The Effect of Cobalt 60 Gamma Rays Irradiation on Anatomical Characters and Chlorophyll Content of Winged-Bean (*Psophocarpus tetragonolobus* (L.) DC)

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Abstract. Winged bean (*Psophocarpus tetragonolobus* (L.) DC) is an alternative food source that can be cultivated optimally. The application of mutation techniques on winged bean can provide genetic variations and have an essential role in the agricultural industry, especially in the assembly of superior cultivars. This research aimed to determine the anatomical response and chlorophyll content of winged plants undergoing mutations due to Cobalt 60 gamma-ray radiation and finding out the differences in the anatomical character between mutated and unmutated winged leaves. The experiments used a factorial, Completely Randomized Design (CRD). The factor I was winged plants and factor II was the concentration of Cobalt 60 radiation (0, 25, 50, 75, and 100 grays), each with 5 replicates. The results showed that the Cobalt 60 gamma-ray radiation increased the cuticle and leaf mesophyll thickness, but decreased the stomatal length and density of winged leaves. Cobalt 60 ray radiation affects the chlorophyll content of winged leaves. The total chlorophyll content has increased significantly, followed by an increase in the amount of gamma-ray radiation. The exposure radiation time of 40 minutes gives a response of high anatomical character and chlorophyll content than the exposure for 20 minutes.

1. Introduction

Winged-bean (*Psophocarpus tetragonolobus* (L.) DC) is a potential source of vegetable protein as it contains a protein of 37.26% [1]. Scientific study on winged-bean is necessary to cultivate them optimally. Application of mutation technique on winged-bean can provide appropriate genetic variation for selection. Irradiation of gamma-ray using Cobalt 60 may result in the sufficiently high polymorphism of winged-bean; thus, high genetic variation can be produced [2]. An induced mutation on a plant species can be applied to obtain new cultivars of better genetic quality. The use of Cobalt ray was found useful in producing mutant cultivars in plant breeding [3]. Winged bean has a long time been cultivated in Indonesia utilized mainly as vegetables, thus less favor than other grain crops to make low productivity of 0, 7-tonnes only. Increasing production might be reached by utilizing in various ways [4].

Prospect of winged-bean development in Indonesia is promising enough regarding the various usages, nutrition contents, and ecological aspect that considerably meets tropical condition [5]. Efforts to obtain superior cultivars of winged-bean can be performed utilizing creating high genetic variation, followed by analysis on mutants produced to serve as parents inbreeding. High genetic variation is significant as a source of selection because the genetic response in selection depends mostly on the level of genetic variation. Mutation induction with doses range of 0-600 Gy affects the growth and
morphology of plant diversity [6]. Previous studies reported sufficiently high polymorphism in winged-bean. Hitherto, not very many studies on the anatomical response of winged-bean mutated with Cobalt 60 have been reported. Hence, how the anatomical and physiological responses of mutated winged-bean due to Cobalt 60 gamma-ray irradiation are still problems. This study aimed to assess the anatomical response of winged-bean after Cobalt 60 gamma-ray irradiation at various doses and irradiation time. As well, the differences in foliar anatomical characters of irradiated winged-bean and that of control were observed.

2. Methods

The materials used were winged-bean seeds irradiated with Cobalt 60 gamma-ray of 0, 25, 50, 75, and 100 Gy and irradiation time of 20 and 40 minutes. Cobalt 6 gamma-ray irradiation was carried out in BATAN Yogyakarta, Indonesia. The experiments used factorial Completely Randomized Design (CRD). The factor I was winged-bean, while factor II was Cobalt 60 gamma-ray irradiation treatments. Each treatment was replicated five times. Parameters examined included cuticle thickness, mesophyll thickness, stomatal size and density, and foliar chlorophyll content (mg/).

2.1 Winged-bean planting

Irradiated winged-bean seeds were planted using polybags in the greenhouse of the Faculty of Biology Universitas Jenderal Soedirman, almost three months to describe the generative phase.

2.2 Embedding methods using to anatomically characters

Examination on foliar anatomy response was conducted by the use of foliar preparations made with the embedding-paraffin method. The observed anatomical characters included cuticle thickness, epidermis thickness, mesophyll thickness, stomatal size, stomata, and trichomes density per 1 mm² area of epidermis of leaves. The 5th leaf from the shoot bud was taken and cut into one cm pieces. It then was subjected to fixation in FAA solution (FAA: 10% formalin, 5% acetic acid, 50% ethyl alcohol, and aquadest 35%) for 24 hours [7]. Leaves were taken from the same positions (fifth leaf from top). An incision was performed using microtome rotary, with an incision thickness of 10 µm [8].

2.3 Chlorophyll content

Modification of [9], two grams of fresh leaves were homogenized using chilled mortar containing 10 mL of methanol (80% v/v) and some MgCO₃. The sample extract was collected and filtered using the Buchner funnel through Whatman filter paper no. 5. Extract volume was topped up to 50 mL with methanol (80% v/v). Samples were centrifuged at 3000 rpm for 5 minutes. Chlorophyll content was measured with 10 mL, an acetone-ethanol solution (1:1, v/v) [10]. The samples were soaked in the solution for 24 h at 4°C in the dark. The absorbance values at 663 nm (OD663) and 645 nm (OD645) of the solution were measured with a spectrophotometer (UV1100, Shanghai, China). Chlorophyll content was calculated with the following equations:

- Chlorophyll a (µg/g FW) = 12.72 × OD663 – 2.59 × OD645
- Chlorophyll b (µg/g FW) = 22.88 × OD645 – 4.68 × OD663
- Total chlorophyll, Ca + Cb = 7.15 (OD663) + 18.71 (OD646)

2.4 Analysis method

Data were analyzed using ANOVA with the F test, followed by HSD/LSD of 95 and 99% level of confidence.

3. Results

3.1 Anatomical characters of the transversal section due to Co-60 gamma rays irradiation

Examination of leaf anatomical characters after Cobalt 60 gamma-ray irradiation on winged-bean was presented in Table 1. It can be seen from Table 1 that the thickest cuticle (4.20 ± 0.37µm) was in winged-bean irradiated at 100 Gy for 40 minutes, while that of control showed the thinnest cuticle
(1.80 ± 0.24 µm). ANOVA revealed that Co 60 gamma-ray irradiation had a highly significant effect on cuticle thickness in winged-bean. Dunnet test showed that the treatment increased cuticle thickness at 50, 75, and 100 Gy for 20 and 40 minutes.

Table. Leaf anatomical characters of winged-bean mutants after Cobalt 60 gamma-ray irradiation

| No | Co60 gamma-ray doses (Gy x minute) | Cuticle thickness (µm) | Mesophyll thickness (µm) | Stomata number per mm² | Stomata length (µm) | Stomata width (µm) | Total chlorophyll content (mg/l) |
|----|----------------------------------|------------------------|-------------------------|------------------------|--------------------|--------------------|-------------------------------|
| 1  | 0 X 0                            | 1.80 ± 0.24c           | 43.00 ± 1.87c           | 15.6 ± 1.02bc          | 25.50 ± 1.87ab     | 11.00 ± 1.46ab     | 1.60 ± 2.13c                   |
| 2  | 0 X 20                           | 2.20 ± 0.19bc          | 45.50 ± 3.32c           | 17.6 ± 2.58b           | 26.50 ± 0.94a      | 11.50 ± 0.94a      | 1.67 ± 0.34c                   |
| 3  | 0 X 40                           | 2.25 ± 0.16bc          | 43.50 ± 3.00c           | 22.2 ± 2.32a           | 21.50 ± 1.22b      | 13.00 ± 1.00a      | 1.87 ± 0.43c                   |
| 4  | 25 X 20                          | 2.20 ± 0.19bc          | 46.00 ± 3.39c           | 19.6 ± 2.65ab          | 22.00 ± 1.00b      | 11.00 ± 1.22ab     | 3.08 ± 0.86bc                  |
| 5  | 50 X 20                          | 2.35 ± 0.20bc          | 46.50 ± 3.00c           | 14.2 ± 1.60c           | 21.75 ± 1.50b      | 11.25 ± 1.12ab     | 3.16 ± 0.88bc                  |
| 6  | 50 X 40                          | 3.00 ± 0.61b           | 51.00 ± 2.25b           | 14.8 ± 1.47c           | 20.00 ± 1.58bc     | 11.00 ± 1.22ab     | 3.24 ± 0.62b                   |
| 7  | 75 X 20                          | 3.00 ± 0.61b           | 52.00 ± 2.92bc          | 17.6 ± 2.33b           | 22.00 ± 1.87b      | 10.10 ± 0.96c      | 3.58 ± 0.76b                   |
| 8  | 75 X 40                          | 3.00 ± 0.61b           | 51.50 ± 3.39bc          | 12.4 ± 2.58cd          | 22.25 ± 2.00b      | 10.10 ± 0.96bc     | 3.95 ± 0.51ab                   |
| 9  | 100 X 20                         | 3.90 ± 0.56a           | 55.00 ± 2.74b           | 14.0 ± 2.19            | 19.25 ± 1.00c      | 10.25 ± 1.22bc     | 4.04 ± 0.53a                   |
| 10 | 100 X 40                         | 4.20 ± 0.37a           | 56.50 ± 4.64a           | 13.4 ± 1.20            | 19.75 ± 1.66c      | 10.50 ± 0.61bc     | 4.15 ± 0.61a                   |

Figure 1. Average cuticle thickness of winged-bean leaves after Co60 gamma ray irradiation. 1:0Gy/0minute; 2:0Gy/20minute; 3:0Gy/40minute; 4:25Gy/20minute; 5:50Gy/20minute; 6:50Gy/40minute; 7:75Gy/20minute; 8:75Gy/40minute; 9:100Gy/20minute; 10:100Gy/40minute.

Examination on mesophyll thickness showed that Co 60 gamma-ray irradiation increased the mesophyll thickness of winged-bean leaves. ANOVA revealed that the treatment had a highly significant effect on the mesophyll thickness of winged-bean leaves. Dunnet test showed that Co 60 gamma-ray irradiation increased mesophyll thickness at 50, 75, and 100 Gy for 20 and 40 minutes. [11] noted that plant response against ionizing gamma-ray irradiation could cause a change in mesophyll thickness. Leaves may change, such as necrosis of tissues, distortion of nervation, structure, and size of palisade tissue and enlargement of spongy tissue. The increasing thickness of the mesophyll of winged-bean after Co 60 gamma-ray irradiation is presented in Figure 2.
Figure 2. Average mesophyll thickness of winged-bean leaves after Co-60 gamma ray irradiation.  
1:0Gy/0minute; 2:0Gy/20minute; 3:0Gy/40minute; 4:25Gy/20minute; 5:50Gy/20minute; 6:50Gy/40minute; 7:75Gy/20minute; 8:75Gy/40minute; 9:100Gy/20minute; 10:100Gy/40minute.

[11] Dickison (2000) stated that ionizing gamma-ray irradiation could cause changes in the anatomical structure of leaves. Gamma-ray irradiation is capable of increasing thickness of cuticle, epidermis, palisade. It leaves in some individuals after gamma-ray irradiation, though the increment varies and does not show a congruent pattern with the increment of radiation dose. Cross-section of winged-bean leaves after Co 60 gamma-ray irradiation is depicted in Figure 3.

3.2 Anatomical characters of the paradermal section due to Co-60 gamma rays irradiation

Based on ANOVA of stomata size, it was found that Co 60 gamma-ray irradiation had a highly significant effect on stomata length, but had no significant effect on stomata width. Duncan test showed that Co 60 gamma-ray irradiation resulted in a decrease in stomata length. Lowest stomata length (19.25 µm and 19.75 µm) was found in winged-bean leaves after Co 60 gamma-ray irradiation at 100 Gy for 20 and 40 minutes, respectively. This number was much lower than control, which showed a stomata length of 26.50 µm. The average stomata length and width were depicted in Figures 4 and 5.
Figure 4. Average stomata length of winged-bean leaves after Co60 gamma-ray irradiation. 1:0Gy/0minute; 2:0Gy/20minute; 3:0Gy/40minute; 4:25Gy/20minute; 5:50Gy/20minute; 6:50Gy/40minute; 7:75Gy/20minute; 8:75Gy/40minute; 9:100Gy/20minute; 10:100Gy/40minute.

Figure 5. Average stomata width of winged-bean leaves after Co60 gamma-ray irradiation. 1:0Gy/0minute; 2:0Gy/20minute; 3:0Gy/40minute; 4:25Gy/20minute; 5:50Gy/20minute; 6:50Gy/40minute; 7:75Gy/20minute; 8:75Gy/40minute; 9:100Gy/20minute; 10:100Gy/40minute.

ANOVA on stomata numbers showed that Co 60 gamma-ray irradiation had no significant effect. Plants with a high density of stomata enable higher gas exchange or CO₂ absorption, thus increasing the photosynthetic rate. Consequently, photosynthesize produced will also increase supporting plant growth [12]. Induced mutation can lead to anatomical changes, e.g., a lower number of stomata [13]. Based on research conducted by [14], gamma ray radiation in soybeans can affect the length, width, and density of stomata that influence the process of respiration and photosynthesis. Induced mutation utilizing radiation could reduce stomata size and number. Besides, the stomata number can also be affected by the stomata size. The larger the stomata, the smaller their density. The average number of stomata per 1 mm² epidermis of winged-bean leaves after Co 60 gamma-ray irradiation was shown in Figure 6.
Figure 6. Average stomata number of winged-bean leaves after Co60 gamma-ray irradiation.
1:0 Gy/0 minute; 2:0 Gy/20 minute; 3:0 Gy/40 minute; 4:25 Gy/20 minute; 5:50 Gy/20 minute; 6:50 Gy/40 minute; 7:75 Gy/20 minute; 8:75 Gy/40 minute; 9:100 Gy/20 minute; 10:100 Gy/40 minute.

Figure 7. Longitudinal section of winged-bean leaves after Co60 gamma-ray irradiation

3.3 Effect of Co-60 gamma rays irradiation on chlorophyll content

The results showed that winged bean seed exposed to gamma irradiation (25, 50, 75 a 100 Gy) exhibited an increase in total chlorophyll levels (Figure 8.) compared to non-irradiated treatment. Total chlorophyll increased by 80.5% in seedlings that were irradiated at 25 Gy. The total chlorophyll content of winged leaves has increased significantly, followed by an increase in the amount of gamma-ray radiation. Cobalt 60 radiation (100 Gy/40 minutes exposure) gave the highest chlorophyll content, 4.24 mg/L.
Figure 8. Average chlorophyll content of winged-bean leaves after Co60 gamma-ray irradiation. 1:0Gy/0minute; 2:0Gy/20minute; 3:0Gy/40minute; 4:25Gy/20minute; 5:50Gy/20minute; 6:50Gy/40minute; 7:75Gy/20minute; 8:75Gy/40minute; 9:100Gy/20minute; 10:100Gy/40minute.

Examination on gamma-ray irradiated and non-irradiated plants showed differences in foliar anatomical characters. It can be due to both environmental and genetic factors. [15] Sobrizal (2016) reported, the mutation breeding is considered more effective to improve traits and more efficient to screen new traits. Different genetic factors cause different phenotypic performance, and this depends on the magnitude of environmental changes occurring in the growth condition surrounding the plant genotype. ANOVA revealed that Co 60 gamma-ray irradiation gave affect the chlorophyll content of winged-bean leaves. Cobalt 60 gamma-ray irradiation has different effects on plants to changes in foliar anatomical structures, which is considerably individual. A similar dose of irradiation has not automatically the same effect on plants because of the random effect of a mutagen. [16] Sutapa & Kasmawan (2016) stated that Cobalt 60 gamma-ray irradiation is at random and heritable so that radiation can increase plant growth, or oppositely, cause plant death.

4. Discussion

This research generally results in high observation values when winged bean leaves were treated with a low rate of gamma irradiation (25, 50, 75, and 100 Gy) and times exposure plants when treated (20 an 40 minutes) in comparison to controls. Several studies on the stimulatory effects using gamma irradiation at low and high rates showed depression of phytochemical contents and leaf anatomy [17]. A mutation is one of the ways to improve genetic variation in plant breeding, so the target traits were more easily obtained. Gamma irradiation is a widely manipulated mutation breeding approach in agriculture for producing crops with desired agronomic traits. The technique is particularly advantageous to conventional breeding methods because of minimal labor and time requirement.

Generally, low-rate irradiation produces higher observation values than controls. A rate of 25 Gy resulted in the highest number of stomatal density of winged bean. A rate of 60 Gy/400 minutes exposure resulted in the highest mesophyll thickness compared with controls. Changes in the thickness of the cuticle and mesophyll can be caused by the ionizing nature of gamma rays, which can penetrate the epidermal layer and cause the changes. Other factors affecting the changes in leaf anatomical characters besides the regeneration are the environmental factors such as the availability of water, light intensity, concentration of CO₂, and the temperature, which can affect the density of stomata. [18] Widiastuti et al. (2010) on gamma-ray irradiation of mangosteen showed changes in the leaves' size, which are caused by changes in the number and size of the cells.
Research showed that the Cobalt 60 gamma-ray radiation increased the cuticle and leaf mesophyll thickness, but decreased the stomatal length and density of winged leaves. Cobalt 60 ray radiation gave the chlorophyll content of winged leaves. The total chlorophyll content has increased significantly, followed by an increase in the amount of gamma-ray radiation.

The total chlorophyll content of winged leaves has increased significantly, followed by an increase in the amount of gamma-ray radiation. Cobalt 60 radiation (100 Gy/40 minutes exposure) gave the highest chlorophyll content, 4.24 mg/L. [19] Jan et al. (2013) stated that rate gamma irradiation could stimulate photosynthetic pigment systems, where there is a significant increase in total chlorophyll to 71.66% when compared with controls. In contrast, high rates (15 and 20 kGy) reduce total chlorophyll.

The physiological responses include an increase in leaf area, a ratio of chlorophyll a/b, and anthocyanin pigment, while anatomical changes include decreased leaf thickness, cuticle thickness, and trichomata density [8]. The chlorophyll contents in plants affect growth and development processes. The higher the chlorophyll content is linear, the higher the rate of photosynthesis will be, which causes a more optimal absorption of nutrients from the soil, which will stimulate plant growth and development [9]. Mutations with radiation can increase genetic variation. Cells that can survive well after irradiation will undergo several changes in physiological or genetic.

5. Conclusion

In conclusion, Cobalt 60 gamma-ray irradiation could increase cuticle and mesophyll thickness and decrease stomata length and density of winged-bean leaves. On the other hand, it has a significant effect on the increase of the chlorophyll content of winged-bean leaves. The exposure time of 40 minutes gives a response of high anatomical character and chlorophyll content than the exposure for 20 minutes.

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