Increased genetic variability of sugarcane through gamma ray irradiation

S Suhesti*, M Syukur², A Husni¹ and R S Hartati¹

¹ Indonesian Agency for Agricultural Research and Development, Jalan Ragunan 29 Pasar Minggu, Jakarta Selatan 12540, Indonesia.
² Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Jalan Meranti Kampus IPB Darmaga, Bogor 16680, West Java, Indonesia.

Corresponding author: hesti.khrisnawijaya@gmail.com

Abstract. To increase the genetic diversity of sugarcane can be done through induced mutation using gamma ray irradiation. This research was carried out to determine the response and radiosensitivity of calli sugarcane variety (Kidang Kencana) to gamma ray irradiation, and knowing the diversitie of phenotypic mutant of sugarcane. The research was conducted at BATAN and ICECRD, from August 2012 until March 2013. This research was arranged in Completely Randomized Design with 6 doses of gamma ray irradiation (0, 10, 20, 30, 40 and 50 Gy). Each treatment consisted of 10 replications. Each replication consists of 5 clumps of calli. The observed variables were calli fresh weight, percentage of regenerated calli, number of shoots, shoot height, leaves number, roots number and plantlets number, calli and mutant phenotype. The results showed that the ability of calli to regenerate and shoot growth decreased with increasing doses of gamma ray irradiation. Radiosensitivity (LD20-LD50) of sugarcane calli Kidang Kencana variety to gamma irradiation were in the range of 10 and 30 Gy doses. Gamma irradiation 10 and 20 Gy doses caused the variability mutant phenotype were very high. It means that gamma irradiation can be used to increase the genetic variability of sugarcane.

1. Introduction
Sugarcane is an allopolyploid plant with n = 5, 6,7, 8, 9. More often than not, the basic chromosome of the genus Saccharum is 10 [1]. Conventional genetic improvement of sugarcane has been proven to be difficult due to its high polyploid levels [2]. Thus, making genetic improvement by mutation induction an easier alternative. It is also one of the cheapest and fastest methods to improve plant genes. Mutation induction can be done using physical or chemical mutagens. Among the existing physical mutagens, gamma ray irradiation is the most widely used in plant breeding because of its ability to increase genetic diversity to produce new mutants [3]. Changes in genetic material are usually expressed in plant phenotypes and passed on to the next generation. However, it may not show phenotypically, and this is known as silent mutation. The desired mutation is generally positive and depends on the purpose of breeding, while negative mutations include physiological damage, sterile plants, abnormal plants and death.

Apart from being influenced by the type of culture used, the response of plants to gamma rays also depends on the irradiation dose used. The irradiation dose is the number of doses absorbed per time
unit (rad per second or Gy per second). High doses generally result in death, whereas low doses only cause abnormal changes in the plant phenotype. The effect of radiation dose on growth, fertility and mortality rate has been widely reported. Meanwhile, at moderate to low doses, the adaptability of plants can be maintained and reversed. Radiosensitivity is the level of plants’ sensitivity to radiation [4]. Radiosensitivity tests are carried out to obtain an effective irradiation dose to produce mutants and to determine the frequency and spectrum of mutations [5]. Sensitivity to radiation can be measured based on the lethal dose (LD) value, which is the dose that causes the death of the irradiated plant population. The level of plant sensitivity is influenced by the type of plant, the growth phase, the size, and the material to be mutated, and varies greatly between plant types and genotypes [6]. In mutation induction, several studies have shown that the optimum dose that can produce the most mutants is generally obtained around LD. The highest mutant variability was found in the gamma-ray irradiated mutants around the LD 20 and LD50. To get the LD20 and LD50 values, the best curve-fit analysis program is used, which is a statistical analysis program that can be used to find the best model equation.

Mutation induction using gamma rays has been widely used to induce mutations in several plants, including *Saccharum sp.* [7], *Sorghum bicolor* [8] and *Triticum aestivum* [9]. The mutation induction technique is the best used for plants with high incompatibility problems such as sugarcane. This study aims to determine the response and radiosensitivity of calli sugarcane variety (Kidang Kencana) to gamma ray irradiation, and knowing the diversities of phenotypic mutant of sugarcane.

2. Materials and methods

The research was carried out at the Irradiation and Radioisotope Application Center (BATAN) Jakarta and the Laboratory of Superior Agricultural Seed Management Unit, Indonesian Center of Estate Crops Research and Development, from August 2012 to March 2013.

The primary material used was the organogenic callus of the Kidang Kencana sugarcane variety. The study used a completely randomized design with 6 doses of gamma ray irradiation, namely 0, 10, 20, 30, 40 and 50 gy [7]. Each treatment consisted of 10 replications with each replication containing 5 callus clumps in culture bottles.

Organogenic callus was inserted into the Gamma Chamber 4000A with the active ingredient Co 60 and irradiated according to the treatment dose. Irradiated calli were then immediately sub-cultured on media without growth regulators (MS0) for 2 weeks, then sub-cultured into MS regeneration medium added with IBA 2.46 µM + BAP 1.33 µM [10] to form plantlets. The subculture was carried out over a period of 4 weeks. The cultures were incubated on a culture rack with a TL lamp irradiation intensity of ± 1000 lux for 16 hours a day.

The variables observed were calli fresh weight, percentage of regenerated calli, shoots number, shoot height, leaves number, roots number, plantlets number, calli and mutant phenotype. The percentage of regenerated calli was determined by the presence or absence of shoots growing. After irradiation treatment, radiosensitivity of sugarcane calli was analyzed using curve expert 1.3 program to determine LD20 (the dose that causes 20% of deaths in the gamma-irradiated population) and LD50 (the dose that accounts for 50% of the deaths of the gamma-irradiated population). Analysis of variance was carried out using the SAS 9.1 program. A further test was carried out using DMRT at the 5% level.

3. Results and discussion

3.1. The response of calli and shoots growth of sugarcane to gamma ray irradiation

Increasing the dose of gamma ray irradiation may inhibit calli growth. Irradiation causes inhibition of cell division and growth [11]. Plant cell death due to irradiation is possible. Moreover, there could be DNA damage and indirect consequences, such as the toxic effect of free radicals from H2O2 and OH- ions generated from water radiolysis [12]. Calli damage often decreases plants’ ability to regenerate and can even cause death so that cells cannot regenerate [13].
Calli growth affected by the gamma ray irradiation can be seen in the calli fresh weight. The fresh weight of the calli decreased as the irradiation increased. This decrease was significantly shown in the irradiation treatment at 20 Gy and above, while at 10 Gy it was not significantly different from the control (Figure 1). This result is also demonstrated in a study conducted by [14] in which the patchouli calli irradiated, calli fresh weight decreased at 10 Gy.

Figure 2 shows that the percentage of regenerated calli decreases as the irradiation dose increases. The regenerated calli ability was significantly inhibited at 20 Gy and the inhibition increased as the dose increased. The inhibition of regenerated callus ability was probably caused by the number of damaged cells due to gamma ray irradiation. The cell damage is indicated by the brownish or even black color of the calli at high irradiation doses. According to [15], irradiation is a form of abiotic stress. This result is in line with [14] on patchouli calli, who stated that the regeneration ability of calli decreased with the increase in the dose of gamma ray irradiation.

The dose of gamma irradiation affects the quality of the calli color. At low doses (10 Gy), the calli color did not show any changes compared to control, that was yellowish white (Figure 3). Calli with this color generally have a crumbly structure and are easier to regenerate. This statement is supported with [14] who states that yellowish white calli has good quality and high regeneration ability, whereas calli with white-brown or brownish yellow and brown shows a decrease in calli quality and low regeneration ability.

Changes in calli color began to appear at 20 Gy, where the calli was white with brownish spots. Calli discoloration from yellowish white to mostly brown or even black occurred at 30 Gy to 50 Gy. The higher the dose, the browner the calli color. At 50 Gy, most of the calli was burnt and black. Calli browning and blackening occurred mostly at high irradiation doses (Figure 3). This shows that the higher the irradiation dose, the higher the possibility of stimulating the activity of the polyphenol
oxidase enzyme. According to [16], browning is caused by phenol oxidation after cell membrane degradation or disorganization. This process is indicating the formation of quinones as a result of enzyme activity. [14] stated that yellowish white callus showed high regeneration ability while brownish or black callus showed decreased in regeneration ability.

The unirradiated callus (Figure 4) regenerated better than the irradiated calli. The higher dose of irradiation inhibited the calli regenerating shoots. The highest inhibition occurred particularly at 40 gamma ray where most of the callus was blackened with shoots appeared only at a few points (Figure 4).

![Figure 4. Regeneration of mutant callus of Kidang Kencana variety 4 months after gamma ray irradiation: (a) 0 Gy, (b) 10 Gy, (c) 20 Gy, (d) 30 Gy, (e) 40 Gy, (f) 50 Gy](image)

The effect of gamma ray irradiation is random. Irradiation can cause a positive effect with expected result or negative (unexpected characteristics). It can be either positive (generates the desired character traits) or negative (contradictory with the desired character). Figure 5 shows that the number of shoots, shoot height, leaves number, roots number and plantlets number decreased as the dose of gamma ray irradiation increased. Figures 5a and 5e show that the plantlets number formed is lower than the shoots number formed at all gamma ray irradiation doses. It indicated that not all shoots are able to grow and regenerate properly to form plantlets.

![Figure 5. The effect of irradiation on the number of shoots, shoot height, leaves number, roots number and plantlets number of Kidang Kencana sugarcane variety: (a) number of shoots, (b) shoot height, (c) leaves number, (d) roots number, (e) plantlets number](image)

The decreasing growth and physiological responses of irradiated sugarcane shoots proved that radiation can cause physiological damage, gene mutations or chromosome mutations. The effect of irradiation on DNA is the ionization of nitrogenous bases in the DNA chain, especially DNA synthesis. Ionization of one or more bases with free radicals produced by radiation will change nitrogen base structure. Physiological damage due to radiation that often occurs in plant chromosomes...
is chromosome aberration. The alteration processes that occur in chromosome structures can be in the form of translocation, inversion, duplication and deficiency [11]. Irradiation resulted in decreased growth ability as the gamma ray irradiation dose increased, and also the tendency to lower plant height and leaf number.

3.2. Radiosensitivity of sugarcane callus to gamma ray irradiation

Plants have different responses to gamma ray irradiation. The response is influenced by the type of culture and the irradiation dose used. The plants’ sensitivity level is influenced by the type of plant, the growth phase, the size, and the material to be mutated, and varies greatly between plant types and between genotypes [6].

Radiosensitivity is the sensitivity of genetic material to radiation. This can be measured based on the lethal dose (LD) value, which is the dose that causes the death of the irradiated plant population. A low radiation dose will cause "diploitic selection" so that it is possible for mutants to return to their origin, while high radiation doses can cause sterility or even death. The optimum dose that can produce the most mutants is generally obtained around the lethal dose.

The LD was determined using a curve fit analysis program based on the percentage of regenerated calli. The analysis results on the percentage of regenerated calli showed that the best equation was linear fit with the equation $Y = 98.55 - 1.74 X$ so that the lethal dose $LD_{20} = 10.67 \text{ Gy}$ and $LD_{50} = 27.93 \text{ Gy}$ (Figure 6). The results above showed the tissue radiosensitivity of callus ability to regenerate is higher than that of the live callus. At lower irradiation doses, the callus’ ability to regenerate is more easily inhibited than live callus, as demonstrated in callus that does not experience necrosis or browning.

The analysis showed that the radiosensitivity ($LD_{20} - LD_{50}$) of calli of Kidang Kencana sugarcane variety to gamma ray irradiation was in the dose range of 10 and 30 Gy. The mutant calli resulting from gamma ray irradiation between $LD_{20}$ and $LD_{50}$ is expected to have high variability so that it provides a higher chance of obtaining certain traits expected from breeding objectives. This is confirmed by [4] statement that 10-30 Gy gamma ray irradiation to calli causes an increase in soma-clonal diversity. These results are in line with the research of [7] and [17], who stated that the $LD_{50}$ in sugarcane calli at 20 Gy

![Graph of the percentage of regenerated calli mutants of Kidang Kencana sugarcane varieties at various doses of gamma ray irradiation](image)

**Figure 6.** Graph of the percentage of regenerated calli mutants of Kidang Kencana sugarcane varieties at various doses of gamma ray irradiation

3.3. Phenotypic diversity of mutant sugarcane generated from gamma ray irradiation

The identification of phenotypic diversity in mutants was carried out as an initial screening for possible variations among the formed sugarcane plantlets. The phenotypic appearance of the Kidang
Kencana plantlets after gamma ray irradiation showed that there was a fairly high and varied phenotype variation in all mutants, at 0 to 50 Gy irradiation. The gamma ray irradiation resulted in various phenotypic growth, namely normal-shaped leaves, rosette growth, curly leaves, fan-shaped leaves, straighter leaves, variegated and albinos (Figure 7).

![Figure 7. Penotypic diversity of Kidang Kencana mutant plantlets resulting from gamma ray irradiation: (a) normal growth, (b) rosette, (c) curly leaves, (d) fan leaves, (e) upright leaves, (f) variegata leaves, (g) albino leaves](image)

The doses that generated highest phenotypic mutant variation were at 10 and 20 Gy. In both doses, there were many variations in phenotypic mutant such as rosette growth, curly leaves, fan leaves, upright leaves, variegata and albino (Table 1). Doses of 10 and 20 Gy are doses between LD<sub>20</sub> and LD<sub>50</sub>, which can create a fairly high diversity of mutants. This is consistent with the statement of [4] which states that doses between lethal doses will cause a high increase in genetic diversity.

**Table 1.** The frequency of phenotypic changes of the Kidang Kencana sugarcane mutant at various doses of gamma ray irradiation

| Irradiation Dose (Gy) | The frequency of mutant phenotypic (%) |
|-----------------------|----------------------------------------|
|                       | Normal  | Rosette | Curly leaves | Fan leaves | Upright leaves | Variegata | Albino |
| 0                     | 97.41   | 0.00    | 0.26         | 0.00       | 1.90           | 0.27      | 0.16   |
| 10                    | 88.58   | 0.31    | 0.36         | 0.61       | 6.44           | 3.07      | 0.63   |
| 20                    | 75.65   | 6.91    | 4.66         | 0.07       | 11.86          | 0.07      | 0.79   |
| 30                    | 57.35   | 36.07   | 6.58         | 0.00       | 0.00           | 0.00      | 0.00   |
| 40                    | 51.54   | 36.18   | 11.65        | 0.00       | 0.00           | 0.00      | 0.00   |
| 50                    | 40.00   | 60.00   | 0.00         | 0.00       | 0.00           | 0.00      | 0.00   |

Variations also occurred in no gamma ray irradiation treatment (0 Gy), such as mutants with curly leaf phenotypes, upright leaves, variegata and albino. This is presumably due to soma-clonal variations during the tissue culture process. Soma-clonal variation is a genetic variation that occurs spontaneously as a result of tissue culture using somatic cell populations. The use of a 2.4-D growth regulator at the time of callus induction in this study probably triggered soma-clonal variations. 2.4-D is a strong auxin, so it can increase cell division. Cell division that is too fast can cause errors during the transcription process, causing genetic changes in plants. Also, soma-clonal variations can also cause polysomic networks’ occurrence on sugarcane explants used. Polysomic network has a different genetic structure from the surrounding tissue [18]. This polysomic network is widely found in plants’ leaves or roots, especially monocot plants such as sugarcane. Explants originating from polysomic tissue can also contribute to genetic variations in the resulting plantlets, although the changes frequency is not as high as in gamma ray irradiation.

The frequency of mutant formation with normal phenotypes was decreasing at higher dose of gamma ray irradiation. At 50 Gy, most mutants had rosette growth or no growth at all. This is because...
gamma ray irradiation has high energy and penetration. Higher dose can cause changes in mutant phenotypes and result in growth inhibition so that plants have rosette growth or stunted, and at even higher doses may cause death of the cell or tissue. This is supported by [11] who stated that gamma ray irradiation is ionizing radiation, which causes ionization, releasing ionization energy when it passes through or penetrates the material. When plant material is exposed to radiation, ionization occurs in the tissues and causes changes at the cellular, genomic, chromosome and DNA levels. Changes that occur in genetic material are usually expressed in plant phenotypes and passed on to the next generation.

The phenotypic diversity obtained in this study is possibly a solid mutant as the irradiated material was the callus. This is in line with [4] who states that irradiated callus will cause a solid mutant (intact mutant) so that it is hereditary, whereas irradiated multicellular networks such as seeds, tubers, stolon cuttings or other parts of plant tissue will produce sectoral mutants. The phenotypic diversity obtained in this study needs to be further observed in adult plants or their offspring (clonal).

4. Conclusion
The percentage of growing calli, ability to regenerate and shoots growth decreased with the increasing dose of irradiation. Radiosensitivity (LD$_{20}$ - LD$_{50}$) of calli of Kidang Kencana sugarcane variety to gamma ray irradiation were in the range of 10 and 30 Gy doses. Gamma ray irradiation at 10 and 20 Gy caused significantly higher phenotypic mutant variations. The mutant phenotypes that occurred include the growth of rosettes, curly leaves, fan leaves, upright leaves, variegata and albino.

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