Radio-sensitivity of irradiated seed, plantlets, callus, and in vitro leaves from *Indigofera zollingeriana* Miq by gamma rays

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**Abstract.** Test of radio-sensitivity is important to use as a recognizable effect of radiation. The optimal doses usually create maximum variability. Radio-sensitivity of each part of plants of *Indigofera zollingeriana* Miq to gamma rays had no report yet. This research aimed to know radio-sensitivity, optimal doses, and growth of M1 generation from each material of *I. zollingeriana* irradiated with gamma rays. Seed, plantlets, callus, and in vitro leaves were tested for radio sensitivity by gamma rays. Doses level used of Gamma rays were: 0 until 500 Gy. The value of LD50 of each material was evaluated using Curve-fit Analysis. Growth parameters from each material were observed for six weeks after planting and analyzed using IBM SPSS 22. Research showed that the sensitivity of each doses level was different for each source of the material plant. The seed had radio-sensitivity of gamma rays at dose 183.988 Gy, plantlet at dose 253.677 Gy, callus could not calculate, and in vitro leaves at dose 242.241 Gy.

1. Introduction

In plant breeding programs, gamma rays as one of physical mutagen have improved plants into superior plants and increased plant variability. Gamma-rays are one of the most used mutagens that penetrate deeply into target tissues with high energy. It is less destructive, that making base changes, disrupting hydrogen bonds between complementary stands of DNA [1]. The reason to use gamma rays to improve plant due to simple application with good penetration, reproducibility, high mutation frequency, less disposal problems, lower cost (easy availability), and increased effectiveness [2];[1];[3];[4];[5];[6]. Improvement plant by using gamma rays were proved to increased productivity [7], variability [8], resistant from diseases and pest [9];[10], secondary metabolite [11]; [12]; [13], also stress tolerance of biotic and abiotic [14]; [15].

Jankowicz-Cieslak [16] explained that mutation breeding consists of three processes, (1) inducing mutations, (2) screening for putative mutant candidates, and (3) mutant testing and official release. After the inducing process, the first step to be done is to identify the mutagen and optimum dose to know the effective mutation induction, which is determined by dosimetry or radio-sensitivity tests [6]. Radio sensitivity is a relative measure that indicates the quantity of recognizable effects of radiation on the irradiated objects [17]. Tullmann-Neto [18] explained that the radio-sensitivity of the irradiated
genotype is determined by exposing the material to a range of radiation intensities and selecting those doses that allow visible effects of the radiation that to be observed while maintaining the survival of the tissues.

The effect of a mutagen on plants is fundamentally determined by their survival percentage and the level of regeneration and/or multiplication. Dakha [19] said that the determination of radio sensitivity is a prerequisite for large-scale irradiation for induction of mutation. Each part of plants has sensitivity from irradiation; a test of radio sensitivity is essential to use as a recognizable effect of radiation. To know the optimal doses that are effective to create a mutation in plants, a lethal dose of each part of plants needs to be observed. The mean lethal dose (LD50) needs to be calculated, a widely used parameter to determine the optimal mutation frequency with the least possible involuntary damage. The LD50 was different for each type of plant depending on the stage of growth and development of plants and parts of plants irradiated. Based on the LD50 value obtained, it will be the determine the doses of irradiation that are used to induce diversity in plants and obtain the desired characters [20].

*Indigofera zollingeriana* Miq, is one of the forages that is currently being intensively developed in Indonesia. This forage contains a high source of protein and minerals, a good fiber structure, and a high digestibility value [21]. The average crude protein of *I. zollingeriana* ranges from 26% to 31%, with a protein digestibility rate from 83% to 86.3% [22]. Development of *I. zollingeriana* production is constrained by land designated for food crops. The improvement of *I. zollingeriana* by gamma rays can create a new cultivar of *I. zollingeriana* plant that is tolerance to shade, marginal land, and drought tolerance. This research aimed to know the radio-sensitivity, optimal doses, and growth of M1 generation from each source of materials of *I. zollingeriana* irradiated with gamma rays.

2. Materials and methods

These experiments were done at the Center of Isotope and Radiation of BATAN (National Atomic Energy Agency) for irradiation and at The Laboratory of Plant Production of BPPT (Agency for The Assessment and Application of Technology) for observation and growth of material.

2.1. Materials

Materials used for this research were seed, plantlets, callus, and *in vitro* leaves. Seed was obtained from certificated seed of *I. zollingeriana* from IPB University, plantlet was obtained from elongation of the shoot after induction of multiply shoots in media treatment, and callus was obtained from nodes that was induced to become callus on media of callus treatment. Seed was store at room temperature, plantlet and callus were store at thermostatic room with temperature 24-26°C before used. Media MS [23] without plant growth regulator (MS0) was used as media for elongation of plantlets and for selection of irradiated plantlets and leaves after sub cultured. Media MS with Benzylaminopurine (BAP) and Indolbutyric acid (IBA), was used as callus induction and propagation.

2.2. Methods

2.2.1. Materials preparation

Preparation of each plant material based on the source of the material used. Seed material was prepared from selected seed with criteria: viable, not floating, round and greenish-brown, and no diseases found. Plantlet material was prepared from elongation of the shoot after induction of multiply shoots in media treatment for eight weeks after planting. Callus material was prepared from callus induction in media treatment for callus, and for *in vitro* leaves, the material was prepared from plantlets. Each source of materials was kept in a plastic clip before used for materials for irradiation.

2.2.2. Irradiation with gamma rays

Materials of *I. zollingeriana* were irradiated at Center of Isotope and Radiation of BATAN (National Atomic Energy Agency). Each material was subjected to gamma rays from ⁶⁰Co source. Level of
doses of irradiated were 0, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 Gy. After irradiation, each of the plants' materials was planted in each media from growth. Seed material was planted in wet tissue for induction of germination, plantlets and \textit{in vitro} leaves materials were subculture on media \cite{1} without plant growth regulator. For callus, the material was a subculture to media propagation.

2.2.3. \textbf{Observation of growth from each material}  
Observation of growth for each material was done after six weeks after planting. Parameter observations were the percentage of life and death of each source's materials based on doses level, growth of morphology of each material, and another parameter that appears during observation.

2.2.4. \textbf{Determination of radio-sensitivity and LD50 value}  
The life percentage data obtained from each \textit{I. zollingeriana} were analyzed using the Curve Expert 1.4 software program. From the analysis using the software, the LD50 value was obtained. The subsequent analysis was the manufacture of curve fit; from several fit curvatures formed from each source of plant material, the best curve fit was selected, which could be used as the best model to describe the population mortality pattern from each plant source.

2.2.5. \textbf{Characterization of morphological changes of irradiated from \textit{I. zollingeriana}}  
Each source material of \textit{I. zollingeriana} was observed for changes in morphological characters six weeks after planting. Observation parameters for seed material, and plantlets included: plant height growth, number and changes in leaf shape, number of nodes, and other morphological changes during growth of materials were compared to controls. Several parameters that can be observed quantitatively and qualitatively during the six weeks after planting were recorded.

2.2.6. \textbf{Data analysis}  
The experimental design used was a completely randomized design (CRD) with 1 factor, i.e., the irradiation dose on each plant material source. The data were analyzed using IBM SPSS 22 software. If there was a significant or very significant difference, it then was continued using Tukey test at a level of 5%.

3. \textbf{Results and discussions}  
Exposing plants to gamma rays in all source material had different effects even with the same dose. Our research used seed, plantlets, callus, and \textit{in vitro} leaves as a source of materials from \textit{I. zollingeriana} that gave different responses to gamma rays.

3.1. \textbf{The effect of dose levels on seed material of \textit{I. zollingeriana}}  
Seed irradiation on \textit{I. zollingeriana} showed unsatisfactory results due to very low germinating seeds. We used seed of \textit{I. zollingeriana} about 200-350 seeds (2 g each dose) for germinated seeds. To induce germination rate, we dipped seeds for 12 hours in water and then were sterilized with 10% of sodium hypochlorite solution for 30 minutes. The percentage of germinating seeds in control was only 20%. LD 50 in the seed material was obtained at a dose of 183.988 Gy. The fit curve formed was a polynomial curve with low germination in \textit{I. zollingeriana} seeds (figure 1).

The percentage of germinated seed from this research was low, ranging from 7% to 20%. The low seed germination of Indigofera seed was not only caused by the longtime of harvesting the seeds that decrease the viability of seed, but it was also caused by the large number of fungi that grow and inhibit seed growth. But for this research, we not counting the number of fungi that growth and inhibit seed germination of \textit{I. zollingeriana} because the fungi spread quickly at the surface of seed. Some researchers reported that the germination rate of \textit{I. zollingeriana} seed become a problem because generally, seed of Indigofera has a thick seed coat \cite{24}, and the viability of seed was quickly decreasing until 24% if seeds storage for more than four weeks \cite{25}. The fungal invasion during germination of Indigofera seed that reduces germination rate was also reported by Nair \cite{25}. To increase the germination rate for Indigofera, some researchers reported used 18% of CO\textsubscript{2} injection to
induce germination [27]. Meanwhile, Hutasoit [28] reported that the germination of Indigofera seed was 34% - 42% when dipped into warm water with any temperature (normal, 40°C, 60°C, 80°C, 100°C) before germination. Mulik [29] got 75% seed germination when dipped into 10% sodium hypochlorite solution for 10 minutes.

Our research reported that the effect of irradiation on seed did not significantly different because, if we compared the germination seed for every dose, we could see that in control, the germination rate was 20%, then in irradiated seed at a dose of 200 Gy was only 7% but at 250 Gy was 17.79%, nearly the same as the control. Research reported by Musa et al [26] showed that gamma rays with 1000-200 Gy did not contribute to a significant effect of I. zollingeriana germination. The higher irradiation dosage tends to inhibit the germination process.

Figure 1. The polynomial fit curve of seed material of I. zollingeriana

The observation of M1 generation growth showed that the morphology of shoots from seed germination until six weeks after planting was not different (figure 2). The M1 generation is the first generation that growth after radiation treatment. The statistical analysis of variance also showed that plant length did not significantly differ at each dose of growing shoots. The shoot length ranged from 6.65 to 8.17 cm, with the highest average was in control (table 1). The effect of irradiation for growth of seedling showed that control grew normally with the average of length, number of leaves, number of nodes and length of roots was better than irradiated plant.

Table 1. Growing of shoots germination of I. zollingeriana

| Dose (Gy) | length of shoots (cm) | number of leaves from shoots | number of nodes from shoots | length of roots from shoots (cm) |
|-----------|-----------------------|-----------------------------|-----------------------------|---------------------------------|
| 0         | 8.17                  | 6.16<sup>b,c</sup>         | 4.589<sup>d</sup>          | 2.26<sup>a</sup>               |
| 50        | 7.07<sup>a</sup>      | 4.57<sup>a</sup>           | 3.57<sup>a,b,c</sup>       | 2.21<sup>a</sup>               |
| 100       | 7.13<sup>a</sup>      | 5.75<sup>b,c</sup>         | 3.88<sup>b,c,d</sup>       | 1.81<sup>a</sup>               |
| 150       | 6.65<sup>a</sup>      | 5.47<sup>a,b,c</sup>       | 3.35<sup>a,b,c</sup>       | 1.97<sup>a</sup>               |
| 200       | 7.43<sup>a</sup>      | 6.13<sup>b,c</sup>         | 3.87<sup>b,c,d</sup>       | 1.50<sup>a</sup>               |
| 250       | 7.50<sup>a</sup>      | 6.61<sup>c</sup>           | 4.22<sup>d</sup>           | 1.69<sup>a</sup>               |
| 300       | 7.92<sup>a</sup>      | 5.54<sup>a,b,c</sup>       | 3.92<sup>b,c,d</sup>       | 1.92<sup>a</sup>               |
| 350       | 7.45<sup>a</sup>      | 6.10<sup>b,c</sup>         | 3.60<sup>a,b,c</sup>       | 1.85<sup>a</sup>               |
| 400       | 7.25<sup>a</sup>      | 6.00<sup>b,c</sup>         | 3.33<sup>a,b,c</sup>       | 1.97<sup>a</sup>               |
| 450       | 7.00<sup>a</sup>      | 5.70<sup>b,c</sup>         | 3.20<sup>a,b</sup>         | 2.10<sup>a</sup>               |
| 500       | 6.72<sup>a</sup>      | 5.25<sup>a,b</sup>         | 2.75<sup>a</sup>           | 1.59<sup>a</sup>               |

Note: the numbers followed by the same letter in same column showed no difference according to the 5% level of the Tukey test.
The shape and color of the leaves, stems, and roots were normal and were not different from the control. Some shoots that were growing on seed material showed morphological differences. Morphological differences showed in the presence of branching at the first node of shoots which in control of the shoot’s growth was straight without branches (figure 3). Other differences were found at the first node of leaf growth, the control usually produced two leaves but at some doses, it was recorded three leaves. This morphological difference was starting to be seen in some plants irradiated from a dose of 150 to 400 Gy. When compared to LD50 that was obtained for seed material at 183.988 Gy, it can be assumed that the effect of irradiation of gamma rays appeared in the near LD50 value that effectively created a mutation in Indigofera.

There was no significant change in the number of nodes and the morphological from the shape, diameter, and surface of the stem of the nodes. This means that a rosette plant was not formed. Analysis of variance for the number of nodes showed a significant difference between the doses given. The number of nodes ranged from 2.75 to 4.58 nodes per plant (table 1). The highest average number of nodes was found in control, while the smallest was at a dose of 500 Gy. From these data, it can be assumed that the higher dose given impacted the number of nodes produced, except at a dose of 250
Gy. The assumption is that the higher the dose is given, the greater the plant's height and distance of the inter-node length between the nodes.

The observation of root length and analysis of variance showed no significant difference in root length at each dose. Root length ranged from 1.50 to 2.26 cm with the longest root length was in control, and the shortest roots length was at a dose of 200 Gy with 1.50 cm (table 1). Roots morphology was normal, no different from control and root nodules were found on the root hairs at six weeks of observation.

3.2. The effect of doses level on plantlets material of I. zollingeriana

The irradiated plantlet materials were sub-cultured with cut every nodes and shoot tips with size approximately 1-2 cm then planted to MS0 media. The M1V1 generation, which the first generation from sub-cultured of plantlet after iradiation, were observed for growth. The radio-sensitivity of the plantlet material showed that LD50 value was obtained at a dose of 253.677 Gy with the best curve on the exponential fit curve based on the varians (S) and r values on the analyzed curve (figure 4). The percentage of plantlet material ranges from 27 to 100%.

![Figure 4. Exponential fit curve of plantlet material of I. zollingeriana](image_url)

In plantlet materials exposed to gamma rays showed the growth of explants in the form of nodes and shoots; the nodes were only able to form small shoots with a size of approximately 0.1 cm and could not grow again except the control plant. The ability to initiate shoots from nodes was capable but did not develop further. Meanwhile, in shoot explants, there was no elongation growth from the shoots. In control, both node and shoot explants showed normal shoot growth. The node explants could induce new shoots with normal growth, and the shoot’s explants could elongate shoot normally become plantlets (figure 5).
3.3. The effect of doses level on callus material of *I. zollingeriana*

The radio-sensitivity of callus were observed by counting the percentage of dead or live of callus after sub-cultured in callus propagation media. The M1V1 generation of calluses were observed for growth. Our research showed that all calluses were no died, still fresh and crumbly with a transparent color at all doses given (figure 6) after six week planting.

With this result, we could not calculate LD 50 because the grown of calluses no different with the control. We assumed that maybe *I. zollingeriana* callus had thick of cell wall, so the doses given could not be optimal to make a lethal or callus die or possibly needed more time to know the effect of gamma rays to callus. The result of this research also found in plantlet of *Ficus carica* L that the dose of gamma irradiation found did not cause plantlet mortality [30].

3.4. The effect of doses level on in vitro leaves material of *I. zollingeriana*

Each irradiated leaves cut from plantlets material then planted in MS0 media. The M1V1 generation of leaves were observed for growth. The radio-sensitivity of leaves material showed that the LD50 of leaves at a dose of 242.241 Gy with the best curve formed was the exponential fit (figure 7).

The survival of life from leaves material grown on the media were 15 to 100%. The higher dose was given to leaves material, the lower the live percentage of the leaves, but at a dose of 150 Gy, the
percentage of life was 73.33%, while at 300 Gy it was only 26.67%, whereas at a dose of 350 Gy and 450 Gy the percentage of life was 45% and 40%, respectively. The percentage of live leaves was observed based on the number of yellowing, blackening, or white leaves compared with green leaves from control (figure 8). In control, the leaves were still green and fresh.

![Figure 7. Exponential fit curve of in vitro leaves material of I. zollingeriana](image)

![Figure 8. Growth of M1V1 generation of leaves material of I. zollingeriana](image)

This research showed that every source of plant has different radio-sensitivity of gamma rays. Seed have radio-sensitivity of gamma rays with LD 50 at dose 183.988 Gy, plantlet at dose 253.677 Gy, and leaves at dose 242.241 Gy. LD 50 of callus could not calculate because the grown of calluses no different with the control. This is the first report of irradiated of *I. zollingeriana* using plantlet, callus and leaves as materials and found the LD 50 value from that materials. The irradiated of seed of *I. zollingeriana* had been published by [26] but not calculated LD 50 value. The impact of this current information will be useful for preliminary research of improvement of *I. zollingeriana*, especially for mutation breeding program.

4. Conclusion

Each source of *I. zollingeriana* has different radio-sensitivity to gamma rays. Seed have radio-sensitivity of gamma rays with LD 50 at dose 183.988 Gy, plantlet at dose 253.677 Gy, and leaves at dose 242.241 Gy. LD 50 of callus could not calculate because the grown of calluses no different with the control. The impact of this current information will be useful for preliminary research of improvement of *I. zollingeriana*, especially for mutation breeding program.

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