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PEROXIDASE ISOZYME IDENTIFICATION OF SOME RICE GENOTYPES IN M_1 GENERATION UNDER DROUGHT STRESS LEVEL OF -0.03 MPa

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ABSTRACT

The effort to fulfill the need of rice through the improvement of dryland productivity can be viewed as a more environmentally-friendly way. This research used 36 rice genotypes in M_1 generation that were grown hydrophonically under drought stress level of -0.03 MPa. The identifications were conducted based on peroxidase isozyme marker. The isozyme patterns in zymogram were binary-coded by visual scores for each genotype, based on the thickness and the number in the appearance of bands on certain migration distance. The migration distances were measured based on values of Rf. The similarity coefficients were calculated using Dice's coefficient that were used to construct dendrogram using the UPGMA employing the SAHN from the NTSYSpc 2.02. The results showed that the most resistant genotype under drought stress was R-4, and the genetic relationships among the genotypes were divided into two main groups, aromatic and non-aromatic group, in which some genotypes experienced the reduced levels of aromatic character (R-8 and R-9) and the drought resistance character (IU-2, IU-3, IU-4, IU-5, IU-6, IU-7, and IU-8), but there were some genotypes that were able to improve the resistance under drought stress (R-2, R-3, R-5, R-6, R-7, IT-4, IT-5, and IT-7).

Keywords: drought resistance, gamma irradiation, peroxidase isozyme, rice, sodium azide

INTRODUCTION

The government policy in providing the subsidy on rice all this time has the role to make the rice staple food for the majority of Indonesian people (Panuju *et al.*, 2013). The national need for rice that is higher cannot be fulfilled by domestic production at last (Mulwanyi *et al.*, 2011). It is caused by the development of cultivation technologies that does not adequately consider the fertility of soil and is focused on the wetland (Azwir and Ridwan, 2009). Besides, the wetland is also blamed as the largest contributor of greenhouse gas emissions (Zhang *et al.*, 2011), so the development of cultivation technologies on dryland is viewed as an effort to provide food that is more environmentally-friendly.

The rice productivity on dryland can be improved with genetic improvement. However, the efforts of genetic improvement are often limited by the available natural diversity (Shu, 2009), though the natural diversity can be improved through induced mutation (Rustikawati *et al.*, 2012). The induced mutation is reported to be able to improve the characters of plants, with the treatment of gamma irradiation that is able to improve the yield components around 9-40 % (Shehzad *et al*., 2011), and the treatment of sodium azide that is able to improve the resistance under drought stress until -0.0077 MPa (Aurabi *et al*., 2012). Besides, the combinations of treatment between gamma irradiation and sodium azide is reported to improve the yield components around 7-15 % (Siddiqui and Singh, 2010), and the resistance under drought stress until -0.0021 MPa (He *et al*., 2009).

The drought resistance character is known to collerate with yield components character (Haider *et al*., 2012). Therefore, the selection on yield components character can be started with the selection on drought resistance

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character. The drought resistance character can be identified based on the peroxidase isozyme (Nasr *et al.*, 2009), since the increasing levels of drought stress can encourage the increase of peroxidase activities (Sharma and Dubey, 2005). It is caused by the peroxidase role in osmotic adjustment of cells, so the plant can be more resistant under drought stress (Omidi, 2010). The objectives of this research are to analyze the rice genotypes in M_1 generation that is the most resistant under drought stress, and investigate the genetic relationships among the genotypes based on peroxidase isozyme marker. This study is expected to give information that can be used for genetic improvement of drought-resistant rice.

MATERIALS AND METHODS

M_1 Rice Seedling Materials

The materials used were 36 rice genotypes in M_1 generation induced by gamma irradiation and sodium azide 10^{-3} M (SA) (Herwibawa *et al.*, 2014). The 36 M_1 rice seedlings were IU-1, IU-2, IU-3, IU-4, IU-5, IU-6, IU-7, IU-8, IU-9, R-1, R-2, R-3, R-4, R-5, R-6, R-7, R-8, R-9, IT-1, IT-2, IT-3, IT-4, IT-5, IT-6, IT-7, IT-8, IT-9, C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, and C-9. Remarks: IU = Inpago Unsoed 1 (upland aromatic); R = Rojolele (lowland aromatic); IT = Inpari 13 (lowland non-aromatic); C = Cirata (upland non-aromatic); - = that was treated with; 1 = without mutagen; 2 = gamma 100 Gy; 3 = gamma 150 Gy; 4 = SA 2 hours; 5 = SA 6 hours; 6 = gamma 100 Gy + SA 2 hours; 7 = gamma 100 Gy + SA 6 hours; 8 = gamma 150 Gy + SA 2 hours; 9 = gamma 150 Gy + SA 6 hours.

Drought Stress Application

The location was at Screen House of Seed Technology Laboratory, Vocational Education Development Center of Agriculture, Cianjur, Indonesia. The two-week-old rice seedlings were grown hydroponically (Wang *et al*., 2013), with the source of nutrient from Hoagland solution. The pH condition of the solution was observed once every two days, and the solution was replaced once a week (Santika, 2011). After the adaptation for three weeks, they were kept in Hoagland solution containing 5 % (w/v) polyethylene glycol 6000 to achieve drought (osmotic) stress level of -0.03 MPa. The drought stress was given for six weeks, then the nursery was maintained in optimal condition for four weeks.

Enzyme Extraction

The isozyme analysis was conducted in Plant Biology Laboratory, Study Center of Biological Sciences, Bogor Agricultural University, Bogor, Indonesia. The used analysis method was based on Wendel and Weeden (1989) techniques that were modified. The samples were chosen from the tip of the second leaf under the flag leaf of fifteen-week-old rice that was cut into quarters as the material of enzyme extraction. The cut of the leaf as a sample with a weight of around 200 mg was then cut into pieces, and crushed with the pestle in a mortar that was added with silica sand and 0.5 ml of extraction buffer (0.07045 g of L-ascorbic acid, 0.1939 g of L-cysteine, 0.12 ml of Triton-X-100, 0.25 g of PVP-40, 0.54 g $Na_2HPO_4.2H_2O$, and pH buffer was adjusted to 7.0) until the tissue extract was obtained.

Gel Preparation

The 10% starch gel solution was made of 10 g of potato starch per 100 ml of buffer gel solution (1.048 g of L-Histidine monohydrate that was dissolved with distilled water until one litre, then its acidity was regulated with Tris that was added until pH 6.0). The making process was started with one third of buffer gel that was mixed with two third of the part that was boiled first on hot plate. The solution was then de-aerated with membrane vacuum pump around 30 seconds and poured into the acrylic gel mold with liquid paraffin on its surface, in which electrode strips were sealed with masking tape. The gel was allowed to cool and set for approximately 30 minutes at room temperature and closed with paraffin-coated plastic, and then it was placed for 1 hour in a refrigerator to cool it before use. The wells were then made in the middle of the cooled gel with comb. The filter papers with a size of around 0.5 x 0.5 cm were used as the absorbent paper of tissue extract, and then were inserted into the gel based on the order of wells.

Electrophoresis

The isozyme was separated into discrete bands by horizontal starch gel electrophoresis. The tapes were then removed from the

electrode strips, the mold was then entered into the tray with the electrode buffer solution (10.5507 g of citric acid monohydrate and 18.1650 g of tris (hydroxymethyl) aminomethane in the distilled water until the volume reached one litre, and the last pH was adjusted to pH 6.0). The tray was then stored in the refrigerator in a temperature around 10 ^{0}C, and then it was connected with anode and cathode of power supply. The initial electrophoresis occurred for 3 minutes in the voltage of 100 volt, the voltage was then increased to 150 volt after 1 hour for 4 hours.

Staining

The tray was removed from the refrigerator soon after the electrophoresis finished. The inserted filter papers were then removed from the wells. The gel was then immersed into a peroxidase staining assay (100 ml sodium acetate 50 mM pH 5.0; 50 mg $CaCl_2$; 0.5 ml H_2O_2 3 %; 50 mg 3-amino-9-ethylcarbazole; and 5 ml N,N-dimethylformamide). Furthermore, the stain box was closed with aluminium foil, and then it was incubated in a room temperature for a night. After one night, the enzymatic activity zone was observable, and the gel was cleaned with distilled water to remove the rest of the staining solution.

Scoring

The isozyme patterns interpreted in the zymogram were binary-coded by visual scores for each genotype, based on the thickness and the number in the appearance of bands on certain migration distance (Bon *et al.*, 2006). The measurement of migration distance was conducted based on values of Rf (relative front). Similarity coefficients from electrophoretic banding pattern were calculated using Dice's coefficient. These similarity coefficients were used to construct dendrogram using the UPGMA (Unweighted Pair Group Method with Arithmetic mean) employing the SAHN (Sequential, Agglomerative, Hierarchical, and Nested clustering) from the NTSYSpc 2.02 (Rohlf, 1998).

RESULTS AND DISCUSSION

The drought stress level of -0.03 MPa made R-1 genotype (lowland aromatic without mutagen) unable to survive. There were 35 M_1 rice genotypes that can survive and show high diversity based on the patterns of peroxidase isozyme bands (Figure 1). The diversity was observable based on the thickness and number of bands, and the migration distance (Nurmiyati *et al.*, 2009). The various thickness of the bands was caused by the difference of molecules weight that were migrated, in which the heavier molecules made the worse split of the molecules, so the formed bands became thicker (Cahyarini *et al.*, 2004). Besides, the band thickness also showed the resistance of the plants under drought stress.

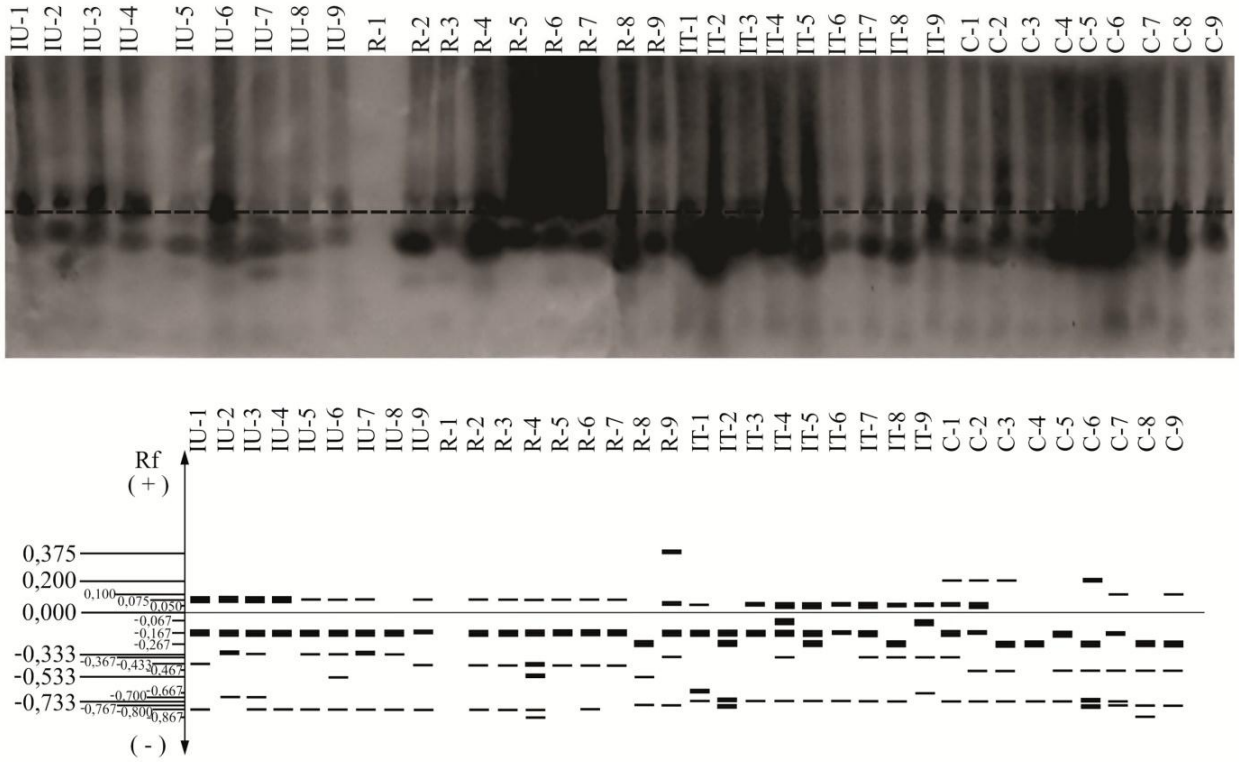
According to Mathius *et al.* (2001), increasing levels of drought stress made the isozyme concentration lower, so the formed bands got thinner. The drought stress will encourage the hydrolysis that can result in the toxic compounds, that causes the inhibition of enzymatic activities (Mahajan and Tuteja, 2005). The different condition happened when the plants were able to neutralize the toxic compounds through the osmotic adjustment of cells, so the formed bands were thicker during the increase of levels of drought stress.

The improvement of resistance under the drought stress was caused by the synthesis and accumulation of organic compounds that caused the reduction of osmotic potential (Omidi, 2010). The reduction of osmotic potential showed the increase of peroxidase activities that was marked with the increasing number in the appearance of bands (Abedi and Pakniyat, 2010). Therefore, the most resistant genotype under drought stress was R-4 since it was the thickest with the maximum number in the appearance of bands (Figure 1). It showed that the treatment of sodium azide 10^{-3} M for 2 hours was able to improve the resistance of Rojolele (lowland aromatic) under the drought stress.

The resistance mechanisms on the drought stress were coded by many genes that can be categorized into the genes that were related to the cell protection on drought, and the genes that were related to the mechanism of response regulation on drought (Shinozaki and Shinozaki, 2007). According to Dixit *et al.* (2014), the resistance mechanisms on drought stress can be categorized into escape, avoidance, and tolerance. Figure 1 showed 32 variations of band pattern (genotypes) from 19 values of Rf. The variations indicated a wide

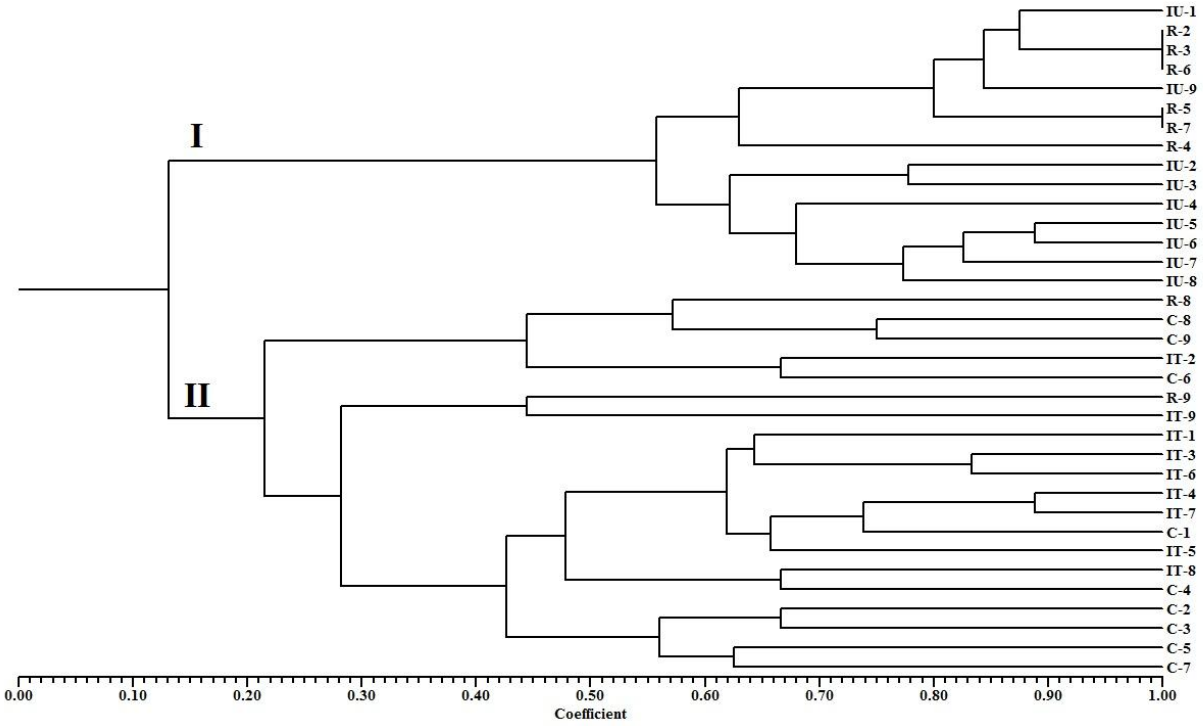
genetic diversity among the genotypes (Khan *et al.*, 2009), which may have corresponded to allelic diversity (Bon *et al*., 2006). It was suitable with the research of Alvarez *et al.* (2000) emphasising that the variations strongly indicated that high genetic variability was generated by mutagen. Besides, the research of Lestari (2006) explained that the variations were generated by mutagen caused different responses to the drought stress that really depended on the resistance of each genotype.

The genetic relationships among the genotypes can be considered far when the coefficient was less than 0.60 (Cahyarini *et al*., 2004). Figure 2 showed that the genetic relationships among the genotypes were divided into two main groups on the coefficient of 0.13, the first group that comes from the aromatic character in general (Inpago Unsoed 1 and Rojolele) and the second group that comes from non-aromatic character in general (Inpari 13 and Cirata). The existence of R-8 and R-9 genotypes on the second group showed that those two genotypes experienced the reduced levels of aromatic character. According to Gehrke *et al.* (2013), the reduced aromatic character was caused by transversion mutation associated with oxidative damage. Besides, other genotypes also experienced the changed of drought resistance character.



Remarks: IU = Inpago Unsoed 1 (upland aromatic); R = Rojolele (lowland aromatic); IT = Inpari 13 (lowland non-aromatic); C = Cirata (upland non-aromatic); - = that was treated with; 1 = without mutagen; 2 = gamma 100 Gy; 3 = gamma 150 Gy; 4 = SA 2 hours; 5 = SA 6 hours; 6 = gamma 100 Gy + SA 2 hours; 7 = gamma 100 Gy + SA 6 hours; 8 = gamma 150 Gy + SA 2 hours; 9 = gamma 150 Gy + SA 6 hours; Rf = relative front

Figure 1. Zymogram and diagrammatic interpretation of M_1 rice genotypes



Remarks : IU = Inpago Unsoed 1 (upland aromatic); R = Rojolele (lowland aromatic); IT = Inpari 13 (lowland non-aromatic); C = Cirata (upland non-aromatic); - = that was treated with; 1 = without mutagen; 2 = gamma 100 Gy; 3= gamma 150 Gy; 4 = SA 2 hours; 5 = SA 6 hours; 6 = gamma 100 Gy + SA 2 hours; 7 = gamma 100 Gy + SA 6 hours; 8 = gamma 150 Gy + SA 2 hours; 9 = gamma 150 Gy + SA 6 hours

Figure 2. Dendrogram of M_1 rice genotypes

The R-4 genotype was the most resistant under drought stress and it had a close genetic relationship with the IU-1 genotype (upland aromatic without mutagen), on the coefficient of 0.63 (Figure 2). Besides, the IU-1 genotype also had the closer genetic relationship with genotypes of R-2, R-3, R-6, IU-9, R-5, and R-7, that explained some genotypes of the first group that were able to improve the resistance under drought stress. On the contrary, the IU-1 genotype had the further genetic relationship with genotypes of IU-2, IU-3, IU-4, IU-5, IU-6, IU-7, and IU-8 on the coefficient of 0.56,that explained the reduced levels of resistance under drought stress on some genotypes.

The changed of drought resistance character also happened in the second group. Figure 2 showed that the C-1 genotype (upland non-aromatic without mutagen) had the close genetic relationship with genotypes of IT-7 and IT-4 on the coefficient of 0.74, it was also close with the IT-5 genotype on the coefficient of 0.66, so it was certain that three genotypes were more resistant under drought stress. However, there was limited observable information on the second group since the genotypes of C-1 and IT-1 (lowland non-aromatic without mutagen) had close genetic relationship on the coefficient of 0.62, so other separated genotypes on the coefficient that was less than 0.48 had very different drought resistance characters with two genotypes of non-aromatic without mutagen.

The difference of the drought resistance character among the genotypes under drought stress explained the change of characters due to the mutation process (Kadir, 2011). It was suitable with the research of Ando and Montalvan (2001) where the treatments of gamma irradiation, sodium azide, and its combinations caused physiological damages and improved the mutation frequency. Besides, the research of Kumar and Srivastava (2013) explained that the chromosomal aberrations happened through different mechanisms among the given mutagenic treatments, so it caused the various changes of each genotype.

CONCLUSION

The most resistant genotype under drought stress was R-4. The genetic relationships among the genotypes were divided into two main groups, aromatic and non-aromatic group, in which some genotypes experienced the reduced levels of aromatic character (R-8 and R-9) and the drought resistance character (IU-2, IU-3, IU-4, IU-5, IU-6, IU-7, and IU-8), but there were some genotypes that were able to improve the resistance under drought stress (R-2, R-3, R-5, R-6, R-7, IT-4, IT-5, and IT-7). The results are expected to be used as the reference, especially in determining the genotypes for the drought-resistant rice-breeding program.

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