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Shade Selection of *Indigofera zollingeriana* Miq Putative Mutant: Evaluation of Plant Growth, Biomass Production, Nutrient Contents, and *In Vitro* Digestibility

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ABSTRACT

The use of gamma rays to improve *Indigofera zollingeriana* is beneficial for developing new superior varieties with genetic characteristics inheritable by other generations. During the development, selecting genotypes from *I. zollingeriana* putative mutant under shaded conditions can create stable shade-tolerant varieties, with the potential to be developed into new cultivars. Therefore, this study aimed to explore the selection of *I. zollingeriana* putative mutant in the M2 generation for assessing and evaluating plant growth performance, biomass production, as well as nutrient content and digestibility under shading. Seedlings of 10 *I. zollingeriana* putative mutants along with 2 control plants, were subjected to 5 levels of shade, namely 0%, 55%, 65%, 75%, and 85%, to identify genotypes with shade tolerance. The results showed that shading significantly ($p<0.05$) increased plant height, chlorophyll content, leaf length, and leaf width, but decreased the number of leaves, nodes, stem diameter, and branches, also leading to decreased biomass production, high nutritional content, and improved digestibility values. Genotypes R4.10 and R5.10 showed enhanced plant growth, stable biomass production, and increased nutritional content, with low digestible neutral detergent fiber (dNDF), and higher *in vitro* true digestibility (IVTD) values compared to control under shaded and unshaded conditions. The identified superior genotypes are promising for breeding programs and practical application in agroforestry or silvopasture systems.

Keywords: inter-cropping systems; I. zollingeriana; putative mutant; shade tolerant

INTRODUCTION

Forage availability is essential for advancing livestock production and productivity. However, the increasing demand for forage and the limited availability of planting land pose significant challenges for the livestock industry (Hisham *et al.*, 2022). In Indonesia, the area available for cultivation is expected to decrease due to the rapid conversion of agricultural land for other uses, with an estimated reduction of 150.000 to 200.000 hectares per year (KATR/BPN, 2020). To address these challenges and optimize land use efficiency, silvopasture, and agroforestry systems have been proven effective by integrating forage with plantation crops or forest trees. These systems can potentially improve land productivity from economic and ecological perspectives (Lista *et al.*, 2019; Malaviya *et al.*, 2020; Tsaniya *et al.*, 2020).

Despite the numerous benefits, implementing these systems in forage production requires in-depth study, particularly due to the need for specific plant adaptations to thrive in the environment. A major issue is that forage may be overshadowed by the main crops, leading to limited growth or production on designated land (Soares *et al.*, 2016). This is because the canopy of main crops creates shade, reducing sunlight intensity and potentially hindering the growth of livestock forage (Paciullo *et al.*, 2017; Franca *et al.*, 2017; Angadi *et al.*, 2022). Since light is essential for plant growth and development, affecting the process of photosynthesis and photomorphogenesis, shade can significantly impact forage quality (Pang *et al.*, 2019; Teixeira, 2020), decreased reproductive growth (Qin *et al.*, 2022) and productivity (Soares *et al.*, 2016; Paciullo *et al.*, 2017; de Santos Neto *et al.*, 2023). The impact of shade has a significant effect on the dry matter of legumes (Angadi *et al.*, 2022), the quality with nutritive value of grass and legumes (Pang *et al.*, 2019), lower tiller population density on grasses (Deepthi & Thomas, 2023; Neto *et al.*, 2023), as well as increased of crude protein contents,

ADF, NDF, nitrate and oxalate (Paciullo *et al.*, 2017; Kumar *et al.,* 2023). Developing forage that is tolerant to these conditions is not easily achievable. Therefore, several studies have reported successful induction of plant tolerance to shade, including perennial ryegrass *Lolium perenne* L. (Li *et al.,* 2016) and legume *Medicago sativa* (Lorenzo *et al.,* 2019), which produced new superior plants cultivated under shaded conditions.

Indigofera zollingeriana Miq is a forage crop valued for its high nutritional content, including protein and minerals, as well as favorable fiber structure and high digestibility (Abdullah & Suharlina, 2010; Suharlina *et al.,* 2016; Hutapea *et al.,* 2018; Ernawati *et al.,* 2023). Despite the significant benefits, the production of *I. zollingeriana* is challenging due to competition for land used for food crops. A potential solution is to grow *I. zollingeriana* in crop plantations or forest areas through silvopasture and agroforestry systems. However, no shade-tolerant cultivars of *I. zollingeriana* have been developed yet. This shows the need for developing new superior shade-tolerant varieties using gamma rays to improve *I. zollingeriana,* with genetic characteristics that can be inherited by other generations. In previous studies, *I. zollingeriana* seeds were mutated using gamma rays from a ^{60}Co source. Genotypes were also selected from *I. zollingeriana* putative mutants under shaded conditions to create stable shade-tolerant varieties that could be developed into new *I. zollingeriana* cultivars (Saijo *et al.,* 2018). The results showed that the crop had moderate tolerance to shade and could adapt to shade intensities of approximately 80%, although optimal growth occurred at 40% shade. Meanwhile, there was no information on the nutrient content and digestibility of *I. zollingeriana* in shaded conditions.

Based on the description, this study aimed to explore the selection of *I. zollingeriana* putative mutant in the M2 generation to assess and evaluate the growth, biomass production, and information on nutrient content and digestibility under shading. M2 generation on an irradiated plant refers to the second generation of plants that are grown from seeds that were irradiated. Some of the putative mutants showed increased productivity and nutrient content in shade and unshaded conditions, which could potentially be used to develop new superior varieties of *I. zollingeriana.*

MATERIALS AND METHODS

The seeds of *I. zollingeriana* putative mutants were obtained from certified providers at IPB University for the M2 generation.

Preparation of Seed Materials

Seeds of *I. zollingeriana* putative mutant in the M2 generation, 1 g per genotype, were subjected to serial sterilization using a bactericidal and fungicidal solution of 2 g/L overnight (18-20 hours). After being rinsed, seeds were soaked in a 10% chlorine solution for 30 minutes, planted in moist tissue paper within plastic containers, and incubated for 2 weeks until germination. These seeds were transferred into polybags measuring with length x width (15x15 cm), containing a mixture of organic fertilizer and soil at a 1:1 ratio, and incubated for 9 weeks after planting (WAP). Meanwhile, non-mutated seeds were used as a control in this study.

Design of Shade Planting and Environmental Conditions

The experimental design used was a completely randomized split-plot with 2 factors. The first factor comprised 5 levels of shade, namely N1: 0%, N2: 55%, N3: 65%, N4: 75%, and N5: 85%. The second factor was the seedling of *I. zollingeriana,* which included 2 genotypes R1.2 and R1.8, as control with 10 genotypes: R2.3, R2.7, R3.1, R3.2, R4.1, R4.10, R5.10, R5.2, R5.7, and R6.2 as a putative mutant. Each treatment was carried out in 3 replications, with shade level as main plots and genotype as subplots. The soil conditions were pH H_2O 5.83 ± 0.35, pH N KCl 4.89 ± 0.30, total nitrogen (N) content 0.18 ± 0.04, P2O5 content 31.18 ± 29.55, and cation exchange capacity 16.42 ± 1.66. The holes for planting were dug with a spacing of 1x1 m, and organic fertilizer was applied two weeks before planting at a rate of 1 kg per hole. Environmental factors in the field were measured using several observation parameters: air temperature, pH, light intensity measurement (Lux-meter), and daily air humidity. Each measurement was taken at 3 points for individual shade treatment at 3 different times, namely morning (08.00-09.00 AM), noon (12.00 AM-1.00 PM), and afternoon (4.00-5.00 PM).

Planting under Shade and Observation of Plant Growth

Seedlings of *I. zollingeriana* putative mutant at 9 WAP and control plants were transplanted into the field. Plant maintenance included applying NPK fertilizer (15:15:15) at 1 g per plant every 4 weeks (Tarigan *et al.*, 2010). Insecticide spraying was conducted as needed when pests or diseases were observed. The observed parameters included plant height (cm), number of leaves, stem diameter (mm), number of branches, number of nodes, chlorophyll contents (%), leaf length (cm), and leaf width (cm) at 8 WAP.

Biomass Production

Biomass production of *I. zollingeriana* putative mutants from all genotypes was assessed at 8 WAP. Each genotype treatment was uprooted, and the total fresh biomass weight was recorded. The stem and leaf biomass were separated, while the fresh weight of each part of the plant was re-weighed and recorded. The separated leaf and stem biomass were dried in an oven at 50 °C for 2-3 days until dry biomass. The dry weight of leaf and stem biomass was measured and recorded. Subsequently, the recorded parameters were total fresh weight/plant (g), fresh and dry weight of leaf biomass/plant (g), as well as fresh and dry weight of stem biomass/plant (g).

Nutrient Analysis

Proximate analysis was performed to evaluate the nutritional content of dry leaves from *I. zollingeriana*

putative mutant, comprising R1.2 as control and R2.3, R3.1, R4.10, R5.10, R5.7, and R6.2 as putative mutant under five different shade levels (0%, 55%, 65% and 75%). Parameters for analysis were dry matter (DM), moisture content (MC), organic matter (OM), ash content (AC), crude protein (CP), crude fat (CF), crude fiber (CFB), and Total Digestible Nutrients (TDN). The analysis of MC and AC was performed using Association of Official Analytical Chemists (AOAC) methods (Horwitz & Latimer, 2005). CP analysis was conducted using the Kjeldahl method with OMNILAB FoodALYT (Horwitz & Latimer, 2005). For the analysis of CF, hydrolysis of fat was conducted using the ANKOMMxt10 Extractor. The analysis of CFB was performed using the ANKOM A200 Fiber Analysis system. All steps of the analysis were based on the manufacturer's procedures. The value of DM and protein/nutrient production was determined using the formula:

DM Production= Fresh weight (g) x DM value (%)

Protein Production= DM production x CP value

In Vitro Digestibility Analysis

Analysis of digestible Neutral Detergent Fiber (dNDF) and *In Vitro* True Digestibility (IVTD) with the batch culture incubation method was performed using the ANKOMXT10 *in vitro* true digestibility DaisyII (ANKOM, 2015). Dry leaf from *I. zollingeriana* (1 genotype: R1.2 as control, and 6 genotypes: R2.3, R3.1, R4.10, R5.10, R5.7, and R6.2 as putative mutants) under 5 different shade levels (0%, 55%, 65% and 75%) were used for the analysis. F57 filter bags to be used were labeled and weighed as the bag weight (W1). The dry leaf of each sample (W2) was weighed 0.45-0.55 grams into the filter bag. Moreover, one bag was weighed as a blank for the correction factor (C1). The next procedures are followed by the instructions of the manufacturer for each analysis. After the analysis, the final samples were weighed to obtain the weight of the sample bag (W3). dNDF and *in vitro* true dry matter digestibility (IVTDMD) were performed using formulas from Rofiq *et al*. (2015).

dNDF (%DM)= {100 x [(W2 x %NDF sampel) - (W3 - (W1 x C1))} / (W2 x %DM sample)

IVTD MD (%DM)= {100 - [(W3 - (W1 x C1)) x 100]} / (W2 x %DM sample)

Notes: digestible NDF (dNDF), Dry matter (DM), weight F57 filter bag (W1), weight of sample (W2), final weight (W3: weight F57 filter bag + weight of sample), the percentage of NDF content in the sample -%DM (NDF sample), the percentage of dry matter in the sample (DM sample), correction factor-NDF value from empty filter bag (C1).

Analysis Data

The data from plant growth and biomass production were analyzed using analysis of variance test (ANOVA). The data were further tested using the Duncan test at a 5% significance level when there were significant differences. Data were analyzed using IBM SPSS ver. 27 software. Meanwhile, the data from nutrient content, NDF, and IVTDMD were analyzed based on the formulas of Rofiq *et al.* (2015).

RESULTS

The data of the environment at 8 WAP are presented in Table 1. The daily temperatures fluctuated between 28 °C and 40 °C, while the pH levels ranged from 6.23 to 7.00, based on the observation time and shade levels. The average light intensity varied from 432.00 to 62.566.67 lux, and humidity from dry to dry+, which varied according to the observation time and shade conditions.

Plant Growth of *I. zollingeriana* Putative Mutant Under Shading

The plant growth parameters from shade levels in *I. zollingeriana* putative mutant in the M2 generation are shown in Table 2. ANOVA ($p<0.05$) showed that shade treatment significantly affected the growth parameters. These included an increase in plant height, chlorophyll content, leaf length, and leaf width until 75% of shade, but a significant decrease was observed at 85% shade. However, there was a decrease in the number of leaves, nodes, stem diameter, and branches for all parameters compared to control plants. Shade at 75% possessed the potential to promote the growth of *I. zollingeriana* putative mutant with some parameters showing increased growth. Furthermore, the effect of genotypes was significant ($p<0.05$) for all parameters except for the number of nodes and leaf length. Genotypes R4.1, R4.10, and R5.10 showed an increase in all parameters compared to the control plants.

The interaction between shade levels and genotypes is shown in Figure 1. Based on the results, there was no significant interaction between shade levels and genotypes for all parameters. Plants without shade showed better growth responses than those in shaded conditions. This showed that shade levels significantly influenced the number of leaves and branches, as higher levels caused a decrease in parameters. Genotypes R4.1, R4.10, and R5.10 showed potential to thrive in both shaded and non-shaded conditions. However, 85% shade led to a decrease in all growth parameters across genotypes.

Biomass Production of *I. zollingeriana* Putative Mutant Under Shade

Biomass production of *I. zollingeriana* putative mutant is shown in Table 3. The ANOVA ($p<0.05$) showed that shade levels significantly affected the biomass production for all parameters. Based on the results, higher levels reduced biomass production on all parameters compared to control plants. Genotypes significantly influenced biomass production ($p<0.05$), except for leaf dry weight. Specifically, genotypes R3.1, R4.1, and R4.10 outperformed the control plants in biomass production. The interaction between shade levels and genotypes on biomass production was not significant, as shown in Figure 2. This was because biomass production decreased as shade levels increased,

Table 1. The environmental conditions during the planting of putative mutants of *Indigofera zollingeriana* under shading at 8 weeks after planting

Treatments	Variables											
	Air temperature (°C)			pH			Light intensity (lux)			Humidity		
	M	N	A	M	N	A	M	N	A	M	N	A
N1	32.67	40.33	36.00	7.00	6.87	6.93	62,566.67	15,106.67	22,023.33	Dry+	Dry+	Dry
N2	30.67	36.00	34.33	7.00	6.97	7.00	23,666.67	3,761.67	1,468.33	Dry+	Dry+	Dry+
N3	29.00	35.33	33.33	7.00	6.83	6.23	21,676.33	7,282.00	1,713.33	Dry+	Dry+	Dry
N4	28.00	33.33	33.67	6.73	6.60	6.87	11,180.00	1,379.67	911.33	Dry+	Dry	Dry+
N5	29.00	33.33	34.00	7.00	6.93	7.00	3,694.00	567.00	432.00	Dry+	Dry+	Dry+

Note: N1 (shade 0%, Control), N2 (shade 55%), N3 (shade 65%), N4 (Shade75%), N5 (85%), M: morning (08.00-09.00 AM), N: noon (12.00-1.00 PM), A: afternoon (04.00-05.00 PM).

Table 2. Growth variables of putative mutants of *Indigofera zollingeriana* in the M2 generation under shaded conditions

Treatments	Variables							
	Plant height (cm)	Number of leaves	Number of nodes	Stem diameter (mm)	Chlorophyll contents	Leaves length (cm)	Leaves width (cm)	Number of branches
Shade level								
0%	74.04±20.24^{ab}	48.94±17.20^{d}	52.81±24.33^{b}	8.66±1.68^{d}	39.51±3.38^{a}	27.04±2.91^{b}	14.44±1.64^{b}	14.92±5.68^{d}
55%	100.56±27.33^{c}	38.08±13.55^{c}	23.83±5.52^{a}	7.63±1.68^{c}	39.24±8.14^{a}	30.47±6.17^{c}	14.92±3.49^{b}	8.47±3.56^{c}
65%	79.92±22.79^{b}	36.94±14.41^{c}	23.17±3.62^{a}	7.13±1.67^{bc}	39.90±3.30^{a}	30.89±2.56^{c}	15.08±1.40^{b}	9.31±3.95^{c}
75%	101.81±29.92^{c}	28.81±10.24^{b}	22.25±3.77^{a}	6.46±1.56^{b}	43.93±4.51^{b}	30.40±4.46^{c}	15.42±2.32^{b}	4.69±2.63^{b}
85%	64.47±18.23^{a}	14.28±3.06^{a}	18.42±2.75^{a}	3.80±0.83^{a}	39.59±3.43^{a}	21.81±3.47^{a}	11.47±1.72^{a}	0.19±0.75^{a}
Genotype								
R1.2	82.10±32.23^{a}	33.07±15.60^{ab}	25.00±11.48	6.43±2.13^{abc}	39.53±3.44^{b}	28.73±5.53	14.61±2.41^{abc}	6.20±4.31^{ab}
R1.8	71.20±31.21^{a}	23.87±10.66^{a}	22.73±10.05	5.60±2.29^{a}	35.91±10.85^{a}	27.30±8.66	14.98±4.74^{bc}	4.60±4.29^{a}
R2.3	81.13±15.93^{a}	32.20±12.70^{ab}	27.13±12.55	6.74±1.61^{abc}	40.27±4.70^{b}	28.31±3.86	14.08±2.25^{abc}	7.33±4.79^{ab}
R2.7	90.47±31.07^{ab}	32.93±16.31^{ab}	26.93±13.54	6.91±2.05^{abc}	41.49±3.85^{b}	27.73±6.17	14.63±3.11^{abc}	7.73±5.50^{b}
R3.1	73.93±30.79^{a}	30.40±22.70^{ab}	31.00±25.87	6.36±2.58^{ab}	41.53±5.24^{b}	27.47±6.33	13.02±2.34^{a}	7.53±7.21^{ab}
R3.2	79.60±26.20^{a}	32.87±15.56^{ab}	28.47±17.07	6.60±2.23^{abc}	41.41±3.62^{b}	27.73±5.21	13.97±1.60^{abc}	8.0±6.71^{b}
R4.1	89.60±22.80^{ab}	36.87±22.08^{b}	31.87±26.88	7.23±2.37^{bc}	41.69±5.55^{b}	28.67±3.90	15.22±1.98^{c}	7.53±7.80^{ab}
R4.10	105.53±33.45^{b}	38.80±18.02^{b}	31.27±18.41	7.77±2.48^{c}	41.06±5.05^{b}	30.06±5.13	15.35±2.72^{c}	8.67±5.97^{b}
R5.10	85.13±21.29^{a}	36.73±17.12^{b}	29.33±18.48	6.77±2.21^{abc}	40.42±3.18^{b}	27.92±4.27	13.99±2.10^{abc}	8.47±8.14^{b}
R5.2	82.13±27.58^{a}	35.47±17.46^{b}	30.00±16.42	6.96±2.20^{bc}	40.19±3.95^{b}	28.39±4.29	13.73±1.98^{abc}	7.87±5.15^{b}
R5.7	84.07±24.88^{a}	37.33±17.22^{b}	24.07±8.85	6.85±2.37^{abc}	41.15±4.34^{b}	26.65±4.82	13.13±2.48^{ab}	8.40±6.29^{b}
R6.2	85.00±28.66^{a}	30.40±16.24^{ab}	29.33±16.22	6.59±2.06^{abc}	40.55±3.77^{b}	28.51±5.37	14.47±2.45^{abc}	7.80±6.99^{b}
Interaction shade and genotypes	ns (p=0.25)	ns (p=0.95)	ns (p=0.87)	ns (p=0.97)	ns (p=0.41)	ns (p=0.62)	ns (p=0.67)	ns (p=0.53)

Note: Means in the same column with different superscripts differ significantly (p<0.05). ns: no significant.

particularly at 85%. Genotypes R3.1, R4.1, R4.10, and R5.10 produced more biomass than the control plants under both shaded and unshaded conditions.

Nutrient Contents of *I. zollingeriana* Putative Mutant Under Shade

Nutrient contents of *I. zollingeriana* putative mutant are presented in Table 4. The results showed no difference in nutritional values between the control plants and putative mutant, as both remained within the standard range for *I. zollingeriana* (Abdullah, 2010). However, some genotypes showed better nutritional values, whether grown in shaded or unshaded conditions. The dry weight of control plants at various shade levels remained consistent at 91%, except for a decrease observed at 75%. Furthermore, the dry weight values from all genotypes ranged from 88.80% to 92.40%. Genotypes R3.1 and R4.10 produced the best DM at 0% shade compared to the control plants, both with 92%.

CP in the control plants increased with shade levels, while the putative mutant showed fluctuations depending on the genotype. The highest CP values were observed at 55% shade, and approximately all putative mutants produced higher values compared to the control plants. At 75% shade, the highest CP was recorded for all genotypes, including both the putative mutant and the control plants, from 28%-31%. CFB in control plants ranged from 22% to 25%, while CF in most putative mutants was higher, except for genotype R4.10 at 55% shade, which recorded 18.76%.

At 75% shade, CF increased to 42% in both genotypes R5.7 and R6.2. TDN values decreased in all genotypes across shade levels, except for genotype R4.10, which remained stable and higher than the control plants at 65% shade. Furthermore, it was discovered that shade reduced both DM and protein production in putative mutants under unshaded conditions compared to the control plants. In shaded conditions, genotypes R5.10, R5.7, and R6.2 showed

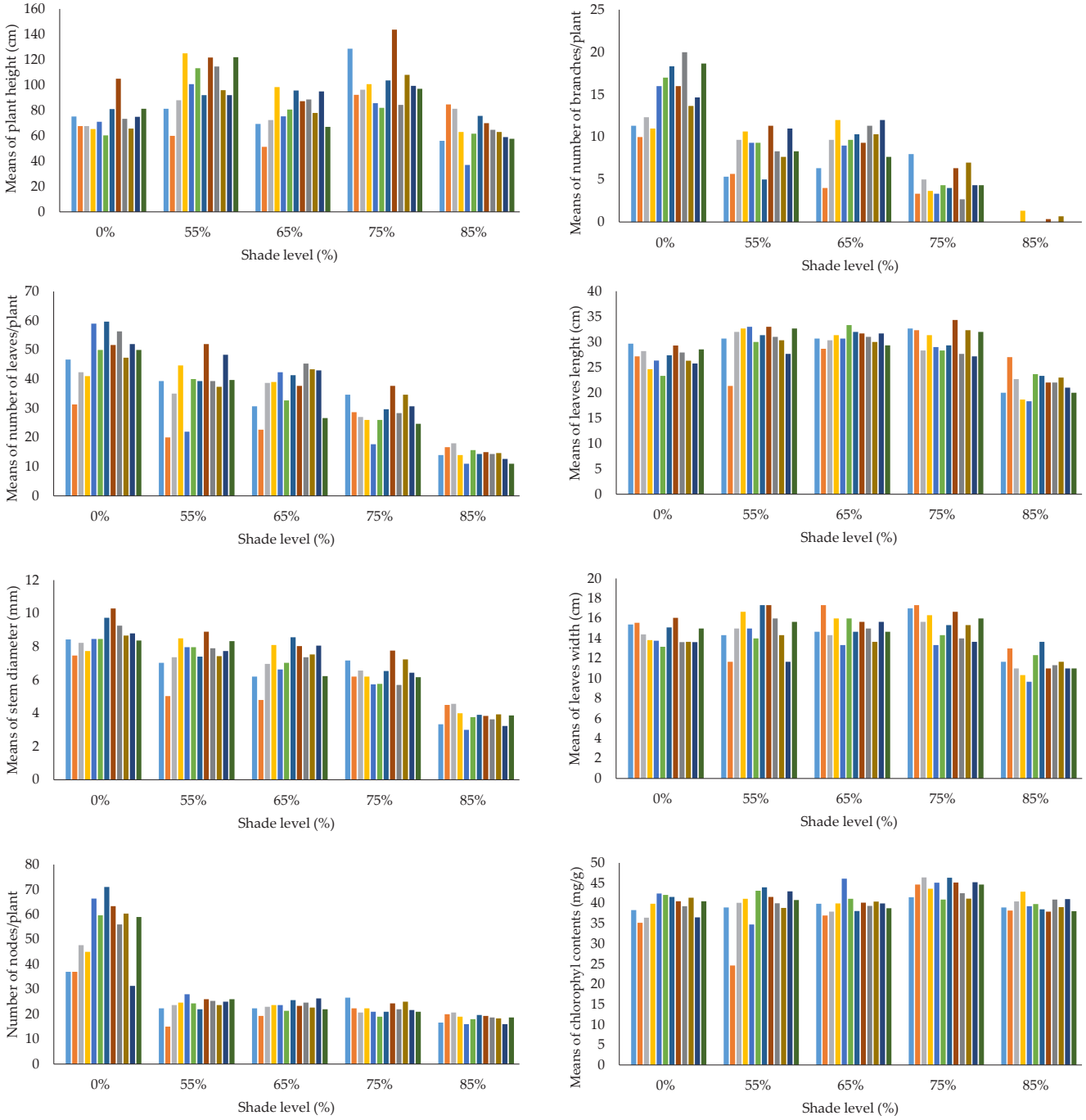

Figure 1. The plant growth variable of 12 genotypes of the putative mutant of *Indigofera zollingeriana* in the M2 generation under various shade levels. Genotypes R1.2 (■), R1.8 (■), R2.3 (■), R2.7 (■), R3.1 (■), R3.2 (■), R4.1 (■), R4.10 (■), R5.10 (■), R5.2 (■), R5.7 (■), and R6.2 (■).

increased DM and protein production compared to the control plants at 55% and 65% but decreased at 75%.

Digestibility Value of *I. zollingeriana* Putative Mutant Under Shade

The data from the digestibility value of *I. zollingeriana* putative mutant are shown in Table 5. The overall dNDF value in the control plants ranged from 13.99 to 22.78, increasing with the level of shade but decreasing at 65%. Several genotypes of putative mutants showed a decrease in dNDF values under both shaded and unshaded conditions. Genotypes R4.10, R5.10, and R6.2 showed lower dNDF values when grown in unshaded conditions compared to the control plants, with dNDF values of 15, 16, and 14, respectively. Meanwhile, R4.10 showed a lower dNDF value at 55% shade but increased at 65% and 75%. R5.10 showed a consistent dNDF value in both shaded and unshaded conditions, ranging from 12 to 16. R6.2 maintained a stable dNDF value of 65% shade, ranging from 14 to 16, but increased to 26 at 75%.

IVTD values in the control plants and *I. zollingeriana* putative mutant ranged from 73 to 86, depending on the

Table 3. Biomass production of putative mutants of *Indigofera zollingeriana* in the M2 generation under shaded conditions

Treatments	Variables					
	Fresh weight (g)			Dry weight (g)		Leaf/stem ratio from dry biomass
	Total	Leaf biomass	Stem biomass	Leaf biomass	Stem biomass	
Shade level						
0%	322.03±162.26^{d}	195.72±95.31^{d}	126.31±69.58^{d}	47.36±20.53^{e}	48.87±28.89^{d}	0.96
55%	241.31±97.35^{c}	135.17±54.56^{c}	106.14±44.76^{cd}	34.22±12.56^{d}	29.55±12.71^{c}	1.16
65%	202.61±97.55^{c}	114.94±48.54^{c}	87.67±50.39^{c}	26.93±12.05^{c}	31.69±17.75^{c}	0.85
75%	128.39±60.61^{b}	72.97±31.21^{b}	55.42±30.15^{b}	17.28±8.11^{b}	19.23±10.67^{b}	0.89
85%	20.89±10.54^{a}	13.19±6.68^{a}	7.69±4.21^{a}	2.49±1.32^{a}	1.34±1.13^{a}	1.86
Genotype						
R1.2	181.87±146.69^{ab}	107.60±90.52^{ab}	74.27±56.97^{abc}	20.76±19.16	28.97±27.02^{ab}	0.87
R1.8	121.93±88.30^{a}	74.93±52.42^{a}	47.00±36.47^{a}	23.82±16.35	16.29±13.92^{a}	1.27
R2.3	160.00±113.43^{ab}	97.13±67.71^{ab}	62.87±46.34^{ab}	24.25±19.84	21.89±20.15^{ab}	1.11
R2.7	178.33±117.61^{ab}	103.20±69.63^{ab}	75.13±49.82^{abc}	24.47±18.76	24.69±18.27^{ab}	1.05
R3.1	201.40±196.10^{ab}	122.27±118.52^{ab}	79.13±78.58^{abc}	24.59±15.93	23.31±15.93^{ab}	1.17
R3.2	187.67±126.81^{ab}	111.73±71.49^{ab}	75.93±57.37^{abc}	25.23±16.26	24.84±17.11^{ab}	1.03
R4.1	216.93±216.82^{b}	121.07±126.50^{ab}	95.87±91.38^{bc}	25.41±28.36	33.29±26.92^{bc}	0.95
R4.10	238.73±159.45^{b}	129.27±85.12^{b}	109.47±75.92^{c}	25.57±19.36	42.13±34.98^{c}	0.69
R5.10	173.80±134.31^{ab}	104.00±81.05^{ab}	69.80±55.44^{ab}	25.89±21.44	25.15±23.64^{ab}	1.01
R5.2	183.40±123.62^{ab}	104.40±67.35^{ab}	79.00±58.61^{abc}	27.18±16.59	25.73±25.55^{ab}	0.95
R5.7	183.60±129.92^{ab}	107.13±76.77^{ab}	76.47±54.54^{abc}	28.99±18.29	22.89±15.86^{ab}	1.07
R6.2	168.87±120.07^{ab}	94.07±66.47^{ab}	74.80±54.88^{abc}	31.70±17.15	24.45±19.21^{ab}	0.97
Interaction shade and genotypes	ns (p=0.73)	ns (p=0.80)	ns (p=0.60)	ns (p=0.85)	ns (p=0.42)	

Note: Means in the same column with different superscripts differ significantly (p<0.05). ns: no significant.

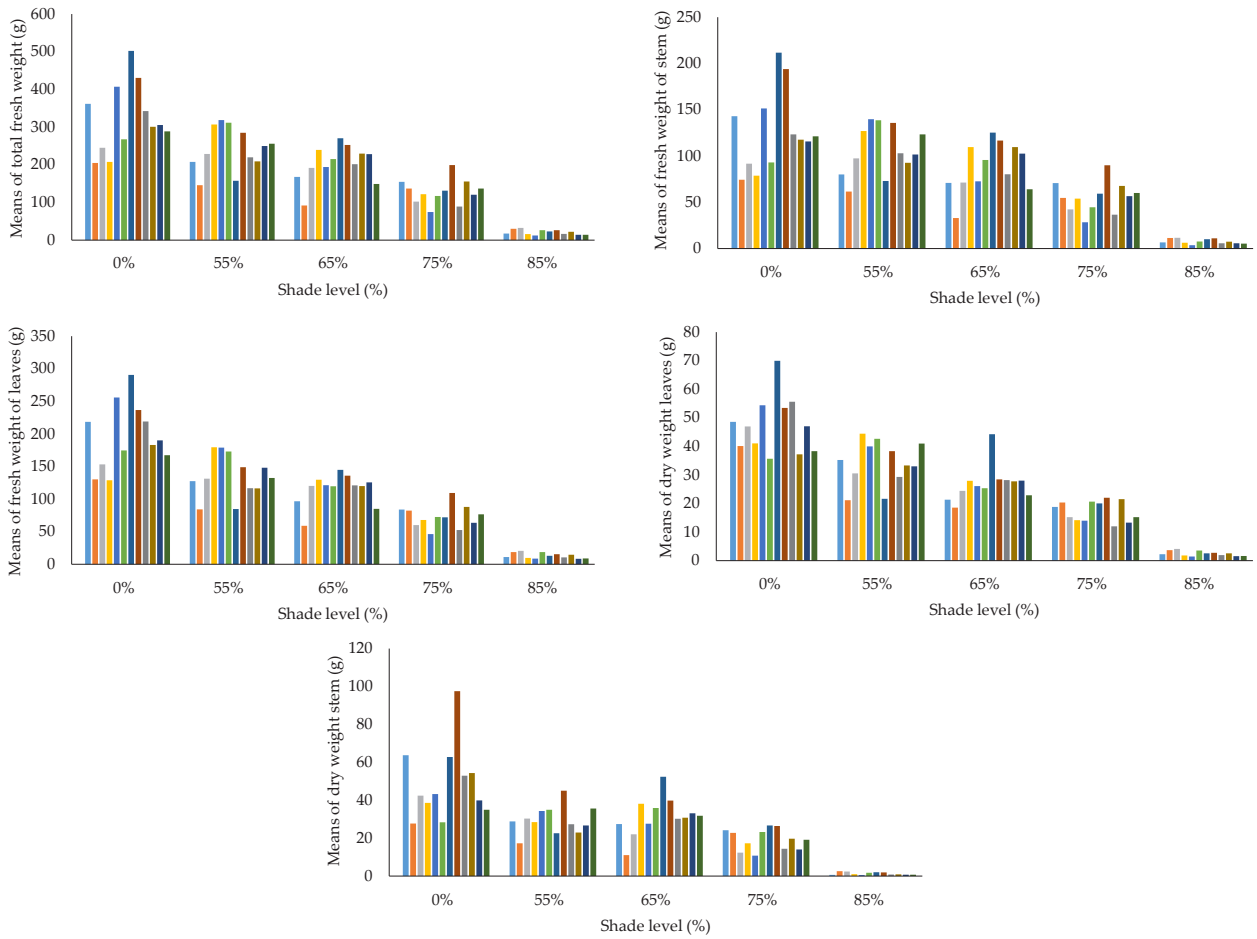

Figure 2. Biomass production variable of 12 genotypes of the putative mutant of *Indigofera zollingeriana* in the M2 generation under various shade levels. Genotypes R1.2 (■), R1.8 (■), R2.3 (■), R2.7 (■), R3.1 (■), R3.2 (■), R4.1 (■), R4.10 (■), R5.10 (■), R5.2 (■), R5.7 (■), and R6.2 (■).

Table 4. Nutrient contents of 4 control plants and 24 putative mutants of *Indigofera zollingeriana* in the M2 generation

Shade level	Genotype	Variables									
		Dry matter	Moisture content	Organic matter	Ash content	Crude protein	Crude fat	Crude fiber	TDN	Dry matter production	Protein production
		%	%	%	%	%	%	%	%	g	g
N1 (0%)	R1.2	91.94	8.06	89.47	10.53	24.10	3.61	22.83	62.62	20104.21	484511.54
	R2.3	88.80	11.20	85.11	14.89	27.39	2.12	26.05	53.84	9353.60	256195.10
	R3.1	92.40	7.60	88.32	11.68	24.52	3.98	30.50	56.23	13398.00	328518.96
	R4.10	92.07	7.93	91.22	8.78	19.93	4.02	23.31	66.31	12245.31	244049.03
	R5.10	91.90	8.10	91.10	8.90	21.73	3.78	28.87	61.32	12467.77	270924.57
	R5.7	91.93	8.07	91.20	8.80	23.17	3.50	31.59	58.64	11583.18	268382.28
	R6.2	91.27	8.73	90.76	9.24	21.60	3.40	29.87	60.18	15485.48	334486.30
N2 (55%)	R1.2	91.00	9.00	91.87	8.13	23.48	4.54	25.89	63.55	11617.67	272782.81
	R2.3	91.81	8.19	91.92	8.08	27.10	4.79	25.99	61.76	12210.73	330910.78
	R3.1	91.02	8.98	90.13	9.87	26.30	3.97	27.09	59.33	9223.36	242574.37
	R4.10	90.45	9.55	91.44	8.56	25.19	3.23	18.76	66.50	10944.45	275690.70
	R5.10	91.12	8.88	91.40	8.60	30.69	4.49	26.11	59.15	13637.63	418538.76
	R5.7	90.70	9.30	90.97	9.03	27.37	4.19	29.29	58.16	15237.60	417053.11
	R6.2	91.39	8.61	90.33	9.67	26.44	3.54	35.20	53.64	19100.51	505017.48
N3 (65%)	R1.2	91.32	8.68	90.70	9.30	26.13	3.03	23.17	62.17	8827.60	230665.19
	R2.3	91.19	8.81	91.37	8.63	24.47	3.88	26.31	61.94	5076.24	124215.67
	R3.1	91.14	8.86	90.49	9.51	24.68	3.48	28.24	59.48	8597.54	212187.29
	R4.10	91.51	8.49	92.03	7.97	24.74	3.74	24.58	63.54	9852.58	243752.75
	R5.10	91.67	8.33	91.95	8.05	25.35	3.43	27.88	60.71	12467.12	316041.49
	R5.7	90.23	9.77	91.00	9.00	25.69	2.16	31.49	56.52	12451.74	319885.20
	R6.2	90.50	9.50	90.26	9.74	29.34	2.51	35.74	51.16	13786.17	404486.13
N4 (75%)	R1.2	89.82	10.18	88.88	11.12	29.07	2.94	24.02	58.30	7544.88	219329.66
	R2.3	89.74	10.26	88.69	11.31	29.43	3.29	29.59	54.28	6700.59	197198.27
	R3.1	90.64	9.36	89.93	10.07	28.79	2.44	31.34	54.13	8731.65	251384.30
	R4.10	90.49	9.51	89.06	10.94	27.28	3.10	31.06	54.64	4826.13	131656.92
	R5.10	90.34	9.66	88.12	11.88	25.70	2.40	35.00	51.52	5330.06	136982.54
	R5.7	90.06	9.94	88.63	11.37	27.72	3.41	42.16	46.52	6334.22	175584.58
	R6.2	90.53	9.47	91.00	9.00	31.20	3.95	42.87	46.71	6186.22	193009.96

Note: N1 (shade 0%, Control), N2 (shade 55%), N3 (shade 65%), N4 (Shade75%), TDN (Total digestible nutrients).

shade conditions. In the unshaded condition, genotypes R4.10, R5.10, and R6.2 showed increased IVTD values compared to the control plants. At 55%, most genotypes showed improved IVTD values, except for R3.1, similar to the control plants. In the shaded conditions, treatment at 75% enhanced the IVTD values, except for R6.2, which showed a decrease. Genotypes R5.10 and R5.7 showed high IVTD values that remained stable under both shaded and unshaded conditions. Specifically, the results showed that R5.10 reached an IVTD value of 87 at 55% shade.

DISCUSSION

In different environments, particularly shaded conditions, plants often use shade avoidance or tolerance as adaptation strategies (Xu *et al.*, 2021; Liu *et al.*, 2022; Martinez-gracia & Rodriguez-Concepcion, 2023). Shade avoidance is associated with changes in leaf anatomy and morphology to enhance photosynthesis efficiency, promote elongation, and reduce branching in response to high far-red light environments. This strategy readjusts growth and development to escape potential shade conditions, even at the expense of potentially detrimental effects on photosynthesis and defense. Meanwhile, shade tolerance is linked to a reduced light compensation point and efficient respiration by optimizing light interception and maximizing the efficiency of absorbed light, allowing plants to grow under low light levels while adopting a more conservative growth strategy (Liu *et al.*, 2022; Martinez-gracia & Rodriguez-Concepcion, 2023).

During observation of plant growth, *I. zollingeriana* putative mutant did not show any morphological changes but had alterations in growth. This suggested that the shade tolerance mechanism was evident in the putative mutant. According to Xu *et al.* (2021), shade-tolerant species enhanced their survival in shaded conditions by showing traits such as lower growth rates, thinner leaves, an altered chlorophyll a:b ratio, reduced apical dominance, and increased branching.

Despite a decrease in some growth parameters, 55% and 75% shade showed the best performance by tolerating and surviving compared to the control plants and some at 85% shade level. This outcome exceeded expectations, as a previous study by Saijo *et al*. (2018) achieved similar results at 80% shade. Genotypes R4.1, R4.10, and R5.10 showed the highest tolerance and survival in shaded conditions, suggesting significant improvements in plant growth compared to control plants. This could be attributed to a mutation in *I. zollingeriana* putative mutant that modified a gene responsible for enhancing shade tolerance. Plant mutation caused by gamma rays directly altered molecules within cells (Barela *et al.*, 2022) and primarily led to DNA breakage (Di Pane *et al.*, 2018), generating

Table 5. Digestible neutral detergent fiber and *in vitro* true digestibility values in 4 control plants and 24 putative mutants of *Indigofera zollingeriana*

Shade level (%)	Genotype	Variables	
		dNDF value	IVTD value (%/500mg DM)
N1 (0%)	R1.2	19.17±0.12	80.83±0.12
	R2.3	19.63±4.42	80.37±4.42
	R3.1	20.07 ± 4.58	79.93±4.58
	R4.10	15.81±1.19	84.19±1.19
	R5.10	16.54±5.23	83.46±5.23
	R5.7	20.18±2.04	79.82±2.04
	R6.2	14.10±2.57	85.90±2.57
N2 (55%)	R1.2	21.10±6.83	78.90±6.83
	R2.3	19.57±5.11	80.43±5.11
	R3.1	21.10±3.51	78.90±3.51
	R4.10	16.69±2.77	83.31±2.77
	R5.10	12.74±1.17	87.26±1.17
	R5.7	19.79±0.57	80.21±0.57
	R6.2	16.25±1.29	83.75±1.29
N3 (65%)	R1.2	13.99±0.95	86.01±0.95
	R2.3	13.54±1.13	86.46±1.13
	R3.1	15.98±1.33	84.02±1.33
	R4.10	20.38±1.99	79.62±1.99
	R5.10	14.99±2.55	85.01±2.55
	R5.7	16.45±3.19	83.55±3.19
	R6.2	15.73±1.61	84.27±1.61
N4 (75%)	R1.2	22.78±5.42	77.22±5.42
	R2.3	17.66±2.59	82.34±2.59
	R3.1	14.69±2.01	85.31±2.01
	R4.10	20.75±5.38	79.25±5.38
	R5.10	16.59±5.73	83.41±5.73
	R5.7	16.31±0.20	83.69±0.20
	R6.2	26.14±2.08	73.86±2.08

Note: N1 (shade 0%, Control), N2 (shade 55%), N3 (shade 65%), N4 (Shade75%), dNDF= digestible Neutral Detergent Fiber, IVTD= *In Vitro* True Digestibility.

radicals from water almost instantly. These radicals can affect either single or double DNA strands and react with one another or nearby unaffected molecules. The reaction can lead to breaking chemical bonds or the oxidation of molecules (Elsherbiny *et al.,* 2024), which induces specific changes in the genome (Caplin & Willey, 2018), potentially modifying the gene.

I. zollingeriana putative mutant showed high plant height relative to the control plants at 75% shade, but a decrease was observed at 85%. This study showed that the maximum limit of plant height at the shade level was 75%. Similarly, Deepthi & Thomas (2023) reported that the Bajra Napier Hybrid reached maximum plant height under 50% shade, and increased shading adversely affected growth. In comparison, Saijo *et al*. (2018) found that plant height in *I. zollingeriana* decreased with increasing shade levels until 10 WAP. High plant height under low light intensity during development can lead to etiolated symptoms due to auxin hormone activity, although there is a limit to this increase (Deepthi & Thomas, 2023).

The effect of shade on chlorophyll content was similar across all levels except for 75%. However, all genotypes of the putative mutant showed higher chlorophyll content compared to control plants. Similar results were reported by Deepthi & Thomas (2023), where the photosynthetic rate increased as shade levels decreased in the Bajra Napier hybrid. It was also reported that moderate shade promoted chlorophyll accumulation by triggering an adaptive response to the stress of intense light, thereby mitigating chlorophyll damage (Wang *et al.,* 2019). However, Saijo *et al.* (2018) stated that chlorophyll content in *I. zollingeriana* decreased with increasing shading.

Biomass production decreased as shade levels increased, with higher shade leading to reduced biomass yields. However, plants in 75% shade achieved a total fresh weight of 128.39 grams per plant, approximately 39.86% lower than unshaded treatment. In contrast, biomass production significantly decreased in all parameters at 85% shade. Light intensity can also impact plant metabolism and forage production (Wang *et al.,* 2017). Generally, photosynthetic activity decreases under shaded conditions due to inhibition of photosynthesis, which is associated with reduced enzyme activity in low light. This decrease is significant for biomass production as the carboxylation enzyme is not fully active when plants are shaded. Consequently, the net photosynthetic rate (Pn) substantially decreases after shade (Yuan *et al.,* 2022). The limited availability of photosynthates under shaded conditions mainly affects cell size and the development of secondary cell walls in plants (Angadi *et al.,* 2022). The increased cell wall content observed in shaded environments can be associated with decreased non-structural carbohydrates, such as starch and sugars. Additionally, reduced photosynthesis inhibits CO_2 uptake by the leaf and disrupts the metabolic processes essential for effective photosynthesis (Elango *et al.,* 2023). In this study, the results were consistent with Herdiawan (2016), where the fresh weight of *I. zollingeriana* under shaded conditions in oil palm estate showed a significant decrease in biomass and leaf with increasing shade levels. Similarly, Saijo *et al*. (2018) and Soares *et al.* (2016) reported that forage production decreased under shade conditions.

Regarding the effect of genotypes, R3.1, R4.10, and R5.10 showed the highest biomass production compared to control plants. The ability of these plants to cope with shade stress depends on their capacity to continue photosynthesis under low light conditions. These genotypes also produced significantly more biomass compared to control plants in unshaded treatments, serving as potential candidates for superior forage and feedstuff availability.

Shade conditions can impact the carbon balance of plants, as the demand for carbohydrates (sugars) increases while their production decreases. This leads to an increase in the rate of physiological processes while the yield of photosynthesis decreases (Yang *et al.,* 2018). Plant growth in low-light conditions is disrupted due to insufficient energy supply and ATP needed for photosynthesis (Niinemets, 2010), affecting nutritional content. However, this study showed that some nutritional contents, including DM, OM, and CF, were nearly similar to the control plants. CP, CFB, and TDN

varied depending on genotypes and shade levels. Based on the results, CP levels in all genotypes increased under 75% shade. This increase was used by plants to enhance enzyme levels, ensuring that their metabolism continued to function optimally. Moreover, the CP content of forage shows the total nitrogen (N) in the feed, which includes both true protein and non-protein nitrogen, such as urea and ammonia (Saha *et al.,* 2023). Nitrogen is an essential component of every amino acid, and non-protein nitrogen can potentially be used in protein synthesis by rumen microorganisms.

This study showed that some genotypes outperformed the control plants in terms of nutritional content in both conditions. Specifically, R4.10 consistently had higher and more stable nutritional content, showing the potential to be developed as a superior plant.

Neutral detergent fiber (NDF) is a chemical measure used to estimate the plant cell wall content, showing the amount of feed that will pass through to the hind-gut of the gastrointestinal tract (Springer *et al.,* 2023). dNDF is the fraction of the NDF fermented by rumen microbes and converted to volatile fatty acids (VFA) to provide energy. Additionally, dNDF significantly influences feed intake, digestibility, and the use of nutrients in ruminants. The digestibility of NDF is an important parameter of forage quality, comprising the largest amount of nutrients in the ruminant diet and varies widely in degradability in the rumen. The measurement of dNDF is essential for its inclusion in the summative equation, which predicts the energy content of forage and mixed rations (Mahyuddin & Purwantari, 2009).

The evaluation of dNDF offers valuable insights for assessing forage quality when making purchasing decisions. It also provides essential information for characterizing forage materials and predicting animal performance. Therefore, evaluating dNDF values of *I. zollingeriana* putative mutants will provide insights into their capability to digest forage after mutation treatment, which can be used to select genotypes with dNDF values suitable for feed. This study showed that some genotypes had low dNDF values in both shaded and unshaded conditions. Genotypes R4.10, R5.10, and R6.2 had dNDF values of 15, 16, and 14, respectively. R5.10 genotype showed a consistent dNDF value in both shaded and unshaded conditions. This suggested that R5.10 could ferment quickly and serve as an additional energy source for ruminal microorganisms (Azevedo *et al.,* 2012).

IVTD refers to anaerobic fermentation conducted in the laboratory. It simulates the digestion process that occurs in the rumen and is often referred to as real digestibility, which is used to assess the actual digestibility of forage. The IVTD value shows the results of testing to determine the *in vitro* rumen digestibility. In this study, the average IVTD value ranged from 73.86 to 87.26 based on genotypes. Under unshaded conditions and at 65% and 75% shade, the IVTD values of genotypes R4.10, R5.10, and R6.2 were higher than the control plants. At 75% shade, the values increased slightly but remained high.

The evaluation of *I. zollingeriana* putative mutant in the M2 generation under shade conditions showed genotypes with improved survival, biomass production, nutrient content, and digestibility values compared to the control plants. The low dNDF and high IVTD values in several genotypes showed that the feed ingredients were easily degraded by rumen microorganisms (Sandi *et al*., 2020), leading to improved digestibility levels. This provided additional information that *I. zollingeriana* putative mutant, despite being subjected to nonspecific mutation, did not alter the dNDF content. Moreover, several genotypes with reduced dNDF and high IVTD values could be developed as new superior candidates for both shaded and unshaded conditions.

Our study revealed a large standard deviation in the data. We believe several factors contribute to this, including scattered data with significant variability, likely due to genetic differences in putative mutants of *I. zollingeriana*, which exhibit genetic variation when analyzed using SSR markers (data not shown). A study on putative mutant rice (*Oryza sativa*) found that gamma-ray irradiation caused a wide range of genetic variability among individual plants in the M3 generation progeny, impacting their growth parameters (Ishak, 2023).

One method for characterizing gamma-ray-induced mutations is RNA sequencing, which allows the exploration of genome-wide single-nucleotide polymorphisms (SNPs), insertion/deletion (InDel) variants, other genome-wide variations, and whole genome sequencing (Tan *et al*., 2019; Li *et al*., 2019; Eun *et al*., 2024). Gamma-ray-induced mutations in barley dwarf mutants were analyzed using RNA sequencing, identifying 1,193 genetic mutations in gene transcription regions. Nearly 97% of these mutations were concentrated in specific regions of chromosomes 5H and 7H. Among the 26,745 expressed genes, 140 were affected by the radiation, with their biological functions linked to cellular and metabolic processes (Tan *et al*., 2019). In the Miyagawa-wase (*Citrus unshiu* Marc cv. Miyagawa-wase) mutant line, induced by gamma irradiation, a total of 3,344 SNPs, 3,154 InDels, 465 SNPs, and 709 InDels were detected in genes annotated in the gene ontology database from wild-type and Gwonje-early plants. The two SNPs were annotated in the glutamate receptor 3.2 gene of *C. sinnensis* and the hypothetical protein CUMW_259270 of *C. unshiu*, and the one InDel was annotated in NO-associated protein 1,a chloroplastic/mitochondrial isoform X1 gene of *C. clementina* (Eun *et al*., 2024). Whole-genome resequencing of rice (*Oryza sativa* L.) mutant lines at the M5 generation revealed that, on average, each gamma-ray-irradiated mutant had 57.0 single base substitutions (SBS), 17.7 deletions, and 5.9 insertions, whereas each C-ion-irradiated mutant had 43.7 SBS, 13.6 deletions, and 5.3 insertions. Structural variation (SV) analysis detected an average of 2.0 SVs (including large deletions or insertions, inversions, duplications, and reciprocal translocations) per C-ion-irradiated mutant, while an average of 0.6 SVs was detected per gamma-ray-irradiated mutant (Li *et al*., 2019).

Understanding the molecular mechanisms of shade tolerance is less well understood compared to shade avoidance (Xu *et al*., 2021; Liu *et al*., 2022; Martinez-Garcia & Rodriguez-Concepcion, 2023). Shade tolerance may be achieved by optimizing light capture and reducing the dark respiration rate, which

in turn increases the maximum potential carbon gain (Niinemets & Valladares, 2004). It is also linked to various traits, and many plants can survive in low light conditions (Valladares & Niinemets, 2008). Martinez-Garcia & Rodriguez-Concepcion (2023) explained the molecular mechanisms of shade tolerance in plants, focusing on the regulation of hypocotyl elongation in shade-avoiding species. Shade inhibits phytochrome B (phyB) activity by shifting the phytochrome photoequilibrium toward its inactive form, allowing Phytochrome Interacting Factors (PIFs) to activate the expression of shade-avoidance-related genes, such as Long Hypocotyl in Far-Red Light 1 (HFR1). HFR1 then heterodimerizes with PIFs, blocking their DNA-binding ability and thereby reducing hypocotyl elongation. The abundance of HFR1 is regulated through its interaction with Constitutive Photomorphogenic1 (COP1), which leads to its degradation. Additionally, shade promotes the accumulation of phytochrome A (phyA), further inhibiting hypocotyl elongation.

At the transcriptional level, gene expression related to shade tolerance is influenced by cells, cell parts, and organelles, which contribute to the plant's response through various physiological, biochemical, and morphological traits associated with shade tolerance. Most of the differentially expressed genes (DEGs) were related to photosynthesis, plant hormone signal transduction, chlorophyll synthesis pathways, nitrogen metabolism, biosynthesis of secondary metabolites, free radical scavenging, glycolysis/gluconeogenesis, carbon metabolism, fatty acid metabolism, glutathione metabolism, ribosome, and protein biosynthesis in the endoplasmic reticulum by functional enrichment analysis. (An *et al.,* 2022; Wu *et al.,* 2020; Zhang *et al.,* 2022a; Jiang *et al.,* 2023). A genome-wide association study (GWAS) integrated with transcriptome sequencing demonstrated that shade tolerance is regulated by multiple interacting genes involving various biological functions organized into a gene network (Su *et al.,* 2024). Comparisons of the gene expression and structure reveal that differential transcriptional regulation together with an increased copy number of photosynthesis-related genes (e.g., electron transfer and carbon fixation), may improve the photosynthetic efficiency (Zhang *et al.,* 2022b).

Combined transcriptome and metabolome analysis of soybean (*Glycine max*), a legume, identified mechanisms of shade tolerance associated with ATP phosphoribosyl transferase (ATP-PRT2), phosphocholine phosphatase (PEPC), auxin-responsive protein (IAA17), and purple acid phosphatase (PAP) (Jiang *et al.,* 2023). The integrated transcriptomic and metabolomic analyses of *Carex adrienii* (herbaceous) revealed that the DEGs and differentially accumulated metabolites (DAMs) were enriched in pathways related to photosynthesis, plant hormone signal transduction, and flavonoid biosynthesis (Guo *et al.,* 2024). Genes involved in plant hormone signaling were significantly upregulated in response to shading induction. In the leaves of foxtail millet (*Setaria italica* (L.) P. Beauv.) under shaded conditions, several photosynthesis genes were also identified. These genes may enhance the efficiency of light-harvesting molecules and the photosynthetic electron transport chain in shade-tolerant varieties, thereby maintaining a more stable photosynthetic rate and yield under low light conditions (electronic supplementary material) (Liu *et al.,* 2022).

In bowl lotus (*Nelumbo nucifera*), comparative transcriptomic and proteomic profiling of light signal regulation in shade tolerance reveals that several transcription factors (MYB90, MYB1R1, bHLHs, and WRKYs) and hormone signaling pathways (auxin, gibberellin, and ethylene) play a role in mediating light signaling, which regulates downstream biological processes such as metabolism, secondary metabolite production, fatty acid and protein biosynthesis, flowering, and flavonoid biosynthesis (Sheng *et al.,* 2022). In shade-tolerant Swarnaprabha rice, genes involved in ethylene and cytokinin signaling pathways were upregulated in the shade-exposed panicles (Panigrahy *et al.,* 2019). Additionally, the dwarf shade-tolerant mutant of perennial ryegrass (*Lolium perenne* L.) exhibited downregulation of gibberellin (GA) biosynthesis genes in shade-exposed plants, with gibberellin 20-oxidase (GA20ox) expression reduced to 3.3% (a 96.7% decrease) of the wild-type level under shade conditions (Li *et al.,* 2017).

CONCLUSION

In conclusion, shade levels significantly increase plant height, chlorophyll content, leaf length, and leaf width, but decrease the number of leaves, nodes, stem diameter, and branches in the *I. zollingeriana* putative mutant. While biomass production decreased, the nutritional content was high, and digestibility values showed improvement. The identified superior genotypes are promising for breeding programs and practical application in agroforestry or silvopasture systems.

CONFLICT OF INTEREST

L. Abdullah serves as an editor of the Tropical Animal Science Journal but has no role in the decision to publish this article. The authors also declare that there is no conflict of interest in any financial, personal, or relationships with organizations regarding the material discussed in this study.

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