Australian Journal of Crop Science AJCS
AJCS 6(4):704-710 (2012) ISSN:1835-2707

Effects of ear shading on the anthocyanin contents and quality of kernels in various genotypes of maize

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Abstract

This study aimed to investigate anthocyanin accumulation and the effects of shading on the quality and anthocyanin contents of kernels in various genotypes of maize (*Zea mays* L.). The ears of four differently-colored cultivars of maize, (Xixingheinuo NO1 [black], Zinuoxiang [purple], Xixingchinuo NO1 [red] and Yuxiangnuo [white]) were shaded after anthesis using opaque bags. Changes in grain colors of maize seeds during development under shading treatment and the location of coloration were observed. Anthocyanin, total phenolic compounds, starch, soluble protein and sugar contents, as well as phenylalanine ammonialyase (PAL) activity, were also measured. Over 99% of the anthocyanin content in Xixingchinuo NO. 1 was found in its pericarp and seed coat. In Zinuoxiang and Xixingheinuo NO1, over 99% of their anthocyanin contents were found in the aleurone layer. Shade treatment (The ears shaded after anthesis using opaque bags from anthesis until harvest) decreased the intensity of the kernels' apparent color. Shade treatment significantly reduced anthocyanin production during the early grain filling stage of growth, but the difference in percentage of reduction between the control and shade-treated plants decreased during development. In contrast, differences of total phenolic contents between the control and shade-treated plants were increased, except Yuxiangnuo, Shade treatment decreased anthocyanin and total phenolic contents by 68.6%, 68.8%, 60.0% 56.1% and 20.6%, 3.6%, 6.6%, 10.0%, respectively, in Yuxiangnuo, Xixingchinuo NO1, Zinuoxiang, and Xixingheinuo NO1 15 d post-anthesis (DPA). Further decreases to 9.7%, 15.8%, 31.7%, 37.0% and 8.3%, 12.6%, 13.7%, 20.4%, respectively, were observed in the same plants at 50 DPA. These findings show significant differences in anthocyanin and total phenolic syntheses in maize. Shade treatment decreased PAL activity, soluble sugar and starch contents but increased soluble protein contents compared with control kernels. The results of this study indicate that fresh ears could be picked at about 35 DPA, at which point anthocyanin contents would be at maximum levels. Dissimilar farina pollination, as well as good lighting receipt by plants, not shaded ears, and the high grain filling rate, could improve anthocyanin contents in maize

Keywords: Shading; anthocyanin; maize (*Zea mays* L); kernel; total phenolic.
Abbreviations: PAL: phenylalanine ammonialyase; DPA: days post anthesis; W: Yuxiangnuo, R: Xixingchinuo NO. 1, P: Zinuoxiang, B: Xixingheinuo NO. 1； CK: the controls, S: the shading treatment; ha: hectare.

Introduction

The planting area of maize is largest in terms of size in China, far exceeding even the planting area of rice. Maize has applications in many fields, including medicine, and maize quality is a factor that affects maize applications (Ministry of Agriculture, 2002). The color of the kernel correlates well with the commercial value of maize and its capacity for germination (Tian, 2006). Anthocyanins perform favorable biological functions, including antioxidant activities, inhibition of lipid peroxidation in the liver, inhibition of inflammation and carcinogenesis, and free radical scavenging activities (Russo et al., 2009; Hilleber et al., 2004; Wang et al., 2009; Cheng et al., 2006). Accumulation of pigments in fruits and vegetables (Ashraf et al., 2002; Besseau et al., 2007; Chutintrasri et al., 2007; Bai et al., 2008) and studies on the purification methods and stability of anthocyanins (Cao et al., 2009; Sun et al., 2009) have recently sparked significant interest from various research communities. Several studies have reported on the effects of shading on the content of anthocyanins in fruit skin and fruit color (Wang et al., 2000; Randhir et al., 2005). Arakawa et al. (1994) reported that shading reduces anthocyanin content. Shading restrains the synthesis of other flavones, aside from anthocyanin (Kubo, 1988). Lighting is necessary to synthesize anthocyanins in apple but is not necessary for the synthesis of anthocyanins in grape (Li et al., 2006). Shading reduces anthocyanin contents of guava, cara orange, pear, and so on (Wang et al., 2002; Li et al., 2004; Wang et al., 2006; Li et al., 2003). Few studies have investigated the effect of shade on pigment accumulation in maize kernels. The present study thus aimed to investigate anthocyanin accumulation and the effects of shade on the quality and anthocyanin contents of the kernels of four differently colored maize varieties.

Results

Changes in grain colors of maize seeds during development under shading treatment and location of coloration

Although shading reduced the intensity of color in the kernel, the kernel size remained unchanged compared with the control. Anthocyanins were detected in the pericarp and seed coat of the Xixingchinuo NO. 1, while those in the aleurone layer of Zinuoxiang and Xixingheinuo NO. 1 were demonstrated (Fig. 1).Over 99% of the anthocyanin content determined in Xixingchinuo NO.1 was found in its pericarp and seed coat; in Zinuoxiang and Xixingheinuo NO.1, over 99% of their anthocyanin contents were found in the aleurone layer (Table 1).

Table 1. Anthocyanins contents of different parts of maize

Parts	Xixingchinuo NO. 1	Zinuoxiang	Xixingheinuo NO. 1	Yuxiangnuo
Pericarp and seed coat (mg grain^{-1})	20.8±1.92	0.04±0.001	0.05±0.003	0.04±0.003
Aleurone layer (mg grain^{-1})	0.10±0.003	21.5±1.34	56.8±2.95	1.34±0.27
Starch layer (mg grain^{-1})	0.07±0.002	0.07±0.003	0.09±0.003	0.05±0.003
Embryo (mg grain^{-1})	0.08±0.002	0.07±0.003	0.08±0.003	0.05±0.003

Table 2. Effects of shading on phenylalanine ammonialyase activity in kernels

Varieties	Treat-ments	Days after pollination (d)							
		15	20	25	30	35	40	45	50
Yuxiangnuo (U grain^{-1} h^{-1})	Control	0.132 ± 0.003	0.238 ± 0.007	0.285 ± 0.005	0.341 ± 0.011	0.452 ± 0.017	0.468 ± 0.016	0.457 ±0.021	0.451 ± 0.019
	Shading	0.034 ± 0.001	0.127 ± 0.003	0.212 ± 0.004	0.281 ± 0.007	0.331 ± 0.018	0.374 ± 0.014	0.387 ± 0.017	0.362 ± 0.011
Xixingchinuo NO.1 (U grain^{-1} h^{-1})	Control	1.379 ± 0.024	2.430 ± 0.067	4.678 ± 0.134	5.789 ± 0.209	4.123 ± 0.197	3.988 ± 0.167	3.256 ± 0.133	2.810 ± 0.109
	Shading	0.415 ± 0.016	2.010 ± 0.102	3. 456 ± 0.203	4.898 ± 0.249	3.320 ± 0.181	3.124 ± 0.106	2.870 ± 0.067	2.411 ± 0.124
Zinuoxiang (U grain^{-1} h^{-1})	Control	1.865 ± 0.112	2.307 ± 0.103	4.076 ± 0.164	4.819 ± 0.221	5.827± 0.194	6.498 ± 0.267	5.275 ± 0.226	4.014 ± 0.134
	Shading	0.748 ± 0.013	1.894 ± 0.167	3.783 ± 0.234	4.375 ± 0.197	5.267 ± 0.237	5.985 ± 0.249	4.848 ± 0.193	3.984 ± 0.134
Xixingheinuo NO.1 (U grain^{-1} h^{-1})	Control	2.110 ± 0.137	3.450 ± 0.238	4.513 ± 0.269	5.789 ± 0.206	6.267 ± 0.264	7.394 ± 0.276	6.670 ± 0.207	5.246 ± 0.172
	Shading	0.872 ± 0.067	2.539 ± 0.164	3.874 ± 0.191	4.789 ± 0.224	5.945 ± 0.213	6.405 ± 0.241	5.705 ± 0.211	4.715 ± 0.143

Effects of shade treatment on anthocyanin contents

Anthocyanin contents increased gradually in the controls (no-shading condition of normal) until 35 to 40 DPA, with peak values obtained at 35 DPA for Xixingchinuo NO. 1and Zinuoxiang and at 40 DPA for Yuxiangnuo and Xixingchinuo NO. 1. Anthocyanin contents of control and shade-treated plants showed similar trends during the experiment for all varieties (Fig. 2). The anthocyanin content of the varieties was in the following order: Xixingheinuo NO. 1> Xixingchinuo NO. 1 > Zinuoxiang > Yuxiangnuo. Shade-treatment reduced anthocyanin contents (Fig.2), and contents were significantly lower than the controls during the early grain filling stage. However, the difference in percentage of reduction between the control and shade-treated plants became less pronounced during later stages of development. Anthocyanin contents of the control plants began to decrease at the late grain filling stage, in contrast to shade-treated maize, in which anthocyanin contents did not appear to decrease during later development. The reduction in anthocyanin contents followed the order: Xixingheinuo NO. 1 > Zinuoxiang > Xixingchinuo NO. 1 > Yuxiangnuo. Thus, among the cultivars studied, the Xixingheinuo NO. 1 was the most sensitive to shade. In conclusion, shading reduces the synthesis of anthocyanins especially during the early grain filling stage but shading appears to reduce the decomposition of anthocyanins during the late grain filling stage.

Effects of shade treatment on total phenolic contents

The total phenolic contents of kernels from control and shade-treated plants were determined. In the control plants, total phenolic accumulation changed during development but remained similar amongst all cultivars, with the contents increasing gradually until 35 to 40 DPA. Total phenolic contents peaked at 30 DPA in all cultivars except for the Yuxiangnuo (which peaked at 35 DPA) and then decreased gradually. Total phenolic accumulation trends were similar in the shade-treated plants (Fig. 3). The reduction in total phenolic contents followed the trend Xixingheinuo NO. 1 > Zinuoxiang > Xixingchinuo NO. 1> Yuxiangnuo, indicating that the Xixingheinuo NO. 1 was the most sensitive among the cultivars to light limitation whereas the Yuxiangnuo was the least sensitive. Shading reduced the synthesis of total phenolics during the early grain filling stage, while the shade treatment reduced the decomposition of phenolics during the late grain filling stage, but the decomposition of phenolics during the late grain filling stage. Except for Yuxiangnuo, the difference in total phenolic contents between the control and shade-treated plants increased gradually during the early grain filling stage and then decreased gradually during the late grain filling stage.

Effects of shade treatment on the PAL activity

PAL activities of the kernels are shown in Table 2. PAL activities varied from 0.415 grain^{-1} h^{-1} to 7.394 U grain^{-1} h^{-1}. PAL activities peaked at 40 DPA and then decreased gradually until the end of the growing season for all maize varieties (except Xixingchinuo1, which showed peak PAL activity 30 DPA). Shade treatment reduced the PAL activities in all maize varieties. PAL activities amongst the cultivars tested followed the order Xixingheinuo NO. 1 > Zinuoxiang > Xixingchinuo NO. 1> Yuxiangnuo.

Effects of shade treatment on the soluble protein, soluble sugar and starch contents of maize

Soluble protein contents increased during development in control and shade-treated plants to different extents, but the difference in percentage of reduction between groups of plants gradually decreased (Table 3). In both control and shade-treated plants, the soluble sugar contents increased to maximum levels at 40 DPA and then decreased. Shade treatment significantly reduced the soluble sugar contents to

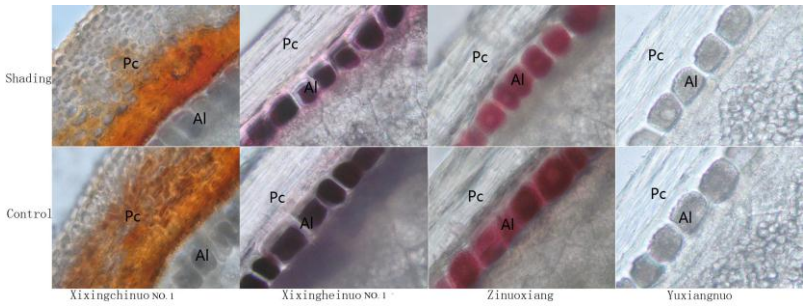

Fig 1. Changes in grain colors of maize seeds during development under shading treatment and the location of coloration. Pc：Pericarp and seed coat，Al: Aleurone layer.

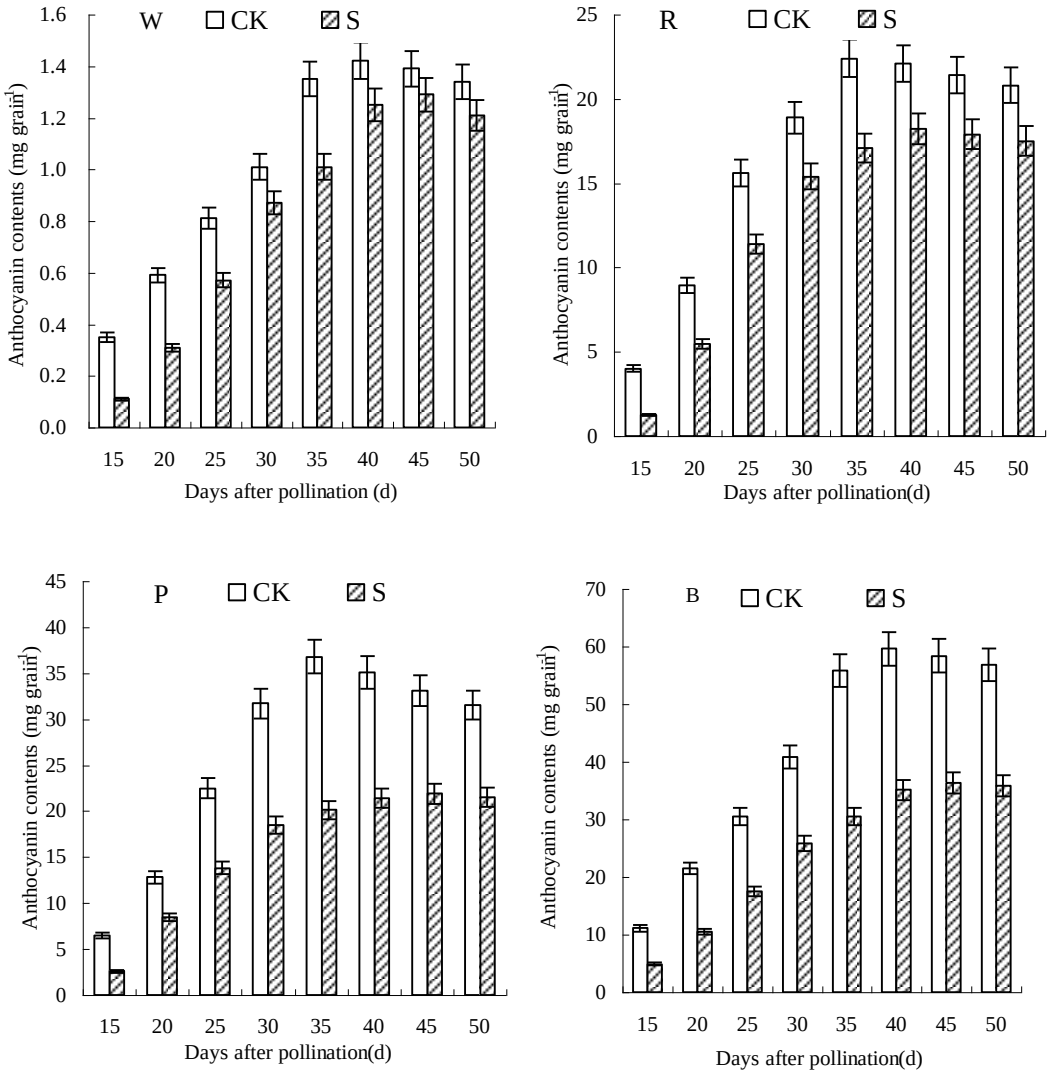

Fig 2. Effects of shading on anthocyanins content of maize seeds. W: Yuxiangnuo, R; Xixingchinuo NO.1, P: Zinuoxiang, B: Xixingheinuo NO.1, CK: the controls, S: the shading treatment. The same were as follows.

different extents during the early grain filling stage, but the difference in percentage of reduction between groups of plants gradually decreased during later stages of development (Table 4). Starch contents increased continually during development in both control and shade-treated plants. Shade treatment decreased starch contents to different extents during the early grain filling stage, but the difference in percentage of reduction between groups of plants gradually decreased during later stages of development (Table 5).

Discussion

Anthocyanins spread in the correlative cells of the most plants equably (Tsuda et al., 1994). Anthocyanins of colorized rice have been found to accumulate in several cells of the seed capsule (Lv et al., 2005). In this study, over 99% of the anthocyanins detected in Xixingchinuo NO. 1 was found in its pericarp and seed coat; in Zinuoxiang and Xixingheinuo NO. 1, over 99% of their anthocyanin contents were found in their

Table 3. Effects of shading on the soluble protein content of maize seeds.

Varieties	Treat-ments	Days after pollination (d)							
		15	20	25	30	35	40	45	50
Yuxiangnuo (mg grain^{-1})	Control	3.295± 0.146	6.798± 0.264	9.254± 0.301	11.348± 0.267	13.245± 0.264	14.148± 0.211	15.135± 0.119	15.986± 0.311
	Shading	4.623± 0.137	7.012± 0.267	10.156± 0.22	12.461± 0.291	14.015± 0.197	16.237± 0.234	17.125± 0.214	17.845± 0.246
XixingchinuoNO.1 (mg grain^{-1})	Control	3.110± 0.164	5.501± 0.192	9.801± 0.194	12.201± 0.311	15.912± 0.261	16.612± 0.316	18.101± 0.315	19.120± 0.349
	Shading	4.901± 0.192	7.104± 0.282	12.801± 0.209	15.109± 0.351	17.411± 0.229	18.914± 0.247	19.802± 0.229	20.712± 0.358
XixingheinuoNO.1 (mg grain^{-1})	Control	3.302± 0.143	7.309± 0.237	9.803± 0.216	11.701± 0.246	13.501± 0.193	14.501± 0.169	15.209± 0.432	16.012± 0.427
	Shading	4.508± 0.164	9.312± 0.411	10.810± 0.167	12.612± 0.231	14.303± 0.209	16.411± 0.314	17.310± 0.391	18.124± 0.361
Zinuoxiang (mg grain^{-1})	Control	2.212± 0.091	5.611± 0.203	7.119± 0.114	10.910± 0.211	13.309± 0.214	15.609± 0.226	17.219± 0.346	18.029± 0.426
	Shading	3.109± 0.106	6.409± 0.261	9.219± 0.191	10.812± 0.316	14.612± 0.245	16.803± 0.239	18.117± 0.442	18.987± 0.228

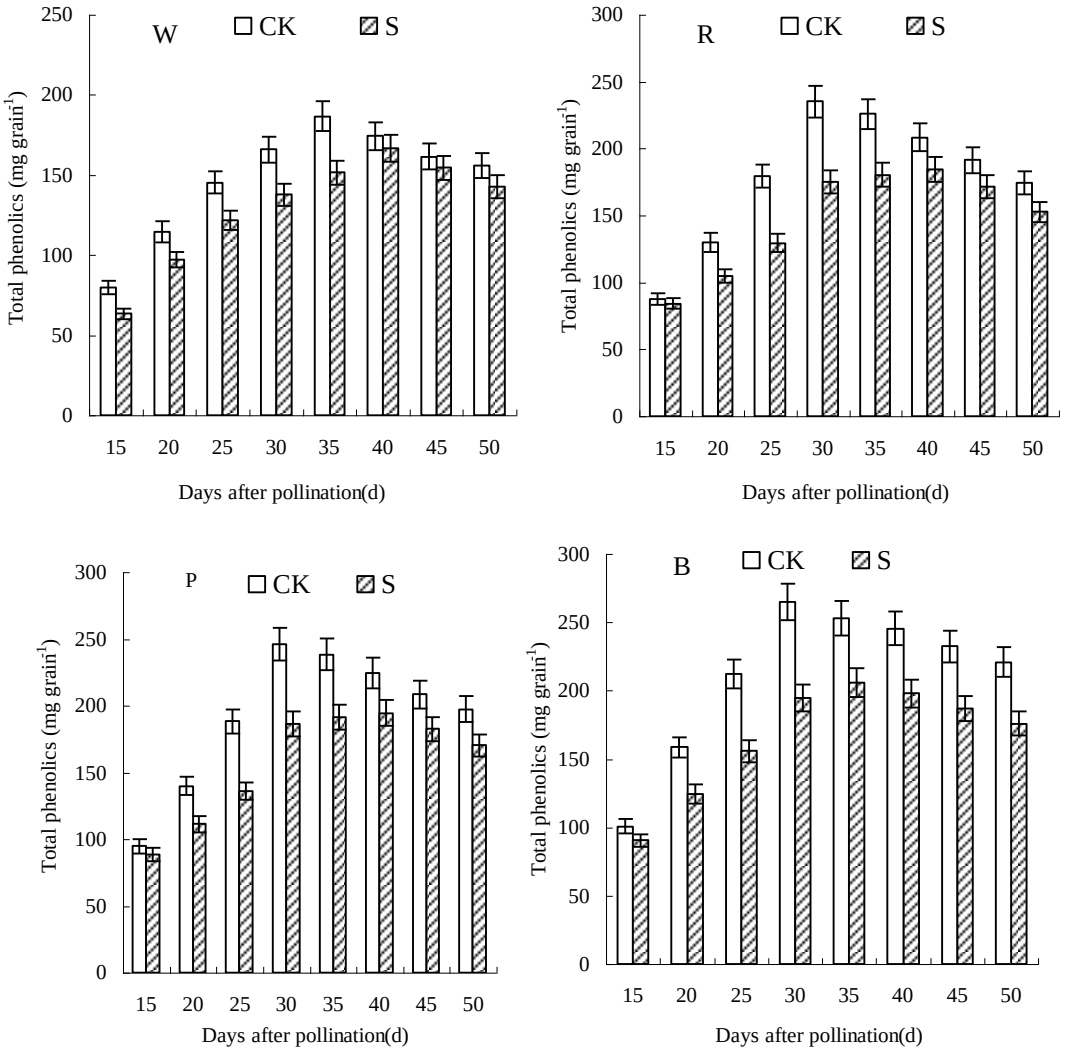

Fig 3. Effects of shading total phenolics content of maize seeds. W: Yuxiangnuo, R; Xixingchinuo NO.1, P: Zinuoxiang, B: Xixingheinuo NO.1, CK: the controls, S: the shading treatment.

Table 4. Effects of shading treatment on the soluble sugar contents of kernel.

Varieties	Treatment	Days after pollination (d)							
		15	20	25	30	35	40	45	50
Yuxiangnuo (mg grain^{-1})	Control	4.01± 0.147	6.57± 0.346	7.12± 0.261	7.71± 0.321	9.75± 0.316	9.74± 0.244	9.21± 0.167	9.03± 0.216
	Shading	3.34± 0.211	6.05± 0.216	6.78± 0.221	7.33± 0.311	8.82± 0.291	9.56± 0.305	8.94± 0.264	8.65± 0.167
Xixingchinuo NO.1 (mg grain^{-1})	Control	4.15± 0.114	6.74± 0.241	7.23± 0.167	7.89± 0.267	9.89± 0.345	10.01± 0.223	9.34± 0.259	9.11± 0.138
	Shading	3.45± 0.167	6.13± 0.167	6.98± 0.164	7.45± 0.301	9.01± 0.354	9.89± 0.264	9.14± 0.197	8.97± 0.221
Xixingheinuo NO.1 (mg grain^{-1})	Control	4.54± 0.213	7.14± 0.231	7.89± 0.234	8.70± 0.349	10.78± 0.262	10.98± 0.324	10.01± 0.224	9.83± 0.264
	Shading	4.01± 0.134	6.54± 0.232	7.34± 0.261	8.11± 0.319	10.3± 0.211	10.69± 0.228	9.58± 0.254	9.33± 0.322
Zinuoxiang (mg grain^{-1})	Control	3.89± 0.133	5.45± 0.167	7.12± 0.223	8.93± 0.334	9.71± 0.309	9.94± 0.196	8.57± 0.238	8.31± 0.268
	Shading	3.45± 0.142	5.11± 0.134	6.58± 0.194	7.94± 0.264	8.99± 0.234	9.17± 0.256	8.16± 0.243	8.04± 0.206

Table 5. Effects of shading on the starch content of maize seeds.

Varieties	Treatments	Days after pollination (d)							
		15	20	25	30	35	40	45	50
Yuxiangnuo (mg grain^{-1})	Control	9.74± 0.341	61.2± 1.23	87.7± 2.11	131.8± 3.66	155.4± 3.94	197.6± 6.11	241.7± 6.11	260.1± 4.67
	Shading	8.41± 0.237	56.8± 2.36	81.4± 2.61	124.7± 4.23	141.8± 2.64	184.7± 5.43	235.8± 4.98	249.3± 5.61
Xixingchinuo NO.1 (mg grain^{-1})	Control	10.98 ± 0.264	69.1± 3.69	98.6± 2.61	143.0± 3.29	167.4± 3.77	211.0± 6.09	256.4± 5.55	271.4± 6.37
	Shading	8.91± 0.323	60.1± 2.66	89.4± 2.66	131.0± 3.48	150.0± 3.62	198.0± 5.82	238.0± 3.99	252.1± 4.62
Xixingheinuo NO.1 (mg grain^{-1})	Control	9.80± 0.261	60.7± 2.36	89.5± 4.23	134.0± 4.11	157.0± 5.16	201.0± 6.11	245.0± 4.29	265.8± 5.37
	Shading	8.91± 0.249	56.5± 1.96	81.4± 3.11	123.0± 2.66	143.0± 4.38	189.0± 4.95	232.0± 5.07	251.4± 4.22
Zinuoxiang (mg grain^{-1})	Control	13.4± 0.311	78.9± 3.22	114.0± 2.69	165.0± 3.46	198.0± 5.34	234.0± 6.22	289.0± 4.32	297.8± 6.09
	Shading	12.6± 0.314	67.8± 1.34	101.1± 3.42	148.8± 3.66	187.9± 4.91	213.9± 5.09	271.9± 2.83	281.5± 5.64

aleurone layer. Thus, dissimilar farina pollination could improve anthocyanins contents. For example, XixingchinuoNO.1 could be used as the female parent and either Zinuoxiang or XixingheinuoNO.1 could be used as the male parent. The effects of shading on anthocyanins content did not differ significantly among the cultivars. Lighting is necessary to synthesize anthocyanins in apple, but not necessary for the synthesis of anthocyanins in grape (Li et al., 2006). Shading reduced anthocyanin content of guava, cara orange, pear and so on (Wang et al., 2002; Li et al., 2004; Wang et al., 2006; Li et al., 2003). Anthocyanins synthesis correlated with closely between the activity of PAL activity and soluble protein content of many plants. Light can activate PAL activity and enhance anthocyanins contents (Hirner et al., 2001), whereas shading also reduces the synthesis of other phenolics besides the anthocyanins (Ju et al., 1995; Kubo 1988). Similar finding were found in this study. Increasing the activity of PAL and decreasing soluble protein contents were beneficial for the synthesis of anthocyanins. However, results vary among existing studies (Ju et al., 1992; Zhou et al., 1997). For example, Zhou et al. (2005) found that shading of grape decreased soluble sugar contents, whereas Liu (2010) reported that shading on fruit of apple increased soluble sugar contents. In this study, shading decreased both soluble sugar and starch contents. Sugar forms part of anthocyanins molecule (Bureau et al., 2009) and any factors which affected sugar content could also affect anthocyanins synthesis. Shade treatment affected the PAL activity and other photosynthetic products, all of which influenced anthocyanins synthesis. Given the current inconsistencies in the findings thus far, further studies are necessary to investigate the relationships between these parameters and their precise effects on the mechanisms of anthocyanin synthesis. To ensure that maize is abundant in anthocyanins, ears could be picked about 35DPA, during which anthocyanins are at their maximum levels. Dissimilar farina pollination, as well as ensuring that plants receive good lighting, ears are not shaded, and the grain filling is good, could also improve anthocyanin contents in maize.

Materials and methods

Experimental materials and design

The field experiments were conducted from 2007 to 2008 at the Corn Research Center of Shandong Agricultural University, China (35°50´N, 117°30´E; 100 m above sea level). Laboratory tests were carried out at the State Key Laboratory of Crop Biology, Shandong Agricultural University, China.

Plant materials

Four waxy maize varieties, Xixingchinuo NO. 1 (red), Zinuoxiang (purple), Xixingheinuo NO. 1 (black) and Yuxiangnuo (white), which were used as initial materials, are widely cultivated as the major varieties in the local area. Xixingchinuo NO. 1, Zinuoxiang, Xixingheinuo NO. 1 and Yuxiangnuo ears were respectively harvested 95, 90, 95 and 88 days after sowing.

Sampling

The soil in the planting field was brown, had a pH of 6.85, and contained 1.34 mg kg^{-1} organic matter, 90.90 mg kg^{-1} alkaline nitrogen, 0.076% total nitrogen, 74.86 mg kg^{-1} available phosphorus, 83.64 mg kg^{-1}rapid-efficient potash, and 118.50 mg kg^{-1} total potash. The maizes were planted on 13 June at a density of 67,500 plants ha^{-1}. The plots consisted of five rows situated 0.60 m apart. Before sowing, 375 kg ha^{-1} of complete fertilizer (N, P, K=15%, 16%, 17%) was applied to the soil. About 45 days after sowing, the soil was ground-dressed with urea nitrogen (375 kg ha^{-1}). Ears were shaded from anthesis until harvest using opaque bags (100% light reduction at full sunlight) as the shade treatment. Ears exposed to natural light (i.e., no shade) were used as controls. At 30 d post-anthesis (DPA), middle kernels were taken to investigate the distribution of anthocyanins by hand section. Exactly 15, 20, 25, 30, 35, 40, 45 and 50 DPA, middle kernels were again obtained to determine their anthocyanin, total phenolic, soluble protein, soluble sugar and starch contents and PAL activity. Fresh middle kernels were kept at -70°C to determine anthocyanin contents, total phenolic contents and PAL activity. At maturity, middle kernels were killed at105°C for 30 min, dried at 85°C to a constant weight, ground, and then passed through a 100-mesh sieve to assess grain quality by determining their soluble protein, soluble sugar and starch contents.

Measurements

Distribution of anthocyanins

Hand sections were prepared according to the method of Kenneth (2000) to observe the distribution of anthocyanins in the maize grain. Sections 0.1 to 0.2 mm in thickness were prepared with a double-faced razor blade and observed under a light microscope (Stenri2000-c, Carl Zeiss Co., Stuttgart, Germany).

Determination of total phenolic contents

Total soluble phenolic contents were assessed using the Folin–Ciocalteu reagent procedure. Briefly, extracts of 100 μL were diluted with 500 μL of water. Up to 700 μL of 0.2 mol equivalent L^{-1} Folin–Ciocalteu reagent was added to the extracts, and the mixture was left to stand for 3 min at room temperature. Afterwards, 900 μL of 1 mol equivalent/L Na_2CO_3 was added to the mixture. The mixture was left for 90 min, after which absorbance readings at 765 nm were taken using a photodiode array spectrophotometer (model 8452A; Hewlett-Packard Co., Waldbronn, Germany). Acidified methanol was used as a blank. Total phenolic contents were expressed as milligrams of gallic acid per 100 g extract, based on a standard curve of 0 μg to 600 μg of gallic acid mL^{-1}(Swain et al., 1959).

Determination of anthocyanin contents

Fresh kernel sample (about 1.0g) were homogenized in 25 mL of an acid-ethanol solution (0.225 mol equivalent L^{-1} HCl in ethanol-water [95:5, v:v]). The tube containing the kernel mixture was flushed with nitrogen gas, agitated for 30 min, and then centrifuged at 3000 × g for 15 min. Supernatants were collected and absorbance readings were taken at 535 nm (corrected for background turbidity at 700 nm) using a photodiode array spectrophotometer. Anthocyanin content was expressed as milligrams of cyanidin-3 glucoside equivalents/100 g of extract, using a molar extinction coefficient of 25965 mol cm^{-1} and a molecular weight of 449.2 Da (Abdel-Aal, 1999).

Determination of PAL activity

3 mL of 0.05 mol L^{-1} boracic acid buffer (pH 8. 8) (composition: l mmol L^{-1} β-mercaptoethanol, 0. l mmol L^{-1} EDTA, 5% glycerin, 0.1% TritonX-100) was added to fresh kernel samples, and then homogenization was begun. The liquid was decanted into a centrifuge tube and centrifuged at 10000 rpm for 20 min at 4 °C. The supernatant was then tested for PAL activity. 300 μL of the supernatant was added to l mL of 0.02 mo1 L^{-1} L-phenylalanine, 2 mL of 0.05 mol L^{-1} boracic acid buffer (pH 8.8) and l mL of distilled water. The control mixture contained 300 μL of supernatant, 2 mL of 0.05 mol L^{-1} boracic acid buffer (pH 8.8) and 2 mL of distilled water. All mixtures were kept in a water bath for 30 min at 30 °C, after which 0.2 mL of 6 mol L^{-1} HCl was added to them to stop the reaction. Enzyme activity was determined by measuring absorbance at 290 nm using an ultraviolet spectrophotometer and then comparing with a blank containing of all the reagents except the kernel supernatant. Enzyme activity was measured in absorbance unit and expressed as U $grain^{-1}$ h^{-1}. (Zhang, 2003).

Determination of soluble protein, soluble sugar and starch contents

Soluble protein, soluble sugar and starch contents were determined using semi-micro Kjeldahl methods (conversion coefficient of 6.25) (He, 1985).

Statistical analysis

The experiment was arranged in a randomized block design with three replicates (n=3) for each treatment. As the field experiments were consistent between 2007 and 2008, the data for these 2 years were averaged. All measurements were taken three times and subjected to analysis of variance testing using DPS v.3.01 and Sigmaplot v.10.0.

Acknowledgments

We are supported by the State Key Program of Basic Research of China (2011CB100105), the National Natural Science Foundation of China (30871476, 31171497), System of Maize Modern Industrial Technologies (nyhyzx07-003) and Ministry

of Agriculture System of Maize Industrial Technologies (CARS-02).

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