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ORIGINAL PAPER

Study of pomological traits and physico-chemical quality of pomegranate (*Punica granatum* L.) genotypes grown in Italy

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Abstract
Pomegranate is considered a functional food but several local accessions and cultivars are widespread in different countries. The characterization of local germoplasm allows to identify genotypes that possess the highest nutraceutical value compared to standard cultivars (cvs.) and that are well-adapted to local climatic conditions and could be used in the breeding programs. The aim of this study was the characterization of pomological and physico-chemical traits as well as antioxidant system in local pomegranate accessions ('Mondrone Dolce', 'San Pietro', 'Granato' and 'Roce'), comparing to an Italian ('Dente di Cavallo') and international cvs. ('Wonderful'). A high variability of the pomological traits resulted among the cultivars. 'Wonderful' showed the highest value of anthocyanins (554.99 ± 0.05 mg C_3gE L^{-1}), total phenols (1494.00 ± 116.20 mg GAE L^{-1}) and antioxidant activity (EC_{50} values 21.21 ± 0.05 µL mL^{-1}), whereas 'Granato' had the highest values among local accessions. Furthermore, the antioxidant enzymes activities varied with genotypes. Principal component analysis revealed great differences in all investigated parameters among pomegranate genotypes. 'Mondrone Dolce', 'San Pietro' and 'Dente di Cavallo' showed similar pomological and nutraceutical traits compared to 'Granato' and 'Roce'. Conversely, 'Wonderful', due to its peculiar traits, revealed significant differences with respect to other genotypes.

Keywords Pomegranate · Germoplasm · Anthocyanins · Phenols · Antioxidant activity · Enzymes activity

Introduction

Pomegranate (*Punica granatum* L.) is a temperate climate species, mainly cultivated in the Mediterranean area, Southern Asia, and several countries of North and South America. It belongs to the monogeneric family *Punicaceae*, subclass *Rosidae*, believed to be native to the region between Iran and northern India [1]. It is one of the oldest cultivated species among fruit trees. The name pomegranate comes from the Latin "pomum" meaning "apple" and "granatus" meaning "full of seeds". The edible parts of pomegranate are the arils, which are seeds covered by a red pulp, that is a juice sac, called sarcotesta. The arils are surrounded by the mesocarp, or albedo, a white fleshy substance separating the arils from the fruit peel [2]. The arils are a source of bioactive compounds such as flavonoids, phenolic acids and vitamin C and, for this reason, the fruit has gained popularity due to its nutritional value for human health. It is considered a functional food because it might induce health benefits against cancer, cardiovascular and other health problems [3]. Pomegranate genotypes differ in sensory and agronomic characteristics and in their application in food processing in terms of sweetness–sourness, soft–hard seeds and juiciness [4]. The arils can be consumed fresh or in the preparation of juices, jellies, jams and colorings for drinks.

However, despite the impressive growth of its market, pomegranate is still an underutilized species if compared to other domesticated fruit crops. In particular, the Italian germplasm i has been scarcely studied, despite the wide distribution of *P. granatum* in many rural areas and the presence of various genotypes, in particular in Southern Italy [5–7].

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At present, few studies provide pomological and physico-chemical data on ancient and local accessions [5–8]. Thus, the objective of the present study was aimed to investigate the pomological and physico-chemical properties of four local germplasm accessions, identified in Campania region (Italy) ('Mondrone Dolce', 'San Pietro', 'Granato' and 'Roce') and an Italian cv. 'Dente di Cavallo' compared with the known international cv. 'Wonderful' to identify any relationships that could exist in all the traits of the fruit. The results of this study will contribute to the evaluation of pomegranate biodiversity, assist with future breeding efforts, and allow us to detect accessions with high fruit quality attributes for potential commercial production.

Materials and methods

Plant materials

Six different pomegranate (*P. granatum* L.) genotypes were selected for this study: four local germplasm accessions, identified in Campania region (Italy) ('Mondrone Dolce', 'San Pietro', 'Granato' and 'Roce') and an Italian cv. 'Dente di Cavallo' were compared with the international cv. 'Wonderful' (Fig. 1a–f). The pomegranates accessions 'Mondrone Dolce' and 'San Pietro' and cvs. 'Dente di Cavallo' and 'Wonderful' were grown in the same experimental orchard in Caserta (Caserta, Italy; 41°04′N, 14°19′E with an altitude of 61 m above sea level and the average annual rainfall of 650 mm), at the CREA-Research Centre for Olive, Fruit Tree

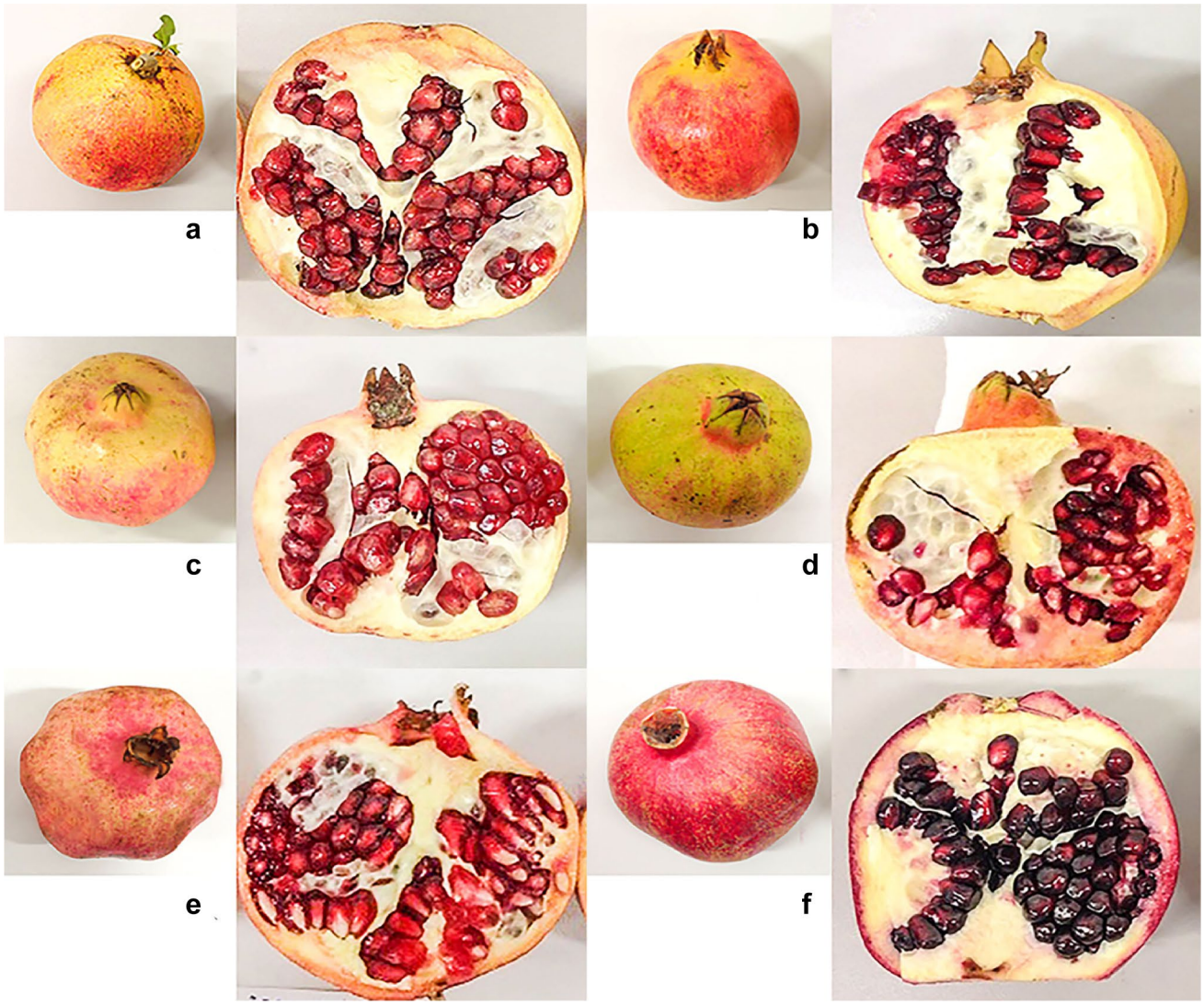

Fig. 1 'Mondrone Dolce' (**a**), 'San Pietro' (**b**), 'Dente di Cavallo' (**c**), 'Granato' (**d**), 'Roce' (**e**), 'Wonderful' (**f**) pomegranate genotypes

and Citrus, whereas 'Granato' and 'Roce' accessions were grown in a local farm Giovomel (Aiello del Sabato, Avellino, Italy; 41°53′N, 14°49′E with an altitude of 404 m above sea level and the average annual rainfall of 850 mm). The samples were harvested, from five adult trees (~ 10-year-old) as suggested by Sarckhosh et al. [9], in October at the commercial ripening stage in agreement with Ferrara et al. [7], transported to the laboratory, screened for uniformity, appearance, and the absence of physical defects or decay and stored at 4 °C for 24 h. To minimize the environmental effects on all of the agronomic and qualitative traits, the data were collected across two consecutive years (2015–2016).

Pomological characterization of fruit

Twenty fruit from each genotype (four per plant) were used to determine the pomological and qualitative traits according to the International Union for the Protection of New Varieties of Plants [10]. The weight of each fruit, aril and tegument was determined on a precision digital balance with an accuracy of 0.001 g (Practum 213-1S, Sartorius, Goettingen, Germany). The length and width of each fruit, the length of calyx crown, thickness of skin, the length and width of aril, the length and width of tegument were determined by an electronic digital caliper (PCE-DCP 300, PCE Instruments, Lucca, Italy). Fifty arils were used for each genotype.

Physico-chemical analyses

Sample preparation

The selected fruits were initially washed in 100 ppm NaOCl solution followed by cold distilled water, drained and then cut. The arils were hand-separated from the skin and pith and collected to form a homogeneous mixture for each genotype. The juice was extracted by squeezing the pomegranate arils using electric extractor (HR1869/80, Philips, Amsterdam, Netherlands) and it was used for the study of physico-chemical properties.

Titratable acidity, pH, total soluble solids, maturity index and reducing sugars

The juice was separated in two aliquots: one was immediately frozen at − 20 °C for HPLC analysis and on the other aliquot of the juice, the main physico-chemical characteristics were measured as described below.

Titratable acidity (TA), expressed as g of citric acid equivalent per L of juice (g citric acid L^{-1}), was determined by acid-base titration of the fruit juice (10 mL) with NaOH 0.1 N to the end point of pH 8.2 using a digital pH meter (Model 2001, Crison, Barcelona, Spain). Furthermore, the pH value of each genotype was estimated on fresh juice using the same digital pH meter at 20 °C.

Total soluble solids (TSS) content was measured by hand refractometer (Model N-10; Atago, Tokyo, Japan). Measurements were conducted at 20 °C, and results expressed as °Brix.

The maturity index (MI) was calculated for each sample by TSS/TA ratio, after converting the TA values in percentage. Reducing sugars were evaluated by Felhing assay, according to previous established methodologies [11]. This method is a volumetric method to define the reducing sugars in food. A mixed Fehling's solution and methylene blue as indicator were used in titration. Fehling solution A is made from copper (II) sulfate pentahydrate ($CuSO_4$, $5H_2O$) dissolved in water and Fehling solution B contains potassium sodium tartrate tetrahydrate (Rochelle salt) ($KNaC_4H_4O_6$, $4H_2O$) and sodium hydroxide (NaOH) in water. 5 ml of Fehling A and 5 ml of Fehling B were put into a 200 mL Erlenmeyer flask and 40 ml of water were added. This mixture was placed in a small furnace and heated to the boiling point in the presence of a few glass beads to prevent bumping. A 25 mL burette was filled up with diluted sample. From the burette the sample solution was added slowly to mixed Fehling's solution as boiling continued. When the colour was becoming brick red, three drops of methylene blue indicator was added and the titration was continued. The titration was finished when the colour was became strong brick red or cherry red. The results were expressed in g of reducing sugars in 100 mL of juice.

Total phenols and individual phenolic compounds

The total phenols (TP) were quantified using Folin–Ciocalteu assay [12–15]. The juices were centrifuged at 5000×*g* for 10 min and an adequate dilution of the juice was mixed with the Folin–Ciocalteu reagent in sodium carbonate solution (20% w/v), stored in the dark for 2 h. The absorbance was read at 765 nm using a UV–Vis spectrophotometer (Lambda Bio 40; Perkin Elmer, Waltham, MA, USA). Calibration curve, with a concentration range between 50 and 350 mg of gallic acid L^{-1}, were used for the quantification of TP ($y = 0.0011x - 0.0031$; R^2 0.9858). The results were expressed as mg of gallic acid equivalents per L of juice (mg GAE L^{-1}).

For the analysis of individual phenolic compounds by HPLC-DAD, pomegranate juices were centrifuged at 8000×*g* for 10 min and filtered through 0.22 µm filter (Millipore). Samples were diluted 1:2 with water/metanol mixture (1:1 v:v) and analyzed by HPLC system (Agilent 1200 series, Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a diode array detector. The separation was carried out with an analytical column Sperisorb S5 ODS2

(250×4.6 mm). The diode array detector was set at an acquisition range of 200–600 nm.

Individual phenolic compounds were quantified using calibration curves of the respective reference compounds. For this purpose, stock solutions (1.000 mg L^{-1}) were diluted to concentrations of 0–100 mg L^{-1} gallic acid, protocatechuic acid, punicalagin and o-coumaric acid, respectively.

When reference compounds were not available, the calibration was based on structurally related substances, using a molecular weight correction factor [16] (gallic acid for galloyl hexose). The injection volume for all samples was 50 µL. The mobile phase consisted of 0.1% (v/v) trifluoroacetic acid in water (eluent A) and of 0.1% (v/v) trifluoroacetic acid in acetonitril (eluent B). The flow rate was 1.3 mL min^{-1}, and the gradient with eluent B was optimized as follows: 2–7% linear gradient in 5 min, 7–8% linear gradient in 2 min, 8% isocratic for 6 min, 8–12% linear gradient in 8 min, 12–17% linear gradient in 5 min, 17–25% linear gradient in 5 min, 25–35% linear gradient in 10 min, 35–100% linear gradient in 1 min and 100% isocratic for 5 min. Total run time was 47 min. Simultaneous monitoring was performed at 280 nm and 320 nm.

Total anthocyanins content and antioxidant activity

Total anthocyanins content and antioxidant activity were determined on the juices after a centrifugation at 5000×*g* for 10 min. Total anthocyanins content (TAC) was quantified spectrophotometrically according to Adiletta et al. [4] using the pH differential method. Two buffer systems comprising of potassium chloride (pH 1.0, 0.025 M) and sodium acetate (pH 4.5, 0.4 M) were used.

An aliquot of diluted juice sample (1 mL) was mixed with 9 mL of the two buffer solutions, separately. After 10 min of dark incubation, the absorbance of the two mixtures was measured at 520 and 700 nm using a UV–Vis spectrophotometer (Lambda Bio 40; Perkin Elmer, Waltham, MA, USA). Total anthocyanins content was expressed as cyanidin-3-glucoside equivalents per L of juice (mg $C_3gE\ L^{-1}$) and was calculated according to the following equation:

$$C_3gEL^{-1} = (A \times MW \times DF)/(\varepsilon \times L) \quad (1)$$

where $A = (A_{520}–A_{700})_{pH1.0} – (A_{520}–A_{700})_{pH4.5}$ absorbance (nm); MW (molecular weight) = 449.2 g mol^{-1} for cyanidin-3-glucoside; DF = dilution factor; L = cell path length (1 cm); ε = 26.900 molar absorptive coefficient for cyanidin-3-glucoside [17].

Total antioxidant activity (AA) was determined by the DPPH method [18–20]. Pomegranate juice was centrifuged at 4000×*g* for 10 min and then filtered through 0.45µm filter (Millipore). Successively, different volumes (µL) of the juice were mixed with 3 mL of a 6×10^{-5} M methanol solution of DPPH in cuvettes. These solutions were left to stand for 30 min in the dark.

The bleaching of DPPH was measured at 517 nm by UV–Vis spectrophotometer (Lambda Bio 40; Perkin Elmer, Waltham, MA, USA) at 25 °C (A_{sample}). A solution without the pomegranate juice was used as blank (A_{blank}) and the absorbance was recorded.

The AA was calculated by the following equation:

$$\%\ \text{Inhibition of DPPH} = \left[(A_{blank} - A_{sample})/A_{blank}\right] \times 100 \quad (2)$$

where A_{blank} is the absorbance of the control at initial time (t = 0 min) and A_{sample} is the absorbance of sample after 30 min. The free radical scavenging activity (AA), determined by DPPH, was expressed as EC_{50} value. It is defined asthe volume (µL) required to decrease the initial DPPH radical activity by 50%.

Enzyme extraction and activity assays

Catalase and ascorbate peroxidase(APX) activity

The pomegranate arils (1 g) were frozen in liquid nitrogen followed by grinding with 5 mL of ice-cold phosphate buffer (100 mM potassium phosphate buffer (pH 7.8), 1 mM sodium EDTA (pH 7), 2 mM DTT, 1 mM PMSF, 0.2% Triton X-100, 5% (w/v) PVPP) and, 5 mM ascorbic acid (the ascorbic acid has been used only for APX enzyme extraction). The homogenate was collected and centrifuged at 18,000×*g* for 10 min at 4 °C. Bradford assay [21] has been used to estimate the concentration of total protein using bovine serum albumin as a standard.

Catalase (EC 1.11.1.6) activity (CAT) was analysed as described by Pasquariello et al. [22]. The decrease in absorbance at 240 nm, caused by H_2O_2 breakdown, was monitored. The CAT activity was expressed as µmol mg $protein^{-1}$.

Ascorbate peroxidase (EC 1.11.1.11) activity (APX) was determined according to Pasquariello et al. [22]. The oxidation of ascorbic acid was monitored at 290 nm. The APX activity was expressed as µmol mg $protein^{-1}$.

Guaiacol peroxidase and superoxide dismutase activity

The pomegranate arils (1 g) were frozen in liquid nitrogen followed by grinding with 5 mL of ice-cold phosphate buffer (50 mM potassium phosphate buffer pH 7.8, 1 mM sodium EDTA pH 7, 2% (w/v) PVPP). The homogenate was collected and centrifuged at 14,000×*g* for 10 min at 4 °C and used for GPX (guaiacol peroxidase) and SOD (superoxide dismutase) activity determinations.

Guaiacol peroxidase (EC 1.11.1.7) activity (GPX) was assayed according to Pasquariello et al. [22]. The reaction mixture contained 250 µL of crude enzyme extract in a final

volume of 1 mL. Guaiacol peroxidase activity was detected at 470 nm monitoring the formation of tetraguaiacol. The GPX activity was expressed as nmol mg protein^{-1}.

Superoxide dismutase (EC 1.15.1.1) activity (SOD) was determined from the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT), as described by Pasquariello et al. [22]. The reaction was started by adding riboflavin, and after 15 min of incubation at room temperature under continuous light, the absorbance at 560 nm was measured. One SOD unit was defined as the amount of enzyme that inhibits the rate of NBT reduction by 50% under the above assay conditions. The SOD activity was expressed as U mg protein^{-1}.

Polyphenoloxidase activity

Polyphenoloxidase activity (PPO) was determined following the method described by Pasquariello et al. [22]. Arils (2.5 g) were homogenized in100 mM sodium phosphate buffer pH 6.4 containing 0.125 g PVPP (5 ml). Extract (20 µL) was incubated with a 500 mM catechol in 100 mM sodium phosphate buffer pH 6.4 in a final volume of 1.5 mL and monitored by evaluating the increase in absorbance at 398 nm. The PPO activity was expressed as µmol mg protein^{-1}.

Statistical analysis

All data, collected across two consecutive years (2015–2016), are expressed as the mean ± standard deviation. Four fruits collected from each tree were referred to as one sample for physico-chemical and enzymatic analyses and three technical replicates were carried out for each one.

To determine differences between pomegranate genotypes, one-way ANOVA and Duncan's test were used. Differences at $P < 0.05$ were considered significant and are indicated with different letters.

Principal component analysis (PCA) was applied to describe the relationship among the pomological, physico-chemical and nutraceutical traits and the enzymatic activities to identify the principal components contributing to the majority of the variation within the dataset. All analyses were performed using the SPSS software package, Version 20.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Pomological characteristics of pomegranate fruit

Mean values of pomological traits of fruit, collected across two consecutive years (2015–2016), are showed in Table 1. The average fruit weight varied significantly among

Table 1 Mean values of pomological traits of pomegranate genotypes

Genotypes	Weight (g)	Fruit length (cm)	Fruit width (cm)	Calyx crown length (cm)	Thickness of skin (mm)	Aril length (mm)	Aril width (mm)	Aril weight g	Tegument length (mm)	Tegument width (mm)	Tegument weight (g)
'Mondrone Dolce'	407.5^{ab}	8.2^{b}	9.1^{bc}	1.7^{b}	5.1^{b}	11.3^{cd}	8.8^{bc}	0.52^{e}	9.5^{c}	4.6^{c}	0.09^{e}
'San Pietro'	407.75^{ab}	9.2^{b}	10.3^{cd}	1.8^{b}	5.2^{b}	11.7^{d}	8.9^{bc}	0.46^{d}	8.1^{b}	3.7^{b}	0.07^{d}
'Dente di Cavallo'	559.6^{b}	8.9^{b}	10.5^{cd}	1.6^{ab}	6.0^{b}	13.7^{e}	9.5^{c}	0.58^{f}	9.5^{c}	3.6^{b}	0.04^{c}
'Granato'	246.3^{a}	5.6^{a}	7.9^{ab}	1.4^{a}	3.0^{a}	10.0^{b}	7.3^{a}	0.32^{b}	6.8^{a}	2.8^{a}	0.02^{a}
'Roce'	244.3^{a}	6.15^{a}	7.3^{a}	1.3^{a}	3.0^{a}	8.5^{a}	6.8^{a}	0.22^{a}	6.2^{a}	2.6^{a}	0.02^{a}
'Wonderful'	513.25^{b}	8.9^{b}	10.8^{d}	1.9^{b}	3.9^{a}	10.6^{bc}	7.6^{ab}	0.34^{c}	8.3^{b}	3.3^{b}	0.03^{b}

Values followed by the same letter within the same column were not significantly different according to Duncan's test ($P < 0.05$)

Values followed by different letters (a, b, c, etc.) within the same row are statistically different according to Tukey's multiple range tests ($p < 0.05$). All were significant at $p < 0.001$

genotypes, from a minimum of 244.3 ± 23.23 g ('Roce') to a maximum of 559.6 ± 35.5 g ('Dente di Cavallo'). A high variability has been observed in fruit weight among pomegranate genotypes of different geographical origin [6, 7, 9, 23]. In this study, the average weight of the pomegranate fruit (403.1 g) was comparable to that reported for some Italian genotypes [7] and Spanish cultivars [23] but higher than Iranian ones [9]. Furthermore, we observed a large variation among the genotypes and the average fruit length and fruit width varied from 6.15 to 9.2 cm and, from 7.3 to 10.8 cm, respectively. Our values are similar to that reported by Ferrara et al. [6, 7] for other Italian accessions cultivated in Puglia region (Italy). Calyx crown length showed a significant difference among genotypes and ranged from 1.3 to 1.9 cm, with lower values to that reported for some Iranian cultivars [9] and similar than Italian genotypes [6, 7]. 'Granato', 'Roce' and 'Wonderful' showed a thin thickness of skin (3–3.9 mm), while 'Dente di Cavallo', 'San Pietro' and 'Mondrone Dolce' were ranked among pomegranate genotypes possessing thick skin (5.1–6 mm). The average weight of the arils varied from 0.22 in 'Roce' to 0.58 g in 'Dente di Cavallo', with statistical differences in aril length and width. Furthermore, genotypes showed significant differences in length, width and weight of tegument. Our results fall within the range of values obtained from previous studies in pomegranate genotypes of different geographical origin [6, 7, 9, 23].

Although the pomegranate has a narrow genetic base, since ancient times an intense flow of genetic materials from Persia towards different countries has been occurred thus explaining the variability of the accessions/varieties [24]. Several studies have demonstrated that different pomegranate genotypes from Spanish, Iranian, Turkish or Moroccan areas displayed a considerable phenotypic diversity with variations in fruit traits [25, 26]. Most of the fruit traits are greatly affected by the orchard management and environmental conditions, although in pomegranate these traits are also controlled by multiple genes [27].

Physico-chemical analyses

Titratable acidity, pH, total soluble solids, maturity index and reducing sugars

The mean values of pH, TA, TSS, MI and reducing sugars of the juice obtained by squeezing arils are shown in Table 2. The genotype significantly affected ($P < 0.05$) all five parameters. The lowest pH value was measured in cv. 'Wonderful' (3.32), while cv. 'Dente di Cavallo' was characterized by the highest value (3.97). This latter result is in the same range (3.88–4.00) of two genotypes of 'Dente di Cavallo' studied by Todaro et al. [6] and growth in the experimental farm of the Catania University (Italy) located near the eastern coast of Sicily. Furthermore, the pH values of our local accessions 'Mondrone Dolce', 'San Pietro', 'Granato' and 'Roce' were ranging from 3.49 to 3.91.

Our data (3.32–3.97) and the pH values measured by Ferrara et al. [7], for Italian and Israeli genotypes (2.93–3.59) were in a narrower range with respect to the values of accessions grown in other countries such as in Spain 3.35–4.28 [23], in Iran 3.10–4.13 [9] and in Tunisia 2.93–4.60 [28].

The pomegranate fruit with a lower pH, showed a correspondingly higher acids content [29]. In particular, 'Wonderful' characterized by the lowest pH, had the significantly highest TA (15.05 citric acid g L^{-1}) among the genotypes analyzed. In previous study, Beaulieu et al. [30] found similar TA value (13.2 g citric acid L^{-1}) for the evaluated germplasm 'Wonderful' grown in California (USA). On the other hand, in this research, all other genotypes showed TA value significantly lower than 'Wonderful', ranging from 3.88 to 4.54 g citric acid L^{-1} (Table 2). Acidity varied from 4.9 to 38.6 g L^{-1} in Italian and Israeli genotypes grown in Southeastern Italy (Puglia region) [7], from 2.1 to 23.9 g L^{-1} in Greece [31], from 2.2 to 29.2 g L^{-1} in Spain [32], from 1.5 to 24.4 g/L in Iran [9], from 13.3 to 16.9 g L^{-1} in Tunisia [28] and from 5.0 to 38.0 g L^{-1} in Turkey [33].

Table 2 Mean values of physico-chemical characteristics of pomegranates genotypes

Genotypes	pH	Total titratable acidity (citric acid g/L)	Total soluble solid content (°Brix)	Maturity index (MI)	Reducing sugar (g 100 mL^{-1})	Total phenolics content (mg GAE L^{-1})	Total anthocyanins content (mg C_3gE L^{-1})	EC_{50} values (μL/mL)
'Mondrone Dolce'	3.91^{e}	3.88^{a}	18.00^{c}	46.39^{e}	11.09^{c}	797.16^{a}	194.92^{c}	143.94^{d}
'San Pietro'	3.89^{d}	4.54^{d}	18.50^{d}	40.78^{c}	11.35^{cd}	814.80^{a}	203.04^{d}	197.42^{e}
'Dente Di Cavallo'	3.97^{f}	4.12^{b}	17.50^{b}	42.49^{d}	9.91^{b}	708.40^{a}	102.47^{a}	229.96^{f}
'Granato'	3.73^{c}	4.45^{cd}	17.00^{a}	38.20^{b}	8.44^{a}	1190.00^{b}	251.98^{e}	79.61^{b}
'Roce'	3.49^{b}	4.22^{bc}	17.00^{a}	40.28^{c}	8.30^{a}	1140.30^{b}	159.49^{b}	94.28^{c}
'Wonderful'	3.32^{a}	15.05^{e}	18.50^{d}	12.30^{a}	11.78^{d}	1494.00^{ac}	554.99^{f}	21.21^{a}

Values followed by the same letter within the same column were not significantly different according to Duncan's test ($P < 0.05$)

Total soluble solids (TSS) estimate the level of dissolved sugars but also the presence of other soluble compounds, such as acids, salts, water-soluble vitamins, and other chemical compounds [2]. In the investigated genotypes, the TSS range was from 17.0 to 18.5°Brix; in particular 'Granato' and 'Roce' had the lowest total soluble solid content, corresponding to the lowest reducing sugars.

In previous studies, values for different pomegranate genotypes were in the range 13.6–18.5°Brix in Southern Italy [7], 14.4–17.0°Brix in Greece [31], 14.0–16.8°Brix in Spain [32], 11.4–16.2°Brix in Iran [9], 14.7–19.0°Brix in Turkey [33] and 15.90–17.70°Brix in California [30]. These differences may be not only due to the different genotypes but also to environmental conditions and harvesting times.

Titratable acidity (TA) and total soluble solid (TSS) content are important components of fruit organoleptic quality and their ratio (TSS/TA), also called the Maturity Index (MI), is responsible for the taste and flavor of the pomegranate [23]. The MI value for the investigated genotypes was in the range 12.30–46.39 and 'Wonderful' pomegranate is clearly separated from the other samples.

According to Martinez et al. [23] who discriminated Spanish cultivars by maturity index in sour, in sour–sweet and in sweet, the investigated local accessions were classified as sweet fruit. Conversely, cv. 'Wonderful' with MI ratio of 12.30 was sour–sweet pomegranate, as reported by Ben-Arie et al. [34].

Quantification of total phenols and individual phenolic compounds by HPLC-DAD

Polyphenols represent the predominant class of phytochemicals of pomegranate arils which have beneficial effects as free radical scavenging and antioxidant activity. Polyphenols are important constituents regarding the organoleptic properties of pomegranate since they contribute to the appealing red colour and provide the characteristic of mild astringency [35].

In the present study, among the investigated genotypes, 'Granato' and 'Roce' showed a similar total phenols (TP) content (about 1100 mg GAE L-1) and significantly higher ($P < 0.05$) than 'San Pietro', 'Mondrone Dolce' and 'Dente di Cavallo' (about 750 mg L^{-1}); the highest concentration was found in cv. 'Wonderful' (Table 2). The TP amount of pomegranate juice is very wide depending on genotypes. In fact, Ferrara et al. [7] found a TP amount ranging from 627 to 2839 (mg GAE L^{-1}) in Italian and Israeli genotypes; lower values were detected in Greek accessions (225–697 mg GAE L^{-1}) [31]; instead, some Turkey cultivars had an amount in the range 1245–2076 mg GAE L^{-1} [33]. In this study, the TP results were similar to those reported for Chilean genotypes (676–1280 mg GAE L^{-1}) by Sepulveda et al. [36] and for Italian genotypes (303–1328 mg GAE L^{-1}) by Ferrara et al. [6]. For the qualitative screening of pomegranate phenols, they were characterized by comparison of their UV–Vis spectra and retention times with those of reference substances. The concentrations reported in this study represent only the free forms of phenolic compounds since no hydrolysis was applied to the samples before HPLC analysis. Polyphenols identified were galloyl hexose at 3.9 min retention time (r.t.), gallic acid at 4.3 min r.t., protocatechuic acid at 6.9 min r.t., punicalagin at 13.6 min r.t., and o-coumaric acid at 26.3 min r.t. Galloyl hexose, punicalagin and o-coumaric acid were the main phenolic compounds detected. By HPLC analysis, 'Wonderful' and 'Granato' fruit resulted the most rich in polyphenols. 'Wonderful' showed the highest content of gallic acid (10 mg L^{-1}), galloyl hexose (32 mg L^{-1}), protocatechuic acid (12 mg L^{-1}) and *o*-cumaric acid (77 mg L^{-1}), while 'Granato' showed the highest content of punicalagin B (1175 mg L^{-1}) (Table 3). Significant differences ($P < 0.05$) were found among the samples. Comparison of the individual phenolic compounds with literature is difficult because of variability due to factors such as cultivar type, weather conditions and ripeness. However, all samples showed a content of gallic acid varying from 2.3 to 10.43 mg L^{-1} and higher than data reported in Fischer et al. [35]. Galloyl hexose, a common constituent of plants containing hydrolyzable tannins, varied from 16 to 32 mg L^{-1}. It was higher than data reported by Fischer et al. [35] but lower than data reported by Gil et al. [37]. Protocatechuic acid showed values (from

Table 3 Mean values of individual phenolic compounds (mg L^{-1}) of pomegranate genotypes

Genotypes	Gallic acid	Galloyl hexose	Protocatechuic acid	Punicalagin B	*o*-cumaric acid
'Mondrone'	6.192 ± 0.599^{b}	19.238 ± 0.590^{b}	$6.652 \pm 1.113^{b,c}$	329.589 ± 4.244^{a}	35.863 ± 90.019^{d}
'San Pietro'	6.857 ± 1.5690^{b}	24.550 ± 1.580^{c}	7.732 ± 0.508^{c}	434.336 ± 7.315^{b}	37.318 ± 0.246^{e}
'Dente di Cavallo'	2.297 ± 0.091^{a}	16.980 ± 0.100^{a}	2.25 ± 1.252^{a}	536.284 ± 9.808^{c}	15.605 ± 0.012^{a}
'Granato'	8.815 ± 0.395^{c}	23.313 ± 0.395^{c}	5.308 ± 0.007^{b}	1175.574 ± 1.581^{e}	26.620 ± 0.116^{b}
'Roce'	8.903 ± 0.442^{c}	16.120 ± 0.500^{a}	9.412 ± 0.248^{d}	539.199 ± 2.088^{c}	31.546 ± 0.087^{c}
'Wonderful'	10.431 ± 0.372^{d}	32.777 ± 0.430^{d}	12.263 ± 1.539^{e}	844.324 ± 8.458^{d}	77.222 ± 0.075^{f}

Values followed by the same letter within the same column were not significantly different according to Duncan's test ($P < 0.05$)

0.8 to 12 mg L^{-1}) similar to literature data [37, 38], while punicalagin B varied from 329 to 1175 mg L^{-1}.

Finally *o*-cumaric acid showed values ranging from 31 to 112 mg L^{-1}. The presence of *o*-cumaric acid in pomegranate juices has also been reported previously by Poyrazoglu et al. [39].

Total anthocyanins content and antioxidant activity

Anthocyanins are members of phenolic compounds that contribute to the attractive red and violet-blue colors of many fruit including pomegranate arils and they exhibit considerable antioxidant activity [39]. There were significant differences ($P < 0.05$) in total anthocyanins content as determined spectrophotometrically (Table 2) ranging from 102 ('Dente di Cavallo') to 555 ('Wonderful') mg $C_3gE\ L^{-1}$. In particular, 'Wonderful' and 'Granato' had the highest anthocyanins content than the other genotypes. Similar data were published for other pomegranate genotypes [8, 40]. The antioxidant activity of the studied pomegranate genotypes are shown in Table 2 and is expressed in terms of EC_{50}. The EC_{50} value is the effective concentration which is required to decrease the initial DPPH concentration by 50% and lower EC_{50} value reflects better protective action. The EC_{50} values varied from 21 to 230 µL mL^{-1} (Table 2) and it was significantly different in all evaluated samples ($P < 0.05$). The lowest values were exhibited by 'Wonderful' and 'Granato'. These results confirmed the data of total phenols and total anthocyanins content as discussed above.

Antioxidant enzymes activity

To evaluate the different response to oxidative stress in the arils of different genotypes, the enzymatic antioxidants such as CAT, APX and SOD, which serve as frontline defence against reactive oxygen species (ROS), were analyzed.

CAT and APX regulate the level of H_2O_2, formed by SOD with several reaction mechanism. In each genotypes, we observed a negative correlation between APX and CAT activity, this could be due to a compensatory mechanism as reported by Apel and Hirt [41]. CAT activity displayed the lowest value in 'Wonderful' and its higher values in 'Mondrone Dolce'. Other genotypes showed similar values with slight differences (Fig. 2a). In APX we observed low activity, with no significant difference in 'Mondrone Dolce', 'Granato' and 'Roce' and high activity with no significant difference in 'San Pietro', 'Wonderful' and 'Dente di Cavallo' (Fig. 2b). The differences in APX activity observed in these genotypes may be due to different ascorbic acid content [42]. This compound is involved in several physiological process such as growth, photosynthesis and photoprotection [43].

CAT and APX showed different affinities to the substrate because they belong to two different classes of H_2O_2-scavenging enzymes. CAT has low substrate affinity and does not require any reducing power for H_2O_2 detoxification while APX has high substrate affinity and reduces H_2O_2 by utilizing ascorbic acid as reducing agent [41].

In addition, to our knowledge, few studies reported the influence of cultivar on antioxidant enzymes activities of pomegranate fruit [42]. In this study, we observed that the pomegranate genotypes showed a different antioxidative defense system that influence the protective mechanisms against ROS activity.

SOD is the first ROS scavenging enzyme, that catalyses the dismutation of superoxide radical (O_2^-) to H_2O_2 and molecular oxygen (O_2). 'Wonderful', 'Dente di Cavallo' and 'San Pietro' showed high level of SOD activity with its highest value in 'Wonderful', while significant low values were registered in 'Granato' and 'Roce' (Fig. 2c).

PPO and GPX enzymes are involved in aril tissue browning due to direct oxidation and polymerization of phenolic compounds [44, 45]. PPO catalyses the oxidation of phenols to o-quinones that can undergo non-enzymatic secondary reactions to produce undesirable brown pigments as melanins [46]. GPX is involved in quality deterioration and in oxidation of several compounds in the presence of hydrogen peroxide as an electron acceptor [44].

Differences for PPO and GTX activities were observed among pomegranate genotypes. Significantly differences in GPX activity were registered, with the activity values ranged from 5.91 ± 0.34 to 0.37 ± 0.03 nmol mg $protein^{-1}$ in 'Granato' and 'Dente di Cavallo', respectively (Fig. 2d). PPO activity displayed the highest value in 'Wonderful', followed by 'Granato', 'Roce' and 'Dente di Cavallo', while a significantly lower value were registered in 'Mondrone Dolce' and 'San Pietro' (Fig. 2e).

Principal component analysis

The overall variability in pomegranate genotypes was analyzed by PCA approach (Fig. 3). Two principal components are necessary to explain the total variability of the characteristics analysed, using the cross-validation technique. Analyzing the eigenvalues of the covariance matrix we observed that the set of the two principal components (PCs) accounted for 72.40% of the total variance in the dataset. PC1 explained 37.24% of the variance in the dataset, whereas PC2 explained an additional 35.16% of the variance.

The variables were distinctly oriented towards four PCA quadrants as showed in Fig. 3. Fruit traits were highly positively correlated with PC1, while analyzing the physicochemical properties we observed that TSS and reducing sugar were highly positively correlated with PC1, while pH and TA were negatively and positively correlated with

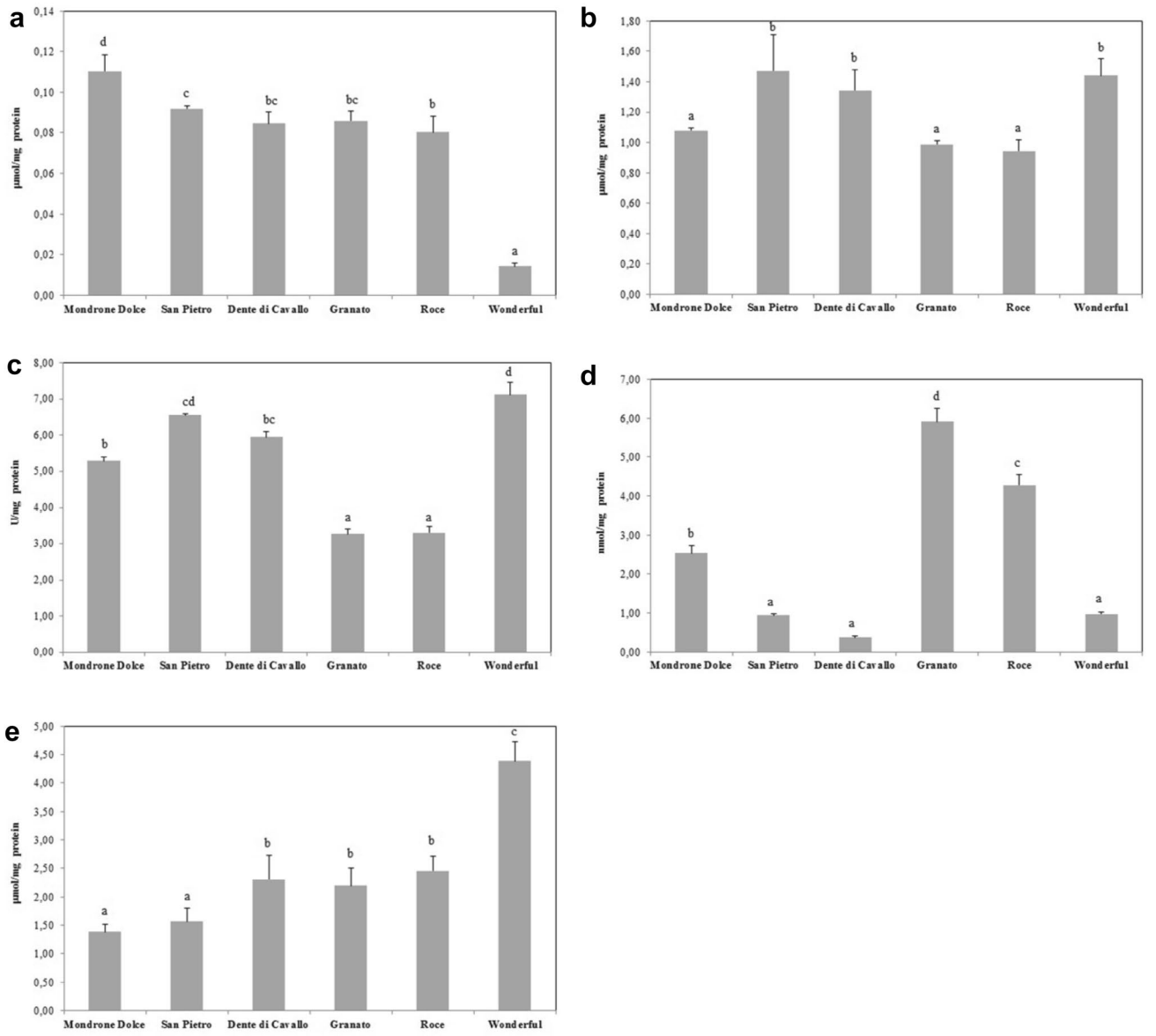

Fig. 2 Antioxidant enzyme: catalase activity (CAT) (**a**), ascorbate peroxidase activity (APX) (**b**), superoxide dismutase activity (SOD) (**c**), guaiacol peroxidase activity (GPX) (**d**), polyphenol oxidase activity (PPO) (**e**)

PC2, respectively. Bioactive compounds were positively correlated with PC2 with the exception of punicalagin B that was negatively correlated with PC1. Antioxidant enzymes showed a different correlation with PCs; CAT and PPO were negatively and positively correlated with PC2, respectively. APX and SOD were positively correlated with PC1 while GPX was negatively correlated.

On the basis of analyzed traits, pomegranate genotypes were grouped differently in 2D-PCA plot (Fig. 3). 'Mondrone Dolce', 'San Pietro' and 'Dente di Cavallo' were plotted in the PC1 positive and PC2 negative quadrant, 'Granato' and 'Roce' were projected on the quadrant defined by PCs negative while 'Wonderful' was plotted in the PCs positive. PCA analysis is a valid tool to discriminate the pomegranate genotype, showing the relationship among similar samples.

PCA is widely applied to evaluate differences among several cultivars of fruit crops at harvest and during storage, as previously demonstrated in other studies [47, 48].

Conclusions

This study provides information on the fruit traits and physico-chemical characteristics of local accessions ('Mondrone Dolce', 'San Pietro', 'Granato' and 'Roce') of Southern Italy compared with cvs. 'Dente di Cavallo' and 'Wonderful',

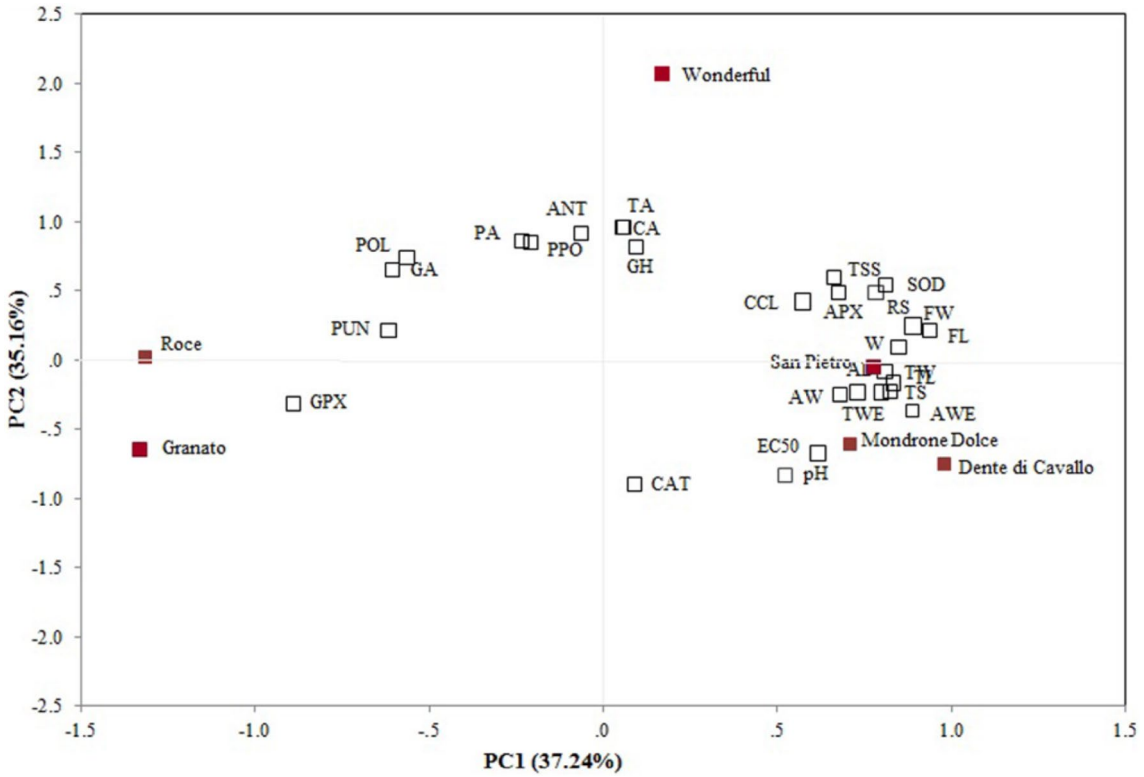

Fig. 3 2D-principal component analysis plot of the pomological, nutraceutical and enzymatic attributes in pomegranate genotypes: 'Mondrone Dolce', 'San Pietro', 'Dente di Cavallo', 'Granato', 'Roce' and 'Wonderful'. *pH* pH, *TSS* total soluble solid content, *RS* reducing sugars, *TA* total titratable acidity, *ANT* total anthocyanins, *POL* total polyphenol content, *EC50* antioxidant activity, *GA* gallic ac., *GH* galloyl hexose, *PA* protocatechuic ac., *PUN* punicalagin B, *CA* *o*-cumaric ac., *CAT* catalase, *APX* ascorbate peroxidase, *GPX* guaiacol peroxidase, *PPO* polyphenol oxidase, *W* weight, *FL* fruit length, *FW* fruit width, *CCL* calyx crown length, *TS* thickness of skin, *AL* aril length, *AW* Aril width, *AWE* aril weight, *TL* tegument length, *TW* tegument width, *TWE* tegument weight

grown in Italy. A significantly variability has been observed among pomological, qualitative and enzymatic traits in the pomegranate genotypes.

'Mondrone Dolce' and 'San Pietro' can be considered as promising pomegranate genotypes destined for the fresh market because of fruit size, qualitative traits and sweet taste, whereas 'Roce' and 'Granato' can be destined for the production of juices because of their high phenolic compositions. The high punicalagin content in these two latter pomegranate accessions contributes to the antioxidant activity of juice, that has gained in the last years a high reputation for its health benefit effects.

Further studies are needed for improving the knowledge of pomegranate accessions, collected in local germplasm of different Italian regions, as promising genotypes for either breeding program or commercial exploitation as fresh or processed fruit.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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