In-vitro selection of sugarcane (*Saccharum officinarum* L.) putative mutant for drought stress

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Abstract. In-vitro selection through tissue culture followed by field testing is one of the breeding methods to improve sugarcane varieties with drought-tolerant. This research aimed to obtain putative mutant sugarcane physically mutated using gamma-ray irradiation that passed the in vitro drought selection using PEG-selecting agents. This study was conducted from March to December 2016 using embryogenic calli of PSJT 941, PS 862, and BL varieties. The experimental design was the factorial randomized complete design with two factors, i.e. irradiation dose (0, 5, 10, 15, 20, 25, 30, 35 Gray) and PEG concentration (0, 10, 20%). The irradiated calli selected by PEG media had changed in color from yellowish-white to brownish and blackish and indicated the growth inhibition. The higher the dosage of irradiation and the concentration of PEG, the more calli with growth inhibition. Some putative callus mutants successfully passed in vitro selection using 10 and 20 % PEG, which showed tolerance to drought stress. The appropriate irradiation dose to produce tolerant mutants is 5 – 30 Grays for PS 862 and BL varieties, and 5-25 Grays for PSJT 941. To recognize the mutant response further, it is necessary for in-vivo selection at the greenhouse and in the field.

1. Introduction

Drought or lack of water is one of the factors that cause a decrease in sugarcane productivity by up to 40%. Currently, sugarcane development is shifted to dry land because paddy fields are prioritized for food crops. The problem faced in developing sugarcane on dry land is the limited variety of adaptive varieties. Inappropriate varieties is one of the causes for the low productivity of national sugarcane, which ranging from 65 - 75 tons/ha, much lower than the varieties yield potential which ranging from 90 - 147 tons/ha.

Sugarcane has high biomass, so they need a lot of water [1]. The use of drought-tolerant varieties will be more profitable in the long term. Besides that, the availability of adaptive varieties does not require high costs compared to environmental manipulation. The drought-tolerant sugarcane varieties could adaptation in sub-optimal land such as dry land with little water.

Creating of drought-tolerant sugarcane varieties can be carried out through conventional methods (hybridization), mutation, in vitro breeding, genetic engineering or a combination of the purposes mentioned above. Hybridization as a conventional method has several obstacles to sugarcane, including its complex genomes, low fertility, and an extended selection cycle [2]. Besides, genetic
improvement efforts in conventional sugarcane are complicated because of the high polyploidy rate of sugarcane [3].

Mutation induction that has the potential to increase genetic diversity, followed by in vitro or in vivo selection has been widely used and has resulted in new genotypes that are tolerant of drought, salinity, aluminum, pests and diseases in various crops. Several mutant soybean plantlets resulting from 400 rad gamma-ray irradiation successfully passed the in vitro selection at the PEG level of 20% [4]. The MT58 rice mutant lines that were mutated using EMS showed the lowest yield reduction in drought stress conditions [5]. Four mutants of sugarcane originating from the CP 48-103 variety showed a lower decrease in plant dry weight under more tropical salinity stress conditions than their original parents [6].

Physical and chemical mutations in plants are two types of methods to produce the mutant, using physical mutagens such as x-ray radiation or gamma rays, and chemical mutagens such as colchicine and EMS. Callus or seed is the appropriate subject to treat. Callus cells are meristematic. So, they are more responsive to radioactivity and mutagen exposure than adult cells. The new genotypes as a result of mutation induction are very diverse because the mutations are random. It is possible to select mutants using the specific select agents in the in-vitro selection method.

In-vitro selection using a selective agent in the form of an osmotic compound that can simulate drought conditions in the field is the method to select drought-tolerant variants. The most widely used osmotic compounds to act drought stress is polyethylene glycol (PEG) [7], [8], [9], [10], [11], [12].

According to [13], in vitro selection is instrumental in accelerating the obtaining of soma-clones or mutants that are tolerant of abiotic stress and resistance to biotic stress. Some research on drought tolerance includes rice [14], sorghum [15], sugar cane [16], [17], wheat [18], soybean [19]. The availability of selective agents for drought simulations and the availability of practical standards to induce callus and regenerate it into plantlets, allow mutation induction and in vitro selection using PEG to be carried out to obtain mutants of sugarcane that are tolerant to drought stress. In-vitro selection then followed by field selection. This research aimed to obtain putative mutant sugarcane from gamma-ray irradiation that passed the in vitro drought selection using PEG-selecting agents.

2. Material and methods

The research was conducted from February to December 2016 in the laboratory of tissue culture and greenhouses of Agriculture Source Seed Management Unit, Bogor, West Java. The plant materials were three varieties of sugarcane, i.e. PS 862, Bululawang and PSJT 941.

2.1. Mutation induction

Mutations aimed to increase the genetic diversity of sugarcane using physical mutagens through gamma-ray irradiation was carried out at the Irradiation and Radioisotope Application Center, BATAN, Pasar Jumat, Jakarta. Gamma-ray irradiation uses the active ingredient Co 60 in the Gamma cell 220 irradiator. The callus then put into the Gamma cell 220 Chambers and shot, according to the treatment dose. The irradiated callus was immediately transferred to the MS media without growth regulators for two weeks. The period of sub-culture was three weeks. The culture was placed on a culture rack using a TL lamp with an irradiation intensity of ± 1000 Lux for 16 hours a day.

2.2. In vitro selection

In vitro selection was carried out using a drought-selecting agent, namely PEG 6000, by growing embryogenic callus from irradiated gamma-ray sugarcane on MS medium added with PEG 6000. The duration of selection was four weeks with two sub-cultures in the same media. Calluses that pass the selection were those that are still alive and capable of regenerating. The callus was then sub-cultured in regeneration medium for shoot induction.

The experimental design used was factorial completely randomized design with the first factor was the dose of gamma-ray irradiation (0, 5, 10, 15, 20, 25, 30, and 35 Gray) and the second factor was the concentration of PEG 6000 (0, 10 and 20%). Each treatment consisted of 10 replications, and each
replicate consisted of 5 calluses. The observation is the percentage of life callus, and Sensitivity index (SI) as follows:

\[
\text{Sensitivity index (SI)} = \frac{\text{Percentage of live callus on PEG media}}{\text{Percentage of live callus on non-PEG media}} \times 100\%
\]

Data was analyzed using SAS 9.1 program followed by Duncan's Multiple Range Test (DMRT) at the significance level of 5%.

3. Results

Irradiation and PEG together significantly affect callus of PSJT 941 and Bululawang, while in case of PS 862 each factor had a singular effect. Callus growth was affected by a physical mutation indicated by the color change of the callus (Figure 1). Alive and survived callus was distinguished by its yellowish-white color. In the case of succumbing callus, the color was brown or black. A callus that has turned brown or black is tough to develop. These are damaged or died callus. In the three varieties, the higher the irradiation dose, the more callus was damaged and even died, as indicated by the percentage of callus that survived. Change in color from yellowish-white to brownish, even partly black, shows the damage that occurred in callus. Based on the callus performance, the highest growth inhibition occurred in the PSJT 941 variety, followed by PS 862 and Bululawang (figure not shown).

The damage that occurred in calli as a result of gamma-ray irradiation occurred from 5 Gray dose as indicated by a decrease in the percentage of live callus in non-PEG media. The more the irradiation dose, the damage was getting more prominent. The highest reduction in the percentage of live callus was in the PSJT 941 variety. At the 5 Gray irradiation dose, the percentage of alive callus decreased from 98.40% to 50.20% (Table 1), while in PS 862 it was only from 86.1% to 80% (Table 2) and in BL was from 89.8% to 82.6. % (Table 3). The decrease in the percentage of live callus continued to increase with the higher the irradiation dose. However, an increase in irradiation dose does not always correspond to the rise in callus damage. The percentage value of live callus seemed to fluctuate with an increase in the irradiation dose.

The PEG treatment effect and make callus damage, both in mutant and non-mutant callus. At the same irradiation dose level, an increase in PEG concentration resulted in more severe callus damage, as indicated by the lower percentage of live callus at the higher PEG concentration.

The sensitivity index (SI) shows growth inhibition as a result of drought stress on the media given PEG. Non-irradiated callus showed higher growth inhibition than irradiated callus on media provided PEG 10 and 20%. SI value of non-irradiated callus in PEG 10 and 20% were 30.28 and 53.76% for PSJT 941, 28.92 and 46.34% for PS 862, and 17.59 and 21.27% for BL, respectively.

In the case of irradiated callus, growth inhibition varies among varieties. SI value of irradiated callus range from 3.71 - 54.55% in 10% PEG and 26.82 - 68.18% in 20% PEG for PSJT 941 (Table 1), 3.32 - 53.48% in 10% PEG and 23.68 - 85.03% in PEG 20% for PS 862 (Table 2), and 1.72 - 41.45% in 10 % PEG and 5.54 - 67.09% in 20 % PEG for BL (Table 3).

The SI value also showed that at the dose level of 5 -25 Gray in PSJT 941 and 5 – 30 Gray in PS 862 and BL, the amount was smaller than that of the non-irradiated callus (0 Gray). At the 30-35 Grays for PSJT 941 and 35 Gray for PS 862 and BL, the value of the SI increased sharply and was much higher than the non-irradiated treatment. The highest growth inhibition occurred in PSJT 941, and the lowest was in BL.
Figure 1. Representation of callus mutant resulted of irradiation on several doses, after in-vitro selection for drought using PEG

Table 1. The percentage of live callus and sensitivity index of PSJT 941 variety sugarcane putative mutant affected by irradiation dose and PEG in in-vitro selection media

| Irradiation dose (Gray) | Percentage of live callus (%) | Sensitivity index (%) |
|-------------------------|-------------------------------|-----------------------|
|                         | 0 %                           | 10 %                  | 20 %                  |
|                         | Irradiation dose              | PEG concentration (%) | PEG concentration (%) |
| Irradiation dose (Gray) | 0 %                           | 10 %                  | 20 %                  |
| 0                       | 98.40 a                       | 68.60 b               | 45.50 c               |
| 5                       | 50.20 c                       | 48.34 c               | 31.40 de              |
| 10                      | 35.80 d                       | 29.50 def             | 26.20 efg             |
| 15                      | 22.20 fgh                     | 16.20 hi              | 12.10 ijk             |
| 20                      | 30.80 def                     | 22.40 fgh             | 18.60 ghi             |
| 25                      | 29.94 def                     | 25.50 efg             | 21.90 fgh             |
| 30                      | 28.90 def                     | 16.30 hi              | 12.20 ijk             |
| 35                      | 13.20 ij                      | 6.00 jk               | 4.00 k                |
| CV (%)                  | 29.35                         |                       |                       |

*The numbers followed by the same letter in one column are not significantly different (P < 0.05)
Irradiation also causes changes in the ability of callus to overcome drought stress. The effect of irradiation is very efficient in causing changes in the genetic material [22]. The results of in vitro selection using PEG 10 and 20% showed that some calluses survived in drought stress conditions. In non-irradiated callus (control), the sensitivity index of callus grown in PEG 10 or 20% selection media was relatively higher compared to the putative mutant callus irradiated at various doses. The sensitivity index of irradiated putative mutant callus in the dose range 5 - 25 Grays in PS 862 and BL were lower than the control. There was an increase in tolerance to drought stress indicated by SI that was lower than the control.

Mutation affect the genetic changes in some callus cells as a result of gamma-ray irradiation, which resulted in changes of cells’ characteristics and their response to environmental stress. At the relatively low doses of irradiation, the genetic changes that occur only change the character of some callus cells, which result in changing the cell's response to environmental stress. But at higher doses, the changes that occur are more significant, so that callus cells are no longer able to adapt to stress conditions caused by PEG.

### Table 2. The percentage of live callus and sensitivity index of PS 862 variety sugarcane putative mutant affected by irradiation dose and PEG in in-vitro selection media

| Irradiation dose (Gray) | Percentage of live callus (%) | Sensitivity index (%) |
|-------------------------|-------------------------------|----------------------|
|                         | PEG concentration (%)        |                      |
|                         | 0                             | 10                   | 20                   | 10 | 20 |
| 0                       | 86.1                          | 61.2                 | 46.2                 | 28.92 | 46.34 |
| 5                       | 80                            | 58.2                 | 51.8                 | 27.25 | 35.25 |
| 10                      | 71.6                          | 52.4                 | 49.8                 | 26.82 | 30.45 |
| 15                      | 69.2                          | 66.9                 | 41.0                 | 3.32  | 40.75 |
| 20                      | 66.1                          | 53.3                 | 35.8                 | 19.36 | 45.84 |
| 25                      | 61.7                          | 44.2                 | 33.2                 | 28.36 | 46.19 |
| 30                      | 60.4                          | 56.8                 | 46.1                 | 5.96  | 23.68 |
| 35                      | 18.7                          | 8.7                  | 2.8                  | 53.48 | 85.03 |
| CV (%)                  | 31.03                         |                      |                      |      |

*The numbers followed by the same letter in one column are not significantly different (P < 0.05)*

### Table 3. The percentage of live callus and sensitivity index of BL variety sugarcane putative mutant affected by irradiation dose and PEG in in-vitro selection media

| Irradiation dose (Gray) | Percentage of live callus (%) | Sensitivity index (%) |
|-------------------------|-------------------------------|----------------------|
|                         | PEG concentration (%)        |                      |
|                         | 0                             | 10                   | 20                   | 10 | 20 |
| 0                       | 89.8 a                         | 74.0 bc               | 70.7 bcd             | 17.59 | 21.27 |
| 5                       | 82.6 ab                        | 77.9 bc               | 71.1 bc              | 5.69  | 13.92 |
| 10                      | 77.0 bc                        | 73.6 bc               | 72.3 bc              | 4.42  | 6.10 |
| 15                      | 76.2 bc                        | 74.2 bc               | 71.8 bc              | 2.62  | 5.77 |
| 20                      | 75.7 bc                        | 74.4 bc               | 69.9 bcd             | 1.72  | 7.60 |
| 25                      | 72.2 bc                        | 70.2 bcd              | 68.2 cd              | 2.77  | 5.54 |
| 30                      | 68.6 cd                        | 66.9 cd               | 58.1 de              | 2.48  | 15.31 |
| 35                      | 55.0 e                         | 32.2 f                | 18.1 g               | 41.45 | 67.09 |
| CV (%)                  | 18.27                         |                      |                      |      |

*The numbers followed by the same letter in one column are not significantly different (P < 0.05)*

### 4. Discussion

Irradiation using gamma-ray causes damage in callus cells which indicated by a change in callus colour and inhibition of callus growth, especially in high dose. Similar results were reported by [20] and [21].
The results of previous research stated that the dose of 20 Gray is a lethal dose (LD50) of callus sugarcane treated with gamma-ray irradiation after observation four weeks after culture [23]. It was reported that irradiation at doses 30 and 40 Grays harmed the embryogenic callus of sugarcane [24]. Meanwhile, in the case of this study, 30-35 Grays resulted in a high percentage of callus mortality in varieties PSJT 941, PS 862, and BL. There was a significant mutation that resulted in cell damage at the dose of 30-35 Grays.

Callus induction, proliferation and regeneration of plantlets decreased with increasing PEG concentrations. The inhibition of callus growth on PEG media directly so that only tolerant cells will pass the selection.

Although mutations are generally destructive, in-vitro selection could select some of the callus cells undergoing genetic changes to specific purposes [26] and [27]. The advantage of in vitro selection is that each section faces the selective media directly so that only tolerant cells will pass the selection. Furthermore, tolerant callus cells will develop into plantlets. Mutation induction combined with in vitro culture techniques is proven to accelerate the breeding program because it can cause genetic diversity, besides the genotypes obtained can be multiplied to be reproduced quickly [28] and [29].

5. Conclusion
There was an increase in drought tolerance in putative mutant callus of PS 862 and BL varieties irradiated at a dose of 5 - 30 Grays, and putative mutant callus of PSJT 941 variety irradiated at a quantity of 5 - 25 Gray. To further determine the mutant response, it is necessary to select in vivo drought at the greenhouse level and the field.

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