Isozyme banding patterns of drought tolerant sugarcane putative mutants

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Abstract. Genetic characterization based on isozyme is useful for plant breeding studies. Isozyme marker may be used to understand genetic variability of irradiated plant. The objective of this research was to recognise banding pattern variabilities of 34 putative irradiated mutants that survival drought screening, based on isozyme analysis. The research was conducted in March – April 2015 in Biology Laboratory, PAU IPB Bogor. Vertical polyacrylamide gel electrophoresis was used to analyse three enzymes: peroxidase (PER), Esterase (EST), Aspartate Aminotransferase (AAT). Isozyme analysis was carried out on gamma rays irradiated mutants of sugarcane “Kidang Kencana” that have been selected for drought tolerant. The result showed that there was a genetic variability on 34 putative mutant of drought tolerant sugarcane, that was shown by polymorphic banding patterns on PER and EST enzymes. AAT was monomorphic, no variation was shown amongs the mutants. PER enzyme had 17 banding patterns and EST enzyme had 7 banding patterns. Dendogram based on PER and EST on 34 putative mutants could be grouped into two clusters at 81.14% genetic similarity. The isozyme analysis can be used as a specific identifier of a new mutant that will be developed as new varieties.

1. Introduction

Increasing genetic diversity is the main capital for plant breeders to get new superior varieties according to the desired characteristics. High genetic diversity in germplasm will provide high opportunities for genetic improvement in plants [1]. The use of mutation techniques with gamma ray irradiation is known to increase plant genetic diversity [2] The new properties that are formed by mutation induction are very diverse, because the mutations that are formed are random. Therefore, selection is needed to get new mutants according to the desired breeding goals. To find out genetic changes due to mutation treatment it is necessary to identify mutants to determine genetic diversity in the population of mutants produced.

Genetic variability of plant population or specific character can be observed through morphological, biochemical, and molecular markers. At perennial plant, morphological marker are
less successful because the character to identify vegetative phase are limited and requires more time until the plant reach generative phase. Furthermore, the appearance of morphological characters often ambiguous because of environmental factors [3].

An alternative method to observe the genetic variability is using isozyme or isoenzyme [4]. Isoenzymes are relatively stable to environment and presence high polymorphism [4]. Isozyme is enzymes that have different chemical molecule, but catalyzed the same chemical reaction. Isozyme polymorphism in the form of different protein molecules, the phenotype are showed as different bands and banding pattern. Polymorphism is obtained through gel electrophoresis with specific color for each enzyme. Therefore, the isozymes have been used (1) to observe genetic variability inside and among the plant population, (2) as marker for breeding or mutation, (3) as marker for plant endurance or vulnerable against disease in selection program [5].

Some characteristic and benefit of using isozyme are [6] [7] [8]: (1) product from different allele are moving to different position in the gel, (2) different allele usually codominant, free of epistasis, so that the homozygote are distinguished from heterozygote, (3) the band position often as product of one locus so that it is possible to detect the number of genes coding for one enzyme by analyzing its banding pattern, (4) required tools and materials are relatively cheap in laboratory, (5) lots of sample can be analyzed faster, as well as (6) possible to be done at germs stages to save time, place and cost. According to the above characteristics, isozyme have been used for identification and clarification some plant species, such as triploid identification on orange using four enzyme system (MDH, 6-PGD, SKDH, and PGI) [9], sugarcane germplasm [10]. Isozyme markers can be used to see genetic diversity in irradiated mutants. The objective of this research was to observe isozyme banding pattern variability of some drought tolerance sugarcane putative mutants from gamma irradiation treatments.

2. Materials and Methods
Variability analysis of induced mutation sugarcane was conducted in Biologi Pusat Antar Universitas (PAU) Laboratory in IPB Bogor at March - April 2015. Material used in this research was the leaves of 34 drought tolerance sugarcane putative mutants from gamma irradiated Kidang Kencana variety. The sugarcane mutants were selected from in vitro drought test and have been acclimatized for six months in the screen house. These mutants were as follow (Table 1).

Isozyme analysis carried out using 3 enzymes were AAT (Aspartate aminotransferase), EST (Esterase) and PER ( Peroxidase). Isozyme electrophorecy was carried out using 2 layers of polyacrilamid gel, were stacking gel (4% acrylamid, 0,5 M Tris-HCL pH 6,8, amonium persulfat, TEMED) and separating gel (6-7,5% acrylamid, 1,5 M Tris-HCL pH 8,8) (Triest dan Kabir 2000). Electrode buffer used was Tris-glicine pH 8,3.

The young leaves of 0,5 g were crushed in low temperature, then added 1 ml extraction buffer. The extract sample was centrifuged in low temperature at 10.000 rpm, the pellet are disposed while the supernatant was used for electrophoreosis. The electrophoreosis carried out at temperature 4-10°C, voltage 125-175 V, and current 18-34 mA for 4-5 hours. After electrophoreosis, gel was replaced to the tray and colored according to analyzed enzyme system. Coloring was done on shaker at chamber temperature for 30-60 minutes [9]. The data was cluster analyzed using multivariate cluster observation with single linkage method, euclidean distance using Minitab 1.5 software.

3. Results and Discussion
Analysis result of three system enzymes on 34 sugarcane drought tolerant putative mutant of Kidang Kencana variety resulted from gamma irradiated treatments showed that not all enzyme has banding pattern variability. The enzymes that showed variability in banding pattern were PER and EST (Figure 1, and 2), while AAT was not express banding pattern variability (Figure 3).
Table 1. Sugarcane drought tolerant Putative Mutant of Kidang Kencana variety gamma irradiated population

| Irradiation Dosage (Gy) | Code of drought tolerance sugarcane putative mutant from gamma irradiated Kidang Kencana variety | Total |
|------------------------|---------------------------------------------------------------------------------------------|-------|
| 0                      | K1(MK22), K2(MK24), K3(MK25), K4(MK26), K5(MK27), K6(MK28), K7(MK29) (somaclone)          | 7     |
| 10                     | K8(MK10), K9(MK12), K10(MK13), K11(MK15), K12(MK16), K13(MK18), K14(M31), K15(MK32)     | 8     |
| 20                     | K16(MK19), K17(MK20), K18(MK21), K19(MK33), K20(MK34), K21(MK35), K22(MK37), K23(MK38), K24(MK40), K25(MK46), K26(KMK47), K27(MK54), K28(MK57), K29(MK58) | 14    |
| 30                     | K30(MK42), K31(MK51)                                                                      | 2     |
| 40                     | K32(MK60), K33(MK68), K34(MK64)                                                           | 3     |
|                        | Total                                                                                     | 34    |

In the PER enzyme system there were three active areas controlled by three locus (Figure 1). The structure of PER enzyme is dimeric. The banding patterns were pulled in two directions namely cathoda (+) and anoda (-). In the three active areas were drawn the banding pattern and there were 17 band patterns (Figure 4). The band pattern one was the parent of Kidang Kencana both from tissue culture (KK) and plants from the fields (KL). Mutants which has the band pattern one were K17, K18, K21, K23, K24 and K28 (20 gray). Band pattern two was found in K1, K6, K7 (0 gray) and K30 (30 gray). Band pattern three contained in mutants K2 (0 gray) and K10, K14, K15 (10 gray). The band pattern four was found at mutants K3 and K4 (0 gray). The band pattern five was found at K5 (0 gray). Band pattern six found at mutant K8 and K11 (10 gray). Band pattern seven found at mutant irradiated with 10 gray were K9 (10 gray) and K32 (40 gray). Band pattern eight and nine contained in 10 gray irradiated mutants were K12 and K13 respectively. Band pattern 10 up to 14 contained in mutant 20 gray irradiated were K16 and K29 (band pattern 10), K19 (band pattern 11), K20, K26 and K27 (band pattern 12), K22 (band pattern 13) and K25 (band pattern 14). Band pattern 15 observed at mutant irradiated with 30 gray was K31. The band pattern 16 and 17 observed at mutant irradiated with 40 gray were K33 and K34.

The mutants irradiated with gamma using dosage 20 gray produce the most variated banding pattern mutant were six types of banding patterns (band pattern 1, 10, 11, 12, 13 and 14), followed by dosage 10 gray with five types of banding pattern (3, 6, 7, 8 and 9), dosage 0 gray with four types of banding pattern (2, 3, 4 dan 5), dosage 40 gray with 3 types of banding pattern (7, 16 and 17) as well as dosage 30 gray with dua types of banding pattern (banding pattern 2 and 15). This showed that both tissue culture and gamma irradiation can cause genetic changes which correspond with statement [12] [13] that tissue culture can cause somaclonal variation due to acceleration of cell division as effect of the use of plant growth regulator mainly auctin such as 2,4-D. Besides, the genetic changes also caused by gamma irradiation treatment [2].

In the Esterase system there were four actives area controlled by two locus that are attracted to the cathode (+). According to [14] states that the structure of the Esterase enzyme is monomeric with a large number of loci. Monomeric is an enzyme with a single polypeptide chain. In the four active area, the band pattern drawn and there were seven band patterns (Figure 4). Band pattern one was the Kidang Kencana parents, both from tissue culture (KK) and plants from the field (KL). Mutants that have the same banding pattern as the parents were non-irradiated mutants (K1, K2, K3, K4, K6, K7), 10 gray irradiated mutants (K9, K10, K11, K15), 20 gray irradiated mutants K18, K23, K25, K26, K27, K29), 30 gray (K30) and 40 gray (K32) irradiated mutants. Two banding patterns found in mutants K5 (0 gray), K8, K12, K13, K14 (10 gray) and K16, K28 (20 gray). The three band pattern was found in 20 gray irradiated mutants (K17, K21 and K22). The four band pattern found in 20 gray
irradiated mutants (K20 and K24). The five band pattern found in 30 gray (K31) irradiated mutants. The band pattern six was found in 40 gray (K33 and K34) irradiated mutants. The band pattern seven was found in mutants (K19) with 20 gray irradiation. The band pattern was not expressed in the band pattern seven. The absence of band pattern polymorphism in K19 mutants detected by electrophoresis may be due to the pattern of bands that appear or were detected based only on differences in charge on soluble proteins. This was consistent with the statement of [3] that the band pattern detected through electrophoresis was only read the difference of soluble proteins. Yet according to [15] of 30% of protein dissolved in plant cells, only 25% have different charges that can be detected by electrophoresis.

**Figure 1.** Variation of isozym peroxidase banding pattern at 34 drought tolerance sugarcane putative mutants from gamma irradiated Kidang Kencana variety

Isozyme analysis using Esterase enzyme expressed genetic variation among mutants gamma irradiated Kidang Kencana variety. Mutants irradiated with gamma dosage 20 gray have highest genetic variability among other mutants. Mutants irradiated with gamma dosage 20 gray has five types of banding pattern were 1, 2, 3, 4, 7 followed by dosage 0, 10 and 30 gray with two types of banding pattern were 1 and 2 banding pattern at mutant 0 gray and 10 gray as well as banding pattern 1 and 5 at mutant 30 gray while mutant 40 gray only clustered in one banding pattern was banding pattern 6. This showed that gamma irradiation dosage 20 gray were the most caused genetic variation in sugarcane.

**Figure 2.** Variation of Esterase Isozyme Banding pattern at 34 drought tolerance sugarcane putative mutants from gamma irradiated Kidang Kencana variety

The structure of AAT is dimeric. In the AAT enzyme system there is only one active area with one monomorphic band pattern (Figures 3). In these active areas the banding pattern was less clear because of rapid migration. This caused difficulty in the scoring process. This means that the diversity among putative mutants of Kidang Kencana irradiated using several doses unable expressed using the AAT enzyme.
Figure 3. Variation of AAT Isozyme banding pattern at 34 drought tolerance sugarcane putative mutants from gamma irradiated Kidang Kencana variety

Figure 4 showed that variability of isozym banding pattern using higher peroxidase enzyme than Esterase and Aspartate aminotransferase. Kidang Kencana mutant has 17 types of banding pattern using peroxidase enzyme, seven banding pattern using esterase enzyme and one banding pattern using aspartate aminotransferase enzyme. This showed that peroxidase and transferase enzyme can be used to detect variation on sugarcane mutant genetic of Kidang Kencana variety due to irradiation and tissue culture (Table 2).

Figure 4. Variability of peroxidase (PER), Esterase (EST) and Aspartate Aminotransferase (AAT) Isozyme banding pattern at drought tolerance sugarcane putative mutants from gamma irradiated Kidang Kencana variety

There was fairly clear polymorphic band pattern in the peroxidase enzyme in distinguishing genotypes among putative mutants of drought tolerant sugarcane. This is related to the role of peroxidase enzymes in plants experiencing drought stress. This peroxidase is one of the important components in the system of eliminating reactive oxygen species (ROS) which is damaging to plant cells / tissues when experiencing lack of water. Peroxidase is a family of isozymes found in all plants, to catalyze the final reaction of the lignin formation and other phenols related to plant resistance through strengthen the cell walls. According to [16] and [17], peroxidase is a group of PR (pathogenesis related) proteins that accumulates when the plant is infected or in an unsupportive environment and its expression strongly appears. This is why the use of the peroxidase enzyme to distinguish drought tolerant sugarcane mutants can produce the clearest banding pattern compared with esterase and aspartate aminotransferase. This means that the peroxidase enzyme can be used to detect the adaptation of mutants to drought.

The role of esterase is as an enzyme in hydrolyzing esters and organic acids, so that only produce few band patterns, whereas the use of aspartate aminotransferase does not produce polymorphic band patterns. This is due to the synthesis of proline derived from the glutamate aminotransferase (GAT) rather than through aspartate aminotransferase (AAT). Cluster analysis result based on PER and EST enzymes in 34 sugarcane putative mutant drought tolerant gamma irradiated of Kidang Kencana showed that there were two large cluster distinguished among parents of Kidang Kencana (no 35 and 36) with 34 mutant gamma irradiated (Figure 5).
Table 2. Variability of Isozyme banding pattern at 34 drought tolerance sugarcane putative mutants from gamma irradiated Kidang Kencana variety

| No | Code | Mutant | Banding pattern number | PER | EST | AAT |
|----|------|--------|------------------------|-----|-----|-----|
| 1  | K1   | MK 22  (0 Gy) (somaclone) | 2   | 1   | 1   |
| 2  | K2   | MK 24  (0 Gy) (somaclone) | 3   | 1   | 1   |
| 3  | K3   | MK 25  (0 Gy) (somaclone) | 4   | 1   | 1   |
| 4  | K4   | MK 26  (0 Gy) (somaclone) | 4   | 1   | 1   |
| 5  | K5   | MK 27  (0 Gy) (somaclone) | 5   | 2   | 1   |
| 6  | K6   | MK 28  (0 Gy) (somaclone) | 2   | 1   | 1   |
| 7  | K7   | MK 29  (10 Gy)              | 2   | 1   | 1   |
| 8  | K8   | MK 10  (10 Gy)              | 6   | 2   | 1   |
| 9  | K9   | MK 12  (10 Gy)              | 7   | 1   | 1   |
| 10 | K10  | MK 13  (10 Gy)              | 3   | 1   | 1   |
| 11 | K11  | MK 15  (10 Gy)              | 6   | 1   | 1   |
| 12 | K12  | MK 16  (10 Gy)              | 8   | 2   | 1   |
| 13 | K13  | MK 18  (20 Gy)              | 9   | 2   | 1   |
| 14 | K14  | MK 31  (20 Gy)              | 3   | 2   | 1   |
| 15 | K15  | MK 32  (20 Gy)              | 3   | 1   | 1   |
| 16 | K16  | MK 19  (20 Gy)              | 10  | 2   | 1   |
| 17 | K17  | MK 20  (20 Gy)              | 1   | 3   | 1   |
| 18 | K18  | MK 21  (20 Gy)              | 1   | 1   | 1   |
| 19 | K19  | MK 33  (20 Gy)              | 11  | 7   | 1   |
| 20 | K20  | MK 34  (20 Gy)              | 12  | 4   | 1   |
| 21 | K21  | MK 35  (20 Gy)              | 1   | 3   | 1   |
| 22 | K22  | MK 36  (20 Gy)              | 13  | 3   | 1   |
| 23 | K23  | MK 38  (20 Gy)              | 1   | 1   | 1   |
| 24 | K24  | MK 40  (20 Gy)              | 1   | 4   | 1   |
| 25 | K25  | MK 46  (20 Gy)              | 14  | 1   | 1   |
| 26 | K26  | MK 47  (20 Gy)              | 12  | 1   | 1   |
| 27 | K27  | MK 54  (20 Gy)              | 12  | 1   | 1   |
| 28 | K28  | MK 57  (20 Gy)              | 1   | 2   | 1   |
| 29 | K29  | MK 58  (20 Gy)              | 10  | 1   | 1   |
| 30 | K30  | MK 42  (30 Gy0              | 2   | 1   | 1   |
| 31 | K31  | MK 51  (30 Gy)              | 15  | 5   | 1   |
| 32 | K32  | MK 60  (40 Gy)              | 7   | 1   | 1   |
| 33 | K33  | MK 68  (40 Gy)              | 16  | 6   | 1   |
| 34 | K34  | MK 64  (40 Gy)              | 17  | 6   | 1   |
| 35 | KL   | Parents Kidang Kencana (field) | 1 | 1   | 1   |
| 36 | KK   | Parents Kidang Kencana (in vitro) | 1 | 1   | 1   |

Mutants that have the farthest genetic distance with the parents (35 and 36) were mutants in cluster II, namely mutants 19 (20 gray) with a level of similarity of 81.14% while other mutants were in
cluster I. Cluster I was divided into two sub clusters sub I and sub II. Sub I was divided into two groups, namely sub-I and sub-II. Mutants that have the same banding pattern with their parents in the three types of used enzymes were mutants irradiated with 20 gray gamma irradiation treatment mutant number 18 (MK 21) and number 23 (MK38) with 100% genetic similarity with Kidang Kencana parents.

![Dendogram](image.png)

**Figure 5.** Dendogram in 34 drought tolerance sugarcane putative mutants from gamma irradiated Kidang Kencana variety based on peroxidase and Esterase banding pattern isozyme

Banding pattern variation of mutants compared with its parent showed that the tissue culture process (0 gray) and irradiation (10 – 40 gray) can lead genetical changes on plants. Some clustered mutants from some dosage of irradiation showed that irradiation can cause the genetic changes that randomly happen. This result is appropriate with [2] statement, the genetic changes caused by irradiation treatment are random. **Gamma irradiation at doses of 10, 20, 30 and 40 gray can produce several sugarcane mutants.** Gamma ray irradiation at a dose of 20 gray gives the highest variation of mutants compared to other doses. This 20 gray dose is a lethal dose 50 (LD 50) in sugarcane callus. This is consistent with [18] ‘s statement which states that the dose of irradiation around LD 50 will provide the highest mutant genetic variability.

4. **Conclusion**

Genetic variability of some sugarcane putative mutant drought tolerant from gamma irradiation can be detected through isozyme analysis using Peroxidase (PER) and Esterase (EST) enzyme that showed polymorphic banding pattern inter mutants and the original parents, whereas Aspartate Aminotransferase (AAT) enzyme has monomorphic banding pattern so that unable to show variability. PER enzyme produced 17 banding patterns and seven banding patterns on EST enzyme. Dendogram based on PER and EST enzyme on 34 putative mutants resulted from *in vitro* test obtained two groups at 81.14% genetic similarity.
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