IOP Conf. Series: Materials Science and Engineering **193** (2017) 012038 doi:10.1088/1757-899X/193/1/012038

Identification of Morphological Character and Esterase Isozyme Pattern in Second-Generation Black Rice Plant Irradiated to Gamma Rays

R S Hartanti[1], T A N Putri[1], F Zulfa[1], Sutarno[1]*, and Suranto[1]

[1]Department of Biology, Faculty of Mathematic and Natural Science, Sebelas Maret University, Jl. Ir. Sutami 36A Surakarta, Central Java 57126 Indonesia

*E-mail: nnsutarno@staff.uns.ac.id

Abstract. Black rice is one of the functional foods due to its high anthocyanin content. Black rice grain was irradiated by gamma rays with a dose of 200 Gy and 300 Gy. The main purpose of this irradiation is to induce mutation to the black rice plant in order to achieve the improved organism. This study was undertaken to elucidate the morphological character and esterase isozyme pattern of black rice plant after irradiated by gamma rays. There were morphological differences on leaves, stems and grains between irradiated and non irradiated black rice plant. Gamma radiation dose of 200 Gy showed the significant influence of the length of the stem, number of internodes, and length of leaves. The radiation dose of 300 Gy showed the significant influence of the decrease value of diameter of 3rd internodes, number of branches and width of leaves. Flowering time is getting faster as increasing radiation dose. At the age of 74 days after planting there are 9.15% plants of 200 Gy radiation dose that have flowered faster than normal plants. This value increased into 11.45% at the dose of radiation 300 Gy. There were differences in the esterase banding pattern between radiation dose of 200 Gy and 300 Gy than the control plants, indicated that randomly mutation has occurred.

1. Introduction

Black rice is potential as functional food because it contains of vitamins, minerals and antioxidant [1]. This rice has been recorded to have high flavonoids contents, and one of the flavonoid type contained in the black rice is anthocyanin. Anthocyanins are color pigments that have antioxidant activity and it has proved to be an effective antioxidant against the negative effects of free radicals [2]. Anthocyanins identified in the black rice variety were cyanidin-7-O-galactoside, cyanidin-3-Oglucoside, cyanidin-3'-O-glucoside, cyanidin-5-O-glucoside, cyanidin-3, 7-O-diglucoside, cyanidin-3, 5-O-diglucoside and peonidin-4'-O-glucoside [3]. The antioxidant activity of the extract of black rice has been reported to prevent or reduce the risk of many degenerative diseases such as cancer, hypertension, and diabetes. They also can reduce the risk of hepatic steatosis, lowering LDL cholesterol (Low Density Lipoprotein) or the bad cholesterol in the blood and may help fight heart disease [4, 5]. Black rice also has fibers which are higher compared to most other types of rice. Dietary fiber is very good for the body, plays a role in lowering cholesterol. They could decrease blood sugar levels, and has a chemo-protective effect on colon [6].

Besides of the advantages, black rice plant has shortage of challenges in the business of its cultivation, such as planting time which is longer than white rice and the habitus is very tall, therefore the plant so easily collapse [7]. Therefore, it needs plant breeding efforts to improve the quality of black

IOP Conf. Series: Materials Science and Engineering **193** (2017) 012038 doi:10.1088/1757-899X/193/1/012038

rice plants in order to obtain the superior black rice. One way of plant breeding could do is by inducing mutation. Plant synthetizes the new protein and DNA under radiation stress as an adaptation to environment [8]. This mutation induction can be done using a physical mutagenic agents such as radiation of gamma rays.

Rice grains irradiated with gamma rays yielded by core of Cobalt 60. Gamma rays radiation dose used in this study are 200 Gy and 300 Gy, respectively. Those doses have been used because they are the most effective dose in rice breeding [9, 10]. Those doses caused little physical damage but produce higher mutation probability in the plant [9]. Indications biochemical changes in seed after irradiation with gamma rays such as changes in enzyme activity and respiration rate, and chromosome damage. Radiation is expected to induce mutations that lead to the improvement of plant characters such as shorter in stem length and flowering time. Furthermore, identification of the morphological and molecular alteration is done to ensure that the changes occur in plants as the result of mutation induction by gamma rays radiation. Isozyme analysis can be used as molecular markers to determine differences between mutant and control plants [11].

2. Material and Methods

2.1. *Plant Samples Material*

This study was conducted used 2000 irradiated seeds of rice which were cultivated in field. Control plants were also plotted side by side in order to look at wheter any difference between the treated seed and the control one. These plants were then used for electrophoretic purposes as well as for morphological observations. The morphological character observed when plants aged 4 months, counted from planting time. For molecular identification, the fresh leaves of *Oryza sativa* L. were used for electrophoretic procedures and esterase staining were conducted according to Suranto's [12].

2.2. *Observation of morphological character*

The length of the stem was measured from the base of the stem to the tip. The number of internodes was calculated from the base of the stem to the tip. The length of internode and stem diameter was measured at the third internode from the base. The number of branches on the main stem also calculated. The length and width of the first, second, and third leaves were measured with a ruler. Flowering time was determined by the interval time from the planting time to the first flower appeared. The length and width of the seeds were measured following the procedure from Chang [13]. The number of seeds per panicle is whole calculated.

2.3. *Identification of esterase isozyme pattern*

All methods used in the analysis of isozyme banding pattern was following the procedure of Suranto's [12, 14]. In this study, isozyme banding pattern was detected using polyacrylamide gel electrophoresis method. Staining process of electrophoresis results for esterase isozyme banding pattern, and gel visualization used dye solution with the composition as follows: 0.0125 g of α-naphthyl acetate dissolved in 2.5 ml acetone, then added with 50 ml of 0.2 M phosphate buffer pH 6.5 and 0.0125 g of Fast Blue BB Salt.

3. Result and Discussion

3.1. *Morphological Analysis*

3.1.1. Stems

Table 1. The average length of stem and internodes of black rice after radiated using different gamma rays

Parameters	Dose of Radiation (Gray)($\bar{x}$± SD)		
	0	200	300
Length of stem (cm)	94,04^a ± 0,48	106,61^b ± 1,66	93,66^a ± 1,01
Number of internodes	5,16^a ± 0,17	6,05^b ± 0,21	5,43^a ± 0,38
Length of 3rd internodes (cm)	18,07^a ± 0,74	17,04^a ± 0,70	17,92^a ± 1,08
Diameter of 3rd internodes (cm)	0,57^b ± 0,06	0,54^{ab} ± 0,03	0,50^a ± 0,02
Number of branches	1,07^a ± 0,07	1,17^a ± 0,13	1,70^b ± 0,50

Note: Value with same small letter annotation means has no significant difference in 95% DMRT (Duncan Multiple Range Test)

The average length of stems employed the 3 doses of radiation were 0 Gy is 94.04 cm, 200 Gy is 106.61 cm, and 300 Gy is 93.66 cm. The radiation dose of 200 Gy showed a significant difference compared to the control plant (0 Gy), whereas the radiation dose of 300 Gy showed decreasing the stem, although the shorter growing than the control plant was not significantly different. The average number of internodes resulting from the 3 doses of radiation were 0 Gy was 5.16, 200 Gy (6.05), and 300 Gy (5.43) respectively. It was interested to note that using radiation dose of 200 Gy the result again showing a significant different number of internodes compared to the control, meanwhile no significant difference were recorded when radiation doses of 300 Gy was applied. The average diameter of the third segment on the radiation dose 0 Gy was 0.57 cm, 200 Gy was 0.54 cm, and 300 Gy was 0,50 cm. These results confirmed that treated seed have no effect on the development of stem diameters. The number of branches has increased along with increasing radiation dose. The average number of branches on the control plant was 1.07, and 1.17 after a treatment with dose of radiation 200 Gy has increased significantly to 1.70 after dose of radiation 300 Gy was conducted.

3.1.2. Leaves

Table 2. Morphological character of leaves of black rice plant

Parameters	Dose of Radiation (Gray)($\bar{x}$± SD)		
	0	200	300
Length of 1st leaf (cm)	29,88^a ± 2,44	32,77^b ± 0,77	29,48^a ± 1,67
Length of 2nd leaf (cm)	41,38^b ± 3,77	46,47^c ± 0,52	37,19^a ± 1,18
Length of 3rd leaf (cm)	45,28^a ± 2,46	49,88^b ± 0,83	43,45^a ± 1,40
Width of 1st leaf (cm)	1,42^b ± 0,04	1,50^b ± 0,04	1,26^a ± 0,09
Width of 2nd leaf (cm)	1,29^b ± 0,05	1,33^b ± 0,03	1,15^a ± 0,06
Width of 3rd leaf (cm)	1,17^b ± 0,04	1,26^c ± 0,06	1,07^a ± 0,06

Note : Value with same small letter annotation means has no significant difference in 95% DMRT (Duncan Multiple Range Test)

Observations on the morphological character of leaves were the length and width. These parameters were opserved at the third leaf from the top.The radiation dose of 200 gy create a longer leaf with length of 1st leaf is 32.77 cm, length of 2nd leaf is 46.47 cm and length of 3rd leaf is 49.88 cm, while the control plant have length of leaf in a row are 29.88 cm, 41.38 cm and 45.28 cm respectively, while the radiation dose of 300 Gy have almost the same length leaves of control that are 29.48 cm, 37.19 cm and 43.45 cm. Beside the significant difference in leaf length, the width of leaf result the opposite instead. There is no significant difference found in the width of leaf where the value appears to be in a close range each other.

3.1.3. Grains

The parameters used in measuring the grains were length and width of grains, shape of grain (ratio between length and width of grain) and number of grains per panicle. The data of morphological difference of grains that was analyzed with SPSS (Statistical Package for the Social Science) software were presented at table 3.

Table 3. Morphological character of grains of black rice plant

Parameters	Dose of Radiation (Gray)($\bar{x}$± SD)		
	0	**200**	**300**
Grain Length (cm)	$0{,}98^a \pm 0{,}00$	$0{,}98^a \pm 0{,}00$	$0{,}98^a \pm 0{,}00$
Grain Width (cm)	$0{,}30^a \pm 0{,}00$	$0{,}3^a \pm 0{,}00$	$0{,}30^a \pm 0{,}00$
Grain Shape (L:W)	$3{,}27^a \pm 0{,}06$	$3{,}28^a \pm 0{,}06$	$3{,}26^a \pm 0{,}05$
Number of grains per panicle	$126{,}21^a \pm 12{,}19$	$130{,}23^a \pm 8{,}10$	$124{,}73^a \pm 10{,}07$

Note : Value with same small letter annotation means has no significant difference in 95% DMRT (Duncan Multiple Range Test)

Gamma radiation seems to have no effect on grains morphology. Where the results appeared no one show the significant differences among all the parameters of measurement. However, radiation dose of 200 Gy produce the highest number of grains per panicle. There were no significant difference on the treated seeds and the control one. And this may be due the existance of seed coats.

3.1.4. Flowering time

Flowering time was measured from the day of first flower appears. The data shown below is flowering time of black rice plant irradiated by gamma rays. The control plant doesn't include in the table because they flowers later. So this table just recorded the flowering time that appear faster than normal plant (c.a. 80 days).

Table 4. Flowering time of black rice plant

Age of plant	Number of flowers appear per 2000 plants	
	200 Gy	**300 Gy**
54-60	16	41
61-67	95	114
68-74	72	74
Total flowers appear	183	229

There are remarkable results of observation that radiation could initiate the flowering of black rice plant. From total amount of 6000 black rice plant, 412 plants could flower faster than normal plants (80 days after planting). The radiation dose of 300 Gy have more influence on the early time plant flowering than the 200 Gy. This could be proved by many number of flower appear faster than the normal black rice plant. In the age of 54-60 days after planting, while the radiation dose of 200 Gy produce only 16 flowers while using radiation dose of 300 Gy 41 flowers have been produced. Interestingly, more flowers (114) have been produced when 300 Gy was applied compared to the 200 Gy (95). The radiation dose of 300 Gy obtained the number of plants are flowering faster than the control are 229 plants from a total amount of 2000 plants. The radiation dose of 200 Gy obtained the number of plants that flowering faster than the control plants are 183 plants from a total amount of 2000 plants. The percentage (%) of early time of flowering plants were quite high (9.15 %) at the dose of radiation 200 Gy. This value increased into 11.45 % at the dose of radiation 300 Gy. This indicated that both two different doses of gamma radiation (200 and 300 Gy) had resulted a number of plants flowering early. We do believe that this early flowering time was significantly difference to control plants. In this prelimenary report showed very promising molecular evidence that those radiation to have affects on the alteration of enzyme, protein or DNA of the plants as also shown by De Britto [8].

3.2. *Molecular Analysis*

Figure 1. Zymogram of esterase isozyme patterns of the black rice after radiated using different gamma rays. Gel is loaded by samples in following orders; lane 1 and lane 10 were samples from control plants, lane 2 to 5 were samples from plants with radiation dose of 200 Gy, and lane 6 to 9 were samples from plants with radiation dose of 300 Gy.

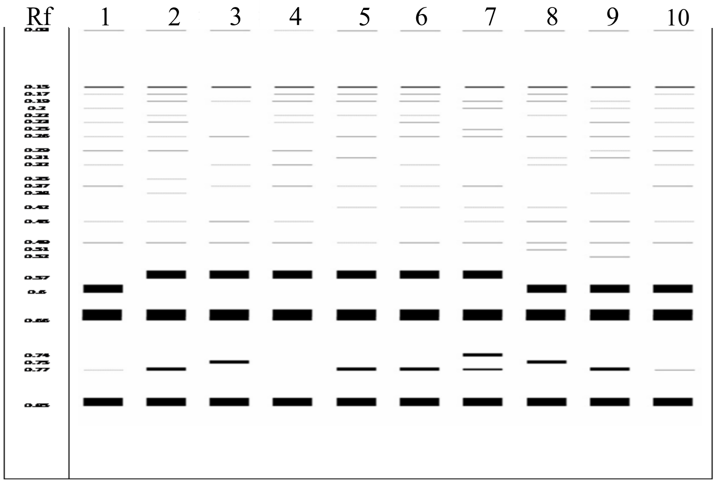

Figure 1. Zymogram of esterase isozyme patterns of the black rice after radiated using different gamma rays.

Result of isozyme banding pattern analysis show 27 bands. Those 27 bands located in the Rf between 0.03 and 0.85 i.e. 0.03; 0.15; 0.17; 0.19; 0.20; 0.22; 0.23; 0.25; 0.26; 0.29; 0.31; 0.32; 0.35; 0.37; 0.38; 0.42; 0.45; 0.49; 0.51; 0.52; 0.57; 0.60; 0.66; 0.74; 0.75; 0.77 and 0.85. There were 12 bands appear from sample of mutated plant that do not exist in the sample of normal plant. From those 12 bands, there was one (1) band (i.e. Rf = 0.35) appears only in sample of mutated plant with 200 Gy radiation dose, 4 bands (i.e. Rf= 0.25; 0.51; 0.52; 0.74) appear only in sample of mutated plant with 300 Gy radiation dose, and 7 bands (i.e. Rf = 0.19; 0.22; 0.31; 0.39; 0.42; 0.57; 0.75) appear in both samples. There were 2 bands (i.e. Rf = 0.20; 0.60) appeared in sample of normal plant and mutated plant with 300 Gy dose of radiation. Besides, there was one (1) band (i.e. Rf = 0.77) showed different thickness among other band in the same line. This band showed thicker band in both sample of mutated plant. This very preliminary evident could prove that radiation leads to mutations that trigger the alteration in isozyme banding pattern. And this could be appeared as new isozyme bands or different thickness of the bands. With increasing radiation dose, it could potentially produce more additional bands or possibly causing a few bands disappeared.

The result of this study was consistent with the previous study which showed addition in band number of protein pattern and DNA pattern of *Zea mays* L. [2]. Exposure of radiation with different dose and time leads to alteration of isozyme banding pattern, and it could be increased or decreased the number of band. This was happen because radiation may resulted mutation which could activated some gene expressions or distract the expression of the gene [15, 11].

4. Conclusion

This preliminary study showed the differences in the morphology of rice plants after treated with gamma rays. That could be noted on stem length, number or internodes, number of branches, leaf length, and flowering time. The dose of radiation 200 Gy have better value than 300 Gy. This first evident of isozyme banding pattern of irradiated plants which differ from the control plant would possibly to do further test molecularly (DNA and protein). And hopefully the induction of gamma rays would give another clue that mutation could occur in mutated rice plants.

Acknowledgements

We would like to thanks to National Nuclear Energy Agency of Indonesia for the support. And our thanks went to Mrs. Atik and Mrs. Nina (Molecular Biology Lab) for the technical assistance.

References

[1] Kristamtini, Taryono, Basunanda P, Murti R H dan Supriyanta 2012 *J. Agric. Biol. Sci*. **7** 982
[2] Miksusanti, Elfita dan Hotdelina 2012 *Jurnal Penelitian Sains* **15** 60
[3] Ali H A, Cho I K, Kim S J, Kim S N, Kim S Y, Cho Y S, Baek H J and Kim B J 2011 *Korean J. Environ. Agric.* **30** 111
[4] Ling W H, Cheng Q X, Ma J and Wang T 2001 *J. Nutr.* **131** 1421
[5] Ling W H, Wang L L and Ma J 2002 *J. Nutr*. **132** 20
[6] Santoso A 2011 *Magistra* **75** 35
[7] Kristamtini 2009 *Tabloid Sinar Tani* (Jakarta : Sinar Tani)
[8] De Britto A J, Jeevitha M and Raj T L S 2011 *J. Stress Physiol. Biochem*. **7** 232
[9] Sobrizal 2008 *Jurnal Ilmiah Aplikasi Isotop dan Radiasi* **4** 1
[10] Utami S 2013 *Agrium* **18** 158
[11] Talukdar D 2010 *Int. J. Genet. Mol. Biol*. **2** 112
[12] Suranto 2001 *Biodiversitas* **2** 85
[13] Chang T T, Bardenas, Eliseo A, Rosario D and Arnulfo C 1965 *Technical Bulletin* (Manila: The International Rice Research Institute)
[14] Suranto 2002 *Biodiversitas* **3** 257
[15] Hamouda M, Saad-Allah K M and Kasim W A 2014 *Int. J. Agric. Res*. **4** 46