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Pigmented maize - a potential source of β-carotene and α-tocopherol

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Abstract
Among cereals, maize has the highest content of bioavailable micronutrients in grain, particularly β-carotene and α-tocopherol, which makes this crop the most appropriate for biofortification. Great genetic variability is a valuable source of micronutrients, and genotypes with enhanced grain content could be used for improvement of commercial hybrids or synthetic populations creation. Three populations with dark orange, dark red and red grain, five elite lines, and their crosses were evaluated for β-carotene and α-tocopherol content. Based on obtained results, line (L5) could be further used in breeding for increased β-carotene content, and population with dark orange grain (P1) is recommended as a good source for multi-nutrient biofortification for both β-carotene and α-tocopherol. Three lines (L1, L2 and L5) had significantly higher value of α-tocopherol in crosses with dark red population (P2), compared to lines *per se*, and require several cycles of back-crossing for increase nutrient content.

1. INTRODUCTION

Biofortification is the process of increasing content and/or bioavailability of essential nutrients in crops during plant growth (Bouis et al., 2011). Nutritional goals for biofortification implicate: increased vitamin and mineral content, elevated amino acid and antioxidant values, and favorable fatty acid composition in edible parts of crops (Hirschi, 2009). It can be realized through three main approaches: conventional (crop breeding), transgenic (genetic engineering) or agronomic (use of fertilization). Agronomic biofortification include physical application of fertilizers to the soil or directly to the plant leaves. The method temporarily improves nutritional status of crops and contributes to human health by consumption. Nowadays, transgenic biofortification is widely studied and researched, but with low utilization because of regulatory and biosafety concerns. From that perspective, conventional breeding method for nutritional improvement is the most accepted, sustainable and cost-effective alternative to transgenic and agronomic-based strategies (Garg et al., 2018). The main limitation could be absence of sufficient genetic variability for the trait of interest, but usually desirable sources could be found in germplasm collections within gene banks worldwide. One of the most cultivated crops with a large genotypic and phenotypic variation is maize (corn). This cereal has numerous types of grain texture, with colors such as white, yellow, orange, violet, red, black, and blue. The great diversity of maize was the basis for the breeding programs resulting in high yielding genotypes worldwide. Nowadays, pigmented maize is receiving more interest since it contains bioactive phytochemicals such as carotenoids, tocopherols, phytic acid and phenolic compounds. The significant maize variability in grain composition is a source of desirable genes for grain micronutrient content improvement by conventional breeding or by biotechnology, and thus makes maize as an apparent target for biofortification projects. Among cereals, maize possesses the greatest natural genetic variation for carotenoids, suitable for provitamin A biofortification (Chander et

al., 2008; Menkir et al., 2008). The yellow maize contains carotenoid isoforms, including two carotenes (α-carotene and β-carotene) and three xanthophylls (β-cryptoxanthin, zeaxanthin and lutein, Cazzonelli and Pagson, 2010). In some studies genotype x environment (G × E) interaction was very low indicating that carotenoids contents are stable across locations (Muthusamy et al., 2015). Higher carotenoid content could be estimated based on grain color, but not determineted, because of possible accumulation in different parts of maize kernel (seed coat, endosperm or germ). However, maize is not only a good source for provitamin A carotenoids but also a source of vitamin E, tocochromanols and phenolic compounds, which have antioxidant properties (Muzhingi et al., 2017). Vitamin E is common name for eight naturally occurring compounds, lipid-soluble antioxidants with two distinct groups, tocopherols and tocotrienols (Della Penna, 2006). Tocopherols are essential micronutrients, but unable to be synthesized by the human body. Among cereals, maize grain has the highest content of tocopherols and the two predominant isomers are γ-tocopherol and α-tocopherol (Rocheford et al., 2002). Although γ-tocopherol is naturally most abundant in maize, α-tocopherol has higher biological activity and is considered more desirable for consumption. Therefore, a breeding goal in biofortification is increased level of α-tocopherol (Péter et al., 2016). Maize Research Institute Zemun Polje gene bank (https://mrizp.rs/emdb/default.htm) is one of the largest maize collections in the world (FAO STAT, 2010). It encompasses about 2200 landraces collected in former Yugoslavia, and about 4000 accessions introduced from different countries. For the present study we chose one population with dark orange grain, from Argentina (P1), another one with dark red grain, from Serbia (P2), and a third one with red grain, from Bosnia and Herzegovina (P3). The aim was to estimate differences in β-carotene and α-tocopherol content in the populations and in five commercial lines, as well as in their crosses and reciprocal crosses, in order to enhance nutritional content in lines. The best combination will be selected for further breeding for increasing single β-carotene, or α-tocopherol content, as well as for obtaining multinutrient-rich maize cultivars i.e. maize genotypes with increased content of combination of both micronutrients.

2. MATERIAL AND METHODS

2.1. Plant material

Three maize populations, P1, P2 and P3 (Figure 1.) and five yellow grain lines, L1, L2, L3, L4 and L5 (Figure 1.), parental components of commercial hybrids, were grown in the experimental field in Zemun Polje in 2017. Each genotype was planted in five rows with two replications in complete randomized block design (RCBD). Plants were sown in single row plots with 15 hills per row and spaced 0.75 m apart with intra-row distance of 0.4 m between hills. Plots were overplanted and thinned to two plants per hill after seedling establishment. Standard agronomical practice was applied. At flowering, plants from one row were selfed, and the others were used either as male or female in crosses. After harvest, ears were dried, shelled, bulked within subsample, and stored for further analysis.

2.2. Chemicals and materials

Acetonitrile, 2-propanol and ethyl acetate purchased from Sigma Aldrich (Germany), were HPLC grade as well as methanol obtained by J.T. Baker (Netherlands). β-carotene and α-tocopherol standards, were also purchased from Sigma Aldrich (Germany) while syringe filters (PTFE membrane 0.45 μm) were purchased from Thermo Scientific (Germany).

2.3. Sample preparation

Acetonitrile, 2-propanol and ethyl acetate purchased from Sigma Aldrich (Germany), were HPLC grade as well as methanol obtained by J.T. Baker (Netherlands). *β*-carotene and α-tocopherol standards, were also purchased from Sigma Aldrich (Germany) while syringe filters (PTFE membrane 0.45 μm) were purchased from Thermo Scientific (Germany).

2.4. Analysis of β-carotene

For *β*-carotene extraction, maize sample (0.2 g) was homogenized twice with 5 mL of mixture methanol-ethyl acetate (6:4, v/v) for 30 min at 25ºC (Sol Rivera and Ramon Canela, 2012). If the residue was colored, extraction was repeated until it became colorless. Collected extracts after centrifugation were evaporated (in the stream of nitrogen) to dryness and redissolved in 1mL of the mobile phase. After filtration through syringe filter, the extracts were injected in the Dionex UltiMate

3000 liquid chromatography system (Thermo Scientific, Germany) fitted with photodiode array detector (DAD) and analytical column, Acclaim Polar Advantage II, C18 (150×4.6 mm, 3 µm). Methanol-acetonitrile mixture (9:1, v/v) (Kuhnen et al., 2011) was used as mobile phase at flow 1 mL/min, column temperature was 25ºC, while injection volume was 50 µL. Tested *β*-carotene was recorded at 450 nm, and the content was express as micrograms (µg) per gram (g) of dry weight (DW).

2.5. *Analysis of* α-tocopherol

Maize sample (0.2 g) was mixed with 2-propanol (4 mL) and homogenised for 30 min at room temperature in order to extract the tocopherol (Gliszczyńska-Świgło et al., 2007). The extracts were then centrifuged at 3000 rpm for 5 min, filtered through a 0.45 µm membrane filter and the clear supernatant was directly injected in the same liquid chromatography system equipped with fluorescence detector (FLD–3100) and the same analytical column maintained at 30ºC. Mixture of acetonitrile and methanol (1:1, v/v) was used as a mobile phase (1 mL/min), injection volume was 5µL, and the wavelengths for excitation and emission were set at 290 nm and 325 nm, respectively. The α-tocopherol content is expressed as µg/g DW.

2.6. Statistical Analysis

A factorial analysis of variance (ANOVA) according to RCBD was performed separately for *β*-carotene and α-tocopherol, for groups I, II and III, related to each population. Significant differences between mean values were determined by the Fisher's least significant difference test (LSD) at the 0.001 probability level. All analyses were carried out by using the statistical program M-STAT-C (Michigan State University).

3. RESULTS AND DISCUSSION

Thus far, the main priorities of agriculture have been increasing grain yield and crop production which hasin widening micronutrient deficiency and malnutrition among consumers. Nowadays, the aim of agriculture production is not only to produce adequate quantities of crops, but to enrich their nutritional content, too. Projections of global population growth and climatic changes in coming decades make food security the main focus for researchers worldwide (Bazuin et al., 2011). Accordingly, World Health Organization (WHO) and the Consultative Group on International Agricultural Research (CGIAR) set the development of nutritionally enhanced high-yielding biofortified crops as one of the most important goals (Bouis, 2010). Humans and animals are unable to synthesize carotenoids and tocopherols, and consequently depend on dietary sources of these nutrients. Mean squares from analysis of variance (ANOVA) for all genotypes and both traits were significant at 0.001 level. Average values of *β*-carotene and α-tocopherol content for the analyzed genotypes are given in Tables 1,2, and 3, as well as the results of LSD tests among them. Table 1 presents a subset of the following samples: population with dark orange grain (P1), inbred lines, and their crosses and reciprocal crosses. The content of *β*-carotene ranged from 0.47 µg/g DW for L1 to 26.9 µg/g DW for P1. The average content for all crosses was 8.36 µg/g DW, and that was the highest level in all three groups (data not shown). The highest significantly different content (16.48 µg/g DW) was obtained for cross P1 x L5. Crosses with line L4, either as male or female, had similar *β*-carotene content. This group has the highest level of α-tocopherol, in the range of 1.66 µg/g DW (L5) and 27. 2 µg/g DW (P1). The highest content was obtained in both crosses with L4, and the similar level was in the cross P1 x L3 (13.21 µg/g DW). The lowest α-tocopherol content was obtained in both type of crosses with line L5, although it was more than in line *per se*.

Table 1. Mean values of nutrient content in grain of maize population P1, inbred lines, and their crosses

Genotypes	β-carotene[a] (µg/g DW)	α-tocopherol (µg/g DW)
P1	26.90^{a}	27.2^{a}
P1xL1	10.22^{d}	8.75^{de}
P1xL2	13.41^{c}	6.85^{ef}
P1xL3	8.14^{de}	13.21^{b}
P1xL4	7.94^{de}	11.98^{bc}
P1xL5	16.48^{b}	3.07^{gh}
L1xP1	3.23^{fgh}	6.49^{f}
L2xP1	4.75^{f}	10.51^{cd}
L3xP1	4.12^{fg}	5.43^{f}
L4xP1	8.00^{de}	13.29^{b}
L5xP1	7.33^{e}	5.16^{fg}
L1	0.47^{i}	6.08^{f}
L2	1.21^{hi}	6.95^{ef}
L3	2.01^{ghi}	6.76^{f}
L4	2.98^{fgh}	7.21^{ef}
L5	2.79^{fghi}	1.66^{h}
$LSD_{(0.001)}$	2.322	2.199

[a]Values with different letters in the same column are significantly different at 0.001 level; $LSD_{(0.001)}$ – last significant difference value at 0.001 level.

Table 2 gives the results for the second group of samples (P2-dark red grain population, inbred lines, and their crosses and reciprocal crosses). For the population *per se*β-carotene was not detected and the highest value was 7.61 µg/g DW for cross L5 x P2. Average value of *β*-carotene in all the crosses was the lowest in all the examined groups, and was even lower than in lines *per se*. The exception is cross P2 x L5 with significantly different value (7.61 µg/g DW). Within these groups α-tocopherol ranged from 1.66 µg/g DW (L5) to 8.35 µg/g DW (L1 x P2). Inbreds L3 and L5 had higher content in both types of crosses compared to lines *per se*. Table 3 gives the results for the third group: a subset of red grain population-P3, inbreds, and their crosses and reciprocal crosses. The content of *β*-carotene was in the range of 0.47 µg/g DW (L1) and 7.00 µg/g DW (P3 x L2). The average content for all the crosses was the lowest, 2.96 µg/g DW. Significantly higher contents, compared to population and inbred lines *per se*, were obtained in crosses P3 x L2 and L4 x P2. Higher *β*-carotene content was obtained in both kinds of crosses with L5, with no significant deference between them. The lowest level of α-tocopherol was obtained in group III, ranging from 1.28 µg/g DW (P3 x L5) to 8.06 µg/g DW (P3 x L1). All the crosses within this group had grain content of α-tocopherol lower than in the corresponding inbreds. The only exception was cross P3 x L1, with the highest content of the examined nutrient.

Table 2. Mean values of nutrient content in grain of maize population P2, inbred lines, and their crosses

Genotypes	β-carotene[a] (µg/g DW)	α-tocopherol (µg/g DW)
P2	0.00^{h}	4.87^{g}
P2xL1	0.48^{fg}	5.23^{fg}
P2xL2	0.62^{f}	2.07^{h}
P2xL3	0.27^{g}	7.63^{abc}
P2xL4	0.62^{f}	6.10^{def}
P2xL5	3.07^{bc}	1.99^{h}
L1xP2	1.23^{e}	8.35^{a}
L2xP2	1.08^{e}	7.97^{ab}
L3xP2	1.02^{e}	5.59^{fg}
L4xP2	3.16^{b}	7.07^{bcd}
L5xP2	7.61^{a}	5.22^{fg}
L1	0.47^{fg}	6.08^{ef}
L2	1.21^{e}	6.94^{cde}
L3	2.01^{d}	5.63^{fg}
L4	2.98^{bc}	7.21^{bc}
L5	2.79^{c}	1.66^{h}
$LSD_{(0.001)}$	1.92	5.72

[a]Values with different letters in the same column are significantly different at 0.001 level; $LSD_{(0.001)}$ – last significant difference value at 0.001 level

Table 3. Mean values of nutrient content in grain of maize population P1, inbred lines, and their crosses

Genotypes	β-carotene[a] (µg/g DW)	α-tocopherol (µg/g DW)
P3	2.51^{de}	4.95^{ef}
P3xL1	1.13^{f}	8.06^{a}
P3xL2	7.00^{a}	2.96^{g}
P3xL3	2.34^{de}	4.95^{ef}
P3xL4	2.00^{e}	4.88^{ef}
P3xL5	3.70^{c}	1.28^{h}
L1xP3	0.72^{fg}	4.66^{f}
L2xP3	2.06^{e}	5.17^{def}
L3xP3	1.88^{e}	5.22^{def}
L4xP3	4.85^{b}	5.72^{de}
L5xP3	3.92^{c}	1.57^{h}
L1	0.47^{g}	6.08^{cd}
L2	1.21^{f}	6.95^{bc}
L3	2.01^{e}	5.65^{de}
L4	2.98^{d}	7.21^{ab}
L5	2.79^{d}	1.65^{h}
$LSD_{(0.001)}$	0.652	0.959

[a]Values with different letters in the same column are significantly different at 0.001 level; $LSD_{(0.001)}$ – last significant difference value at 0.001 level

The least abundant of the three carotenoid components (*β*-carotene, zeaxantin and lutein) is *β*-carotene and breeding for its higher content is beneficial for human health. In the comparative analysis of bioactive phytonutrients in five pigmented maize landraces from Italy, the *β*-carotene content was lower than in our populations and ranged from 0.27-0.3 µg/g DW (Bacchetti et al., 2013). However, the average value of *β*-carotene content of 2.09 µg/g DW obtained in testing nutritional content of 16 landraces from MRIZP gene bank (Vančetović et al., 2016) is in line with the obtained value for population P3. The range of *β*-carotene content in commercial lines from our study was from 0.47-2.98 µg/g DW. Similar average content of 2.5 µg/g *β*-carotene was observed in the analysis of ten maize inbreds with yellow to orange kernel (Egesel et al., 2003). Another research comprehends 87 inbred lines from the main heterotic groups in China and revealed β-carotene content in range from 0.016-1.726 µg/g DW (Chander et al., 2008), much lower than obtained in inbreds in our study. In maize hybrids biofortified for vitamin A (Kurilich and Juvik, 1999) *β*-carotene content was 0.55 µg/g DW, and in new 36 provitamin A biofortified hybrids (Muzhingi et al., 2017) *β*-carotene was within range from 1.3-8.00 µg/g DW. Provitamin A (α-carotene, β-carotene, β-cryptoxanthin) and non-provitamin A carotenoids (lutein and zeaxanthin) in maize are primarly located in the endosperm, but tocopherols (α-

tocopherol, γ-tocopherol and δ-tocopherol) are located in the embryo. Nutritional study in 16 maize landraces differing in origin, obtained the average content of α-tocopherol in grain of 4.95 μg/g DW, and the highest two were with 13.3 μg/g DW and 9.14 μg/g DW (Vančetović et al., 2016). Populations P2 and P3 had α-tocopherol content in line with the average level in the stated study. However, population P1 had a very high α-tocopherol content, likewise Mexican landraces (22.6 μg/g DW, Wyatt et al., 1998). SNP markers were applied on two segregating populations, made from elite line K22 with medium content of α-tocopherol (5.13 μg/g DW), line D (low content-2.98 μg/g DW), and line C17 (high content-6.76 μg/g DW, Shutu et al., 2012). The value of α-tocopherol in inbred lines used in our study was in the similar range, namely from 1.65-7.21 μg/g DW, where four lines, out of five, had content of this nutrient above 5.65 μg/g DW. The contents of α-tocopherol in our inbreds were very low, compared to the nutritional composition of 87 elite inbred lines in China (Chander et al., 2008). Although their content of β-carotene was low, α-tocopherol was significantly higher with the average value of 23.98 μg/g DW (the range was 3.98-74.81 μg/g DW). The contents of α-tocopherol in provitamin A biofortified maize hybrids vary from 3.4 to 34.3 μg/g DW (Muzhingi et al., 2017). Based on present knowledge and experiance, two approaches in breeding maize for increased micronutrient content in grains could be recommended: a) selection of genotypes with increased concentration of particular nutrients and boosting their content through several cycles of selection by creating synthetic populations, and b) development of hybrids from parental lines with enriched grain micronutrient content and with good combining ability (Maqbool et al., 2018).

Figure 1. Different coloured maize grain: populations (P1, P2 and P3) and inbred lines (L1, L2, L3, L4 and L5)

Both approaches aim to produce micronutrient enhanced maize genotypes, keeping tolerance to biotic and abiotic stresses, high yield production, and acceptance of final consumers (Ortiz-Monasterioer al., 2017). Previous results confirmed that maize lines with increased β-carotene content, maintain that trait across different locations and for several years, which makes them suitable for biofortification breeding programs (Egesel et al., 2003; Suwarno et al., 2015). Numerous inbreds with medium of high content of provitamin A are successfully used as components in hybrids and synthetic population, without reducing grain yield and stress resistance (Menkir et al., 2017). Five elite inbreds from our study showed different concentrations of grain β-carotene content, depending on the population and male/female position in crosses: all crosses with P1 obtained higher β-carotene content, compared to lines *per se*; crosses within the second group had only four combinations with higher content than inbreds; in the third group, only two crosses had lower content compared to appropriate inbreds. The only inbred line that had higher content of β-carotene in all crosses with all three populations, than in line *per se*, was line L5. Considering α-tocopherol content, there were variability between groups and lines: in the first group, the highest content in crosses, higher that in line *per se*, has inbred L4; L3 obtained higher content in both types of crosses, than inbred *per se*; and in the third group combination P3 x L1 has higher α-tocopherol content than P3 and L1 *per se*.In provitamin A biofortified maize hybrids, significant correlations between β-carotene and α-tocopherol were obtained (Muzhingi et al., 2017), and similar and relatively high values of both nutrients were measured in dark orange population P1 (26.9 versus 27.2 μg/g DW) in our study. Biofortified maize cultivars, the so-called 'first generation', were enhanced for single nutrient, but new scientific achievements are directed to 'second generation' of biofortified maize genotypes, with increased content of combination of more micronutrients (Gupta et al., 2015). Simultaneous breeding for multinutritional traits in maize, could increase utilization of maize grain in nutrient rich meals instead of using synthetic carotenoids and tocopherols.

CONCLUSIONS

Improving maize grain with provitamin A and vitamin E, e.g. β-carotene and α-tocopherol, as the most important bioavailable compounds for humans, is a significant issue nowadays. In addition to the increase of single nutrient content,

simultaneous breeding for multi-nutritional traits has numerous beneficial effects. Population with dark orange grain (P1), with high content of both β-carotene and α-tocopherol, is a good source for multi-nutrient biofortification. Inbred line L5, in crossing with all three populations, is a good recipient for enhanced value of β-carotene, and could be used for improvement of this trait in commercial maize hybrids. Lines L1, L3 and L4 have different content of α-tocopherol in crosses with different pigmented populations, and thus require further testing and estimation before being included in biofortification programs. Breeding strategy for the development of maize genotypes enhanced with micronutrients could be either to develop inbred lines that can be used as parental lines for biofortified hybrids or to create synthetic populations with increased values of nutrients. The choice depends on the available genetic resources, potential utilization, and end-user needs. Although transgenic approach in biofortification has had some success ('Golden rice'), it has been less prosperous in maize. Thus, recommendation for maize biofortification is the exploitation of genetic variations within available germplasm that have higher variability in micronutriens content than transgenic, and further application of conventional breeding strategies.

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