Increased Anthocyanin Content in Seven Furrows of Cempo Ireng Black Rice with Mutation Induction

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Abstract. Anthocyanins are flavonoid pigments providing colors which vary from blue, dark purple, red, brown to black. Accumulation of anthocyanins occurs in the cells of pericarp on black rice. Anthocyanins are very important for human health, but the consumption of black rice is low. This study aims to determine the increase of anthocyanin content in furrows of mutant black rice. This research conducted on 17-30th September 2019. The treatments used were various numbers of furrow: GH 8, GH 13, GH 44, GH 46, GH 51, GH 52, and control strain without irradiation. Anthocyanin content analysis was using pH differential method. The data obtained were analyzed by analysis of variance (ANOVA) and followed by the Duncan Test (DMRT) of 5%. The variable observed were pericarp color of black rice and total anthocyanin content. The results showed that mutations can increase anthocyanin content in cempo ireng black rice with the highest anthocyanin content found in furrow number 44 is 5.95 ppm and has the blackest pericarp.

1. Introduction
Rice is the main food of most countries around the world, especially in Indonesia. The need for black rice variety in Indonesia is increasing as people realize its potential in nutrition and healthy improvement. Cempo Ireng black rice (Oryza sativa L.) variety is Indonesian local rice which has a high anthocyanin content. The data results shows presence the level of anthocyanin were higher in black rice variety [1]. Currently, black rice with anthocyanin content is considered as important as antioxidant for health and becoming popular as a functional food (foods that are naturally containing or through a special process that contains one or more of functional compositions for human physiology and health) [2].

Anthocyanins are flavonoid pigments providing colors that vary from blue, dark purple, brown to black. Accumulation of anthocyanins in the caryopsis of cereals occurs in the cells of the fruit (pericarp) and seed (testa) coats as well as in the aleurone layer of the endosperm [3]. The pigmentation of rice is primarily concentrated in the seed coat and pericarp [4] which why the material commonly used for extraction is rice. A food source containing anthocyanin is now widely used as a natural food coloring agent and functional food ingredients [5][6]. Pigmented rice has been reported to possess important health promoting properties, such as antioxidant, antiglycation and anticancer...
properties [7]. Like in the sweet potatoes anthocyanins have been reported to have high antioxidant properties which are correlated with the prevention of chronic diseases in humans [8].

Cempo Ireng black rice is not widely supported on the market because it has many weaknesses in its cultivation which drive the requirement to overcome such problem trough mutations by gamma rays. Mutations can be determined as sudden changes in genetic material inherited in the next generation, and those changes are not caused by general phenomena of genetic segregation or recombination [9]. The furrows that have gone through a trial of yield (preliminary and advanced) will show superior performance [10] which then can be used as a requisite for analyzing anthocyanin content. Mutations not only affect the morphological character of Cempo Ireng black rice in pericarp color properties but also affect the anthocyanin content. This study aims to determine the increase of anthocyanin content in furrows mutant black rice.

2. Method

2.1 Sample Preparation

Black rice obtained from seeds variety of Cempo Ireng which is irradiated using gamma rays in the National Nuclear Energy Agency (BATAN), Jakarta. Irradiated seeds were stored in a refrigerator at a temperature of 4°C until use. The seeds planted in the field was in the seven generations that planted at the Agricultural Extension Center on February-June 2019. The existence of anthocyanins is located inside the vacuole cells of the plant, so that most anthocyanins can be taken from several parts of the plant, such as crowns of flowers, leaves, fruits, grains, up to the tubers [11]. The material used for the extraction of anthocyanin analysis were in the form of black rice, while the material used for identify the pericarp color in the form of grain samples. This research conducted at the Laboratory of Food Chemistry, Faculty of Agriculture, Sebelas Maret University on 17-30th September 2019. The tools used for analysis are spectrophotometer, centrifuge, vacuum evaporator, reaction tube, tube shelf, black glass bottle, mortar, pestle, flask measure, and refrigerator.

2.2 Extraction of black rice samples

Black rice anthocyanins extract was made by soaking a black 250 g black rice powder into 1000 ml methanol 70% acid (performed with an addition of 1% of the HCl concentrated) for 1 hour. After soaking for 1 hour, the extract was filtered using a coarse filter paper. Filtrate was then udiated (vacuum) at 60 rpm for 1.5 hours. Filtrate stored at a temperature of 40°C before use [12].

2.3 Analysis of Anthocyanin Content

The anthocyanin content was analyzed by the pH differential method [13]. The principle of this method is anthocyanins discoloration based on pH changes. In the condition of pH 1 anthocyanins were in the form of oxonium or flavylum which has strong color intensity, while in the condition of pH 4.5 anthocyanins in the form of carbinol are colorless. Buffer of pH 1 and buffer of pH 4.5 were added on different extract samples to match the desired dilution factor (DF) state. Anthocyanins were absorbed at 250 – 700 nm wavelengths and the main peak as anthocyanins (aglikon) around the wavelength of 490-535 nm [14], so the measurement of absorbance data were done at wavelengths of 510 nm and 700 nm.

2.4 Make buffer solution of pH 1.0 and buffer solution of pH 4.5

The buffer solution of pH 1.0 was made by using KCl as much as 1.86 g mixed with 980 ml of distilled water (aquades) and was adjusted to its pH to reach 1 by using concentrated HCl. Furthermore, the solution was moved into the 1 L flask measure and added distilled water until the volume of solution 1 L. As for the pH buffer solution 4.5 used Na-acetate as much as 54.43 g mixed with 960 ml of distilled water. Then, the pH was measured and set with a concentrated HCl until the solution is obtained with pH 4.5. Subsequently the solution was transferred into the 1 L flask measure and diluted with distilled water up to 1L volume.
2.5 Measurement of Total Anthocyanin Content

There were some methods included in the measurement of total anthocyanin content: first, the right dilution factor for the sample should be determined in advance by dissolving the sample with a buffer KCl PH 1 to the absorbance obtained less than 1.2 at a wavelength of 510 nm. Second, the first sample solution was prepared used KCl buffer with pH 1 and second sample solution used Na-acetate buffer with pH 4.5. Each sample was dissolved with a buffer solution based on the DF predefined. The samples dissolved using buffer of pH 1.0 were left for 15 minutes before measurement, whereas for samples dissolved with buffer of pH 4.5 were measured after being left mixed for 5 minutes. Third, the absorbance of extracted was assessed using a spectrophotometer [15] at 510 nm and 700 nm in buffers of pH 1.0 and 4.5. Maximum absorption point was reached at wavelength $\lambda_{\text{vis-max}} = 510$ nm [16] for cyanidin-3-glucoside, while the wavelength of 700 nm was used to correct deposits that still exist in the sample. Cyanindin-3-glucoside are all the major anthocyanin compounds which were present in the extracts of black rice [17]. Furthermore, the formula of absorbance the samples ($A$) is represented as follows:

$$A = (A_{510} - A_{700})_{\text{pH 1.0}} - (A_{510} - A_{700})_{\text{pH 4.5}}$$

The concentration of anthocyanin content (mg/l) from the extracted samples was calculated with standard formula and also result has expressed as cyanin-3-glucoside equivalents.

$$\text{Anthocyanin content (ppm)} = \frac{A \times V}{\epsilon \times L \times \text{DF} \times Wt} \times \frac{1}{\text{MW}}$$

Notes:
- $A$ = absorbance
- $\epsilon$ = molar absorptivity Cyanidin-3-glucoside = 26900 L/(mol.cm)
- $L$ = cell path length = 1 cm
- MW = molecular weight of cyanidin-3-glucoside = 449.2 g/mol
- DF= dilution factor
- $V$ = solvent volume (L)
- $Wt$= weight of starting material (gr)

The formula applied for 2 samples of extract black rice in GH 8 is:

$$A1 = (A_{510} - A_{700})_{\text{pH 1.0}} - (A_{510} - A_{700})_{\text{pH 4.5}}$$

$$= (0,648 - 0,111) - (0,201 - 0,081)$$

$$= 0,417$$

$$A2 = (A_{510} - A_{700})_{\text{pH 1.0}} - (A_{510} - A_{700})_{\text{pH 4.5}}$$

$$= (0,618 - 0,121) - (0,203 - 0,080)$$

$$= 0,374$$

$$\text{Anthocyanin content (ppm)} = \frac{A \times V}{\epsilon \times L \times \text{DF} \times Wt} \times \frac{1}{\text{MW}}$$

$$\text{TAC 1} = \left(\frac{0,417 \times 26900}{1}\right) \times 449,2 \times 100 \times \frac{5,0982}{1} = 3550,10 \text{ mg}$$

$$\text{TAC 2} = \left(\frac{0,374 \times 26900}{1}\right) \times 449,2 \times 100 \times \frac{5,0719}{1} = 3167,50 \text{ mg}$$

$$\text{TAC GH 8} = \left(\frac{\text{TAC 1} + \text{TAC 2}}{2}\right) = \left(\frac{3550,10 + 3167,50}{2}\right) = 3.36 \text{ ppm}$$

2.6 Statistics Data Analysis

The data of pericarp color are presented as the mean and standard deviation (SD) and analyzed through one-way analysis of variance (ANOVA) using SPSS statistical software (SPSS 16.0 for
Windows). The confidence level of Duncan test with the confidence level of 95% (p < 0.05). The data of anthocyanin content were presented in descriptive.

3. Result and Discussion
According to the research before in the six generations, mutant genotype has a relatively homogeneous population, so selection activities can be carried out on rice color parameters and the anthocyanin content [18]. Pericarp color observation was performed morphologically/visually by the scoring method as shown in Table 1.

| Characteristics | Score | Traits |
|-----------------|-------|--------|
| Black           | 1     | Black colour domination in one grain of rice 100%; categorized as Black (B) |
| Brown-black     | 2     | Black colour domination in one grain of rice is ≥ 50 % than brown colour; categorized as Brown-Black (BL) |
| Black-brown     | 3     | Brown colour domination in one grain of rice is ≥ 50 % than black colour, categorized as Black-Brown (BR) |
| White           | 4     | White colour domination in one grain of rice 100%, categorized as White (W) |

Source: [18]

Mutation induction by gamma rays influences the pericarp color of black rice. Each furrow number has a different density level. The difference in pericarp color of black rice can be seen in Figure 1.

![Figure 1. Pericarp color of cempo ireng black rice in different furrow numbers](image)

Accordance with the study in barley, the variability for grain color exists due to the presence of many spontaneous and induced mutants [19]. The changing nature of the random mutation makes it necessary for several generation to stabilize the anthocyanins content higher than the control. Analysis of variance showed that the treatment of furrow numbers has a significant effect on the pericarp color of cempo ireng black rice. The furrow numbers treatment also shows a significant statistical value on GH 51. The results of the pericarp color can be seen in Table 2.
Table 2. Pericarp color and total anthocyanin content of Cempo Ireng black rice mutant

| Furrow Number | Pericarp Color | Total Anthocyanin Content (ppm) |
|---------------|---------------|---------------------------------|
| GH 8          | 2.33b         | 3.36                            |
| GH 13         | 1.33a         | 4.60                            |
| GH 44         | 1.00a         | 5.95                            |
| GH 46         | 1.33a         | 3.60                            |
| GH 51         | 1.67ab        | 3.63                            |
| GH 52         | 2.33b         | 2.90                            |
| CONTROL       | 1.00a         | 0.85                            |

Note: the number followed by the same letter in the same column of pericarp color is not significantly different based from the DMRT test level 5%.

The different rice varieties possessed different anthocyanin contents which depends on genotypes, and possibly also differences in their location in the outermost layer of rice [20]. The first color compounds of anthocyanins synthesis, plays an important role in the formation of fruit coloration [21], so pericarp color can be relate to total anthocyanin content. Like in the GH 44 (Table 1), the pericarp color is the blackest within all treatment but also has the highest total anthocyanin content. Through a decrease in the total anthocyanin content, inbredd expression can have a non-specific effect on the composition of detected anthocyanins or segregation influence as the result was obtained after planting seven generations. Anthocyanin content test will change the color of extract black rice when add with buffer of pH 1.0 and buffer of pH 4.5. It can be seen in Figure 2.

![Figure 2. Anthocyanin content test in different furrow numbers.](image)

A: The color in pH 1.0; B: The color in pH 4.5

The combination of polar solvents with the appropriate organic acids to obtain a very acidic pH condition (pH 1) can further solidify the stability of anthocyanins in the form of red flavium cation, whereas when the solvent is combined with a weak acid, then anthocyanins color alteration will change to a purplish red color at pH 4.5 [22][23]. Higher concentration or strong color produced in the plant shows that the greater the concentration of anthocyanins was occur in the plant. Accordance with Figure 2, anthocyanin discoloration indicate that stronger the color of extract samples has higher the anthocyanin content.
4. Conclusion

Based on the value of pericarp color, the GH 51 furrow number has a significant difference in the pericarp color of Cempo Ireng black rice. The color change of rice pericarp can be induced from mutations with gamma-ray irradiation that established into seven generations to get the highest total anthocyanin content than control. GH 44 has the highest of total anthocyanin content of 5.95 ppm.

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