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An integrative approach to developing new tomato varieties with elevated fruit antioxidant content

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Abstract

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Carotenes have been identified as the most essential carotenoids in the human body, together with lycopene, lutein, and β-cryptoxanthin due to their beneficial antioxidant activities. Because of these properties, we have designed and conducted in 2018–2021 an experiment where various tomato accessions were metabolically profiled for their antioxidant content and other fruit parameters for further usage in crop improvement programs. Same genotypes were phenotyped and profiled with DNA markers to characterize their relative genetic distance. Aggregated data from metabolic and genetic profiling were complemented by the phenotypic data to select for the most promising cross (Pl. karotina × L1116) that was performed and developed into F1 and F2 generations. After phenotyping and genetic profiling of the 104 F2 individuals obtained from that cross, 24 individuals were selected for metabolic profiling for antioxidant contents. In relation to carotenoids accumulated in fully matured fruits the highest lycopene concentration detected in our study was 587.03 μg/g DW, while β-carotene reached 440.29 μg/g DW. Our results support the use of such integrative approach to accelerate tomato breeding when improved metabolic content of the fruits is aimed.

Keywords: tomato; breeding; antioxidants; β-carotene; lutein; lycopene; polyphenolics

Introduction

Tomato (Solanum lycopersicum L.), is one of the most important vegetable crops grown worldwide. It belongs to the family Solanaceae, which includes more than 2500 species (Motti, 2021). The genetic diversity in wild tomatoes, especially the self-incompatible species such as S. chilense and S. peruvianum (Park and Westclair, 2004; Tanksley and McCouch, 1997) is still not well utilized in spite of the various tools used (Zsögön et al., 2018) as barriers to crossing different species hamper the efficient transfer of traits to cultivated tomato (Bedinger et al., 2011). The genetic improvement of new varieties of tomatoes and their enrichment with novel traits is mainly achieved through interspecies and intraspecies hybridization, use of the mutant gene pool, and, in the last two decades, based on the application of modern biotechnological methods (Zsögön et al., 2018). The main traits of interest are the yield, resistance to biotic and abiotic stresses and the quality of the fruits.

Fruit quality of the crop is explained by its color, size, shape, and sensory stimuli such as sweetness, acidity and flavor (Baldwin et al., 2000; Tieman et al., 2017). There are multiple compounds that contribute to the overall fruit quality of which organic acids are considered important as they regulate

basic cellular processes such as modification of cellular pH and redox state (Çolak et al., 2020; Drincovich et al., 2016). The other important components are volatile compounds, which contribute to aroma (Baldwin et al., 2008). Further compounds of interest are the health promoting substances, such as the vitamin A precursor β-carotene, which represents an important nutritional source of this vitamin in the human diet. In recent years, because of the expanding knowledge about the benefit of carotenoids for human health, the attention of researchers to the nutritional qualities and antioxidant properties of tomato fruits has increased significantly. It expanded to other carotenoids (such as lycopene) that are not converted into vitamin A, but that may still have health-promoting properties. For example, the consumption of lycopene-rich foods has been reported to be inversely associated with the incidence of cardiovascular diseases and cancer (Borel, 2003; Schweiggert et al., 2014). Carotenes, as a valuable class of dietary carotenoids, have been increasingly appealing due to their beneficial antioxidant activities in health care. α-/β-Carotenes are the major isomers of carotenes and have been identified as the most essential carotenoids in the human body, together with lycopene, lutein, and β-cryptoxanthin. The xanthophyll carotenoid lutein, on the other hand, has been widely used as supplement due to its protective effects in light-induced oxidative stress. Its antioxidant and anti-inflammatory features suggest that it has a neuroprotective role as well (Black et al., 2020). Recently, intensive research has been initiated in several countries to identify and create tomato genotypes with an increased content of anthocyanins in the fruit, the antioxidant effect of which is significantly higher than that of lycopene (Li et al., 2022).

Tomato fruit quality is directly associated to metabolite content profiles; however, a full understanding of the genetics affecting metabolite content during tomato domestication and improvement has not been reached due to limitations of the detection methods employed so far (Yang et al., 2022). The available literature shows that studies on the use of DNA-based marker systems for the identification of polymorphisms in tomato have developed in several ways (Frary et al., 2005; Gonias et al., 2019; Osei et al., 2018; Sato et al., 2012; Shirasawa et al., 2010; Shirasawa and Hirakawa, 2013; Su et al., 2021; Suliman-Pollatschek et al., 2002). Some have concentrated on the analysis of the main genetic regions underlying variation in antioxidant contents (Capel et al., 2015; Ohyama et al., 2017) with the attention on factors encoding and regulating antioxidant production (Gago et al., 2017; Miura et al., 2012; Orchard et al., 2021) expanding to genes and regulatory sequences covering wide interactions (Yang et al., 2022). So far, a complex evaluation of tomatoes has not been carried out in Bulgaria and no genotypes have been identified as donors of multiple antioxidant compounds. In particular, anthocyanins in tomato fruits have not been studied in our country, and until recently there were no sources of these biochemical components with high antioxidant activities. As deciphering the complex genetic interactions in the specific background of the available metabolite diversity in Bulgaria is a paramount task, in this study we have designed and conducted an experiment during the 2018-2021 growing seasons where accessions of various origin were metabolically profiled for the antioxidant content in their fruits. Some of these genotypes were phenotyped and profiled with DNA markers to characterize their relative genetic distance (Bojinov and Danailov, 2009; Ivanova and Bojinov, 2009; Ohyama et al., 2017; Todorovska et al., 2014). Based on the combined information from the three types of analyses we devised an integrative approach to developing new tomato varieties with improved fruit antioxidant content. It relies on phenotypic, DNA-based and metabolic profiling of a selected number of accessions, followed by developing segregating crossing population (F1 and F2), where optimization of the number of metabolically profiled individuals is done based on the clustering of the individuals from F2 population. This approach was aimed at assuring that promising genotypes can be identified within a segregating population from a single cross that would have high probability of carrying improved antioxidant content in the fruits for at least one such compound.

Materials and Methods

1. Growing and phenotyping of accessions and segregation populations

Six accessions (Plovdivska karotina, L 21β, L1116, L 1140, IZK Alya, and L 53β) were selected for the purposes of this study, of which Plovdivska karotina and IZK Alya are commercial varieties. After selection of parent pairs based on metabolic profiling and establishment of genetic diversity between putative parental forms, crosses were made between genotypes identified as potential best donors. Plants from selected accessions (in 2018–2021), hybrids (in 2019–2021) and F2 segregating population (in 2020–2021) were grown in the fields of the “Maritza” vegetable crops institute, Plovdiv, Bulgaria. During the vegetation season, usual agricultural practices were followed for field production, while measurements of the main plant characteristics, such as average fruit weight, pericarp thickness, pressure resistance, etc. were taken periodically.

2. DNA analyses

DNA was isolated from the last young, fully developed leaf of the identified and tagged plants grown under field

conditions. The Plant DNA extraction kit (Omega Bio-tek, Georgia, United States) was used for DNA isolation, following the manufacturer's recommendations. PCR amplification with Inter-Simple Sequence Repeats (ISSR) primers (Table 1) was performed for genotype profiling. The use of ISSR markers for genotyping the potential donor genotypes was performed to detect polymorphisms in that group. Based on the initially collected information, the level of genetic diversity between the analyzed tomato genotypes was determined and used for selecting appropriate parents for crossing.

Table 1. Primers used for ISSR marker detection in studied tomato genotypes

Primers	DNA sequences (5' -> 3')
ISSR 1	$(CA)_8$AA+GG
ISSR 2	$(CA)_8$A A+GC+T
ISSR 3	$(GA)_8$C+TC
ISSR 4	$(AG)_8$C+TC
ISSR 5	$(AC)_8$C+TA
ISSR 6	$(AC)_8$C+TG
ISSR 7	$(AG)_8$C+TG
ISSR 8	$(AC)_8$C+TT
ISSR 9	$(AG)_8$C
ISSR 10	$(GA)_8$T
ISSR 11	$(GA)_9$C
ISSR 12	$(GA)_9$T
ISSR 13	$(GA)_9$A

PCR reactions were carried out in 25 µl volume, and for each reaction the following were used: MyTaq HS Red Mix (Meridian Bioscience, Cincinnati, USA) – 12.5 µl; ISSR primer – 1.5 µl; H_2O – 10.0 µl; 1 µl genomic DNA. ISSR PCR reactions were performed as follows: denaturation at 94°C for 3 min, 40 cycles at 94°C – 1 min, primer annealing – 45 sec, extension at 72°C – 45 sec, followed by a final extension at 72°C – 4 min, where primer annealing temperature was calculated according to (Kochieva et al., 2002).

3. Extraction procedures

Fruits, from 2–5 inflorescences, at technological maturity were selected from each investigated plant. The fruits were lyophilized at -60°C, the lyophilized mass was homogenized in a blender, separately for each fruit and stored in a freezer at -24°C before the further analyses were performed.

3.1. Carotenoids

Two mL of methanol and 5 mL of a mixture of tetrachloromethane and methanol in a ratio of 3:1 were added to 0.1 g of the lyophilized sample, and 0.5% butylated hydroxytoluene (BHT) was added to the solution. The sample was placed for 15 min in an ultrasonic bath (35 kHz) and after extraction, 1 mL of 10% NaCl solution was added. The samples were centrifuged for 10 min. at 5000 rpm. The tetrachloromethane fraction was separated and passed through a column of anhydrous Na_2SO_4. Samples were collected in a 5 mL volumetric flask (Georgieva et al., 2013).

3.2. Tocopherols

One g of lyophilized sample was extracted with 10 mL of saponification solution (0.1 g of NaCl, 12.0 g KOH, and 0.5 mg of BHT previously dissolved in 20 mL of ethanol were added to a 50 mL volumetric flask) on a water bath for 30 min. at 70°C. After extraction, 15 mL of 1% NaCl solution and 15 mL of a 9:1 n-hexane: ethyl acetate solution were added to the sample. The organic phase was separated and evaporated to dryness with a rotary evaporator at 40°C, then dissolved in 1 mL of 98% methanol (Georgieva et al., 2013).

3.3. Phenolic content and antioxidant activity

For analysis of total phenolic content and antioxidant activity each of the analyzed samples (1.0 g of dried leaves) was extracted three times with 10 mL 70% ethanol (v/v) under reflux-heat at 70°C for 20 min according to (Ivanov et al., 2014). The residue of plant material was removed through filtration, and the combined extracts were stored in a refrigerator at 4°C for further analyses.

4. Analyses

The total phenolic contents were measured using a Folin-Ciocalteu assay according to the procedure described by Ivanov et al. (Ivanov et al., 2014). Folin-Ciocalteu reagent (1 mL) (Sigma) diluted five times was mixed with 0.2 mL of sample and 0.8 mL 7.5% Na_2CO_3 (Sigma) and kept for 20 min at room temperature in darkness. After reaction time, the absorption of sample was recorded at 765 nm against blank sample, developed the same way but without extract. The results were expressed in mg equivalent of gallic acid (GAE per g dry weight), according to calibration curve, build in range of 0.02–0.10 mg gallic acid (Sigma) used as a standard.

HPLC analyses were carried out using methods and equipment described in our previous publications for carotenoids and tocopherols (Georgieva et al., 2013).

The antioxidant activity was measured by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric ion reducing anti-oxidant power (FRAP), and CUPric ion Reducing Antioxidant Capacity (CUPRAC) assays (Ahmed et al., 2021;

Benzie and Strain, 1996; Ivanov et al., 2014; Özyürek et al., 2011; Sharma and Bhat, 2009; Zhen et al., 2016). DPPH assay was performed by mixing freshly prepared 4 × 10-4 mol methanolic solution of DPPH with the samples in a ratio of 2:0.5 (v/v). The light absorption was measured at 517 nm. For ABTS assay, ABTS radical cation (ABTS+) was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. Afterward, the ABTS+ solution was diluted with ethanol to an absorbance of 0.7 ± 0.02 at 734 nm and equilibrated at 30°C. After the addition of 1.0 mL of diluted ABTS+ solution to 10 mL of samples, the absorbance was taken after 6 min at 30°C. For FRAP assay, the FRAP reagent was prepared fresh and was warmed to 37°C prior to use. Next, 150 µL of plant extracts were allowed to react with 2,850 µL of the FRAP reagent for 4 min at 37°C, and the absorbance was recorded at 593 nm against a reagent blank. The reaction for CUPRAC assay was initiated by mixing 1 mL of $CuCl_2$ solution (1.0 × 102 M), 1 mL of neocuproine methanolic solution (7.5 × 103 M), 1 mL of ammonium acetate buffer solution (pH 7.0), 0.1 mL of sample followed by 1 mL of water. Absorbance against a reagent blank was measured at 450 nm after 30 min. The results for antioxidant activity were expressed as µM Trolox® equivalents (TE) per g dry weight (DW) plant material (Vrancheva et al., 2019).

Three independent extracts from each of the analyzed samples were prepared and each extract was analyzed in triplicate for total phenolic content, individual flavonoids, phenolic acids, and antioxidant activity. The presented values are means with standard deviations (± SD).

Results

1. Homogeneity analysis of the studied accessions

Upon reaching full maturity, essential fruit characteristics were measured for the selected accessions (Table 2). The established contrasting differences between some of the initially selected genotypes in basic fruit characteristics were used to assign them as suitable parental components for inclusion in hybridization in order to identify donors of the relevant traits, according to the objectives of this research. In addition to the initial selection based on the phenotypic identity of the plants with the descriptor indicators for the respective accession, a determination of the typical molecular profile for each accession was also carried out for this purpose.

Screening of the individual plants using Inter-Simple Sequence Repeats (ISSR) markers at this stage (Figure 1) aimed to reveal deviating genotypes within the selected accessions. The use of the ISSR primers led to the revealing of some polymorphic bands. These polymorphisms were insufficient for the unambiguous identification of each plant of the respective variety. However, they were sufficient to identify individuals with deviating genetic profile, which were discarded from further studies and propagation. Only plants having typical profile for the respective accession were used from this point onwards for metabolic profiling and inclusion in crosses.

After the individual reproduction of the selected individuals has been ensured, the obtained homogenized parental forms were grown in a specialized hybrid nursery. After

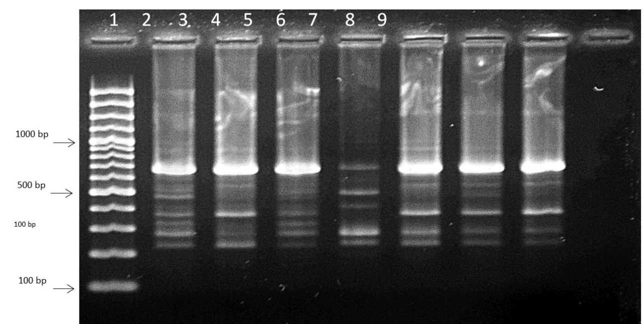

Fig. 1. Detection of polymorphisms among individuals of a single genotype using ISSR primer 1. Lane #1 - Standard sized DNA, in which the bands of size 500 and 1000 bp are brighter. Lanes No. 2–8 – products of PCR reactions carried out with DNA of individual plants from a breeding line with anthocyanin fruits (L1116). Lane #9 – control (all PCR mix components without genomic DNA)

Table 2. Morphological characterization of the fruits of selected tomato accessions

Accession	Average fruit weight (g) ±sd	Index I = h/d ±sd	Pericarp thickness (cm) ±sd	Pressure resistance (kg) ±sd
Pl. karotina	72.88 ±8.44	0.90 ±0.04	0.60 ±0.54	4.7 ±0.4
L 21β	167.46 ±47.27	1.04 ±0.02	0.69 ±0.74	5.6 ±1.0
L1116	97.64 ±32.97	0.90 ±0.04	0.51 ±0.40	4.5 ±0.5
L 1140	17.69 ±3.67	0.99 ±0.02	0.30 ±0.56	3.9 ±0.4
IZK Alya	19.47 ±2.56	1.43 ±0.10	0.27 ±0.29	4.0 ±0.2
L 53β	145.59 ±36.53	0.84 ±0.04	0.76 ±0.65	7.5 ±1.2

flowering, directed crosses were started for the development of F1 and F2 hybrid populations.

2. Antioxidant activity of the initial set of accessions

The initial set of accessions were substantially different for their antioxidant activity in methanol extracts of their fruits (Table 3).

Additional metabolite profiling showed that they also differed significantly in their carotenoid contents (Table 4).

3. Selection of parental forms and characterization of F1 hybrids

Based on the results from the detailed characterization of not only the accessions as bulk representatives of varying phenotypic, genetic and metabolic profiles, but also of the individual plants, several promising crosses were selected for further development. They were thoroughly discussed by the study participants and specific individuals were selected for further crossing as carrying contrasting metabolic profiles. The resulting F1 hybrid populations were phenotyped in the field and screened with the combination of ISSR markers selected in the initial stage to confirm their hybrid nature (data not shown). This was done to assure that only the true hybrids would be metabolically characterized as the antioxidant profiling proved to be both the most highly time and labor-intensive effort in the study.

The results showed that the antioxidant activities in methanol extracts of the fruits varied significantly between the F1 progenies obtained (Table 5).

As demonstrated in the table above, the radical scavenging activity measured through DPPH varied more than fourfold, showing highest values in the Pl. karotina × L1116 cross with the lowest ones in L 1140 × IZK Alya F1 hybrid. Similarly, radical scavenging activity measured through

Table 3. Antioxidant activity of methanol extracts from lyophilized tomato fruits of the selected accessions

Accession	DPPH*, µM TE/g DW ±sd	ABTS, µM TE/g DW ±sd	FRAP, µM TE/g DW ±sd	CUPRAC, µM TE/g DW ±sd	Total phenolics, mg/g DW ±sd
IZK Alya	23.7±8.2	21.4±4.9	26.5±12.4	10.5±4.9	4.6±0.6
L 1116	35.5±5.6	43.5±8.6	30.2±2.7	12.2±2.2	7.8±1.8
L 1140	17.5±4.6	14.2±4.5	22.9±4.5	6.7±1.0	6.0±0.5
L 21β	13.6±3.1	17.0±1.0	18.1±1.4	5.0±0.2	4.2±1.2
L 53β	14.2±0.9	16.5±3.1	19.6±1.4	5.7±0.5	4.8±1.5
Plovdivska karotina	21.6±2.6	22.5±8.7	33.2±9.2	10.1±1.5	7.0±1.7

* DPPH – 2,2-diphenyl-1-picrylhydrazyl; ABTS –2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); FRAP – ferric ion reducing antioxidant power; CUPRAC – CUPric Reducing Antioxidant Capacity; DW – dry weight.

Table 4. Carotenoids accumulated in fresh tomato fruits of initial accessions

Accession	Lutein µg/g DW ±sd	Lycopene µg/g DW ±sd	β-carotene µg/g DW ±sd
IZK Alya	51.1±11.1	993.7±63.7	436.6±51.9
L 1116	62.6±13.6	1128.6±81.0	434.9±44.5
L 1140	60.2±6.1	573.4±51.8	362.9±40.1
L 21β	49.9±8.2	450.8±96.5	309.4±27.0
L 53β	traces	200.0±48.8	1346.6±40.4
Plovdivska karotina	55.1±10.2	458.7±64.2	1637.5±327.8

Table 5. Antioxidant activity of methanol extracts from lyophilized tomato fruits of the F1 hybrids

F1 hybrid	DPPH*, µM TE/g DW ±sd	ABTS, µM TE/g DW ±sd	FRAP, µM TE/g DW ±sd	CUPRAC, µM TE/g DW ±sd	Total phenolics, mg/g DW ±sd
L 1140 × IZK Alya	11.99 ± 2.6	35.09 ± 1.15	161.33 ± 0.15	72.72 ± 1.11	1.82 ± 0.03
L1140 × L1116	21.91 ± 2.61	33.84 ± 4.94	112.66 ± 12.49	77.08 ± 14.33	1.92 ± 0.22
Pl. karotina × L21β	17.62 ± 1.78	35.25 ± 0.69	104.33 ± 3.35	104.53 ± 2.07	1.97 ± 0.07
L1140 × L53β	18.08 ± 2.00	31.97 ± 0.77	85.87 ± 4.83	69.97 ± 1.36	2.20 ± 0.06
L1140 × Pl. karotina	16.98 ± 1.25	27.99 ± 0.67	128.63 ± 1.05	64.87 ± 0.59	1.91 ± 0.14
Pl. karotina × L1116	51.26 ± 2.13	63.82 ± 7.84	119.00 ± 34.84	86.62 ± 7.30	1.52 ± 0.34

* DPPH – 2,2-diphenyl-1-picrylhydrazyl; ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); FRAP – ferric ion reducing antioxidant power; CUPRAC – CUPric Reducing Antioxidant Capacity; DW – dry weight

ABTS was several fold higher in the Pl. karotina × L1116 cross as compared to the contents of fruits from L1140 × Pl. karotina F1 hybrid. The highest ferric reduction power (FRAP) was measured in the L 1140 × IZK Alya F1 hybrid (161.33 μM TE/g DW) while the lowest was detected in the fruits of L1140 × L53β cross (85.87 μM TE/g DW). For cupric reducing antioxidant capacity (CUPRAC) and total phenolic content the differences between hybrids are less pronounced.

The same hybrids were further profiled for their other metabolites, which showed that they also differed significantly in their carotenoid contents (Table 6).

The analyses of the carotenoid content (Table 6) of the fresh fruits of F1 hybrids demonstrated that β-carotene content could be substantially improved in growing hybrid tomatoes. While in the initial set of accessions the concentrations of β-carotene varied from 309.4 μg/g DW in line L21β to 1637.5 μg/g DW in Pl. karotina, in the L1140 × L1116 F1 hybrid the concentration of this important metabolite can reach up to 4092.08 μg/g DW (Table 6) making this a very promising genotype for further evaluation of its yield and taste performances.

Table 6. Carotenoids accumulated in fresh tomato fruits of F1 hybrids

F1 hybrid	Lycopene μg/g DW	β-carotene μg/g DW*
L 1140 × IZK Alya	225.27 ± 81.99	560.96 ± 75.36
L1140 × L1116	682.23 ± 234.31	4092.08 ± 564.59
Pl. karotina × L21β	379.56 ± 11,56	909.31 ± 21.43
L1140 × L53β	438.28 ± 14,23	375.79 ± 12.99
L1140 × Pl. karotina	476.09 ± 31.34	743.25 ± 24.34
Pl. karotina × L1116	604.22 ± 124.12	592.04 ± 119.28

*Lutein was detected in traces in all measured samples and is therefore not shown

4. Antioxidant activities in F2 generation

The genotyping and identification of loci contributing to the expression of quantitative traits in an F2 generation requires rather large numbers of individuals (Capel et al., 2015; Frary et al., 2005; Su et al., 2021; Yang et al., 2022). This would make impractical efforts to metabolically characterize several hundreds (or even thousands) of individuals that would have been the case if we tried to develop all F1 progenies into F2 populations.

Therefore, we chose to concentrate our efforts on studying one of the progenies we thought had the highest potential of producing individuals with particularly attractive combinations of antioxidant compounds. For the purposes of the present study, the Pl. karotina × L1116 cross was selected for developing into a F2 population. One hundred and four F2 plants were produced from this cross and successfully grown to full maturity in the field of "Maritza" Vegetable Growing Institute. They were phenotyped and genotyped using the same methods as described for the initial accessions and the F1 progenies (data not shown). Combined data from phenotyping and genotyping was used for producing dendrogram that represented their relative genetic distance (Figure 2). This information was used for the identification of representative individuals from different clusters for metabolic profiling. The selected group of genotypes was analyzed in detail regarding the composition and concentration of carotenoids, lycopene, lutein and tocopherols by HPLC. In addition, the content of phenolic acids was determined. This allowed for the identification of important groups of antioxidant compounds by which both the initial group of genotypes and the individuals from F1 and F2 generations could be differentiated.

Altogether 24 F2 individuals were selected, together with the two parental lines (Pl. karotina and L1116) for determination of the antioxidant composition. The two parents were added to assure that the data from the F2 progeny is comparable to that of the parents as they were grown together, but

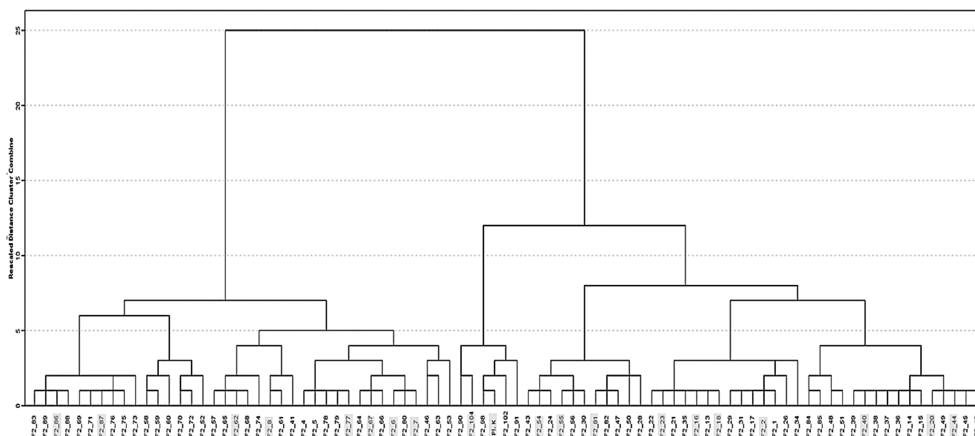

Fig. 2. Clustering of the F2 individuals from Pl. karotina × L1116 cross based on combined data from genotyping and phenotyping. Individuals selected for metabolic profiling are shaded. Clustering is done according to the Ward's method, using squared Euclidean distances (Ward, 1963)

could be also compared to a commercial variety (Pl. carotina) that is currently grown in the country.

The results from the various analyses of antioxidant activity and total phenolic contents of the studied F2 progeny of hybrid combination (Plovdivska karotina × L 1116) are presented in Table 7. The antioxidant activity of the methanol extracts of the analyzed fruits of the F2 progeny varied significantly. The highest amounts of total phenolics were accumulated in the fruits of plant 24 (8.25 mg GAE/g DW), and the lowest in plant 55 (3.30 mg GAE/g DW), while fruits of the parental lines accumulated 6.55 mg GAE/g DW (Plovdivska karotina) and 6.35 mg GAE/g DW (L 1116). The highest radical scavenging activity was detected in the fruit extracts of plant 7 (10.92 µM TE/g DW measured by ABTS and 20.29 µM TE/g DW measured by DPPH). The metal-reducing activity with respect to copper ions was the highest in the fruit extracts of plant 24 (80.56 µM TE/g DW), and with respect to iron ions, the highest activity was identified in the fruit extracts of plant 27 (14.54 µM TE/g DW). It should be noted that the antioxidant activities cited above significantly exceed those detected in the fruit extracts of the parental lines in the same year.

To complement the above data we analyzed the main carotenoids and tocopherols in the ripe tomato fruits of the studied F2 progeny (Table 8). The highest concentrations of lycopene were found in the fruits of plant 20 (587.03 µg/g DW). The highest concentrations of β-carotene accumulated in the fruits of plant 24 (440.29 µg/g DW). It should be noted that the parental lines synthesized significantly higher amounts of lycopene (Line 1116) and β-carotene (Plovdivska karotina).

The fruits of the studied F2 hybrid tomato population mainly biosynthesized α-tocopherol. β-tocopherol was not detected, and γ-tocopherol was detected in low concentrations. The highest amounts of α-tocopherol were accumulated in the ripe fruits of plant 27 (26.72 µg/g DW).

Table 7. Antioxidant activity of methanol extracts from lyophilized tomato fruits of the F2 individuals and parental lines

F2 individual / Parental line	Total phenolics, mgGAE/g DW ±sd	FRAP, µM TE/g DW ±sd	ABTS, µM TE/g DW ±sd	CUPRAC, µM TE/g DW ±sd	DPPH, µM TE/g DW ±sd
2	3.64 ±0.03	3.59 ±0.34	10.25 ±0.24	39.78 ±5.18	12.89 ±1.78
7	6.36 ±0.11	10.59 ±0.81	10.92 ±0.00	69.58 ±8.47	20.29 ±0.09
8	3.35 ±0.66	7.00 ±1.16	9.57 ±0.17	38.34 ±4.76	14.99 ±1.83
27	5.95 ±1.15	14.54 ±0.83	9.16 ±0.00	58.96 ±5.36	17.07±0.09
16	4.21 ±0.90	11.88 ±2.06	10.73 ±0.00	51.19 ±6.05	19.06 ±1.06
18	4.65 ±0.95	10.06 ±0.52	10.64 ±0.00	53.32 ±6.52	15.37 ±3.03
32	5.29 ±0.92	9.81 ±0.52	9.86 ±0.00	56.74 ±3.69	16.70 ±1.05
33	4.44 ±0.84	11.52 ±2.76	10.24 ±0.30	52.44 ±8.88	16.16 ±1.25
62	5.95 ±1.15	11.49 ±0.63	4.51±2.99	72.10 ±11.33	16.23 ±1.68
77	3.57 ±1.56	5.25 ±0.46	10.21 ±0.27	44.11 ±8.45	14.52 ±1.17
87	3.72 ±0.81	5.89 ±0.47	9.24 ±0.66	32.41 ±2.75	10.28 ±0.75
40	8.52 ±1.02	12.29 ±0.09	8.11 ±0.68	58.21 ±12.14	17.49 ±1.36
55	3.30 ±0.29	9.91 ±0.46	9.10 ±0.57	41.05 ±1.95	13.42 ±2.63
104	4.69 ±0.21	11.36 ±0.18	8.62 ±0.57	52.84 ±3.46	16.67 ±1.26
86	5.86 ±0.23	13.11 (±0.38	9.55 ±0.68	65.48 ±11.24	18.78 ±0.7
6	6.97 ±0.72	13.37 ±0.66	8.55 ±0.73	73.82 ±6.85	19.58 ±0.08
81	7.48 ±0.81	12.08 ±0.83	7.55 ±0.68	82.25 ±4.59	17.50 ±0.04
54	4.53 ±1.00	10.47 ±0.31	7.10 ±0.89	48.31 ±5.34	12.52 ±1.54
23	4.59 ±0.97	11.40 ±0.77	6.91 ±0.70	61.34 ±15.43	11.84 (±0.26
31	5.39 ±0.41	12.67 ±0.25	8.23 ±1.11	62.37 ±1.91	17.07 ±1.04
20	4.15 ±0.53	11.06 ±0.15	8.02 ±0.62	46.93 ±5.52	10.54 ±0.31
24	8.25 ±0.98	11.90 ±0.17	8.66 ±1.08	80.56 ±19.48	14.20 ±0.04
44	6.07 ±0.27	13.70 ±0.90	7.51 ±0.69	59.97 ±2.77	14.12 ±5.30
91	5.51 ±1.23	12.82 ±0.49	6.72 ±0.90	68.94 ±6.79	14.25 ±0.21
Pl. karotina	6.55 ±1.00	12.81 ±0.33	3.30 ±0.99	67.01 ±5.04	15.67 ±0.26
L 1116	6.35 ±0.47	12.82 ±0.54	8.79 ±1.34	68.72 ±4.20	18.89 ±0.69

* DPPH – 2,2-diphenyl-1-picrylhydrazyl; ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); FRAP – ferric ion reducing antioxidant power; CUPRAC – CUPric Reducing Antioxidant Capacity; DW – dry weight

Table 8. Carotenoids and tocopherols accumulated in fresh tomato fruits of F2 individuals and parental lines

F2 individuals / Parents	Lutein, μg/g	Lycopene, μg/g	β -Carotene, μg/g	α-tocopherol, μg/g	γ-tocopherol, μg/g*
2	70.50	384.86	178.72	1.98	–
33	62.78	116.48	142.41	–	–
87	75.18	166.11	221.54	4.24	–
8	65.35	464.62	154.70	–	–
27	82.53	266.48	239.33	26.72	–
32	86.48	282.44	251.50	3.90	–
7	57.68	117.44	143.98	–	–
16	83.68	261.33	251.65	2.84	–
62	67.42	136.64	211.22	–	–
18	88.54	162.77	227.62	–	–
77	50.33	165.95	150.42	3.07	–
44	85.08	174.71	244.09	–	–
23	60.99	392.67	150.32	–	–
20	62.11	587.03	159.65	–	–
31	88.18	542.37	203.35	–	–
91	62.22	143.92	146.71	1.85	–
104	82.98	342.38	233.91	3.76	5.82
24	86.22	265.15	440.29	–	0.09
81	51.35	327.47	219.14	–	–
6	81.65	139.73	306.60	9.06	–
54	87.48	209.75	299.76	3.40	–
55	153.60	101.95	213.86	0.02	–
40	66.11	153.57	245.17	2.74	–
86	77.37	149.77	233.86	–	–
Pl. karotina	80.18	415.36	1288.73	–	–
L 1116	77.03	938.18	213.56	2.80	–

* β-tocopherol was detected in trace amounts in all samples and is therefore omitted from the table

Discussion

1. Homogeneity analysis of the initial plant material

When developing a system to identify genotype-specific markers, it is preferable to work with bulk DNA samples (Reyes-Valdés et al., 2013). However, the use of such samples tends to mask the intrinsic levels of polymorphism in the collection samples and thus jeopardizes the reproducibility of the results if heterogeneous material is included in the bulk sample examined (Reif et al., 2005). During the DNA analysis of our accessions special attention was paid to the appearance of unusual bands for the respective variety or line. It is interesting to note that none of the cultivars showed complete identity of the profiles of the individual plants examined. In each of the genotypes studied at least one plant showed the presence of differences in the obtained DNA profile as compared to the other representatives of the same accession. Such plants were later removed and any further reproduction or use in crosses was completed only with the plants fully conforming to the typical profile for given genotype.

As might be expected, the use of different ISSR primers resulted in the detection of different levels of polymorphism within individual samples. However, the use of only 3 ISSR primers proved to be sufficient to obtain a grouping of the individuals that corresponded well to their putative relatedness, which was in an agreement with our previous results (Bojinov and Danailov, 2009) and further supports the use of ISSR markers for assessing genotype purity.

Further analyses of all individuals from the preselected set of genotypes was performed on their morphological and biochemical characteristics resulting in the removal of individuals that showed a deviation in any of them. Combining the information obtained using these different approaches made it possible to select individual plants for development of F1 hybrids suitable in the context of present study.

2. Selection of individual plants for hybridization and of F1 hybrids for further development

As the initial screening of the accessions with the ISSR markers revealed the presence of heterogeneity in all of the accessions, deviating individuals from each accession were discarded based on the aggregate data from phenotyping, metabolic and genetic profiling as described above. The initial set of accessions was comprised of 6 genotypes, which made performing all possible crosses require excessive use of labor and other resources even if performed in a unidirectional manner. Therefore, the number of F1 progenies to be initially produced was reduced to 6 so as to allow for effective characterization of each of them while maintaining the resource utilization at this stage in check. Selection of the specific parent combinations was based on the heuristic approach that prioritizes biological significance instead of statistical significance (Bocianowski et al., 2011) but which was modified in our case to selecting interesting plants (instead of markers, as proposed in the original publication (Bocianowski et al., 2011)) that can be further analyzed with formal methods of statistical inference. Instead of analyzing thousands of plants through parametric methods, using this approach one can analyze a much smaller set.

The initial metabolic characterization established Pl. karotina as a potentially very good parent as it had its antioxidant activity measured by different methods consistently in the upper half of the accessions studied (Table 3) combined with a very high β-carotene content. It is not very often that such advantageous combinations can be found in a single genotype (Otify et al., 2023). The other two accessions with recognizable potential were L1116 and L1140. Therefore, they were selected as main targets for developing F1 generations with the addition of L21β and L53β as other genotypes with a good potential for contributing specific antioxidants to their progeny. Altogether, this resulted in producing the 6 crosses, characterized in Table 5 and Table 6.

The fact that in the F1 progenies the contents of various antioxidant metabolites varied several folds was not surprising as the initial variation of these metabolites in the parental forms was also high. More interestingly, the results of the F1 hybrids substantially exceeded what was obtained from the parental genotypes, i.e. while Pl. karotina had the antioxidant activity of 21.6 μM TE/g DW and L1116 - 35.5 μM TE/g DW as measured by the DPPH method (Table 3) their F1 hybrid had 51.26 μM TE/g DW as detected by the same method in the same year (Table 5). Similar tendencies could be established for several of the other antioxidant activities, measured by different methods. While very promising as an outcome from such an effort, this needed to be confirmed as a result from the individuals from the next generation to confirm the action of essential genetic component(s). Once again, the resource limitations precluded development of all the six F1 hybrids into F2 segregating populations, so our efforts were concentrated on selecting and developing one main F2 population, with one more as a backup.

3. Antioxidant activities in F2 generation

For the reasons outlined above, we have selected the Pl. karotina × L1116 cross as our main hybrid for developing into an F2 population. As metabolically characterizing the obtained 104 F2 plants would have also proved challenging, the aim of the present study was to develop an integrative approach to developing new tomato varieties with improved fruit antioxidant activity so that optimized structure of the efforts could be proposed for further studies involving the improvement of such complex traits. To achieve that we initiated integrative metabolic + genomic characterization of parental lines, F1 and F2 generations as these can be performed (completely or in part) during the early stages of crop development. Based on the data accumulated we were able to obtain characteristic grouping of the F2 individuals (Figure 2) that allowed for the well-informed selection of a sub-set of those that would effectively represent the variability in antioxidant metabolic compounds in the said segregating population. By reducing the number of individuals to be metabolically profiled in F2 we were able to significantly reduce the time and effort needed for the selection (Jamali et al., 2019) of the individuals most likely to carry desired antioxidant composition. This was confirmed by both the wide diversity in the quantities of these compounds in selected individuals and the detection of the ones carrying substantially improved levels of key such compounds.

Several plants carrying particularly interesting antioxidant combinations were identified in the F2 population, namely plant No. 24 that had 25.9% higher total phenolic compounds accumulated in the fruits than the better parental line, plant No. 7 with the highest radical scavenging activity (10.92 μM TE/g DW as measured by ABTS and 20.29 μM TE/g DW – by DPPH), and plant 24 that had the highest metal-reducing activity with respect to copper ions (80.56 μM TE/g DW). Interestingly, while the total antioxidant activity (measured in methanol extracts) was high, the contribution of the subgroup of carotenoids and tocopherols was rather small. As shown in Table 8 the highest lycopene concentration was found in F2 plant No. 20 (587.03 μg/g DW) while the L1116 parental line had 938.18 μg/g DW. The highest concentrations of β-carotene accumulated in the fruits of plant 24 (440.29 μg/g DW), but the parental line Pl. karotina accumulated 1288.73 μg/g DW of the same compound.

Taking into account that the F1 progeny of the same cross had accumulated 592.04 μg/g DW β-carotene would imply that Pl. karotina is a variety that has been very successfully developed for increased β-carotene content while L1116 was developed for high lycopene. The genetic composition of Pl. karotina seems to favor the biosynthesis of β-carotene (where its name also originates from), while significantly reducing the production of lycopene. The opposite apparently is true for the L1116 parent. This was to be expected as the genes involved in the synthesis of the two antioxidants play opposing roles on their concentration (Song et al., 2023; Zunjare et al., 2017).

Another entity with significant antioxidant potential and high variability in our F2 population was lutein. Analyzed lutein content in fruits of initial accessions is relatively high (highest amount was detected in fruits of L1116 – 62.6 μg/g DW). All F1 hybrids accumulated lutein in traces in their fruits, while F2 hybrids accumulated lutein in varying concentrations, as highest amount was detected in plant 55 (153.60 μg/g DW) and the lowest – in plant No. 77 (50.33 μg/g DW). This could be due to the fact that in many tomato genotypes the differences in lycopene, B-carotene, and lutein content may only be due to differences in a single enzyme (Yin et al., 2020) since their biosynthesis occurs at different points in the same metabolic pathway. For example, tomato carotenoid mutants that differ by a single gene from normal genotypes have been well-known for many years (Liu et al., 2016). On the other hand, obtaining of F2 plant with lutein levels above both parents is a promising result that can be exploited in further germplasm development when lutein enrichment is aimed (Wu et al., 2022).

Since the total antioxidant activity depends on the presence in the fruit of a significant number of diverse metabolites, its determination is difficult to perform and requires the conduct of a series of metabolic analyzes. The quantities of such substances are influenced not only by genetic factors. Environmental conditions (temperature, relative air humidity, etc.) in the respective growing periods also can have significant effects, which is why the values of individual components in different years can vary significantly. Therefore, it is necessary to carry out such complex studies that monitor the accumulation of a wide range of antioxidants to be able to identify and (possibly) select genotypes with persistently high levels of one or another antioxidant component. In particular, the discrepancies between the results of the F1 hybrids and the values in the F2 population can be explained both by the presence of heterosis effects in the F1 (a phenomenon well known in tomato for a number of phenotypic traits) and by the fact that the two generations were grown in different years. Which of these factors played a higher role for the observed metabolic differences is the subject of an additional study, which (although indeed of considerable interest) cannot be presented within the scope of this publication. However, with the present study we have demonstrated the usefulness of an integrated approach when the volumes of genotypic and phenotypic information become extremely large and difficult to use when applying classical selection approaches.

Conclusions

The adoption of the integrative approach to developing new tomato varieties with elevated fruit antioxidant content facilitates the identification of proper parental lines for crossing together with simplifying and streamlining the procedures for analyzing F1 and F2 generations. Performed selection of the specific parent combinations based on a heuristic approach that prioritizes biological significance allowed to reduce the total number of analyzed crosses and plants. By applying a combination of phenotyping, genotyping and metabolic profiling the efficient resource use can be achieved that results in adequate characterization of the variability of antioxidant contents in the F2 population so that individuals with desired characteristics can be selected for further development into varieties. In this particular study F2 individuals 20, 24 and 27 expressed notable quantities of lycopene, β-carotene, and α-tocopherol, respectively.

Author Contributions

Conceptualization, B.B., D.G. and A.P.; methodology, B.B., D.G. and A.P.; resources, D.G.; data curation, S.V., D.G. and V.G.; writing–original draft preparation, B.B.; writing–review and editing, B.B., S.V., D.G., V.G. and A.P.; visualization, B.B., S.V.; supervision, A.P.; project administration, A.P.; funding acquisition, A.P. and D.G.. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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