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Extraction and Comparison of Anthocyanin Content in Seeds of Different Black Rice Varieties Using Water Bath Solution Extraction Method

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Abstract

Black rice, rare colored rice, is rich in anthocyanins. Black rice anthocyanin is a kind of natural plant pigment, which has the important effects of anti-oxidation, anti-inflammation and anti-cancer, protecting eyesight and promoting cardiovascular health. Fifteen representative black rice seeds in Southern Henan were used as test materials, and were determined and analyzed by using rice huller, visible light spectrophotometer and water bath solution extraction method and microwave assisted extraction method. The results showed that the anthocyanin content of different varieties of black rice varies greatly, among which the anthocyanin content of black fragrant glutinous rice No. 2 was the highest (0.4258%), and that of black fragrant glutinous rice No.4 was the lowest (0.0207%). The average content of anthocyanin in seeds of black rice varieties tested in Southern Henan Province was 0.1925%. Therefore, the above research results provide important clues for the genetic breeding and improvement of high-quality black rice varieties in Southern Henan. At the same time, varieties with higher anthocyanin content in black rice can be considered as backbone parents for new breeding high-quality black rice.

Keywords: Black rice; Anthocyanin content; Detection method; Compare.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops in the world, with over half of the world's population and two-thirds of China's population relying on rice as their staple food [1]. Meanwhile, rice is also a model organism for functional genomics research and crop genetic improvement [2, 3]. Rice (white) is the most common form of consumption, and people often consume colored rice such as red and black rice in some countries and regions in Asia, Europe, and the Americas [4]. However, with the continuous improvement of people's living standards and consumption levels, it is not only necessary to have enough food but also to eat well. Ordinary rice can no longer meet people's needs for a healthy diet. Black rice has a history of over 2000 years in China. It has a unique color, a soft, sticky, sweet taste, and high nutritional value, making it very precious among rice varieties. Meanwhile, black rice is rich in vitamin E, protein, carbohydrates, B-group vitamin nutrients, as well as nutrients such as calcium, phosphorus, potassium, iron, magnesium, etc. [5]. Black rice is a high-quality and rare rice germplasm. A large number of scientific studies have confirmed that black rice can help prevent or alleviate chronic diseases such as obesity, nonalcoholic steatohepatitis, hyperglycemia, osteoporosis, cancer and arteriosclerosis [6]. Therefore, black rice is very beneficial for human health.

The excellent function of black rice is attributed to the anthocyanin content it contains. Research has shown that black rice anthocyanins are composed of multiple components, including cyanidin-3-glucoside, paeoniflorin-3-glucoside, and malachitin-3-glucoside, etc. However, the types of anthocyanins identified in black rice vary among different varieties, indicating differences in the composition of anthocyanins in different black rice varieties [7]. Anthocyanin is a kind of flavonoid substance, which has many effects such as anti-cancer [8], anti-aging, anti-inflammatory, anti-swelling, antioxidant [9], antidepressant, and protection of nervous system [10]. Meanwhile, anthocyanins are polar compounds that are soluble in both organic solvents and water. Therefore, there are many methods for extracting anthocyanins from black rice, including water extraction, solvent extraction, microwave assisted extraction, ultrasonic assisted extraction, supercritical fluid extraction, ultra high-pressure extraction, and subcritical water extraction. However, currently, solvent extraction is one of the commonly used methods for extracting anthocyanins in industry and laboratories. The solvent extraction method is the operation of grinding raw materials and adding extraction solvents for extraction. The advantages are low equipment requirements, simple process, and higher extraction efficiency compared to water extraction method. The disadvantage is that organic solvents dissolve not only anthocyanins, but also other substances, resulting in high impurity content and long extraction time in the extracted products. Compared to the current new extraction process, the extraction efficiency is low. It has strong flexibility and applicability and commonly used organic solvents include ethanol and methanol [11], etc.

After the extraction of black rice anthocyanins, commonly used methods for the determination of anthocyanin content include UV visible spectrophotometry, high-performance liquid chromatography, etc. Among them, the use of high-performance liquid chromatography requires high equipment requirements, and the UV visible spectrophotometry method for determining the content of anthocyanins in black rice extract is simple and fast [7]. The germplasm resources of black rice in the southern region of Henan are relatively abundant, and the determination, analysis, and evaluation of anthocyanin content in different black rice varieties in the southern region of Henan have important scientific significance and potential application value. Therefore, this study used 15 black rice varieties from southern Henan as experimental materials to determine and analyze the content of anthocyanins in black rice, in order to provide reference for the breeding and improvement of new black rice varieties in southern Henan.

2. Materials and Instruments

2.1. Test Materials and Reagents

Black rice varieties: Hua Mo Xiang (Control variety for the experiment), Black fragrant rice No. 1, Black fragrant glutinous rice No. 2, Black fragrant glutinous rice No. 3, Black fragrant glutinous rice No. 4, Black fragrant glutinous rice No. 5, Black fragrant glutinous rice No. 6, Zheng Hei Zhan No. 7, Early black rice No. 8, Quan Hei Xian Zhan No. 9, Pei Hong Pi Hei No. 10, Full black long-grain glutinous rice No. 11, Black glutinous rice No. 12, Nan Hei No. 13, Hei Bao Nuo No.14, a total of 15 black rice varieties are sourced from the Rice Germplasm

Resource Bank of Xinyang Normal University. Cyanin-3-glucoside (Shanghai Yien Chemical Technology Co., Ltd); Methanol, Ethanol, Hydrochloric acid, all are analytical pure, and procurement from China National Pharmaceutical Group.

2.2. Test Instruments

Rice huller (JLG-II type, China Grain Storage Chengdu Grain Storage Research Institute Co., Ltd); Laboratory cyclone grinding mill (Hangzhou Dacheng Optoelectronic Instrument Co., Ltd); Electronic scale AR2202CN (Aarhus Instruments, Shanghai Co., Ltd); DC-1015 low-temperature constant temperature water tank (Changzhou Feipu Experimental Instrument Factory); Freezing high-speed centrifuge Centrifuge 5810R (Shanghai Beimir Biotechnology. Co., Ltd); V-1000 visible light spectrophotometer (Aoyi Instrument Shanghai Co., Ltd); P70D20AP-TD (W0) microwave oven (Galanz Microwave Oven Electrical Appliances Co., Ltd).

3. Test Methods

3.1. Determination of Methods for Detecting Anthocyanins

3.1.1. Selection of Detection Wavelength

Weigh 2.5 mg of cyanidin-3-glucoside standard into a 25 mL volumetric flask and prepare a 0.8% hydrochloric acid methanol solution. Dissolve the standard and bring to volume to obtain a standard stock solution with a concentration of 0.1 $mg \cdot mL^{-1}$. Transfer 0.5 mL of standard stock solution into a 50 mL volumetric flask and bring to volume with 0.8% hydrochloric acid methanol to obtain a standard dilution solution with a solubility of 0.002 $mg \cdot mL^{-1}$. Measure the absorbance of the standard solution using a visible light spectrophotometer within the wavelength range of 260~600 nm to determine the maximum absorption wavelength.

3.1.2. Standard Curve Drawing

Dilute the standard stock solution to prepare standard solutions with concentrations of 0.002, 0.005, 0.01, 0.02, 0.03, 0.04, and 0.05 $mg \cdot mL^{-1}$ respectively. Measure the absorbance value of the standard solution at the determined maximum absorption wavelength and draw the standard curve.

3.2. Extraction Method of Black Rice Anthocyanins

3.2.1. Black Rice Treatment before Experiment

The 15 black rice grains used in the experiment were shelled to get the whole black rice, and some were milled to get the black rice powder. Store the whole black rice and black rice powder in dark and dry conditions.

3.2.2. Sample Size Test

This experiment used Hua Mo Xiang as the control group to explore the optimal extraction conditions for the experiment. Take 2.000 g of Hua Mo Xiang black rice powder and whole black rice respectively, add 40 mL of 0.8% hydrochloric acid and 50% ethanol to volume at a liquid-solid ratio of 20:1 ($mL \cdot g^{-1}$), and extract with water-bath heating at 75 degrees for 40 minutes. After removal, let the extracting solution chill-down to room temperature and centrifuge at 4000 $r \cdot min^{-1}$ for 15 minutes. After centrifugation, take 1 mL of each supernatant into a volumetric flask and dilute to 20 mL with 50% ethanol of 0.8% hydrochloric acid. Measure the absorbance value of the diluted supernatant at the maximum absorption wavelength.

3.2.3. Comparison of Three Test Methods

The experiment references these methods [7, 12, 13]. Microwave extraction method: Take 2.000 g of black rice powder into a volumetric flask, and add 40 mL of 50% ethanol with 0.8% hydrochloric acid at a liquid-solid ratio of 20:1 ($mL \cdot g^{-1}$) to a constant volume. The microwave action power is 280 W, and the microwave action time is 60 seconds. Solvent extraction method: The first method is to take 2.000 g of black rice powder into a volumetric flask, and add 40 mL of 50% ethanol of 0.8% hydrochloric acid to the volume with a liquid-solid ratio of 20: 1 ($mL \cdot g^{-1}$). Extract with water-bath heating at a temperature of 45 degrees for 60 minutes. The second method involves taking 2.000 g of black rice powder into a volumetric flask and adding 40 mL of 0.8% hydrochloric acid with 50% ethanol to a liquid solid ratio of 20:1 ($mL \cdot g^{-1}$) to a constant volume. Extract with water-bath heating at a temperature of 75 degrees for 30 minutes.

After the extracting solution is cooled to room temperature, the black rice anthocyanin solutions extracted by the three test methods are centrifuged at 4000 $r \cdot min^{-1}$ for 15 minutes. The supernatant fluid is diluted to 20 times with 50% ethanol of 0.8% hydrochloric acid, and the absorbance is measured at the maximum absorption wavelength.

3.3. Determination of Extraction rate of Anthocyanins from different Varieties of Black Rice

Determine the content of anthocyanins in different varieties of black rice based on the adjusted optimal extraction conditions. The cultivated varieties of test include Hua Mo Xiang (Control variety for the experiment), Black fragrant rice No. 1, Black fragrant glutinous rice No. 2, Black fragrant glutinous rice No. 3, Black fragrant glutinous rice No. 4, Black fragrant glutinous rice No.5, Black fragrant glutinous rice No. 6, Zheng Hei Zhan No. 7, Early black rice No. 8, Quan Hei Xian Zhan No. 9, Pei Hong Pi Hei No. 10, full black long-grain glutinous rice No. 11, black glutinous rice No. 12, Nan Hei No. 13, Hei Bao Nuo No. 14, 15 varieties in total. Repeat three times and

compare the average extraction rate of anthocyanins in the extracting solution to compare the content of anthocyanins in different varieties of black rice.

3.4. Data Analysis Methods

Calculate the mass concentration c of anthocyanins in the extraction solution through the standard curve, and obtain the extraction rate x of anthocyanins in black rice.

$$x = \frac{c \times V \times N}{1000m} \times 100\%$$

(x is the extraction rate of anthocyanins in black rice; c is the content of anthocyanins in the extraction solution mg·mL-1; V is the volume of the extracted solution after constant volume, mL; N is the dilution factor; m is the mass of the black rice sample, g)

The experimental data was plotted and calculated using Excel 2019.

4. Test Results and Analysis

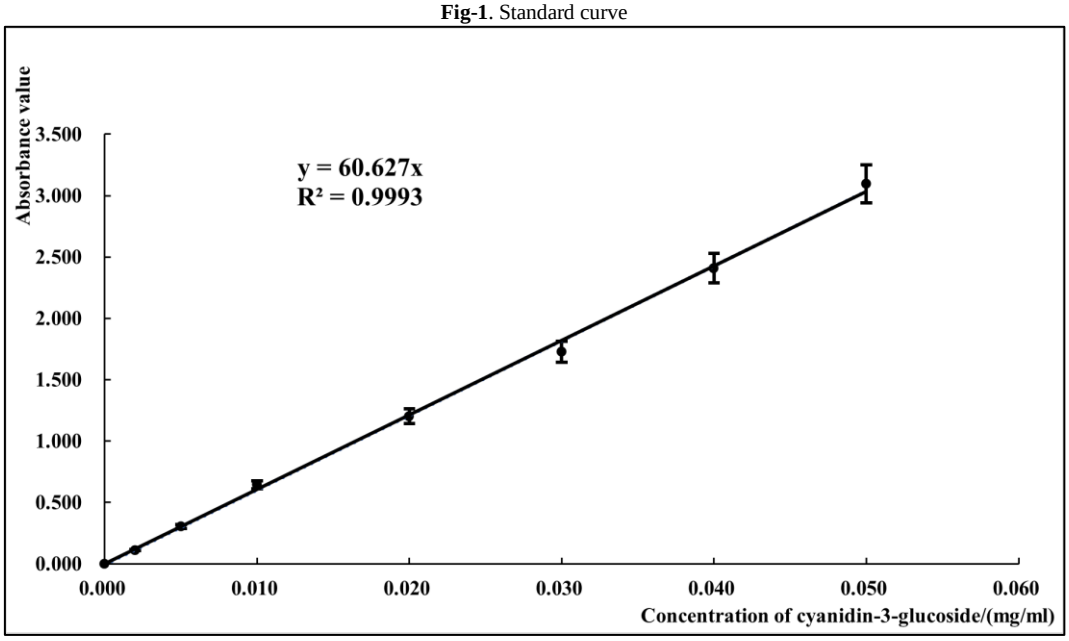
4.1. Determination of Detection Wavelength

According to the method in 2.1.1, use the visible light spectrophotometer to measure the absorbance value of the standard solution in the wavelength range of 260~600 nm. The wavelength gradient is 1 nm. Take the wavelength as the abscissa and the absorbance value as the ordinate to make a scanning map. According to the scanning spectrum, the wavelength corresponding to the highest absorbance value is 520 nm. Therefore, the optimal detection wavelength was determined to be 520 nm.

4.2. Drawing of Standard Curve

The absorbance values of each standard solution were measured with a visible light spectrophotometer, and the results are shown in the table below. Take the concentration of standard solution as the abscissa and the absorbance value as the ordinate, and draw the standard curve using Excel 2019 (Figure 1). The standard curve equation is y= 60.627x, R^2= 0.9993, indicating that the equation has a good linear relationship.

Fig-1. Standard curve



4.3. Determination of Extraction Method for Black Rice Anthocyanins

4.3.1. Extraction Rate of Black Rice Anthocyanins under different Pretreatments

According to the method in 2.2.2, the experimental results (as shown in Table 1) indicate that under the same other conditions, the extraction rate of black rice anthocyanins is as follows: powder>whole grain. Therefore, powdered black rice was selected for subsequent experiments.

Table-1. Extraction rate of black rice anthocyanins under different pretreatments

Black rice processing method	Heating method	Temperature	Time	Anthocyanin extraction rate /%
Powder	Water bath	75 ℃	40 min	0.1444±0.0015
Whole grain				0.1110±0.0021

4.3.2. Compare the Extraction Rate of Anthocyanins from Black Rice under different Test Methods

Three different extraction methods were set up [7, 14] to determine the extraction rate of anthocyanins from black rice. As shown in Table 2, under the first, second and third test methods, the extraction rates of anthocyanins from black rice were 0.0198%, 0.0719% and 0.0996%, respectively. Considering the extraction rate and feasibility of the experiment, the water bath solution extraction method was adopted, and 75 ℃ was selected as the extraction temperature.

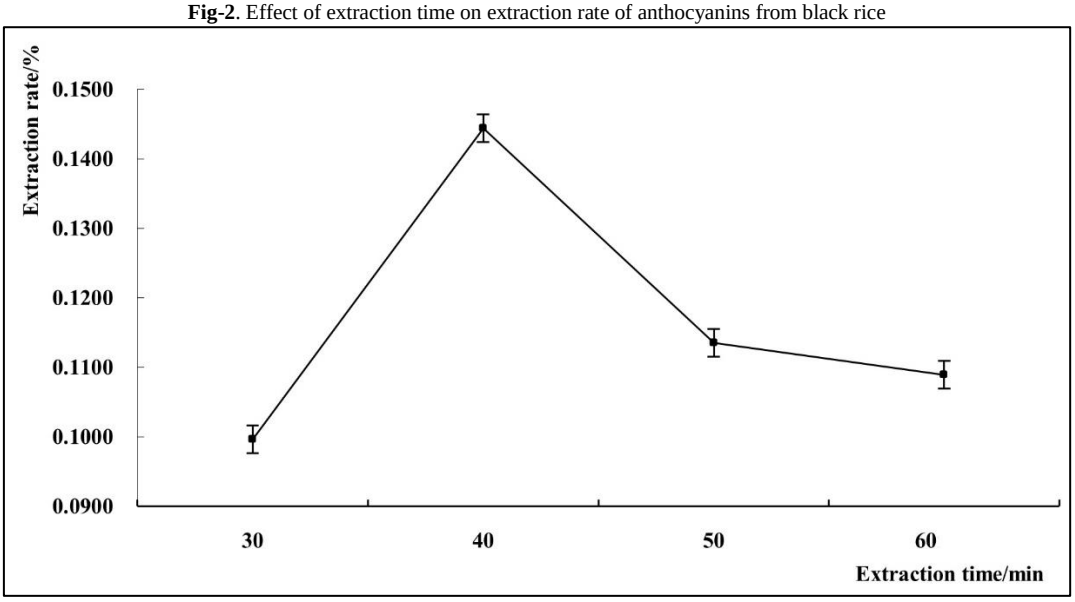
4.3.3. Effect of Extraction Time on Extraction Rate of Anthocyanins from Black Rice

Due to the difference between this test and the black rice varieties used in the test referred to in 3.3.2, the optimal extraction conditions may be changed. In order to ensure the preciseness of the experiment, we added several groups of experiments, the extraction temperature was controlled at 75 ℃, and the extraction rate of anthocyanins from black rice was explored when the extraction time was 30 min, 40 min, 50 min, 60 min. When the extraction time was 40 min, the extraction rate was 0.1444%, which was obviously higher than that of the other three extraction times (as shown in Figure 2). The results showed that short extraction time would lead to incomplete extraction of anthocyanins from black rice, and the extraction rate would decrease; When the extraction time is too long, the extraction rate will also drop. It may be that the stability of anthocyanins in black rice is destroyed and some of them are decomposed by high temperature for a long time. Therefore, the optimal extraction conditions were determined as follows: powder, 50% ethanol with 0.8% hydrochloric acid, liquid-solid ratio 20:1 ($mL \cdot g^{-1}$), extraction temperature 75 ℃, extraction time 40 min, extraction once.

Table-2. Extraction rate of anthocyanins from black rice under different extraction conditions

Heating method	Processing method	Temperature / power	Time	Absorbance value	Extraction rate /%
Microwave	Powder	280 W	60 s	0.030	0.0198±0.0015
Water bath	Powder	45 ℃	30 min	0.060	0.0396±0.0002
Water bath	Powder	45 ℃	60 min	0.109	0.0719±0.0002
Water bath	Powder	75 ℃	30 min	0.151	0.0996±0.0004
Water bath	Powder	75 ℃	40 min	0.219	0.1444±0.0015
Water bath	Powder	75 ℃	50 min	0.172	0.1135±0.0003
Water bath	Powder	75 ℃	60 min	0.165	0.1089±0.0005

Fig-2. Effect of extraction time on extraction rate of anthocyanins from black rice



4.4. Results and Analysis of Anthocyanin Content in different Black Rice Varieties

Under the optimal extraction conditions, the anthocyanin content of 15 black rice varieties was determined, and the results are shown in Table 3. The average anthocyanin content of these 15 black rice varieties was calculated to be 0.1925%.

Table-3. Anthocyanin content of 15 black rice varieties

Black rice varieties	OD value	OD average value	Anthocyanin content /%
Hua Mo Xiang	0.217	0.219	0.1444±0.0015
	0.219		
	0.220		
Black fragrant rice No. 1	0.040	0.041	0.0271±0.0017

	0.043		
	0.040		
Black fragrant glutinous rice No. 2	0.643	0.645	0.4258±0.0032
	0.644		
	0.649		
Black fragrant glutinous rice No. 3	0.460	0.460	0.3033±0.0015
	0.458		
	0.461		
Black fragrant glutinous rice No. 4	0.032	0.031	0.0207±0.0012
	0.030		
	0.032		
Black fragrant glutinous rice No.5	0.467	0.466	0.3077±0.0012
	0.465		
	0.467		
Black fragrant glutinous rice No. 6	0.042	0.042	0.0277±0.0010
	0.043		
	0.041		
Zheng Hei Zhan No. 7	0.388	0.388	0.2558±0.0006
	0.387		
	0.388		
Early black rice No. 8	0.396	0.395	0.2608±0.0012
	0.394		
	0.396		
Quan Hei Xian Zhan No. 9	0.264	0.265	0.1746±0.0012
	0.264		
	0.266		
Pei Hong Pi Hei No. 10	0.105	0.105	0.0691±0.0006
	0.104		
	0.105		
Full black long-grain glutinous rice No. 11	0.328	0.328	0.2166±0.0006
	0.329		
	0.328		
Black glutinous rice No. 12	0.314	0.315	0.2076±0.0012
	0.316		
	0.314		
Nan Hei No. 13	0.284	0.283	0.1865±0.0012
	0.282		
	0.282		
Hei bao Nuo No. 14	0.394	0.394	0.2600±0.0010
	0.393		
	0.395		

By comparing the anthocyanin content of these 15 black rice varieties, the results showed that the anthocyanin content of Black fragrant glutinous rice No.2 was the highest, followed by Black fragrant glutinous rice No. 3 and Black fragrant glutinous rice No. 5, and then Zheng Hei Zhan No. 7, Early black rice No. 8 and Hei bao Nuo No. 14. The anthocyanin content of seven kinds of Hua Mo Xiang, Black fragrant rice No. 1, Black fragrant glutinous rice No. 4, Black fragrant glutinous rice No. 6, Quan Hei Xian Zhan No. 9, Pei Hong Pi Hei No. 10, Nan Hei No. 13, was less than 0.2000%, And Black fragrant glutinous rice No. 4 was the lowest, only 0.0207%. The difference between black fragrant glutinous rice No. 2 with the highest anthocyanin content and black fragrant glutinous rice No. 4 with the lowest anthocyanin content was 0.4051%.

5. Discussion

The main component of black rice anthocyanins is cyanidin-3-glucoside. Select this compound as the standard, The selected wavelength was 520 nm through experiments. The absorbance values of different concentrations of standard solutions were measured at this wavelength and standard curves were plotted. Tian and others explore the effects of various factors on the extraction rate of black rice anthocyanins: extraction time> extraction temperature> liquid material ratio [15]. It can be seen that the extraction time has a significant impact on the experimental results. Therefore, we determined the content of anthocyanins in black rice at different extraction times. Wu and others used a microwave assisted-composite enzyme method to extract anthocyanins from black rice and determined their content [14]. The average extraction rate of anthocyanins was 0.6689%. Liu and others response surface optimization experiment was applied to determine the optimal extraction conditions for black rice anthocyanins using water bath oscillation, with a yield of 1.2490% [7]. The microwave assisted extraction method takes a relatively short time, and the extracted anthocyanins have strong stability, but poor operability. Compared to the

previous method, the water bath oscillation extraction spectrophotometry method for determining the content of anthocyanins in black rice is simple, reproducible, and has high precision in the determination results. Therefore, considering the operability and extraction efficiency of the experiment. This study designed three methods and optimized them to determine the optimal extraction conditions as follows: powdery, 50% ethanol solution of 0.8% hydrochloric acid as the extraction solution, liquid to material ratio 20:1 ($mL \cdot g^{-1}$), extraction temperature 75 ℃, extraction time 40 minutes, extraction once. This experiment adopts a water bath solution extraction method, which saves costs and has high extraction efficiency.

Under the optimal extraction conditions, the content of anthocyanins in 15 black rice varieties was determined and compared. The average value of anthocyanin content in the seeds of black rice varieties tested in the Southern region of Henan is 0.1925%, which is lower than the average value of the experimental results of Wu and Liu [7, 14]. It may be caused by different varieties of black rice. Among them, Black fragrant glutinous Rice No. 2 has the highest anthocyanin content at 0.4258%, while Black fragrant glutinous Rice No. 4 has the lowest anthocyanin content at 0.0207%. The difference in anthocyanin content between the two is 0.4051%. The anthocyanin content of Black fragrant glutinous Rice No. 3 and is equivalent. The anthocyanin content of Zheng Hei Zhan No. 7, Early black rice No. 8, and Hei Bao Nuo No. 14 is essentially equivalent. Therefore, the above experimental results provide important clues for the genetic breeding and improvement breeding of high-quality black rice varieties in southern Henan as backbone parents.

6. Conclusion

The content of anthocyanins in different varieties of black rice varies greatly, with Heixiangnuo 2 having the highest anthocyanin content (0.4258%) and Heixiangnuo 4 having the lowest anthocyanin content (0.0207%). The average anthocyanin content in the seeds of black rice varieties tested in the Southern region of Henan is 0.1925%. Therefore, our results provide important clues for the genetic breeding and improvement of high-quality black rice varieties in southern Henan.

Acknowledgments

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