

Physiological Response Mutant 1 (M1) *Indigofera zollingeriana* at Various Gamma Irradiations

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Abstract. *Indigofera zollingeriana* is known as a high quality forage and supplement for livestock due to its high nutritional content. It is very adaptive to a variety of climatic situations, including acidic and highly saline soils, and it can withstand protracted drought. *Indigofera* plant as a forage for animal feed is able to grow and thrive under environmental stress. It is anticipated that genetic mutation brought on by gamma irradiation will stimulate and activate genetic potential with modifications in the makeup of amino acids, increasing the body's reaction to abiotic stress. The purpose of the study was to determine the effect of gamma irradiation dose on the physiological response of *Indigofera* which includes growth, leaf area, leaf area index, stomatal openings and chlorophyll levels. The study used a completely randomized design (CRD) with 5 treatments and 3 replications for leaf area and stomata while chlorophyll levels used 6 replications. The treatments of gamma irradiation dose were respectively 0 Gy, 50 Gy, 100 Gy, 150 Gy and 200 Gy. After sampling, the data was processed using ANOVA analysis of variance and differences between treatments were tested with the Least Significant Difference (LSD) using SPSS 16.0 software. The results showed that the dose of gamma irradiation affected the number of stomata and chlorophyll content ($P < 0.05$), but had no significant effect on leaf area ($P > 0.05$). The results of the LSD test showed that treatment P1 had the highest stomata number of 74.33.

1 Introduction

Indigofera has many uses as a source of feed for ruminants due to its remarkable adaptability and also as a ground cover crop [1]. *Indigofera*'s widespread growing environment is asserted by Hassen *et al.* (2007) [2] since *indigofera* grows well under full light conditions, but it can also withstand shadow, drought stress, inundation, acid soils, and salinity. This forage has excellent adaptability to diverse environmental conditions, such as acid soils and soils with high salinity, and is tolerant of protracted drought.

In terms of nutritional content, Abdullah (2010) [3] reports that *Indigofera* has a crude fat content of 3.62%, crude protein of 29.16%, crude fibre of 14.02%, Vitamin A 5054 (IU/100

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g), Vitamin D 34.7 mg/100g, and Vitamin E 13.2 mg/100g. Indigofera is highly potential to be developed as a feed source in the future. The high protein level in particular makes for an adequate nutritional content. Akbarillah et al. (2002) [4] reveals the content of crude protein is 27.97%; crude fibre is 15.25%, Ca is 0.22% and P is 0.18%. Furthermore, it is mentioned that as a source of protein, Indigofera leaf meal contains high pigments such as *xanthophyll and carotenoids*. This plant is an excellent replacement for grass in basal feeding.

The development of plant varieties can be done by various methods, such as crossing, mutation induction, somaclonal diversity and *in vitro* selection. The use of nuclear power can be used to increase genetic and phenotypic diversity. Plant parts used as mutation induction treatment are stem cuttings, seeds, pollen, rhizome roots. Genetic improvement through gamma irradiation is aimed to discover superior clones. Gamma irradiation on indigofera plants is carried out to obtain genetic diversity to be applied in the field of plant breeding. Indigofera tolerance to drought and salinity has been reported by [5,6], in the nursery phase with PEG and NaCl stress. Herdiawan and Krisnan (2014) report that at the level of severe drought stress Indigofera can still grow and produce even though it has decreased [7].

The tolerance of indigofera plants to environmental stress can be increased by several efforts, one of them is plant breeding. Hanafiah et al. (2008) reports that plant genetic varieties can be quickly increased by conventional breeding methods such as mutation [8]. Gamma irradiation is a method that has been employed to enhance the genetic diversity of plants. At low doses, or micro-mutations, gamma irradiation has been observed to induce alterations in quantitative characteristics of plants and to induce slight changes in chromosomal structure when compared to macro-mutations, which involve the use of higher doses of gamma irradiation.

Gamma irradiation is understood as an induced (artificial) mutation treatment using gamma rays. Mutations can increase or decrease one or more new special traits without changing the overall superior traits previously possessed [9]. The use of gamma irradiation to improve genetics has been carried out on several plants such as rice can increase plant tolerance to drought.

The recommended dose of gamma irradiation on forage plants of *Stylosanthes guianensis* is 420 Gy irradiation, which is useful for improving the quantitative character of plants for drought tolerance. Furthermore, Premnath *et al.* (2010) [10] reported on the type of legume *Desmethus virgatus* with irradiation doses of 400-450 Gy increased forage biomass production and improved palatability in livestock .

Parameters to be measured for drought adaptability indicators are leaf area, number of stomata, and leaf chlorophyll content. Leaf area is an indicator of plant physiology in analysing plant growth. The leaves function is very instrumental in the process of respiration and transpiration. The number of stomata serves as a gas exchange hole exposed directly to carbon gas, oxygen and water vapour [11]. Chlorophyll functions to capture energy from light (photons) to be transferred to proteins in the photosynthetic centre [12].

Improving plant adaptation towards abiotic stress, particularly water stress conditions, plant response mechanisms to drought differ according to the genetic conditions of each type, varieties or cultivar. Cultivars that are resistant to water stress and drought conditions have higher chlorophyll content [13]. Plant metabolic response to water stress will affect proline accumulation [14]. Proline is involved in oxidative stress tolerance mechanisms and the main strategy of plants to avoid the damaging effects of water shortage [15].

The physiological response of plants to abiotic environments, such as drought stress and salinity is indicated by the parameters of leaf area, number of stomata and chlorophyll content. The ability to adapt to drought, by the treatment of gamma irradiation dose is the purpose of the study, by comparing variations in leaf area, number of stomata and leaf chlorophyll content.

2 Material and method

The study used population of *Indigofera zolingeriana* at Experimental Farm Moncongloe Lappara, Maros and BIOTERNA Laboratory, Faculty of Animal Science, Hasanuddin University.

The tools used in this research are plastic, machete, sickle, hoe, stationery, markers, analytical scales, labels, *Chlorophyll* Meter Konica Minolta SPAD-502 Plus.

2.1 Research Design

This study used a completely randomized design (CRD) with 5 treatments and 3 replication in the observation of leaf area and number of stomata. The observation of leaf chlorophyll content used 5 treatments and 6 replications.

P0 : Control

P1 : Gamma ray 50 Gy

P2 : Gamma ray 100 Gy

P3 : Gamma ray 150 Gy

P4: Gamma ray 200 Gy

2.2 Research Procedures

2.2.1 Plant selection

Plant selection began with the selection of normal-shaped blackish-brown seeds and then the seeds were sent to the National Atomic Energy Agency (PAIR-BATAN), Jakarta. The seeds were irradiated with gamma rays sourced from a device called Gamma Chamber 4000Å.

This research is a follow-up study from previous researchers [16]. Sampling was selected from plants that were 1 year old, had been pruned every 60 days since transplanting.

2.2.2 Maintenance

The study began with pruning all leaves and leaving the main trunk and branches to a height of 1.5 metres. Furthermore, weekly maintenance done by applying liquid organic fertiliser at 20 ml/l (per week) and removing weeds.

2.2.3 Sampling

Sampling was conducted after 60 days of pruning uniformly, in early dry season. Sampling was done simultaneously for leaf area, stomata, chlorophyll and proline before production sampling, under sunny and moderate weather conditions.

2.3 Measured Parameters

The parameters observed in this study were the number of stomata, leaf area, and chlorophyll content of *Indigofera zollingeriana* leaves.

2.3.1 Leaf Area

Observations were made to determine the leaf area by taking samples with gravimetric method. According to Irwan and Wicaksono (2017) [17] the gravimetric method is as follows:

- a) Used leaf patterns (leaf replicas) drawn on plain paper.
- b) The leaf replicas were weighed using an analytical balance.
- c) Made 10 cm x 10 cm paper strips, then weighed them
- d) Calculated the leaf area using the following formula:

$$\text{Leaf area} : \frac{\text{Leaf replica weight}}{\text{Paper weight } 10 \text{ cm} \times 10 \text{ cm}} \times 100 \text{ cm}^2$$

2.3.2 Number of Stomata

Samples were taken without removing the leaves from the twigs, the abaxial part of the leaf was then cleaned with a tissue, then the leaf sample was made by smearing the abaxial surface of the leaf with clear nail polish which was allowed to dry slightly and then given tape. The leaf sample then left for a while and then pulled off to get a stomatal print. Leaf sampling using clear nail polish aims to make imitation of the leaf epidermis and monitor changes in stomatal size [18].

2.3.3 Leaf Chlorophyll Content

Observations were undergone to determine the leaf chlorophyll of *Indigofera zollingeriana* plants. Measurement of leaf chlorophyll can be done using *Chlorophyll Meter Konica Minolta SPAD-502 Plus* as an alternative to determine the adequacy of N nutrients in plants. Chlorophyll is positively correlated with leaf N content [19].

2.3.4 Data Analysis

The data obtained analysed using a complete randomised design (CRD) with analysis of variance (ANOVA), using SPSS software version 16.0. The following is the mathematical model:

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

Description:

Y_{ij} = Observation on i-th treatment and j-th replication

μ = Generalised average

τ_i = Effect of i-th treatment

ε_{ij} = Random effect on the i-th treatment j-th replication.

3 Results and discussion

Gamma-irradiated plants develop varying levels of varieties depending on the treatment. The change produced can be caused by different doses of irradiation as well as the environment around the plants, besides that the weather also have significant impacts. Changes that occur in plants can also be seen in their physiological conditions. Hudson (2012) [20] states that mutation is the process by which genes undergo changes or all types of changes in hereditary

material that cause changes in phenotypes that are inherited from one to the next generation. In an attempt to produce superior varieties in line with breeding goals, new varieties can be developed by applying mutagens or agents that cause mutations. Analysis of variance showed that the dose of gamma irradiation had a significant effect on the number of stomata ($P < 0.05$) and had no significant effect on leaf area and leaf chlorophyll content ($P > 0.05$). The results showed that the number of stomata varied and there was no positive correlation between dose of irradiation with an increase or decrease in stomata, but showed unstable fluctuations from various doses. BNT test showed that the average number of stomata (Table 1) was the highest at a dose of 50 Gy, which was 74.33, compared to without gamma irradiation and doses of 100 Gy, 150 Gy and 200 Gy. The enormous number of stomata indicates that the clone was resistant to drought. According to research findings, the plant with the greatest number of stomata has a low density, which allows it to tolerate drought. This supports the finding of Hepworth et al. (2015) that plants with a large number of stomata have a low stomatal density [21], which results in less water loss from cells and increases drought tolerance.

Table 1. Research results

Treatment	Leaf area (g/cm ²)	Total stomata	Chlorophyll content	Total Chlorophyll Content (mg/g)	Proline (μmol Proline/g)
0 Gy	3.66±128.03	43.33±13.57 ^a	1.20±10.59 ^{ab}	1.319±0.121 ^{ab}	17.004±2.498 ^a
50 Gy	2.33±57.73	74.33±12.58 ^b	1.23±16.61 ^{ab}	1.360±0.041 ^a	13.587±8.311 ^a
100 Gy	1.95±50.17	40.66±10.06 ^a	1.13±11.45 ^a	1.360±0.031 ^{ab}	13.564±2.739 ^a
150 Gy	2.76±57.73	42.00±8.54 ^a	1.30±10.92 ^a	1.333±0.008 ^{ab}	11.137±5.248 ^a
200 Gy	2.42±0.00	53.33±10.21 ^a	1.20±11.42 ^b	1.311±0.017 ^b	17.012±2.731 ^a

Note: different superscript on the same line indicates significant difference ($P < 0.05$).

The number of stomata can be classified into: few (1-50), quite a lot (51-100), many (101-200), very many (201- > 300) and infinity (301 - > 700) [22]. The more the number of stomata, the denser the distribution in the leaf. The number of stomata can be affected by external factors, such as light. The lower the light intensity, the fewer the number of stomata of a plant [23].

In reaction to a water-stressed environment, stomatal closure, a move that affects their physiological and metabolic functions by using less water through transpiration. The case where leaf chloroplasts experience a reduction in the rate of photosynthesis, electron transport, and phosphorylation capability due to water stress, the stomata close.

The results of leaf area research (Table 1) show that the widest leaves are in the control plants (without irradiation). It indicates that the dose of gamma irradiation has no significant effect on the leaf area. The average results of leaf area showed that the higher the dose of irradiation given caused the lower the leaf area. The dose of gamma irradiation used on Indigofera plants caused low leaf area compared to plants without gamma irradiation (control). It is deliberated that environmental influences and naturally occurring mutations are due to this. Ashri (1970) [24] states that changes in leaf area might indicate that the gamma irradiation treatment dose has an impact on the leaf area. Both chemical mutagen treatment and irradiation can cause a reduction in leaf size.

The study outcomes of leaf area from each dose of irradiation are also affected by environmental factors. Shaded plants will have a change in leaf area. The same finding was reported by Anggraeni (2010) [25], who claims that soybean plants that are exposed to shadow lose leaves as a result of the shade. Plants in shade have fewer leaves than those that receive full light (i.e., no shading).

The variety of the results of leaf chlorophyll analysis, showed that there is no correlation with leaf chlorophyll levels in observations at 2 months of leaf age with evenly sunny conditions. It is suspected that the influencing factors on the plants are not yet stable because there is no correlation with high doses and low doses. Chlorophyll content (Table 1) is the highest at 150 Gy irradiation dose. The high chlorophyll content is also influenced by environmental factors. Suherman (2013) [26] states that several factors affect the presence of chlorophyll in a plant, namely the presence of sunlight, carbohydrates, oxygen, nitrogen, magnesium and iron materials, water and temperature. Presuming that all environmental factors are in appropriate conditions, the presence of chlorophyll is higher in a plant.

According to Suherman (2013) [26], a number of factors related to the presence of chlorophyll can lead to a drop in chlorophyll concentrations. Radiation can induce mutations because irradiated cells will be burdened by high kinetic energy, so that it can affect or change the chemical reactions of plant cells which in turn can cause changes in the chromosome arrangement of plants [27]. Sunlight has an impact on the variation in plants with the highest levels of chlorophyll in addition to the irradiation dose. The irradiation component is one of the elements impacting the synthesis of leaf chlorophyll. Since light is essential to plants, leaf pigments serve as a way to both absorb and transmit light throughout the chemical cycle that underpins photosynthesis. Chlorophyll is the most significant pigment in plants. Chlorophyll is the primary pigment in plants.

Hidayat (2008) [28] stated that the age of the leaf also affects the chlorophyll content. In the early stage of leaf formation, leaf meristem activity induces leaf elongation. Subsequent leaf extension occurs as a result of intercalary meristem activity. This means that the base of the leaf should be older than the tip of the leaf which also results in the chlorophyll it contains. The amount of chlorophyll in the leaves will influence the process of photosynthesis. Pratama and Laily (2015) [29] mentioned that low chlorophyll levels will not result in an optimal photosynthetic reaction. It is also impossible to maximize the amount of carbohydrate molecules generated if the photosynthetic reaction is suboptimal.

The effect of gamma irradiation doses on leaf physiological responses in M1 plants is still very high in variability. Increasing irradiation doses do not correlate with high or low numbers of stomata, but produce unstable variants. Irradiation is used to generate new species without altering the plant's genetic makeup. According to Ulukapi and Nasicilar (2015) [30], gamma irradiation can give rise to a wide variety of plant species. After receiving a 100 Gy radiation dose, plants had 40.66 stomata, 1.95 leaf area, and 1.30 chlorophyll content. Sulistyono et al. (2012) [31] state that a reduction in leaf size, which may lead to a reduction in stomata, is the first physiological process impacted by drought stress.

The first mutant plants to be exposed to a 100 Gy irradiation treatment have demonstrated a respectable level of drought adaptability. Analysis results showing that the low number of stomata causes a lower density is in accordance with the opinion of Hepworth *et al.* (2015) [21], which states that plants with low stomatal density have a low transpiration rate, making them more tolerant to drought compared to plants that have a higher stomatal density. Consistent with this statement, the assertion made by Hassen et al. (2007) [2] that *Indigofera* thrives best in full light but can also withstand shadow, drought stress, inundation, acidic soil and salinity.

4 Conclusion

4.1 Conclusion

Based on the results of the study, it was concluded that gamma irradiation on *Indigofera zollingeriana* plants showed that the dose of gamma irradiation had a significant effect on

the number of stomata and leaf chlorophyll content ($P < 0.05$) and had no significant effect on leaf area ($P > 0.05$).

4.2 Recommendation

In conducting research, it is necessary to pay attention to environmental and weather conditions. In addition, it is necessary to carry out intensive maintenance so that the plants maintain their fertility.

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Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contribution statement
Marhamah Nadir: Conceptualization, Methodology, Investigation, Writing- Original draft preparation. Budiman: Data curation, Writing- Reviewing and Editing. Rinduwati: Visualization, Investigation, Validation, Writing- Reviewing and Editing. Ade Irma Rusiana: Validation, Writing- Reviewing and Editing. Muhammad Prawira Anugerah: Validation, Writing- Reviewing and Editing.

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