D/Liquid Chromatography/Mass Spectrometry) and HPLC/MS (High Performance Liquid Chromatography/Mass Spectrometry) approaches reported a battery of proteins including class III-chitinases, selenium-binding proteins, anthocyanidin synthase, chalcone synthase, cinnamyl alcohol

dehydrogenase and flavonol 3-O-glucosyltransferase and other non-storage proteins [30,57,58]. Later, other proteins related to ROS and secondary metabolism were reported [19–24]. Thus far, although the new throughput approaches (mainly iTRAQ and RNA-seq) are contributing to increasing knowledge on the background of pomegranate metabolism [30–35], more research is necessary since these technologies provide data on the gene transcript levels and their corresponding translational products, respectively. However, post-translational events indicate that there is no direct correspondence between transcriptomic and proteomic data and the activity displayed by proteins. Thus, the dynamics and functionality of these proteins (mainly enzymes) have to be investigated to finally understand how the pomegranate enzymatic machinery operates throughout the developmental processes and under unfavorable conditions. In the present work, the activity profile of diverse enzymatic systems involved in ROS and NADPH metabolism and in the peroxisomal glyoxylate cycle of pomegranate arils was determined. The detection of several enzymes, e.g., CuZn-SODs, G6PDH, NADPH-ICDH, and ICL, still not described in this species earlier, is reported here for the first time. Besides, the potential influence of the geographic localization, mainly the altitude and the climatologic agents, in the activity profiles reveals how the metabolic pathways investigated here can be modulated depending on the genotype/variety/cultivar and the natural environmental conditions.

Thus, the electrophoretic analysis of the seed and juice samples in both populations provide a high number of differentially abundant polypeptides, which might be associated to the distinct genotype and to the adaptation of plants to two environments, Alicante and Granada, which differ by about 900 m in altitude, thus affecting temperature, relative humidity, and light intensity, among other aspects. Our data also show the protein content in juice, mainly that from E-A. To date, pomegranate juice has mostly been considered as an enriched source of health-beneficial low molecular weight compounds such a vitamins, antioxidants, tannins, and others [7,58–62]. However, our data demonstrate that the juice is also rich in proteins, which might also influence the organoleptic and nutritional features of this agricultural product. However, the identification of these proteins from pomegranate juice and their beneficial effect need to be addressed in the future.

Likewise, the SOD isoenzimatic pattern displayed important differences depending on the genotype/geographic distribution of samples. Both pomegranate populations share the same isozyme profile but the activity of some of them was much higher in E-A seeds than in F-GR. In previous works, it was found that SOD activity was used as index of the cultivar and ripening stage, and it was described that this enzyme also participated in the response against diverse unfavorable conditions [14–19,55]. Recently, by iTRAQ analysis, the presence of a SOD from the Fe,Mn family has been reported [32], but no CuZn-SODs have been described so far. The identification of at least, five CuZn-SOD isozymes in pomegranate fruits is shown in this work. Also, the data presented here on western blotting using an antibody against Fe-SOD from pepper fruits [54] confirm the existence of such isozyme in this plant material. The molecular weight obtained in the immunoblot assays of about 23 kDa ranges that of other Fe-SODs described in plants [63,64] and is similar to the immunoreactive band found in olive leaves and *Cakile maritima* roots and leaves using this Fe-SOD antibody [53,55]. The absence of Fe-SOD activity in gels suggests that this isozyme could be post-translationally modulated or that the activity is too low to be detected in gel assays, but further specific experiments have to be performed to confirm this.

Due to the multiplicity of the CuZn-SOD isozymes, our results suggest relevant ROS metabolism in pomegranate seeds which may involve diverse cell organelles, as this enzymatic system is mainly located in cytosol, chloroplasts, and peroxisomes [63,64]. This relevance is also supported by the detection of peroxisomal catalase activity in this plant material. CAT, which is the main H_2O_2 scavenging system in plants, has been used in the same situations given above for pomegranate SOD [19–24]. However, unlike SOD, CAT was detected in both seed extracts and juice. This seems logical considering that seed germination involves the mobilization of fatty acids which involves high H_2O_2 production due to the peroxisomal β-oxidation [65].

Globally, our data on the enzymes from the ROS metabolism confirm that both SOD isozymes and peroxisomal CAT could be used as indexes to evaluate the response against diverse topologies, including latitude, altitude and temperature and precipitation conditions.

NADPH is an essential cofactor for cell growth and development and necessary in multiple metabolic pathways including sugar biosynthesis through the Calvin cycle, fatty acid and carotenoid biosynthesis (vitamin A), aromatic amino acid (Phe, Tyr, and Trp) and proline biosynthesis, and ribonucleotide (RNA) conversion to deoxy-ribonucleotide (DNA). NADPH is also involved in cell detoxification processes providing reducing power to the ascorbate-glutathione cycle, NADPH-dependent thioredoxin reductases (NTRs), NADPH oxidase and arginine-dependent nitric oxide (NO) synthase activity [36,37,41–43]. To our knowledge, the only reference which reports the presence of NADPH-generating enzymes in pomegranate was obtained by iTRAQ analysis, where one 6PGDH and one NADP-ME were detected [32]. These same authors also found an isocitrate dehydrogenase, but it was the NAD-depending enzyme. In the present work, besides 6GPDH and ME, the enzyme activity of G6PDH and NADP-ICDH could be determined, and their response under the two environmental conditions where pomegranate plants developed was followed. However, the information about the metabolic pathways where NADPH participates and how they are regulated is still very low. More research needs to address this issue to understand the roles played by the NADPH-generating enzymes in the physiology of pomegranate fruits.

The peroxisomal glyoxylate cycle is a metabolic pathway which mainly occurs in cotyledons from oilseeds and in senescent leaves [66–68], although it has been also shown in pepper fruits [36]. Two of the enzymes participating in this cycle are malate synthase (MS) and isocitrate lyase (ICL), which were proved to operate in both pomegranate seeds and juice. Recently, the MS protein was reported in pomegranate after iTRAQ assays [32]. In the latter paper, MDH was also scrutinized and its presence in this plant crop was confirmed in the present work by following its enzymatic NAD-depending activity. MDH is an enzyme with multiple isozymes which participate in the mitochondrial tricarboxylic acid cycle and in the peroxisomal glyoxylate cycle. In this latter organelle, MDH functions down-cycle the ICL and previous to the MS action. Therefore, and according to the data depicted in this work, a possible regulation at the MS level seems to occur. In oilseeds, the functioning of the glyoxylate cycle is associated to the fatty acid β-oxidation, both processes taking place in peroxisomes. Thus, the β-oxidation provides the acetyl-CoA necessary for the malate synthase activity. This link of the two pathways allows channeling the fatty acid oxidation towards the synthesis of new carbohydrates, necessary for emerging and developing seedlings. The first step of the fatty acid β-oxidation is performed by the enzyme acyl-CoA oxidase (ACOX), which catalyzes the oxidation of fatty acid, previously activated on binding to CoA by an acyl-CoA synthetase (ACOS). Thus, although we could not be able to detect ACOX activity, the presence of both, an ACOX recently reported [32], and the glyoxylate cycle displayed here leads to think that a similar mechanism to that running in oilseeds and in pepper fruits could be also operative in pomegranate fruits.

5. Conclusions

This work reports some data on the functionality of some metabolic pathways and mechanisms of high physiological relevance for pomegranate fruits. These involve ROS metabolism, NADPH regeneration and potential synthesis of carbohydrates through the peroxisomal glyoxylate cycle, all of them being fundamentals for seed emergency and growth and development of future seedlings. The broad number of SOD isozymes and their multiple subcellular localization reported in other plant species [63,64] suggest a very active ROS metabolism at different cell levels and lead us to think about the relevance of the antioxidative (both enzymatic and non-enzymatic) metabolism in this fruit. The NADPH metabolism seems to be also potential target for future studies on pomegranate due to the importance of this nucleotide in the plant redox metabolism. The presence of the glyoxylate cycle enzymes (a pathway totally located in peroxisomes), catalase activity (a peroxisomal marker enzyme), one Mn-SOD, and possibly a Fe-SOD (as typical SOD isozymes which are present in

peroxisomes [63,64,69], assign to these organelles a central role in the physiology of pomegranate fruits which deserves to be investigated further. In juice, enzyme activities were directly assayed in the squash obtained after squeezing arils without any addition of buffering and/or stabilizing substances. This means that, considering the pH of juices and their brix values, enzyme proteins seem to be under stable conditions maintaining their functionality for potential further roles during seed germination. Finally, the distinct enzymatic activity profiles reported for the two pomegranate varieties studied in this work provide evidence that the parameters assayed could be used as differential traits associated to genotypes and the growing environmental conditions. Further research based on the analysis of proteins from pomegranate juice will bestow an additional value to this product.

Author Contributions: M.P., J.I.M. and M.J.C. performed the experimental works. F.J.C. discussed the data and participated to write the manuscript. J.M.P. designed the research, discussed the data, and participated in writing the manuscript.

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