Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
http://dx.doi.org/10.5424/sjar/2014122-5222

Spanish Journal of Agricultural Research 2014 12(2): 467-476
ISSN: 1695-971-X
eISSN: 2171-9292

RESEARCH ARTICLE

OPEN ACCESS

Simulating of Top-Cross system for enhancement of antioxidants in maize grain

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Abstract

Blue maize (*Zea mays* L.) is grown for its high content of antioxidants. Conversion of yellow and white to blue maize is time consuming because several genes affect blue color. After each backcross selfing is needed for color to be expressed. In order to overcome the problem of time and effort needed for conversion to blue kernel color, we have set a pilot experiment simulating a Top-cross system for increasing antioxidants in maize grain. The idea is to alternately sow six rows of sterile standard quality hybrid and two rows of blue maize in commercial production. Five commercial ZP hybrids were crossed with a blue pop-corn population. Xenia effect caused by cross-pollination produced blue grain on all hybrids in the same year. Chemical analyses of the grains of five selfed original hybrids, five cross-pollinated hybrids and selfed blue popcorn pollinator were performed. Cross-fertilization with blue popcorn had different impact on antioxidant capacity and phytonutrients, increasing them significantly in some but not all cross-pollinated hybrids. Popcorn blue pollinator had higher values for all the analyzed traits than either selfed or cross-pollinated hybrids. Selfed *vs*. pollinated hybrids showed significant difference for total antioxidant capacity ($p < 0.1$), total phenolics and total yellow pigments ($p < 0.01$), with the increase of total phenolics and decrease of total yellow pigments in pollinated ones. Total flavonoids showed a little non-significant decrease in pollinated hybrids, while total anthocyanins were not detected in selfed yellow hybrids. Blue maize obtained this way has shown good potential for growing high quality phytonutrient genotypes**.**

Additional key words: anthocyanins; blue kernels; flavonoids; phenolics; xenia; *Zea mays*.

Introduction

Maize (*Zea mays* L.) can have different grain colors such as black, blue, red, orange, yellow, white and brown. Although majority of the dent maize types has a yellow endosperm, white, red and blue dents are also popular in human food products (Cortes *et al*., 2006; Revilla *et al*., 2008). Pigmented maize products have a unique characteristic flavor due to polyphenols and other related compounds (Rooney & Serna-Saldivar, 2003). Anthocyanins, probably the most important group of visible plant pigments besides chlorophyll, are presented in blue, purple and black colored maize grains. Main anthocyanins in these grains are cyanidin glycosides (Pascual-Teresa *et al*., 2002; Escribano-Bailón *et al*., 2004; Del Pozo-Insfran *et al*., 2006) that account for 70% of the anthocyanins (Aoki *et al*., 2000). Anthocyanin glycosides, simple or acylated, are mainly located in the aleurone layer of the grain, affecting its color. Small to medium sized kernels have the darkest blue coloration, since they have a higher proportion of aleurone compared to the starchy endosperm (Betran *et al*., 2000). Anthocyanins, as well as other polyphenols and phenolic acids are thought to be non-nutritive, but interest in them has increased due to health benefits and nutraceutical effects (Setchell & Aedin, 1999). Consumption of these matters is in opposite relationship with the incidence of various

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Received: 31-10-13. Accepted: 01-04-14.

Abbreviations used: BC (backcross); βCE (β-carotene equivalent); CE (catechin equivalent); CGE (cyanidin 3-glucoside equivalent); CMS (cytoplasmic male sterile); DM (dry matter); GAE (gallic acid equivalent); RCBD (randomized complete block design); TAC (total anthocyanins); TEAC (total antioxidant capacity); TF (total flavonoids); TP (total phenolics); TYP (total yellow pigments).

chronic and degenerative diseases, like cardiovascular disease, urinary tract disorders and different types of cancer. All this is due to the high antioxidative and antiradical activities, but also to some other mechanisms, such as antimutagenic, estrogenic activities, inhibition of enzymes, induction of detoxification enzymes (Adom & Liu, 2002; Rondini *et al*., 2002; Tsuda *et al*., 2003; Fimognari *et al*., 2004). Several authors have reported considerable differences in phytochemical contents and antioxidant capacity between a set of maize genotypes with different kernel colors and they found that a higher content of pigment compounds in maize kernels is associated to higher antioxidant capacity (Lopez-Martinez *et al*., 2009; Hu & Xu, 2011; Zilic *et al*., 2012; Rodriguez *et al*., 2013).

Mode of inheritance of grain color is very complex involving many different genes (Betran *et al*., 2000). More than 20 genes play a structural or regulatory role in anthocyianin biosynthesis in maize (Chandler *et al*., 1989; Dooner *et al*., 1991; Pilu *et al*., 2003). Due to this complexity, conversion of commercial yellow or white grain lines to blue color is a time demanding task (Lago *et al*., 2013). Also, antioxidants responsible for maize kernel blue color are mainly concentrated in aleurone, which is triploid in nature due to the fusion of one haploid sperm and two haploid polar nuclei. For this reason kernels on one ear can have several different genotypes and phenotypes, including coloration. This makes converting to blue maize more difficult, because, after the first cross with the source of blue kernel, F1 kernels can have many different colors that are rarely dark blue. That is why selfing must be done in F1 and after each backcross (BC) generation, considerably prolonging the process of convertion of yellow or white commercial inbred lines to blue ones. However, avoiding selfing in some cases is possible, depending on genetic structure of recurrent line.

A variant of Top-Cross[1] system could be performed for enhancement of antioxidants in maize grain that can eliminate the need for converting an inbred line to a blue kernel color. Top-Cross maize production system serves for production of high oil corn (Thomison *et al*., 2002, 2003). It involves planting a mixture of 90% of high yielding sterile hybrid and 10% of special high-oil pollinator that benefits to higher oil content in mother hybrid kernel. This system relies on xenia effect which is a consequence of cross-fertilization of nonrelated lines or hybrids of crops, resulting in grains on mother plants with higher yield and/or better grain quality - larger embryo that contains primarily oil (Letchworth & Lambert, 1998; Liu *et al*., 2010). Male genes involved in xenia effect can affect color, chemical composition and development of the pericarp, mesocarp, endosperm, and the embryo weight in plants (Denney, 1992).

Herein, we present the results of a preliminary research conducted with the objective to evaluate the applicability of Top-Cross system for efficient production of mercantile blue maize hybrids. High yielding commercial male sterile hybrids were used as mothers and a dark blue kernel population as a pollinator. It was expected that due to the xenia effect in the year of fertilization kernels on mother plants would get a partially blue color and thus have higher levels of antioxidants compared to the original hybrids. In this way blue kernel maize could be obtained in just one year, much easier than by converting inbred lines to a blue kernel color by backcrossing at least seven years. This approach could lead to new opportunities for production of value-added varieties rich in health-beneficial components for making functional foods, functional food colorants or/and dietary supplements.

Material and methods

Plant material

Five yellow dent commercial ZP hybrids of standard kernel quality (ZP-1, ZP-2, ZP-3, ZP-4 and ZP-5) were converted into cytoplasmic male sterile (CMS) variants and used as mothers in hand pollination with dark blue popcorn variety used as the father. At the same time, original fertile versions of the sterile hybrids were selfed, simulating mercantile production. Sterile versions of the hybrids were obtained by crossing the sterile mother inbred line with the father inbred line that is a non-restorer of fertility, so pollen fertility restoration was disabled. Original fertile hybrids were obtained by crossing the sterile mother inbred line with fertility restorer version of the father inbred line. For the recurrent parent genotype to be

[1] Top-Cross is registered trademark of DuPont Specialty Grains, DesMoines, IA, USA. Trade name is used in this article solely for the purpose of providing specific information.

restored, sterile, restorer and non-restorer versions of inbred lines (hybrid components) were backcrossed with original inbred line at least seven generations during conversion.

Dark blue popcorn used as a donor of a blue color is an open-pollinated population obtained from Maize Research Institute Gene Bank. It has been chosen because of the fixed alleles for dark blue color and high production of pollen. Additionally, this population pollinates during a long period of time and it could provide adequate amount of pollen for hybrids of different maturity groups used in this research. Gametophyte factor that many popcorn strains carry (Kermicle, 2006) was not of relevance, because the blue population was used in a simulation of production of mercantile blue maize and will not be used in any breeding program. Plants from the blue population were also selfed for measurement of traits *per se.*

The trial was set up according to randomized complete block design (RCBD) in two replications in 2011, at the location of Zemun Polje, Serbia. Blocks comprised of both selfed and crossed standard quality hybrids. Each plot consisted of 10 hybrid plants, sown at plant density of 60,700 plants ha^{-1}. In the second block popcorn pollinator was sown alongside sterile hybrids, with five plants per plot. Standard cropping practices were applied to provide adequate nutrition and to keep the disease-free plots. Chemical analyses were performed on the grains of five selfed original hybrids, five cross pollinated hybrids and selfed blue popcorn pollinator. The maize kernels were finely ground using a Perten 120 lab mill (Perten, Sweden) to obtain fine powder (<500 μm). Ground maize flours were stored at –70°C prior to chemical analyses.

Extraction of total phenolic compounds

Phenolic compounds in maize flour were extracted according to the procedure described by Antoine *et al.* (2004). Total phenolics in 500 mg of maize flour were released by alkaline hydrolysis for 4 h at 25°C using 4 N NaOH. After the pH was adjusted to 2.0 (6 M HCl), the hydrolyzates were extracted with a mixture of ethyl acetate and diethyl ether (1:1, v/v) for four times. The combined extract (5 mL) was evaporated under N_2 stream at 30°C to dryness. The residue was redissolved in 1.5 mL of methanol and kept at –20°C prior to analysis. It was used for the analyses of total phenolic and flavonoid contents.

Analysis of total phenolic content (TP)

The total phenolic content was determined according to the Folin-Ciocalteu procedure. Briefly, the extract (100 μL) was transferred into a test tube and the volume adjusted to 500 μL with distilled water and oxidized with the addition of 250 μL of the Folin-Ciocalteau reagent. After 5 min, the mixture was neutralized with 1.25 mL of 20% aqueous Na_2CO_3 solution. After 40 min, the absorbance was measured at 725 nm against the solvent blank. The total phenolic content was determined by means of a calibration curve prepared with gallic acid, and expressed as mg of gallic acid equivalent (GAE) kg^{-1} in dry matter (DM).

Analysis of total flavonoid content (TF)

The total flavonoid content was determined according to Serpen *et al*. (2008). Briefly, 50 μL of 5% $NaNO_2$ was mixed with 200 μL of the extract. After 6 min, 500 μL of a 10% $AlCl_3$ solution was added. After 7 min, 250 μL of 1 M NaOH was added, and the mixture was centrifuged at 5000 g for 10 min. Absorbance of the supernatant was measured at 510 nm against the solvent blank. The total flavonoid content was expressed as mg of the catechin equivalent (CE) kg^{-1} in DM.

Analysis of total anthocyanin content (TAC)

Anthocyanins were extracted according to the method described by Abdel-Aal & Hucl (1999) with slight modifications. Flour (500 mg) was extracted by mixing with 10 mL of methanol acidified with 1 N HCl (85:15, v/v) and shaking for 30 min at ambient temperature. The crude extract was centrifuged at 8000 g for 20 min and absorbances of supernatant at 535 and 700 nm were measured to detect anthocyanins. Anthocyanin levels were expressed as mg of cyanidin 3-glucoside equivalent (CGE) kg^{-1} of DM, using the molar extinction coefficient of 25965 Abs/M × cm and a molecular weight of 449.2 g mol^{-1}.

Analysis of total yellow pigment content (TYP)

Ground sample (8 g) was extracted with 40 mL of water-saturated 1-butanol by means of homogenization

for 1 min, allowing to stand for 20 min at room temperature followed by a further homogenization to 1 min. The mixture was centrifuged at 5000 g for 10 min, and the absorbance of supernatant was recorded at 435 nm. The total yellow pigment content was calculated using the conversion factor of 1.6632, and expressed as mg of β-carotene equivalent (βCE) kg^{-1} of DM.

Analysis of total antioxidant capacity (TEAC)

The antioxidant capacity of maize samples were measured based on the QUENCHER method described by Serpen *et al*. (2007) using 7 mM aqueous solution of ABTS (2,2-azino-bis/3-ethil-benothiazoline-6-sulfonic acid) with 2.45 mM $K_2O_8S_2$ as the stock solution. The working solution of $ABTS^{\bullet+}$ was obtained by diluting the stock solution in water/ethanol (50:50, v/v). The finely ground sample (10 mg) was mixed with 20 mL of $ABTS^{\bullet+}$ working solution, and the mixture was rigorously shaken at 4°C for 25 min. After centrifugation at 9200 g for 5 min (10°C) the absorbance measurement was performed at 734 nm. The total antioxidant capacity was expressed as Trolox equivalent antioxidant capacity in mmol of Trolox kg^{-1} in DM.

Statistical analysis

Statistical analysis was done according to ANOVA for a two factorial RCBD design for TP, TF, TAC, TYP and TEAC. T-tests were performed for comparisons of the means of selfed *vs*. pollinated hybrids, as well as, pollinated hybrids *vs*. popcorn father, individually and averaged over all five hybrids. Pearson's correlation coefficients were done between the analyzed traits for selfed hybrids on one side, and pollinated mother hybrids together with father population at the other. Comparisons of means among all five hybrids for all the traits were done by LSD test.

Results

Pollinated sterile hybrids produced kernels with different intensity of blue color, indicating the influence of genetic constitution of mother hybrid on the intensity of blue color induced by xenia effect when crossed with dark blue kernel pollinator. The darkest kernels are shown in Fig. 1. A mixture of the same number of kernels from each class of coloration per each mother hybrid was sampled for the chemical analysis, in order to represent an average sample that could be obtained in practice. Three classes of coloration were obtained: light blue, blue and dark blue.

Anthesis-silking interval (ASI) is very important for the Top-Cross system, since it influences final kernel set on mother plant ear. Silking of the five standard quality hybrids was at 4th July (ZP-1) 2011, 5th July (ZP-2) and 7th July (ZP-3, 4 and 5). The dark blue popcorn pollinated from 4th-10th July. Since popcorns are very good pollinators, obtained kernel set on selfed hybrids was the same as in the crossed ones (data not shown).

ANOVA analysis for RCBD design (data not shown) revealed significant differences between blocks ($p < 0.01$) for TEAC, TP, and TYP. The results indicated a significant influence of cross-pollination with blue popcorn on these traits. Non-significant difference between blocks was found only for TF. Significant difference among hybrids was found for TEAC and TP ($p < 0.05$), as well as for TF and TYP ($p < 0.01$). The Hybrid × Block interaction was significant ($p < 0.01$) for all four traits.

Average values of the analyzed traits for selfed *vs*. pollinated hybrids are presented in Table 1. TAC were not detected in selfed yellow hybrids. Significant difference ($p < 0.1$) was found for TEAC. They were 17.58 mmol Trolox Eq kg^{-1} DM for selfed hybrids and 19.89 for cross-pollinated ones. TP and TYP were also significantly different in two types of hybrids ($p < 0.01$), with the increase of TP in pollinated ones (5015.65 for selfed *vs*. 5526.49 mg GAE kg^{-1} DM) and decrease of TYP (14.91 *vs*. 9.22 mg βCE kg^{-1} DM), respectively. However, TF showed a little non-significant decrease in pollinated hybrids (from 143.08 in selfed to 142.88 mg CE kg^{-1} DM in pollinated). Blue popcorn father was superior for all traits except TYP over the average of selfed and pollinated hybrids, but significance was different for each trait: $p < 0.1$ for TEAC and TAC in blue mothers *vs*. father, and $p < 0.01$ for TP and TF for the same comparison.

Hybrids reacted differently on cross-fertilization with blue popcorn regarding antioxidant capacity and phytonutrients (Table 2). TEAC was significantly improved ($p < 0.05$) by cross-fertilization in ZP-2 and ZP-4. TP were increased but not significantly in ZP-1,

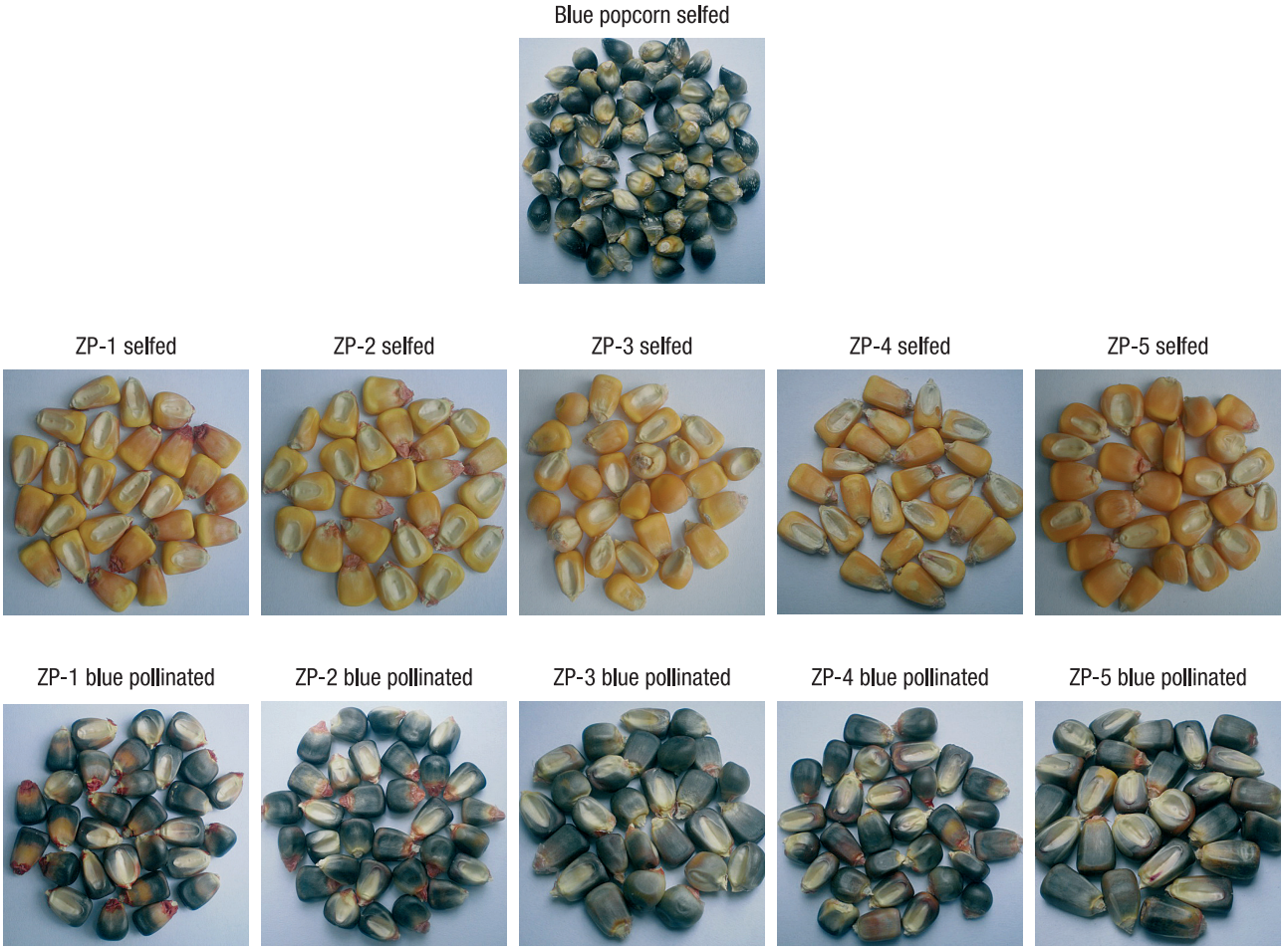

Figure 1. Selfed popcorn father population, selfed yellow hybrids and cross-pollinated hybrids.

Table 1. Average of selfed *vs*. pollinated mother hybrids and father popcorn population for phenolic compounds, yellow pigments and antioxidant capacity

Genotypes	TP (mg GAE kg^{-1})	TF (mg CE kg^{-1})	TAC (mg CGE kg^{-1})	TYP (mg βCE kg^{-1})	TEAC (mmol Trolox Eq kg^{-1})
Selfed hybrids	5015.65	143.08	n.d.	14.91	17.58
Blue pollinated hybrids	5526.49	142.88	162.70	9.22	19.89
Father population	6805.54	234.11	552.27	1.54	24.28
t-test					
t[1]	***	n.s.	—	***	*
t[2]	***	***	—	***	**
t[3]	***	***	*	***	*

TP, total phenolics (GAE = gallic acid equivalent); TF, total flavonoids (CE = catechin equivalent); TAC, total anthocyanins (CGE = cyanidin 3-glucoside equivalent); TYP, total yellow pigments (βCE = β-carotene equivalent); TEAC, total antioxidant capacity (Trolox Eq = Trolox equivalent); [1] Significance of t test between original selfed and blue pollinated mother hybrids; [2] Significance of t test between blue popcorn pollinator and selfed hybrids; [3] Significance of t test between blue popcorn pollinator and blue pollinated mother hybrids; n.s.: statistically non-significant; n.d.: not detected; *, **, ***: statistically significant at 0.1, 0.05, and 0.01 level, respectively.

Table 2. Phenolic compounds and yellow pigments contents and antioxidant capacity of hybrids *per se*, hybrids pollinated with blue popcorn and the father population

	Hybrids										Father population
	ZP-1		ZP-2		ZP-3		ZP-4		ZP-5		
	Y	B	Y	B	Y	B	Y	B	Y	B	
TP (mg GAE kg^{-1})	5898.95	6288.11	4878.21	5655.91	4951.37	4743.54	4630.01	5194.01	4719.72	5750.89	6805.54
t[1]	n.s.		n.s.		n.s.		n.s.		**		—
t[2]	*	**	**	**	**	***	**	*	**	n.s.	—
TF (mg CE kg^{-1})	171.58	176.23	104.10	140.83	160.13	112.53	126.20	132.44	153.40	152.37	234.11
t[1]	n.s.		*		n.s.		n.s.		n.s.		—
t[2]	**	**	**	*	**	*	*	**	n.s.	n.s.	—
TAC (mg CGE kg^{-1})	n.d.	198.14	n.d.	180.37	n.d.	139.83	n.d.	159.59	n.d.	135.58	552.27
t[3]	—	n.s.	—	n.s.	—	*	—	n.s.	—	*	—
TYP (mg βCE kg^{-1})	16.98	6.84	16.86	8.29	14.93	6.06	11.53	10.99	14.23	13.90	1.54
t[1]	**		**		**		n.s.		n.s.		—
t[2]	**	*	***	**	***	*	***	***	**	***	—
TEAC (mmol Trolox Eq kg^{-1})	21.21	21.53	17.89	20.99	18.17	17.53	13.16	21.07	17.48	18.33	24.28
t[1]	n.s.		**		n.s.		**		n.s.		—
t[2]	*	n.s.	n.s.	n.s.	n.s.	**	**	***	n.s.	n.s.	—

TP, total phenolics (GAE = gallic acid equivalent); TF, total flavonoids (CE = catechin equivalent); TAC, total anthocyanins (CGE = cyanidin 3-glucoside equivalent); TYP, total yellow pigments (βCE = β-carotene equivalent); TEAC, total antioxidant capacity (Trolox Eq = Trolox equivalent); Y, selfed yellow hybrid; B, blue pollinated hybrid; [1] Significance of t test between original selfed and pollinated hybrids; [2] Significance of t test between blue popcorn pollinator and selfed, *i.e.* pollinated mother hybrid; [3] Significance of t test between pollinated hybrids and the father population; n.s.: statistically non-significant; n.d.: not detected; *, **, ***: statistically significant at 0.1, 0.05, and 0.01 level, respectively.

ZP-2 and ZP-4, while significant increase was determined in ZP-5 ($p < 0.05$). Surprisingly, TP decreased in ZP-3 (non-significantly). TF significantly increased ($p < 0.1$) only in ZP-2 hybrid. TYP was, as expected, decreased in all the crosses and significantly ($p < 0.05$) in ZP-1, 2 and 3. Popcorn dark blue pollinator had higher antioxidant capacity, phenolics, flavonoids and antocyanins than that of any hybrid, either selfed or cross-pollinated. On the other hand, it had statistically lower value of TYP compared to all the selfed and pollinated hybrids. TAC was significantly higher in father population only compared to ZP-3 and ZP-5 cross-pollinated hybrids.

LSD values of mutual differences among the means of selfed and pollinated hybrids are shown in Table 3. The rank of hybrids for the analyzed four traits was changed after cross-pollination. ZP-1, followed by ZP-3, was the most favorable regarding all four traits within the selfed hybrids. Within the pollinated ones ZP-1, followed by ZP-5, was the best for all traits except TYP. On the other hand, the worst selfed hybrid was ZP-4 and the worst pollinated one ZP-3. The richest in TAC was ZP-1 blue, followed by ZP-2 blue.

For selfed hybrids, significant correlations ($p < 0.01$ and $p < 0.001$) were obtained between TEAC and TP (0.800), and TEAC and TYP (0.848), respectively (Table 4). Correlation was also significant (0.613; $p < 0.05$) between TP and TYP. For pollinated blue hybrids TEAC had significant positive correlation ($p < 0.01$) with TP (0.708), TF (0.796) and TAC (0.738). Significant negative correlation was obtained with TYP (–0.563; $p < 0.05$). TF were highly positively correlated with TP (0.931) and TAC (0.858; $p < 0.001$), and also TP with TAC (0.748; $p < 0.01$). Significant negative correlations were obtained between TF and TYP (–0.558; $p < 0.05$), as well as between TYP and TAC (–0.763; $p < 0.01$).

Discussion

Research presented in this paper revealed that blue pollinated hybrids contained statistically higher antioxidant capacity and total phenolics compared with the yellow ones. These results are in accordance with data from previous studies performed on colored maize

Table 3. Mean comparisons for phenolic compounds and yellow pigment contents and antioxidant capacity of hybrids *per se* and hybrids pollinated with blue popcorn

Traits	TP (mg GAE kg^{1})	TF (mg CE kg^{-1})	TAC (mg CGE kg^{-1})	TYP (mg βCE kg^{-1})	TEAC (mmol Trolox Eq kg^{-1})
Selfed					
ZP-1s	5898.94^{a}	171.58^{a}	—	16.77^{a}	21.21^{a}
ZP-2s	4878.21^{c}	104.10^{e}	—	16.86^{a}	17.88^{b}
ZP-3s	4951.37^{b}	160.13^{b}	—	14.93^{b}	18.17^{b}
ZP-4s	4630.01^{e}	126.20^{d}	—	11.53^{c}	13.16^{c}
ZP-5s	4719.72^{d}	153.40^{c}	—	14.23^{b}	17.48^{b}
LSD	26.45	6.05	—	1.64	2.19
Pollinated					
ZP-1b	6253.31^{a}	176.23^{a}	198.14^{a}	6.84^{cd}	21.53^{a}
ZP-2b	5655.91^{c}	140.83^{c}	180.37^{b}	8.28^{c}	20.99^{a}
ZP-3b	4743.54^{e}	112.53^{d}	139.83^{d}	6.05^{d}	17.53^{b}
ZP-4b	5194.01^{d}	132.44^{c}	159.58^{c}	10.99^{b}	21.07^{a}
ZP-5b	5750.89^{b}	152.37^{b}	135.58^{d}	13.90^{a}	18.33^{b}
LSD	28.62	8.39	5.18	1.56	2.16

TP, total phenolics (GAE = gallic acid equivalent); TF, total flavonoids (CE = catechin equivalent); TAC, total anthocyanins (CGE = cyanidin 3-glucoside equivalent); TYP, total yellow pigments (βCE= β-carotene equivalent); TEAC, total antioxidant capacity (Trolox Eq = Trolox equivalent). Means followed by the same letter within the same column are not significantly different, according to Fisher's least significance difference test ($p < 0.05$).

Table 4. Pearson's correlation coefficients between analyzed traits of selfed hybrids, as well as blue pollinated hybrids and father population

Hybrids	Trait	TEAC	TP	TF	TAC
Selfed hybrids	TP	0.800**			
	TF	0.542 n.s.	0.543 n.s.		
	TYP	0.848***	0.613*	0.123 n.s.	
Blue pollinated hybrids and father population	TP	0.708**			
	TF	0.796**	0.931***		
	TAC	0.738**	0.748**	0.858***	
	TYP	–0.563*	–0.433 n.s.	–0.558*	–0.763**

TEAC, total antioxidant capacity; TP, total phenolics; TF, total flavonoids; TAC, total anthocyanins; TYP, total yellow pigments. n.s.: statistically non-significant; *,**,***: statistically significant at 0.05, 0.01, and 0.001 level, respectively

grains. Moreno *et al*. (2005) characterized pigments in the grains of four native maize varieties from Latin America. These populations contained anthocyanins both in pericarp and aleurone and total anthocyanins content ranged from 54 to 115 mg 100^{-1} g of the sample. In the work of Vazquez-Carrillo *et al*. (2006) blue kernels of 13 maize populations had the highest antioxidative activity, followed by violet and red kernels. Comparing black, yellow and white maize, Hu & Xu (2011) showed that black waxy maize had the highest content of anthocyanins, phenolics and antioxidant activity, while yellow maize a relatively large amount of carotenoids. White maize had the lowest amounts of these compounds. Purple variety in Lopez-Martinez *et al*. (2011) research had the highest phenolic and anthocyianin content, as well as antioxidant activity, followed by red, blue and white maize. Rodriguez *et al*. (2013) concluded that selection for kernel color intensity increased antioxidant capacity and revealed that black kernels had more

anthocyanins than any other class of color, followed by purple and pink kernels.

Zilic *et al*. (2012) determined total phenolic, flavonoids, anthocyanins and antioxidant capacity in whole kernels of ten different colored maize genotypes and found considerable differencies between them. Maize with light blue kernels had the highest content of total phenolics. In colored maize total anthocyanins ranged from 2.50 (red-yellow maize) to 696.07 (dark red maize) mg CGE kg^{-1} DM. In our research these values ranged from 135.58 (ZP-5b) to 552.27 mg CGE kg^{-1} DM (father population).

Total phenolic of all analyzed genotypes in Zilic *et al*. (2012) ranged from 4494.1 to 10528.8 mg GAE kg^{-1} DM. Among the tested anthocyanin-rich genotypes, the highest total phenolic content was found in a light blue, followed by a dark blue genotype (10528.8 and 7352.5 mg GAE kg^{-1} DM, respectively). In our research phenolic content ranged from 4630.01 (ZP-4 selfed) to 6805.54 mg GAE kg^{-1} DM (popcorn pollinator), pointing out that more favorable blue genotypes of maize could be found for proposed Top-Cross scheme. Red and dark red maize in Zilic *et al*. (2012) were lower in flavonoid contents than yellow, lemon yellow and orange maize. The authors have speculated that it was due to the fact that other flavonoid compounds (such as flavonols and flavones) rather than anthocyanins in the yellow and orange maize were higher than in red and blue maize genotypes. This could also be the explanation of our results which showed lower level of flavonoids in blue pollinated than yellow original hybrids.

According to the correlations between the analyzed phytonutrients and antioxidant capacity found in our research, it could be concluded that total antioxidant capacity in yellow maize was largely due to total yellow pigments rather than to total flavonoid content, while the reverse is true for blue maize. Zilic *et al.* (2012) showed that total antioxidant capacity in colored maize is mostly due to anthocyianins. However, the same authors assumed that the antioxidant capacity of studied carotenoids, that mainly make the yellow pigments, was disguised with strong antioxidant capacity of anthocyanins in the samples of different red and blue maize. In our study, a lower content of yellow pigments in the blue genotypes could influence the negative correlation between the total yellow pigment content and the content of total flavonoids. Rodrigez *et al*. (2013) reported high significant correlation between antioxidant capacitiy of anthocyanins and carotenoid extracts ($p < 0.01$), while correlations between carotenoid or anthocyanin contents and their antioxidant capacities were above 0.9 ($p < 0.05$). In Zilic *et al*. (2012) significant correlations were found for total phenolic and antioxidant capacity, total phenolic and total flavonoids ($p < 0.01$), as well as total anthocyanins and antioxidant capacity ($p < 0.05$). In contrast, Del Pozo-Insfran *et al*. (2006) showed that blue genotype with lower total polyphenolics content had higher antioxidant activity.

Many alleles with different mode of inheritance determine blue color of maize grain (Bertran *et al*., 2000). In accordance to this, antioxidant capacity and content of phytonutrients were found to be very dependent on the genetic structure of each individual maize genotype analyzed in our research. Del Pozo-Insfran *et al*. (2006) revealed that the particular polyphenolic composition of each genotype and interaction among their constituents are factors that likely impact the antioxidant capacity levels. Lopez-Martinez *et al*. (2009) have also found that differences in antioxidant activities are due to the unique profile of anthocyanins and other phenolics in each genotype. Antioxidant activity cannot be estimated as a simple sum of antioxidant capacity levels of different phytonutrients in a genotype. Moreover, strong antioxidant capacity level of one phytonutrient can disguise this activity of another one in the genotype (Zilic *et al.,* 2012).

One of the most important preconditions for success of Top-Cross production system is good synchronization between silking of mother hybrids and pollination of blue father populations, so that a full kernel set could be obtained on sterile mother hybrids. In this way, both the effect of CMS (sterile hybrids are often more yielding than their conventional counterparts) and xenia effects would be maximally utilized. The differences between silking of the sterile mothers and anthesis of the blue population was not greater than six days (depending on a mother genotype) which was good enough to provide adequate pollination and full kernel set on mother hybrid ear, according to Westgate *et al*. (2003).

Kroon & Williamson (1999) and Adom & Liu (2002) claimed that differences in polyphenolic composition between the blue corns was a varietal effect with differences due to environmental, seasonal, and geographical growing conditions. According to Hadži-Tašković Šukalović *et al.* (2013), antioxidant compounds can be largely influenced by environment and Genotype × Environment interaction. To minimize

environmental variation, all the analyzed kernels from the different genotypes were produced in the same year. On the other hand, we obtained data only from the single year and single location, so environmental influence, as well as Genotype × Environment interaction was not included. Before any possible introduction in commercial production this system must be examined in different locations and years in larger trials to confirm its benefit.

It is obvious that blue grain maize obtained in the way explained in this paper showed good potential for growing high quality maize. This, of course, has yet to be proven in practice, in large strip trials. The recommendation would be to sow sterile conventional hybrids in six mother rows and donors of blue kernel modification in alternate two rows as fathers. In this way, harvesting and further processing of maize kernel could be done separately for mother and father genotypes, since they could vary very much in grain characteristics (traditional populations of blue corn are often with much softer endosperm than conventional hybrids).

Acknowledgements

This research was supported by the Project TR31028 "Exploitation of maize diversity to improve grain quality and drought tolerance" financed by Ministry of Education, Science and Technological Development, Republic of Serbia.

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