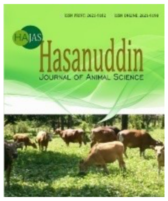

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Physiological Response and Productivity of *Indigofera Zollingeriana* Mutant-2 After Salinity Stress with Different Defoliation

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ABSTRACT

The cultivation of *Indigofera zollingeriana* is carried out using coastal areas to develop livestock farming, and the genetic structure of the plant can be altered through gamma irradiation technology to enhance the level of tolerance and resilience. Therefore, this research aimed to analyze the physiological response and productivity of *Indigofera zollingeriana* mutant-2 plant at several doses of gamma irradiation. A separate plot method was used based on a completely randomized design (CRD). The main plot comprised defoliation duration for 40 (B1) and 70 days (B2), while the subplot consisted of gamma irradiation with dose treatment description A1= Control, A2= 100 Gy, and A3= 200 Gy. Physiological parameters of the *Indigofera zollingeriana* plant consisted of leaf area, index, proline, chlorophyll, and stomata. Meanwhile, productivity parameters, there are dry matter, total leaf, total branch, and stem ratio, were examined using the analysis of variance. The physiological responses of chlorophyll and stomata parameters were also reported in relation to the dose of gamma irradiation (P<0.05) and age of defoliation (P<0.05), respectively. In this context, gamma irradiation treatment affected productivity, specifically on dry matter (P<0.05). The interaction between the doses had a significant effect on the total branch of *Indigofera zollingeriana* mutant-2 (P<0.05). The results showed that the dose of gamma irradiation 200 Gy, with defoliation age 70 days after uniform pruning, was the best in providing physiological responses and productivity of the plant.

Keywords: *Indigofera zollingeriana,* Irradiation, Defoliation Age, Physiology, Productivity

INTROODUCTION

In Indonesia, the coastal and marine areas have great natural resource potential [1]. However, some research explained that these areas possessed land conditions with high salinity. Salinity occurs due to a high concentration of dissolved salts in the soil, which affects the plant's ability to absorb water [2]. The soil affected by high NaCl levels can inhibit water and nutrient absorption, resulting in reduced productivity and plant fertility.

Several studies have shown that the *Indigofera zollingeriana* plant exhibits a fairly high level of tolerance to drought and salinity stress. A significant effort is made to utilize land for planting forage crops. In this context, the coastal area has the potential to develop livestock businesses. *Indigofera zollingeriana exhibit*s high salinity tolerance, which is attributed to its resistance to NaCl stress [3]. The plant has a deep and robust root system, which enables it to adapt to areas with low rainfall, a major factor limiting growth and development [4]. Drought in the plant is related to the low availability of water in the soil. Additionally, it is reported that drought-stressed plants experience a slow decrease in growth rate, resulting in low productivity and eventual death. This shortage of rainfall disrupts plant metabolic processes in the form of inhibition of nutrient absorption, cell division, cell enlargement, enzyme activity, as well as plant growth and development [5]

Several plants, including corn [6], sugarcane [7], *Sesbania grandiflora,* and *Leucaena leucocephala* [8], have been genetically improved to resist salinity. An important type of legume plant used as animal feed with a high level of tolerance to environmental stress is *Indigofera zollingeriana* [3]. This plant is also used as a basis for developing livestock businesses in the coastal area.

Indigofera zollingeriana is drought-tolerant and resistant to pruning. The interval and intensity of pruning affect the anatomical and morphological composition of the plant, as reflected in the leaf-to-stem ratio. Meanwhile, high frequency reduces dry matter production, which in turn affects plant biomass production, nutrient composition, and feed digestibility [9].

Indigofera zollingeriana is adapted to a wide range of environments. The plant exhibits a variety of morphological and agronomic properties, which are important for forage and ground cover [10]. This is due to its high protein content, tolerance to drought and salinity, and roots that respond relatively little to rainfall.

Previous analyses did not thoroughly examine the response of various physiological (leaf area, index, proline, chlorophyll, and stomata) and productivity parameters (dry matter, total leaf, total branch, and stem ratio) of *Indigofera zollingeriana* mutan-2 at different doses of gamma irradiation. Therefore, this study is crucial for analyzing the physiological response and productivity of *Indigofera zollingeriana* mutan-2 at various doses of gamma irradiation, based on the age of defoliation in the coastal area.

MATERIALS AND METHODS

This research utilized gamma-irradiated *Indigofera zollingeriana* mutant-2 after subjecting it to salinity stress. In addition, the materials adopted for proline analysis were 3% sulfosalicylic acid, ninhydrin, glacial acetic acid, H_3PO_4, filtrate, and toluene. Chlorophyll and the number of stomata analyses were carried out using acetone and clear cuticles. The tools used for proline analysis were filter paper, test tubes, an ice bath, a test tube stirrer, and a spectrophotometer. Meanwhile, chlorophyll was analyzed using an Erlenmeyer flask, a desiccator, gauze, and a spectrophotometer.

A split-plot design was used, consisting of 5 treatments and 3 replications, with a completely randomized approach. The main plot was the defoliation time after uniform pruning, comprising 2 treatments (B1 = 40 days and B2 = 70 days after uniform pruning). Meanwhile, the subplot involved gamma ray irradiation dose, consisting of three treatments: A1 (Control), A2 (100 Gy), and A3 (200 Gy).

Research Procedure

Indigofera zollingeriana mutant-2 seedlings were planted in the coastal area at Untia Fishing Port at a distance of 1.5 m with individual plots consisting of 2 rows and 4 m. Plant cultivation was carried out by considering the conditions of water availability, organic fertilizer, and weed control.

Observed Parameters

Physiological parameters include leaf area index, proline, chlorophyll, and stomatal density, while productivity parameters comprise dry matter, total leaf area, total branch area, and stem ratio. Leaf area was measured using the basic method (gravimetry) with a scanner [11]. Leaf area index was measured using the petiole application and calibration paper [12]. This is the ratio between leaf and surface area of the land used for plant growth. Additionally, the leaf area index has no units because it is compared to surface area. The measurement of proline levels was achieved by extracting 0.5 g of leaf in a 10-ml solution of sulfosalicylic acid [13]. Approximately 1.25 g of ninhydrin was dissolved in a mixture of 30 mL and 20 mL of acetic and phosphoric acid until completely dissolved. Subsequently, filtrate and ninhydrin acid were added to glacial acetic acid and heated for 1 hour at 100 ˚C. The extraction was performed using 4 mL of toluene with a "test tube stirrer" for 15-20 seconds. The absorbance at 520 nm was also measured with a spectrophotometer, and toluene was used for a blank solution. The concentration of proline was determined with a standard curve of the pure compound and calculated based on fresh weight. Chlorophyll measurement was conducted by mashing 1 g of leaf against 80% acetone while grinding and stirring [14]. The solution was put into a volumetric flask until 50 ml was obtained. A chlorophyll extract of 1.0 mL was transferred from a volumetric flask to a 10 mL volumetric flask. A spectrophotometer with a wavelength of 645 nm and 663 nm was used to measure absorbance. The calculation of total stomata was performed by observing the number of stomata [15]. This was achieved by using the replica method of cleaning the upper and lower surfaces of the leaf. Additionally, the dry spread is plastered using masking tape before being attached to the preparation glass. A microscope with a 40x magnification is used to count the number of stomata.

The Association of Official Analytical Chemists (AOAC) method was used in 2019 to calculate dry matter. This analysis was performed by weighing the sample and placing it in an oven at 70˚C for 72 hours. The compound formed from each treatment before the uniform pruning process and harvesting time was considered in calculating the total leaf area. Meanwhile, the branch was obtained based on the number of stalks in the stem.

Data Analysis

Microsoft Office 2007 software, analysis of variance, and SPSS version 16 were used to process the data. The mathematical model used was considered in carrying out the analysis of variance, as shown in the following formula:

$$Y_{ijk}= \mu+\alpha_i+\delta_{k(i)+}\beta_j+(\alpha\beta)_{ij}+\epsilon_{ijk}$$

y_{ijk} = The observation value at factor A level i and B level j at the k-th replication.
μ = Additive component of the common mean.
α_i = Main effect of factor A.
$\delta_{k(i)}$ = Random component of the main plot.
β_j = Main effect of factor B.
$(\alpha\beta)_{ij}$ = Interaction component of factor A and factor B.
ϵ_{ijk} = Random component of the subplots.

RESULTS AND DISCUSSIONS

Physiological Response of Gamma-Irradiated *Indigofera zollingeriana* Mutant-2 and Different Ages of Defoliation

Gamma irradiation treatment of *Indigofera zollingeriana* Mutan 2 with different doses gave physiological responses on several parameters. The analysis of variance results showed that gamma ray irradiation had a significant effect on chlorophyll and the number of stomata ($P<0.05$) but had no significant effect ($P>0.05$) on other parameters. Meanwhile, the defoliation age statistically had no significant effect on any of the observed parameters.

Duncan's Multiple Range Test (DMRT) results on chlorophyll and the number of stomata reported that A1 (control) was significantly different ($P<0.05$) from A2 (irradiation dose of 100 Gy) and A3 (irradiation dose of 200 Gy), while A2 and A3 showed no significant difference ($P>0.05$). Statistically, there was no interaction between gamma ray irradiation dose and defoliation age on all observed parameters.

Table 1. Physiological Response of *Indigofera zollingeriana* with Different Irradiation Doses and Age of Defoliation

Treatment		Parameter				
		Leaf area (cm^2)	Leaf Area Index	Proline (µmol/g)	Chlorophyll (mg/L)	Number of Stomata
B1	A1	107.97±42.52[a]	10.80±4.25[a]	0.11±0.09[a]	1.87±0.83[a]	48.00±10.82[a]
	A2	143.53±43.31[a]	14.35±4.33[a]	0.16±0.08[a]	1.15±0.10[b]	54.67±17.67[b]
	A3	107.10±40.91[a]	10.71±4.09[a]	0.14±0.01[a]	1.28±0.19[b]	60.00±21.28[b]
B2	A1	115.13±21.47[a]	11.51±1.55[a]	0.20±0.15[a]	2.19±0.33[a]	40.33±11.37[a]
	A2	108.93±21.80[a]	10.89±2.18[a]	0.22±0.11[a]	2.18±0.13[a]	45.33±1.53[a]
	A3	133.10±10.18[a]	13.31±1.02[a]	0.13±0.04[a]	2.42±0.11[b]	72.33±11.37[b]

Description: Main plots: B1 40 days, B2 70 days; subplots: A1 control, A2 100 Gy, A3 200 Gy. Different superscripts in the same line show significantly different ($P<0.05$)

The average leaf area and index were highest in the A2 treatment with a defoliation age of 40 days after uniform pruning. The highest average proline content was 0.22 µmol/g, observed at a defoliation age of 70 days after uniform pruning. Meanwhile, the average leaf chlorophyll content of 2.42 mg/L and the number of stomata 72.33 were highest in treatment A3. A defoliation age of 40 days with A3 reduced leaf area, leaf area index, and proline, but increased chlorophyll and the number of stomata. Meanwhile, a defoliation age of 70 days with A3 increased leaf area, leaf area index, chlorophyll, and the number of stomata. This demonstrated that A3, with a gamma ray irradiation dose of 200 Gy, affected the physiological response parameters of the observed plants.

Leaf number and area can increase the rate of plant growth and development. The phenomenon occurred due to the assimilation process within the photosynthesis pathway used for cell formation [16]. These new cells are used in the process of growth and development of plant vegetative organs. The large branch number causes the total leaf area and index to increase. This can positively impact the remaining upper leaf area, slowing down the rate of decline in photosynthesis [17]. The measurement of leaf area has a significant influence on plant growth and positively affects photosynthesis. Leaf area with a larger size has greater photosynthesis due to the large number of stomata [18]. In this context, the plant growth is increased due to several energy reserves supporting growth.

Leaf area is also related to the nutrients in the growing medium and the effects of fertilizers, including type, composition, dose, as well as the method and timing of application. Plant growth is largely determined by the nutrients in the soil [19]. A large leaf area index value indicates the amount of sunlight energy converted into biochemical energy during photosynthesis [20].

In Table 1, the gamma irradiation treatment with a dose of 200 Gy produced an average leaf area index of 14.35 at the pruning age of 40 days. This occurs because leaf area is related to the nutrients contained in the growing medium or treatment. Cell division in leaves can occur optimally with the contribution of essential nutrients [21]. The availability of nutrients can increase leaf area index and plant biomass. In this context, nitrogen is essential for stimulating vegetative growth, as well as increasing leaf size and chlorophyll content.

The leaf area index is closely linked to the shape and distribution of the canopy. When this index exceeds the optimal level, it can affect leaf density because a thicker canopy forms. The net assimilation rate (NAR) refers to a plant's ability to produce dry matter per unit of leaf area over time. Additionally, a lower NAR value reduces the speed of plant growth due to competition with the protected leaf [22].

Regarding proline content, the highest level is found in the treatment with gamma irradiation at a dose of 100 Gy, with a pruning age of 70 days, reaching 0.22 µmol/g. The biochemical response of a plant under drought stress involves accumulating proline, an amino acid compound produced in the leaves. According to [23], the high amount of this compound in the leaf is directly related to the level of drought stress caused by a decrease in water potential. Therefore, a large proline content indicates severe drought stress in the plant.

The high accumulation of proline occurs because the compound in the plant with low water availability is synthesized as a consequence of cell osmotic regulation [24]. Proline plays a role in regulating osmosis pressure and the accumulation of water in cells, thereby maintaining water balance. Therefore, the accumulation of proline under stress conditions reduces cell water pressure, allowing the cell to absorb more water and nutrients [25].

The high content of proline was directly proportional to the level of drought stress [26]. Therefore, the large amount of content in the leaves is an indicator of severe drought. The compound interacts with the membrane system, regulates the balance of acidity, and functions as a source of energy. Meanwhile, resistance to drought stress has an important relationship with an increase in proline content, which helps maintain root growth at low osmotic potential [27].

Gamma irradiation at 200 Gy resulted in the highest average chlorophyll content and stomatal density. Therefore, at the time of defoliation, 70 days after pruning, chlorophyll content increases with the gamma irradiation dose from 100 Gy to 200 Gy. The irradiation dose and chlorophyll content can enhance the balance of photosynthetic metabolism during the process [16]. The stomata are influenced by pruning the main stem, and the higher number is related to leaf position. This aligns with Raden et al., who studied iris leaves, where low light reduced the number of stomata [17].

Chlorophyll content and stomata are linked to leaf area or index. At the time of defoliation, 70 days after uniform pruning, the average leaf area of the plants in A3 and A2 treatments reached 133 cm^2 and 108.93 cm^2, respectively. The leaf area index had an average value of 13.31 and 10.89 in the A3 and A2 treatments, respectively. Therefore, there is a positive correlation between leaf area and chlorophyll content in the plant.

The highest and lowest chlorophyll contents of *Indigofera zollingeriana* are observed at treatment doses of 200 Gy and 100 Gy, respectively, during pruning at 70 and 40 days, with concentrations of 2.42 mg/L and 1.15 mg/L. Decreased chlorophyll content is a physiological response of the plant caused by water shortage [28]. Chlorophyll plays a role in the process of photosynthesis, which produces carbohydrates using solar energy, and its deficiency causes stunted growth [29].

The increase in chlorophyll occurs due to the presence of nitrogen (N) in liquid fertilizer. The fertilizer can positively affect the photosynthesis process, growth, and production [30]. Some micronutrients play a crucial role as catalysts in the processes of protein synthesis and chlorophyll formation. Abdullah et al. [31], reported that nitrogen and macronutrients act as constituents of chlorophyll and increase the activity of photosynthesis to develop leaf meristematic tissue.

In Table 1, the highest number of stomata is reported in the 200 Gy treatment with a pruning age of 70 days at 72.33. The lower part of the leaf with reduced exposure to sunlight causes reduced damage to stomata due to strong irradiation [32]. However, the lower part has a cuticle layer lining the epidermis, and this limits the barriers to the transpiration process.

The number of stomata per mm^2 varies in each plant due to stomatal density [33]. The process of opening and closing the stomata is significantly influenced by light, which stimulates the production of chlorophyll for photosynthesis [15]. Meanwhile, the number of stomata influences the amount of transpiration [32].

Productivity of Gamma-Irradiated *Indigofera Zollingeriana* Mutant-2 and Different Ages of Defoliation

Gamma irradiation treatment of *Indigofera Zollingeriana* Mutan 2 plant with different doses affects several productivity parameters. The analysis of variance revealed that gamma ray irradiation treatment had a statistically significant effect (P < 0.05) on dry matter and branches. However, the result did not have a significant effect (P > 0.05) on the leaf-to-stem ratio. Defoliation age statistically had a significant effect (P < 0.05) on the branches but had no effect (P > 0.05) on other observed parameters.

The Duncan test on dry matter and branches showed that A1 (control) was significantly different (P<0.05) from A2 (irradiation dose of 100 Gy) and A3 (irradiation dose of 100 Gy). Meanwhile, A2 was not significantly different (P>0.05) from A3. An interaction was also observed between gamma ray irradiation dose and defoliation age, which significantly affected the branches of *Indigofera zollingeriana* mutant 2 plants (P<0.05). No interaction was found on other productivity parameters.

Table 2. Productivity Level of *Indigofera zollingeriana* Based on Different Doses of Irradiation and Age of Defoliation

Treatment		Parameter			
		Dry matter (%)	Total Leaf	Total Branch	Stem Ratio
B1	A1	19.86±1.17[a]	197.67±69.26[a]	28.00±12.77[a]	7.29±1.40[a]
	A2	21.42±1.77[b]	169.00±86.56[a]	23.67±12.66[b]	7.18±0.51[a]
	A3	21.91±1.42[b]	150.00±70.36[a]	22.33±7.37[b]	6.76±1.91[a]
B2	A1	27.02±1.86[a]	246.00±25.06[a]	29.67±14.57[a]	10.21±5.85[a]
	A2	27.50±0.15[b]	556.33±304.51[a]	121.33±20.11[b]	4.37±1.97[a]
	A3	28.58±0.49[b]	569.00±311.05[a]	144.67±11.24[b]	3.86±2.01[a]

Description: Main plots: B1 40 days, B2 70 days, subplots A1 control, A2 100 Gy, A3 200 Gy. Superscript [a] shows the difference is not significant among the various doses of gamma irradiation, and [b] shows a significant difference among the various doses of gamma irradiation *Indigofera zollingeriana* plant

The highest levels of dry matter, leaves, and stems were found in A3 with a defoliation age of 70 days, with average values of 28.58%, 569, and 144.67. Meanwhile, the lowest stem ratio was reported in the same treatment, at 3.86. The defoliation ages of 40 days and 70 days with A3 increased the percentage of dry matter, leaves, and branches, but decreased the stem ratio. In this context, A3 with a gamma ray irradiation dose of 200 Gy influenced the level of plant productivity observed.

The highest percentage of dry matter in Indigofera plants was observed at a dose of 200 Gy, corresponding to a higher pruning age of 70 days. This is because the plant, at a young age, has active cells undergoing cell division and tissue formation. Meanwhile, old plants can undergo thickening of the cell wall, resulting in an increased dry matter content. Setiyaningrum et al. [34], added that there was a tendency for changes in dry production with the length of pruning age. This is because the proportion of dry matter changes with age. The water content of the plant decreases with age, and the proportion of cell walls becomes higher relative to the contents.

The analysis of variance revealed that the dose of gamma irradiation had a significant effect (P < 0.05) on dry matter, whereas the age of pruning had no significant effect (P > 0.05). Dry matter productivity and the addition of organic fertilizers to increase nutrient content were affected by water and sunlight. Sutaryono et al. [35], reported that plant type, growth phase, time of cutting, as well as soil water and fertility, affected dry matter. In this context, the cell wall and dry matter of old plants increase, while the age of the plant is inversely proportional to its water content.

At a dose of 200 Gy and pruning ages of 40 and 70 days, the lowest and highest leaf numbers of *Indigofera zollingeriana* were 150.00 and 569.00, respectively. Longer pruning provides an opportunity for forage crops to grow by receiving more sunlight, which increases photosynthesis, respiration, and nutrient transportation. The process allows the storage and usage of photosynthates for the growth of new branching, budding, and rooting [26].

The analysis of variance revealed that neither the dose of gamma irradiation nor the age of pruning *had a* significant effect on the total leaf count. This is because the increasing age of the plant provides a comparison between the leaf and the stem. The leaf-to-stem ratio in legume plants is important as a metabolic organ [36]. The total leaf number and the quality of the legume plant are directly proportional, as the nutrients required for metabolic activities are stored and processed.

The highest branch number is 144.67 at a dose of 200 Gy with a pruning age of 70 days. This is because the vegetative growth in the *Indigofera zollingeriana* plant causes an increase in the number of branches on twigs and the weight of the plant biomass. Increased branching, characterized by the development of new twigs in each leaf axil, also affects the leaves. The growth and production of forage crops are influenced by the intensity of branching formation [37]. Increasing the amount of branching after pruning leads to leaf growth overlapping each other. In this context, the leaves become less effective in conducting photosynthesis.

The analysis of variance results showed a significant interaction between the administration of gamma ray irradiation dose and the pruning age of Indigofera plants on the branches. Research by Dewi and Dwimahyani [38] on hibiscus plants (*Hibiscus rosa-sinensis*) reported that gamma irradiation dose had an effect on the branches at the ages of 3 and 6 months after irradiation. Furthermore, an irradiation dose of 10-20 Gy produced more branches than 40 Gy, which resulted in a higher percentage of death and stunted plants. The size of the cuttings and the number of buds also affected the chance of branch formation.

The content of nutrients stimulates the growth rate and activity of the plant, increasing the total number of branches. According to Utami et al. [39], nitrogen in the soil is important in stimulating the growth of plants, specifically the stem and branches. Photosynthetic activity and carbohydrate synthesis are also enhanced by sufficient nutrients, which increases the growth rate of plant height.

At a dose of 200 Gy and a pruning time of 70 days, the lowest and highest stem ratios of the control treatment were 3.86 and 10.21, respectively. Wagiu et al. [40] reported that the increased age of the plant formed a smaller percentage ratio of leaf to stem. This can affect the content because plant nutrients are mostly found in leaf than the stem.

Due to external plant factors, including irrigation and light intensity, the dose of gamma irradiation and pruning time had no significant effect on the stem ratio. The stem ratio affected the quality of the legume as a metabolic organ [41]. This is because the number of leaves and the quality of the legume plant are closely related, and the stem has a reduced nutrient content.

CONCLUSIONS

In conclusion, the physiological parameters of chlorophyll are affected by the dose of gamma irradiation on *Indigofera zollingeriana* plant without any effect on leaf area, index, proline, and stomata. Based on the results, pruning time does not affect the stomata of the leaf. Meanwhile, dry matter productivity and interaction with defoliation age are affected by gamma irradiation treatment and total branch, respectively. At a dose of 200 Gy, leaf area, index, proline, chlorophyll, and stomata provide good results as animal feed nutrition.

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AUTHORS' CONTRIBUTIONS

Conceptualized the study and drafted and revised the manuscript. All Author: Conducted experiments and analyzed data. All authors have read and approved the final manuscript.

COMPETING INTERESTS

We declare that the article has no competing interests.

ETHICAL CLEARANCE

The Authors declare that in this study, they have not used animals as research subjects, either through direct contact with animals or the use of their products in research.

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