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Reciprocal cross performance for anthocyanins and antioxidant activity of waxy corn

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Abstract

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Waxy corn (*Zea mays* var. *ceratina*) is a pigmented corn rich in anthocyanins. Numerous studies have proved various biological activities of anthocyanins, and one of them is antioxidants. Antioxidants will donate electrons to stop or reduce rampaging free radicals in target particles. Several methods have been used to improve pigmented waxy corn's anthocyanin content, and one of them is conventional breeding through the reciprocal cross. Thus, this research investigates the effects of reciprocal cross on the anthocyanin content and its antioxidant activity in waxy corn. The total anthocyanin content (TAC) was determined using the pH differential method. On the other hand, the total phenolic content (TPC) was analyzed by the Folin-Ciocalteu colorimetric method. Then, the antioxidant activity was assayed through 2,2-Diphenyl-2-hydrazyl (DPPH) and 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays. The results indicated that hybrid BL 1-7 x BL 1-8 had the highest TAC and TPC at 71.43 mg/100g and 7.04 mg of GAE/g, respectively. BL 1-7 x BL 1-8 had the lowest IC_{50} values which are 133.68 ug/mL for DPPH assay and 17.13 ug/mL for ABTS assay. It is an exciting finding from this study to utilize inbred BL 1-7 as maternal plant for enhancement of anthocyanin content and antioxidant activity. Furthermore, TAC and TPC were significantly correlated with DPPH and ABTS activity. These correlations helped to add more information on the relationship between phytochemicals and antioxidant activity. The information obtained from this study is useful for making smart selection of inbreds that will result in new waxy corn hybrids that are full of health advantages and provide functional foods like natural colorants.

Keywords: Waxy corn; reciprocal cross; anthocyanin; phenolic; antioxidant

Introduction

Corn or *Zea mays* is one of the members of the Poaceae family. According to Yuan & Sun (2020), corn was first domesticated in Mexico around 7000 years ago and hosted the world's richest diversity of corn. Today, corn is classified as an essential cereal crop and ranked first among other cereal crops globally (Shafiq et al., 2019). In Malaysia, corn is a new national income cash crop (Lee et al., 2021). They are a variety of pigmented corn planted, such as yellow, purple, orange, red and black. The presence of pigments corresponds to a high content of flavonoid-type phenolic compounds (Hernández et al., 2018).

Among pigmented corn varieties, the food industry has recently paid more attention to purple corn because it is high in phenolic compounds with potential health-promoting properties. It is interesting to note the importance of this purple corn as it is currently widely used for food production as a

replacement for artificial colourants (Kalapakdee et al., 2020). The great interest in purple waxy corn is due to its bioactive compounds known as anthocyanins. Naturally, this pigment used in reducing the stress of photoinhibitory light fluxes by absorbing the excess photons that would otherwise be intercepted by chlorophyll b (Gould, 2004). However, findings from numerous research have demonstrated various biological activities of anthocyanins, such as antioxidant, anticancer, anti-inflammatory, and antimutagenic (Khoo et al., 2017; Lao et al., 2017; Pervaiz et al., 2017). Antioxidants will stop or reduce ions caused by free radicals in target particles. The oxidation of protein, nucleic acids and lipids is commonly caused by unstable atmospheric oxygen. In this reaction, the free radicals that consist of unpaired electrons will be able to extract electrons from other molecules, damaging their cellular functions and leading to pathological conditions such as ageing, cancer, stroke, and diabetes (Fuad et al., 2020).

Several methods have been adopted to improve purple waxy corn's anthocyanin content, and one of them is conventional breeding. This strategy become feasible because anthocyanin pigmentation is controlled by genetics. According to Kalapakdee et al. (2020), reciprocal cross effects were significant for anthocyanins accumulation, phenolic content, and antioxidant activities in corn. Thus, this research investigates the effects of reciprocal cross on the total anthocyanin content and its antioxidant activity in purple waxy corn kernels.

Material and Method

The waxy corn ear of four inbred lines, six reciprocal cross hybrids and two commercial varieties were used in this study (Table 1, Figure 1). The inbred line BL 1-7 has dark purple kernels, while BL 1-8 has purple kernels. Both inbred lines BL 1-10 and BL 1-12 have yellow kernels. The hybrids were developed through reciprocal crosses between two inbred lines. Six crosses were generated in the dry season during April-June 2021/2022 at the Green World Genetics research station in Setiu, Terengganu (5.492056, 102.973217). Two commercial F_1 hybrids, F1 A has purple and white bicolour kernels, while F1 B has white kernels.

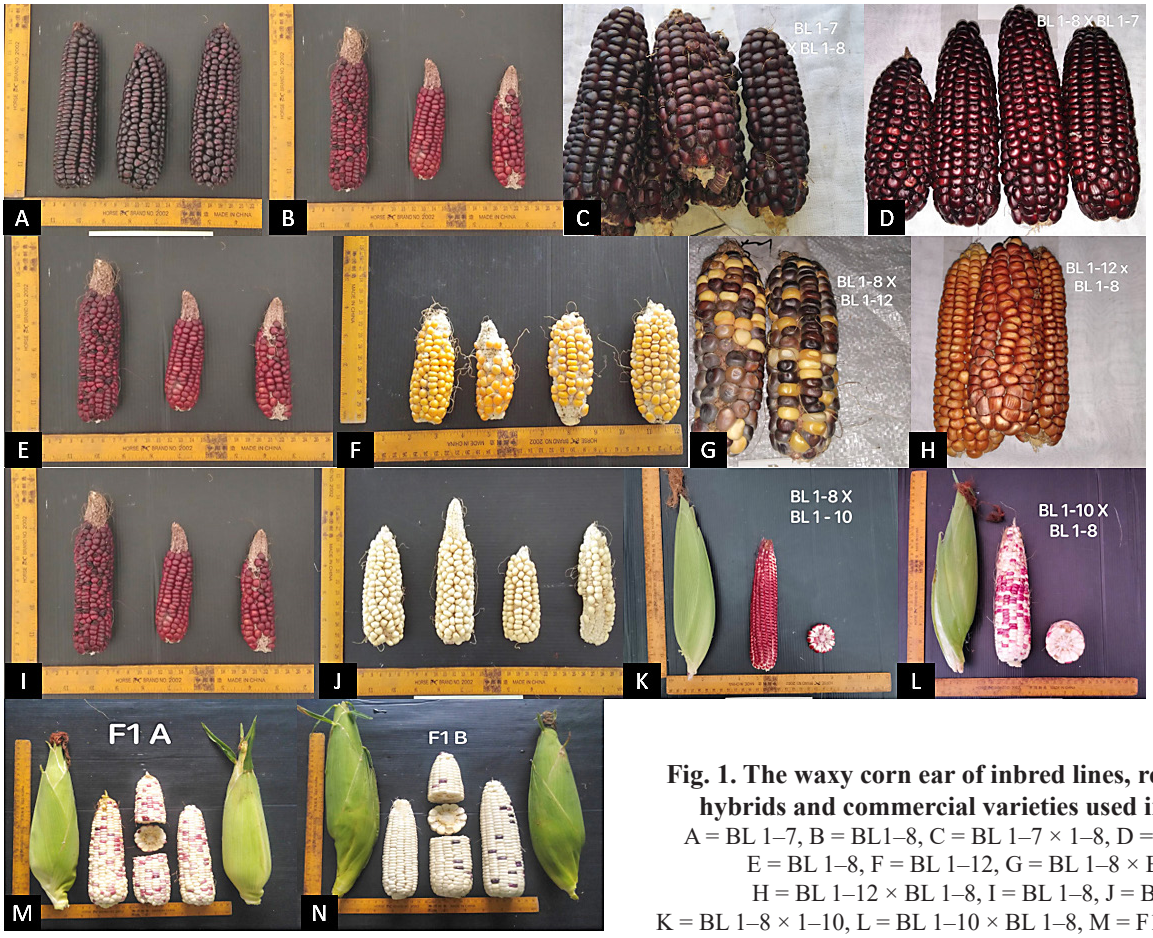

Fig. 1. The waxy corn ear of inbred lines, reciprocal cross hybrids and commercial varieties used in this study
A = BL 1–7, B = BL1–8, C = BL 1–7 × 1–8, D = BL 1–8 × 1–7, E = BL 1–8, F = BL 1–12, G = BL 1–8 × BL 1–12, H = BL 1–12 × BL 1–8, I = BL 1–8, J = BL 1–10, K = BL 1–8 × 1–10, L = BL 1–10 × BL 1–8, M = F1 A, and N = F1 B

Table 1. The inbred lines and hybrids of waxy corn used in this study

Types	Name	Kernel's colour
Inbreds	BL 1–7	Dark purple
	BL 1–8	Purple
	BL 1–10	Yellow
	BL 1–12	Yellow
Hybrids	BL 1–7 × BL 1–8	Dark purple
	BL 1–8 × BL 1–7	Purple
	BL 1–8 × BL 1–12	Dark purple, brown and yellow
	BL 1–12 × BL 1–8	Orange
	BL 1–8 × BL 1–10	Purple
	BL 1–10 × BL 1–8	Purple and yellow
Commercial Hybrids	F1 A	Purple and white
	F1 B	White

Approximately, 100 g of the waxy corn kernel was macerated with ethanol of 400 mL and kept in the dark for 24 h at 4°C. Then, the extract collected and filtered using filter paper. Next, the residues were washed with 200 mL of ethanol, and the filtrate was collected. Ethanol in the crude extract was evaporated using a rotary evaporator until concentrated and then kept at 4°C until further use (Yang & Zhai, 2010)

Total anthocyanin content (TAC)

The total anthocyanin content was determined using the pH-differential method (Yang & Zhai, 2010). A total of 1 mg aliquot sample was placed into a 25 mL volumetric flask, and the final volume of 25 mL was made up of pH 1.0 buffer. On the other hand, 1 mg of the sample was placed into a 25 mL volumetric flask and made up the final volume of 25 mL using a pH 4.5 buffer. Then, the 1.5 mL of each sample was loaded into a cuvette and absorbance was measured using a spectrophotometer (UV-2802, UNICO) at 510 and 700 nm and calculated using the formula below with a molar extinction coefficient for cyanidin-3-glucoside of 26900 L/mol cm.

$$Abs = (A510 - A700)\ pH\ 1.0 - (A510 - A700)\ pH\ 4.5$$

The TAC is calculated using the following equation (Wrolstad et al., 2005) and expressed as milligrams of cyanidin3-glucoside equivalents per 100 g of dry weight. All TAC was made as cyanidin-3-glucoside equivalents.

TAC Equation:

$$TAC\ (mg/100\ g) = AB/eL \times MW \times D \times V/G \times 100,$$

where AB is absorbance, e is cyanidin-3-glucoside molar absorbance (26900), L is the cell path length (1 cm), MW is the molecular weight of anthocyanin (449.2 Da), D is the dilution factor, V is the final volume (mL), and M is the dry weight (mg).

Total phenolic content (TPC)

The total phenolic content of waxy corn kernels was analyzed by the Folin-Ciocalteu colourimetric method (Fuad et al., 2020). First, approximately 0.02 mL of a 2 mg/mL extract solution was mixed with 0.2 mL of Folin-Ciocalteu reagent, followed by 2 mL of distilled water. After 3 minutes, 1 mL of sodium carbonate was added and incubated at room temperature for about 20 minutes. The absorbance at 765 nm was read using a Fisherbrand™ microplate reader. The phenolic content was calculated using the standard curve of gallic acid. Hence, a standard solution of gallic acid of 1 mg/mL was made by dissolving gallic acid in distilled water. Finally, the total phenolic content was expressed in mg of gallic acid equivalent per gram of extracts (Fuad et al., 2020).

2,2-Diphenyl-2-hydrazyl (DPPH) Assay

First, a stock solution of the extracts at 1 mg/mL was prepared with distilled water and diluted to a concentration of 15.625, 31.25, 62.5, 125, 250, 500 and 1000 g/mL. Secondly, ethanol and DPPH were added to a blank solution to serve as a control. The sample was mixed with 0.1 mM DPPH working solution in 96-well plates. Third, it was incubated in the dark at room temperature for 30 minutes. Using quercetin as standard, the absorbance at 515 nm was determined using a Fisherbrand™ microplate reader. Three measurements were made, and the DPPH scavenging activity was calculated using the equation below, where Ac is the absorbance of the control and As = absorbance of the sample (Fuad et al., 2020).

DPPH Equation:

$$DPPH\ scavenging\ activity\ (\%) = [(Ac - As) / Ac] \times 100$$

3-ethylbenzothiazoline-6-sul-fonic acid (ABTS) Assay

The ABTS assay's mechanism is reducing the ABTS+ radical cation from blue green to pale blue in the presence of the antioxidant. The first step was the generation of ABTS+. 0.07 g, 7 mM of ABTS was mixed with 0.0132, 2.45 mM potassium persulfate in 20 mL of methanol. Then, the reaction mixture was incubated in the dark at room temperature for at least 24 hours. The ABTS stock solution needs to be used within 2 days. Before use, the ABTS solution was diluted with methanol to achieve the absorbance 0f 0.7± 0.050 at 734 nm. Secondly, a stock solution of the extracts at 1 mg/mL was prepared with distilled water and diluted to a concentration of 15.625, 31.25, 62.5, 125, 250, 500 and 1000 g/mL. Then the sample was mixed with the ABTS solution in 96-well plates. Thirdly, the samples were incubated for 6

min at room temperature. Lastly, the absorbance was measured at 734 nm using a microplate reader. In addition, ascorbic was used as the standard (Kim et al., 2022).

Data analysis

Each sample of TAC, TPC, DPPH and ABTS assays were measured in triplicate. The mean of the measurements was determined, including the standard deviation using one-way Anova in SPSS software (version 20, SPSS, USA), at the significance level of 0.05. The mean separation was analysed using Tukey post-hoc analysis. Finally, to estimate the correlation between TAC, TPC and antioxidant activity using DPPH and ABTS assays, the Pearson correlation analysis was performed (Fuad et al., 2020).

Results and Discussion

Total anthocyanin content (TAC)

The total anthocyanin value ranges from 36.87 to 88.68 mg/100 g for inbred lines, and 23.51 to 71.43 mg/100 g for hybrids, including commercial hybrids for waxy corn (Table 2). The total anthocyanin content for all samples was significant ($p < 0.05$). Among the inbred lines, BL 1–7 had the highest total anthocyanin content which is 88.68 mg/100 g, while hybrid BL 1–7 × BL 1–8 had the highest total anthocyanin content of 71.43 mg/100 g among the other reciprocal-cross hybrids. BL 1–7 × BL 1-8 likewise surpasses commercial hybrids. BL 1–7 × BL 1-8 was the hybrid produced by crossing Bl 1–7 as the maternal parent with BL 1–8 as the paternal parent. In addition, observation on the colour of the hybrid's kernels were dark purple, just like the mother's. In comparison, the inbred line BL 1–10 with yellow kernels had the lowest anthocyanin content (36.87 mg/100 g), while commercial hybrid F1 B with white kernels was the lowest (23.51 mg/100 g) among the other hybrids. This result supported previous research on purple waxy corn and maize, which showed that coloured corns, such as black, purple, and blue corn, have higher total anthocyanins content than yellow and white corn (Khampas et al., 2013).

Table 2. Total anthocyanin content of waxy corn inbred lines and hybrids

Types	Name	TAC, mg/100 g
Standard	Cyanidin-3-glucoside	44.13 ± 0.955^{e}
Inbred	BL 1–7	88.68 ± 0.322^{a}
	BL 1–8	61.82 ± 0.959^{c}
	BL 1–10	36.87 ± 0.248^{f}
	BL 1–12	43.00 ± 0.519^{e}
Hybrids	BL 1–7 × BL 1–8	71.43 ± 0.519^{b}
	BL 1–8 × BL 1–7	60.02 ± 1.042^{cd}
	BL 1–8 × BL 1–12	47.21 ± 0.244^{e}
	BL 1–12 × BL 1–8	34.41 ± 1.068^{f}
	BL 1–8 × BL 1–10	33.09 ± 0.614^{fg}
	BL 1–10 × BL 1–8	29.30 ± 1.211^{gh}
Commercial hybrids	F1 A	56.76 ± 0.245^{d}
	F1 B	23.51 ± 0.238^{h}

Note: Column is labelled with different alphabets when there is a significant difference ($p < 0.05$)

Total phenolic content (TPC)

The total phenolic content of waxy corn ranged from 3.90 to 7.04 mg of GAE/ g for inbred lines and 0.46 to 7.04 mg of GAE/ g for hybrids, including commercial hybrids (Table 3). The mean difference between all samples were significant ($p < 0.005$). BL 1–7 and BL 1–7 × BL 1–8 had the highest total phenolic content at 7.04 mg of GAE/ g for inbred lines and hybrids, respectively. The mean difference between these two varieties were not significant ($P > 0.05$). However, it was still acceptable as they were entirely two different varieties. In addition, both of these varieties kernel colour were in dark purple. In contrast, BL 1–10 had the lowest total phenolic content for inbred lines at 3.61 mg of GAE/g, while hybrid BL 1–12 × BL 1–8 had the lowest total phenolic content at 0.46 mg of GAE/ g. Moreover, both these inbred lines and hybrids were yellow. The results were supported by a previous study in corn genotypes that darker kernel colour, such as purplish or black kernels have higher total phenolic content than lighter genotypes, such as yellow or white (Khampas et al., 2013). According to Khampas (2013), purple kernel genotypes had the highest phenolic content, followed by red and black kernel genotypes.

Table 3. Total phenolic content of waxy corn inbred lines and hybrids

Types	Name	TPC, mg of GAE/ g
Standard	Gallic acid	6.45 ± 0.005^{b}
Inbred	BL 1–7	7.04 ± 0.000^{a}
	BL 1–8	5.69 ± 0.008^{c}
	BL 1–10	3.61 ± 0.002^{i}
	BL 1–12	3.90 ± 0.003^{h}
Hybrids	BL 1–7 × BL 1–8	7.04 ± 0.000^{a}
	BL 1–8 × BL 1–7	5.22 ± 0.000^{d}
	BL 1–8 × BL 1–12	3.94 ± 0.007^{g}
	BL 1–12 × BL 1–8	0.46 ± 0.006^{k}
	BL 1–8 × BL 1–10	4.00 ± 0.006^{f}
	BL 1–10 × BL 1–8	3.89 ± 0.008^{h}
Commercial hybrids	F1 A	4.48 ± 0.005^{e}
	F1 B	1.52 ± 0.010^{j}

Note: Column is labelled with different alphabets when there is a significant difference ($p < 0.05$)

Antioxidant activity

The antioxidant activity of DPPH and ABTS assay were measured in terms of IC_{50}, the inhibitory concentration at which 50% of radicals are scavenged by the antioxidant (Fuad et al., 2020). Therefore, the lower IC_{50} value indicates higher antioxidant activity, as only a small concentration is needed to scavenge the radicals at 50%. For the DPPH assay, the IC_{50} of all waxy corn samples were significantly different ($p < 0.05$). The DPPH assay is based on the interaction of DPPH with the odd electron, reducing the purple DPPH colour to pale yellow of DPPH-H. Sample BL 1–7 (21.13 ± 0.001 ug/mL) showed the lowest IC_{50} value among the inbred lines, while sample BL 1–7 × BL 1–8 (133.68 ± 0.012 ug/mL) showed the lowest IC_{50} value among the hybrids. In contrast, BL 1–10 and F1 B showed the highest IC_{50} values for inbred lines and hybrids at 827.85 ± 0.002 ug/mL and 948.50 ± 0.014 ug/mL, respectively. The comparison of all sample activities can be observed in Figure 2.

For the ABTS assay, the IC_{50} of all waxy corn samples were significantly different ($p < 0.05$). The ABTS assay's mechanism is reducing the radical cation of the $ABTS^{+}$ ion, changing the colour of the ABTS from blue-green to pale blue in the presence of an antioxidant. As shown in Figure 3, BL 1–7 (6.54 ± 0.000 ug/mL) had the lowest IC_{50} among the inbred lines, while BL 1-7 x BL 1-8 (17.13 ± 0.002 ug/mL) had the lowest IC_{50} among the hybrids. In comparison, BL 1–10 and F1 B showed the highest IC_{50} values for inbred lines and hybrids at 80.99 ± 0.002 ug/mL and 383.18 ± 0.004 ug/mL, respectively. The lowest IC_{50} values indicate the highest antioxidant activity, and vice versa.

Correlation analysis

According to Pearson correlation analysis (Table 4), the TAC was negatively correlated with DPPH ($p < 0.01$, $r = -0.891$) and ABTS ($p < 0.05$, $r = -0.613$), suggesting that enhanced antioxidant activity results from lower IC_{50} and higher TAC values. This result demonstrated that the antioxidant activity of waxy corn was influenced by the total anthocyanins content, in which a similar result has been reported in pomegranate (Zhao & Yuan, 2021).

Correspondingly, the TPC was also negatively correlated with DPPH ($p < 0.01$, $r = -0.874$) and ABTS ($p < 0.05$, $r = -0.620$). Therefore, total phenolic content in waxy corn was directly associated with its antioxidant activity. According to Khampas (2013), black waxy corn reported the highest total anthocyanins, phenolics and excellent antioxidant activity. The DPPH was positively correlated with ABTS ($p < 0.05$, $r = 0.706$), showing that the IC_{50} value of DPPH increases as the ABTS value increases, and vice versa. Even though DPPH and ABTS assays provided a different mean of IC_{50} values, they produced similar information, and the data were consistent between the two methods. This proved that both assays are effective in measuring the antioxidant activity of waxy corn.

Finally, the TPC was positively correlated with TAC, and the value was significant ($p < 0.01$, $r = 0.852$). The result indicates that phenolic content and antioxidant activity increase along with anthocyanin content. Previous research by Kafui & Rui (2002) reported that corn showed the highest total antioxidant activity (181.42 ± 0.86 µmol of vitamin C equiv/g of grain), followed by wheat (76.70 ± 1.38 µmol

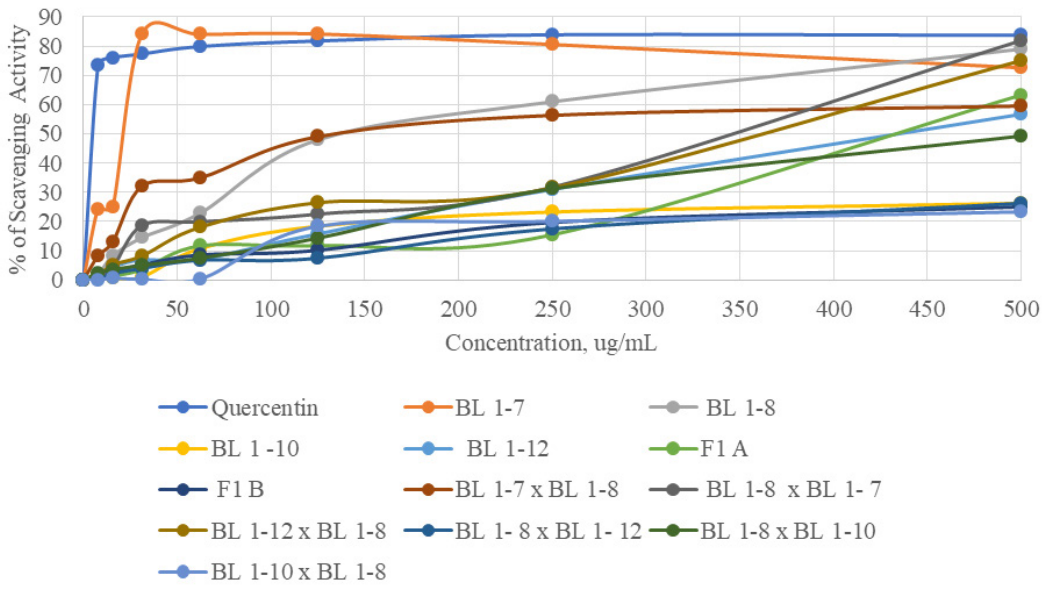

Fig. 2. Comparison of DPPH free radical scavenging activity of waxy corn inbred lines and hybrids

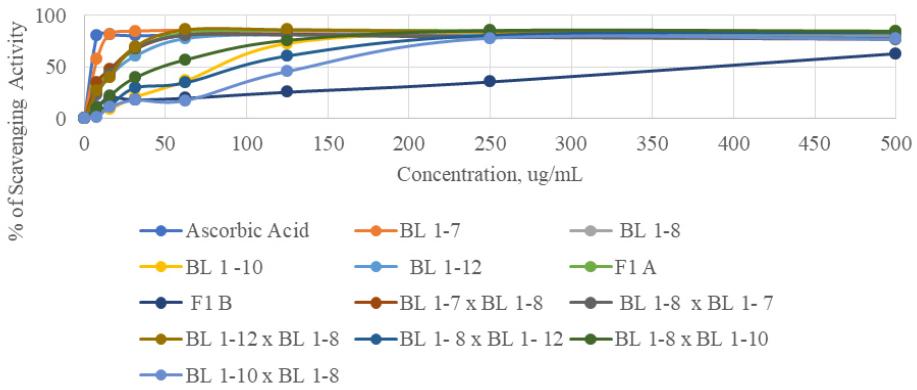

Fig. 3. Comparison of ABTS scavenging activity of waxy corn inbred lines and hybrids

Table 4. Correlations between TAC, TPC, DPPH and ABTS assays of waxy corn varieties

	TAC	DPPH	ABTS	TPC
TAC	1	-.891**	-.613*	.852**
DPPH	-.891**	1	.706*	-.874**
ABTS	-.613*	.706*	1	-.620*
TPC	.852**	-.874**	-.620*	1

**Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

of vitamin C equiv/g of grain), oats (74.67 ± 1.49 µmol of vitamin C equiv/g of grain), and rice (55.77 ± 1.62 µmol of vitamin C equiv/g of grain). Anthocyanin belongs to the class molecules of flavonoid synthesized via the phenylpropanoid pathway, and flavonoid is one of the subgroups of phenolic. Both anthocyanins and phenolic content are proven to have health benefits such as antioxidants (Khampas et al., 2013). Overall, all these correlations supported previous findings, adding more information on the relationship between phytochemicals and antioxidant activity. From this study, it can be observed that only maternal plant enhanced the anthocyanins production, thus, inbred BL 1-7 can be considered to be used as the maternal plant in future breeding efforts. This enhancement then improved the antioxidant activity of the samples (refer to Figures 2 and 3). It also became clear during the crosses between the purple corn BL 1–8 with either yellow corns BL 1–10 or BL 1–12. From these crosses, the hybrids produced from the mother's BL 1–8 showed higher anthocyanins and antioxidant activity as compared to their reciprocal hybrids, showing anthocyanins pigmentation has strong maternal effect. The inheritance of purple pericarp grains in barley was also seen to have a typical maternal effect (Zhang et al., 2017). Zhang et al. (2017) demonstrated that a grain's colour was not determined by its own genotype but rather by that of its mother.

Conclusion

The TAC, TPC, DPPH and ABTS of waxy corn varieties were significantly different among all waxy corn varieties. The hybrid BL 1–7 × BL 1–8 possessed the highest levels of anthocyanins, phenolics and antioxidant activity, as compared to the inbreds and other hybrids. Therefore, to increase anthocyanin, phenolics and antioxidant activity as high as possible in the hybrids, the best way is to use the purple waxy corn inbred BL 1–7 as the maternal plant. Additionally, this knowledge is crucial for creating hybrids with an array of health advantages that can be employed as functional food products, like natural colorants.

Acknowledgments

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