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Gamma ray induced mutagenesis in ricebean [*Vigna umbellata* (Thunb.) Ohwi and Ohashi] for improved forage related traits

S. K. MANTRY, A. P. SINGH, [1]K. JANA, *S. SARKAR AND S. BHATTACHARYYA

Department of Genetics and Plant Breeding, [1]Department of Agronomy
Bidhan Chandra Krishi Viswavidyalaya, Mohanpur-741252, Nadia, West Bengal, India

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ABSTRACT

Research on gamma ray induced mutagenesis in ricebean for trait improvement is limited and only a few literatures are available. Hence, a study on gamma ray induced mutagenesis was carried out in a ricebean variety Bidhan Ricebean-1 (BRB-1) for crop improvement pertaining to forage related traits. Based on LD_{50} calculation, fresh BRB-1 seeds were exposed to a specific dose of gamma rays. M_1 and M_2 were grown according to the recommended package of practices. We identified potential mutants based on improvements in morphological traits as well as enhanced crude protein (CP) and crude fibre (CF) production. Substantial variation was observed in M_1 generation for morphological traits like presence of anthocyanin pigmentation on stem, extra branching, light green leaves, varying pod size and colour. In M_2 generation, a variety of putative mutants were observed. Interestingly, a few of them displayed early maturity, high forage yield, and significant increases in CP and CF values in green leaves. These promising genotypes could be evaluated in M_3 and later generation. Also, these putative mutants will serve as pre-breeding materials for the ricebean improvement programmes.

***Keywords**: BRB-1, Gamma ray, LD_{50}, Mutation, Putative, Vigna umbellata*

Ricebean [*Vigna umbellata* (Thunb.) Ohwi and Ohashi] is a viney warm season legume widely cultivated in South, Southeast, and East Asia (Guan *et al*., 2022). There are many uses for this underutilized pulse crop, including 'daal', vegetables, and fodder (Bhardwaj *et al*., 2021). This crop is nutritionally dense and is used as a cover crop and soil enricher (Pattanayak *et al*., 2019). Green pods and leaves of the plant are used as vegetables, while the seeds are used in 'daal' and sprouts. It exhibits natural resistance to many biotic and abiotic stresses and has high tolerance to bruchid infestation during storage (Somta *et al*., 2006). In addition to fixing nitrogen, this plant prevents erosion on depleted soils. The crop receives relatively few inputs in practice, and it is grown in marginal and exhausted soils using residual fertility and moisture. Ricebean can be grown as an intercrop with maize, pigeon pea, cowpea etc. Ricebean requires a short day to produce seeds. It is grown on a wide range of soils, including shallow, infertile, or degraded soils (Atta *et al*., 2022). It has been considered to be one of the finest nutritionally balanced pulses in the world and has been included in school children's nutritional programme in the Philippines (NAS, 1979). Additionally, fried ricebean seeds are frequently sold as snacks as a substitute of mungbean. This crop has the disadvantage of longer seed to seed duration than the other two Vigna species, *viz. V. radiata* (green gram) and *V. mungo* (black gram). Though it can thrive in the same conditions as cowpea and can better tolerate harsh conditions (including drought, waterlogging, and acid soils), ricebean remains an underutilised legume and there have been limited breeding programmes to improve this crop (Anand and Lingappa, 2020). Thus, the farmers mostly rely on landraces rather than on cultivars (Joshi *et al*., 2008).

Mutation breeding is one of the most efficient and quickest alternatives to conventional breeding for conferring specific improvement in a variety without significantly impairing its desirable traits.

**Email: sutanumax@gmail.com*

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Physical mutagens cause more chromosomal aberrations, and their action on DNA double helices is extremely random, resulting in a high degree of meiotic and mitotic instability. Irradiation has been used in many crops to improve plant type, yield, quality and abiotic stress resilient traits (D'Souza, 2014).

However, very few attempts have been made for rice bean in this regard. Therefore, physical mutagenesis and its influence on ricebean were studied in the present research. The present investigation reports gamma ray-induced mutagenesis of a popular ricebean variety BRB-1. The variety was released in the year 2000 from BCKV, West Bengal, India, for North-Eastern India. This variety yields 350-400 kg/ha green forage and 20qha^{-1} seed. The crop duration for BRB-1 is 180 days, which is considered late for farmers who prefer multiple crops in a given year (Anon., 2018). Hence, in this context, shortening the crop duration along with improvement in forage related traits could be expected from mutation breeding.

MATERIALS AND METODS

Gamma irradiation of seeds

Samples were subjected to gamma radiation in Gamma Chamber 5000 (GC 5000) at Regional Nuclear Agricultural Research Centre (RNARC), Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India. A setup consisting of a set of stationary ^{60}Co sources encased in a cylindrical cage was used for the irradiation process. These sources have been doubly encapsulated in corrosion-resistant stainless steel pencils and have been tested in accordance with international standards. Lead shielding provided around the source was adequate to maintain the external radiation field well within permissible limits. In the GC 5000, the effective dose rate during the experiment was 6.749 KGyh^{-1} with a chamber temperature of 35.1^{0}C.The irradiation dosage and duration of exposure are presented in table-1.

Table 1: Specifications of applied dose and time of irradiation for LD_{50} determination

Doses (Gy)	Radiation time (min)
100	0.88
200	1.77
300	2.40
400	3.33
500	4.26
600	5.20
700	6.22
800	7.11
900	8.01
1000	8.89

Thirty fresh and healthy BRB-1 seeds were taken for exposure to each dose of irradiation. Irradiated seeds were then immediately set for germination test. 30 untreated seeds were also subjected to germination as control. Seeds were allowed to germinate on glass plates lined at bottom with blotting paper. The glass plate arrangement was placed inside a water filled tray in order to facilitate continuous supply of water to blotting paper. Observations were recorded on seed germination percentage, root and shoot length of seedlings at 15 days of initiation of experiment.

Raising M_1 and M_2 generation

The field experiment was carried out at Regional Research Station, BCKV, Gayeshpur West Bengal. The experimental site is located at an altitude of 9.75 m from mean sea level with latitude 23.5 ^{0}N and longitude 89 ^{0}E. Soil of experimental field has sandy loam texture with pH lying between 6.9 and 7.0. Based on calculation of LD_{50}, 300 fresh and healthy seeds were exposed under a specific dose of 6.749 KGyh^{-1} during *kharif* 2017, and the irradiated seeds were immediately sown at Gayeshpur field. Similarly, the control seeds were also sown with a spacing of 30 x 20 cm. The recommended package of practices was followed to raise a good crop stand. Observations were recorded for variation in M_1 generation. All the plants were harvested individually and separately when the crop reached maturity. The harvested seeds were properly packaged, labelled and stored.

All the M_2 seeds from M_1 generation were subsequently sown in *kharif* 2018 along with control BRB-1 seeds following a spacing of 30 × 60 cm. The recommended package of practices was followed to raise a healthy crop. The data were recorded on putative mutants as and when required. Few M_2 plants were allowed for cutting at 30 cm above soil. They were sprayed with a 2% (wv^{-1}) urea solution two days after cutting for early recovery. The remaining plants were harvested, and the seeds were stored after proper labeling. Green forage yield, CF, and CP were estimated for all M_2 plants, individually.

Estimation of CP

Seeds from selected M_2 plants and control were subjected to CP estimation following Kjeldahl's method (Hiller *et al*., 1948). The estimation was carried out at AICRP on FC & U Laboratory, Directorate of Research, BCKV, Kalyani. This method involves the sulphuric acid digestion of organic matter in the presence of a catalyst, resulting in the production of alkaline products. The liberated ammonia is then distilled and titrated to estimate the concentration of N_2. Multiplication of the result by the conversion factor of 6.25 obtains the crude protein content.

Digestion mixture (1:19 mixture of potassium sulphate and copper sulphate), concentrated sulphuric acid, NaOH (40%), boric acid (0.1%), mixed indicator (0.5% Bromocresol green and 0.1% Methyl red in 100 ml of 95% ethanol; neutral point at pH 4.4 to 5.8) were used as reagents Nitrogen percentage was calculated using the formula as follows-

$$\text{Nitrogen (\%)} = \frac{(\text{Sample reading} - \text{blank reading}) \times 0.014 \times \text{strength of H2SO4}}{\text{Weight of sample taken (g)}}$$

Estimation of CF

The CF content of dried samples was determined using the AOAC (2005) method. CF estimation was carried out on M_2 generation and control plants at AICRP at FC and U Laboratory, Directorate of Research, BCKV, Kalyani. The samples were oven-dried at 65^0C and ground after cooling at room temperature. 2 g of powder was added to a 500 ml Erlenmeyer flask containing and 200 ml of 1.25% H_2SO_4. After boiling for 30 minutes on a hot plate, the mixture was allowed to cool and filtered. Residue thus obtained was washed with hot water, transferred back to the flask, and treated with 200 ml of 1.25% NaOH. The resulting mixture was boiled for 30 minutes, filtered, and washed with hot water and 95% alcohol. Resulting residues were then oven-dried, and their weight, along with that of the filter paper, was determined after cooling. The crude fibre content was calculated from the equation:

$$\text{Crude fibre(\%)} = \frac{W}{\textit{Weight of sample taken (g)}} \times 100$$

Where, W = (Weight of filter paper + Residue) – Weight of blank filter paper

Statistical analysis

Statistical analyses for calculation of average, standard deviation, standard error of mean were carried out in MS-Excel.

RESULTS AND DISCUSSION

LD_{50} determination

Thirty fresh BRB-1 seeds for each of the treatments, i.e., 10 gamma doses (100 to 1000 Gy) along with control seeds were set for germination test in order to calculate LD_{50}.

With doses above 600 Gy, the germination percentage of irradiated BRB-1 seeds was drastically reduced. From 200 to 600 Gy, the seeds exhibited higher germination rates than control seeds. The germination percentage is shown in table - 2 against different doses of gamma ray treatment. BRB-1 seedlings showed gradual decrease in mean shoot length with increase in gamma ray doses except in doses of 400 Gy and 600 Gy, where the mean shoot length was increased in comparison to other doses. The seeds treated with doses near to LD_{50} i.e. 900 Gy and 1000 Gy, produced very short shoots. Seedling root length followed the same trend.

Table 2: Germination, shoot length, root length of gamma irradiated BRB-1 seeds and seedlings

Gamma doses (Gy)	Germination (%)	Mean shoot length (cm)	Mean root length (cm)
0	86.67	27.20	24.74
100	70.00	18.58	19.30
200	96.67	16.63	17.83
300	96.67	15.61	14.30
400	100.00	23.46	18.49
500	100.00	18.39	18.57
600	96.67	24.84	21.00
700	70.00	19.56	18.67
800	66.67	19.56	17.40
900	30.00	13.54	12.30
1000	23.37	12.45	10.36

The graph (Fig. 1) depicts the trend line covering different germination percentages affected by various gamma doses. We drew a line parallel to the X axis at 50% germination percentage which touches the trend line. From the joining point a straight line perpendicular to the X axis was drawn and the corresponding value on X axis denoted the LD_{50} value. The equation of trend line was used to calculate the exact LD_{50} dose. Thus we obtained LD_{50} value of 959 Gy for BRB-1.

Study on M_1 generation

A dose of gamma 950 Gy was applied to raise M_1 generation. Immediately after treatment, 300 fresh and healthy BRB-1 seeds were along with 30 control seeds. Out of 300 M_1 seeds, only 170 germinated and 153 plants survived and reached maturity. Out of 30 control seeds, 18 germinated, and they all survived.

Here many variants were observed. The variants were categorized on the basis of major

phenotypic trait in comparison to the control BRB-1 plant. Prakash and Shambulingappa (1999) identified 12 types of ricebean variants. The study also revealed similar variants, including abnormal leaves, extra branches, and white pods.

a) **Pod variant:** Some of the M_1 plants exhibited clear differences in pod colour and size. More darker or light colour pods were observed as compared to the control. Pod size variants were also seen (Fig. 2).
b) **Extra branching variant:** There were more branches on M_1 plants than on BRB-1 control plants, which normally have 4 to 5 branches on their main stem (Fig. 3).
c) **Anthocyanin variant:** Dark purple pigmentation covered the main stem and branches of M_1 plants, but this pigmentation was absent from the control BRB-1 plants.
d) **Variant having light green leaves:** The leaves of M_1 plants were lighter than those of control plants.
e) **Chimeric variant:** A small number of chimeric plants were found. Many of the upper leaves of these plants had yellow spots (Fig. 3).

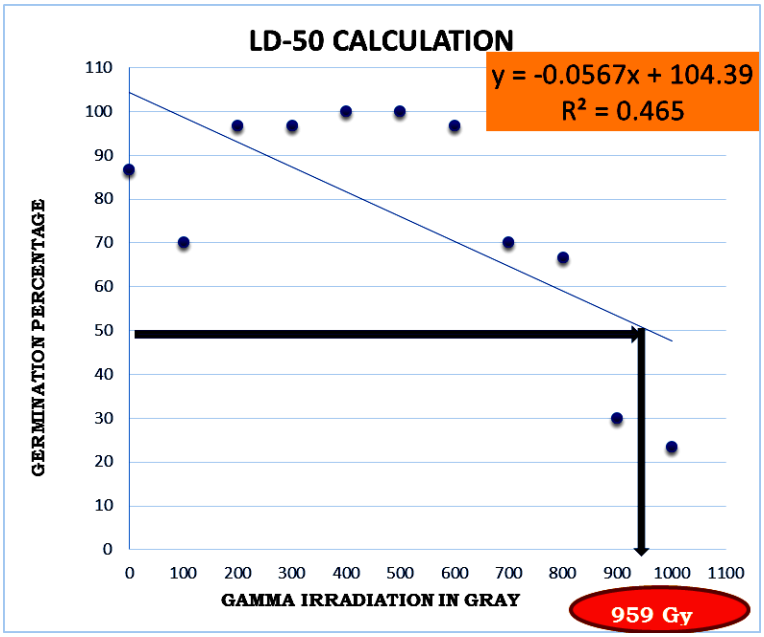

Fig. 1: Graph showing LD_{50} value

Control **Variant**

Fig. 2: Pod colour and shape variants

In spite of the adequate growth of all M_1 plants, most of them did not flower within 120 days. Some even did not flower at all within the normal 180 day period. There were only six M_1 plants capable of bearing only a few flowers, and we harvested 32 pods from these six plants. All control plants flowered regularly and bore pods as well as seeds. As a result, the remaining 147 M_1 plants other than the aforesaid six can also be classified as non-flowering mutants.

Control

Extra branching variant

Chimeric plants in M_1 generation

Fig. 3: Extra branching variant and chimera

Study on M_2 generation

For raising M_2 plants, 146 harvested seeds (from six plants that bore flower) were sown individually at 30 × 60 cm spacing. Out of them, 73 germinated and finally 68 reached maturity. In comparison with control BRB-1 plants, we observed putative mutants on the basis of leaf shape (Fig. 4), non-trailing growth, and pod color. In order to identify dual-purpose plants, we grouped M_2 plants according to flowering time. In general, BRB-1 plants flower 120 days after sowing (DAS). During the 120-day period, the initiation of flowering was observed. Accordingly, fifty-seven M_2 plants were categorized into dual purpose category 1 since they did not flower by 121 DAS, the normal flowering initiation time. Green forage was collected from this category, and once re-growth began, seeds were allowed to set. Additionally, 200 g samples from each cut were isolated for CP and CF estimations to determine the quality of the green forage. A 2% Urea solution was sprayed over them after a 30 cm cut was made above the soil. Despite the plants recovering quickly and setting new branches and leaves, no flowers were produced beyond 180 days. So, they were left in the field for the coming season to bear flowers and set pods so that seeds could be harvested. As a result, we can conclude that the cutting should be performed much sooner than we did in our study. To ensure the plans are rejuvenated and flourish so they can bear a good number of seeds, we recommend making the first cut 45 days after planting. To verify our recommendation, one must evaluate the M_3 seeds harvested the following year.

M_2 plants in dual purpose category 2 showed varying degrees of earliness (from 9 to 12 days) as compared to control plants. The 11 M_2 plants were allowed to set seeds first, and then green foliage was harvested for use as feed for ruminants. To estimate the quality of green forage, CP and CF were estimated immediately after cutting.

These 11 M_2 plants were named as L1P1, L1P4, L1P6, L3P2, L3P4, L4P3, L4P5, L5P2, L6P1, L7P1, L13P5 and their flowering initiation days were 111, 109, 109, 108, 110, 111, 109, 108, 110, 111, 109 respectively.

The control BRB-1 plants started flowering at 121 DAS. The eleven M_2 plants aforesaid were all fifty percent flowering at that time. We can't ignore such a reduction in flower initiation when searching for the early flowering trait in crops like ricebean, even though it might not be statistically significant for long duration crops like ricebean.

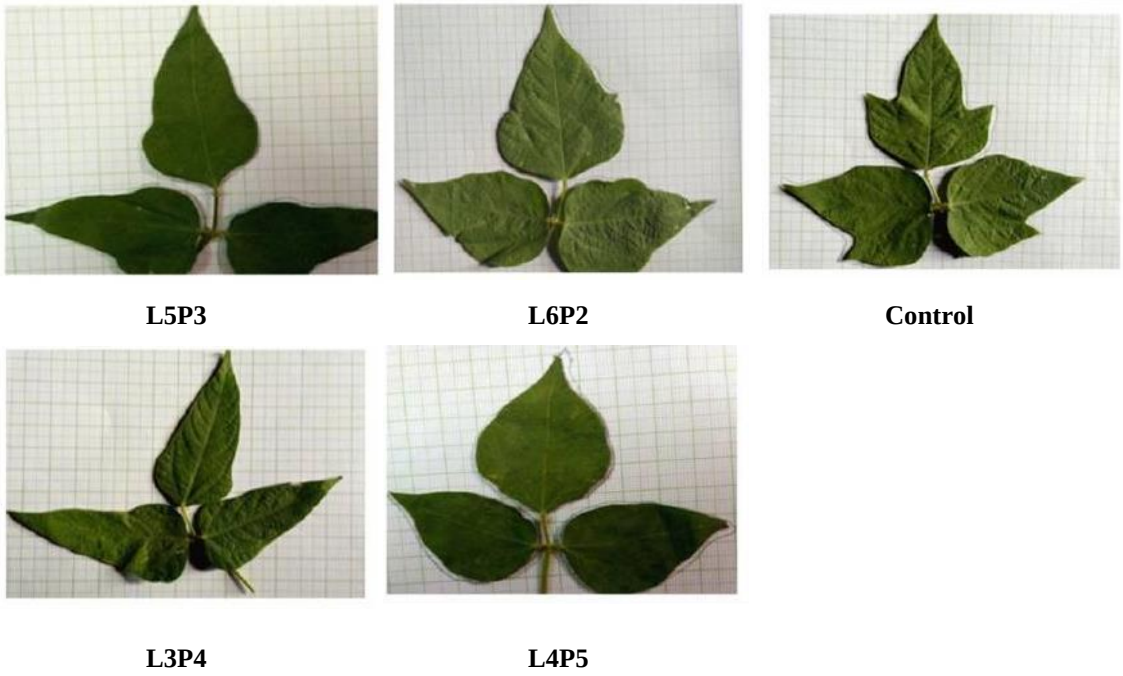

Fig. 4: Leaf shape mutants in M_2 generation as compared to control

CP, CF and yield attributing features of 2nd category plants

Yield attributing parameters such as number of pods per plant, seed yield per plant, pod length along with CP and CF from the 11 putative mutants placed in dual purpose category 2 were recorded. At that time the CP, CF and green foliage yield were from control BRB-1 were 12.56%, 57.5% and 1250 kg per plant; respectively (Table 3).

Putative mutant L4P5 had produced higher number of pods along with higher seed yield as well as higher CP and higher green forge yield than the control BRB-1. The L4P5 putative mutant is therefore suitable for selection for the aforesaid traits.

The M_3 seeds of the L4P5 M_2 plant were therefore stored and will be sown to raise the M_3 generation. The improved mutant will be sorted out after evaluating all the parameters previously mentioned. An additional putative mutant (L3P4) from this category showed higher pod length, seed yield, CP, and CF values than control (Table 4). Therefore, M_3 seeds of the L3P4 M_2 plant will be sown to raise the M_3 generation. The improved mutant will be evaluated after evaluating all the previously listed parameters. Among remaining Category 2 putative mutants L1P1 has a higher CF value, L1P4 and L1P6 have higher forage yield, L3P2 has a higher CP value, L4P3 has higher numbers of pods and higher seed yield per plant, L6P1 has a higher CF value and both L7P1 and L13P5 have higher forage yield compare to control.

Few of their trifoliate leaves are lobular in BRB-1 plants. In contrast, we observed a putative mutant in M_2 (L4P5) having only trifoliate leaves (Fig. 4). This could be regarded as an improved trait as leaf area is noticeably larger as compared to the lobbed leaves if we compare two leaves taken from the same height and position of the respected plants (Table 5).

Putative mutant L4P5 showed higher green forage yield; enhancement in the leaf area due to entireness might be one of the reasons. Further assessment of this finding will confirm this correlation between leaf shapes and green forage yield in ricebean.

Table 3: CP, CF and forage yield data of category 1, M_2 plants

Plant No.	CP (%)	CF (%)	Forage yield (g)
L1P3	12.91	51.32	1100
L1P5	13.02	52.26	600
L1P7	14.13	59.73	1250
L2P1	9.90	51.46	500
L2P2	12.3	57.48	1050
L2P3	10.12	52.55	1050
L3P1	8.49	53.36	1450
L3P3	14.16	58.57	750
L3P5	16.02	51.88	1150
L3P7	12.31	63.82	1000
L4P1	9.57	55.19	650
L4P2	11.25	48.32	1500
L4P6	16.97	51.36	300
L5P1	4.50	58.32	1500
L5P3	13.40	56.29	600
L5P4	6.40	59.34	750
L5P5	13.12	57.71	750
L5P6	7.70	56.32	1100
L5P7	4.40	59.21	800
L6P2	14.87	59.41	600
L6P3	9.50	47.31	1300
L6P4	9.45	51.29	950
L6P5	5.80	52.66	700
L7P2	12.26	49.53	550
L7P3	15.05	49.32	550
L7P4	5.30	57.32	450
L9P1	9.50	50.31	400
L9P2	12.30	48.18	700
L9P3	12.77	55.72	200
L9P4	14.62	63.45	150
L9P5	12.00	57.11	550
L9P6	9.90	52.32	900
L9P7	12.95	61.46	450
L9P8	11.60	44.78	950
L10P1	13.12	50.13	441
L10P2	14.50	52.63	600
L10P4	11.90	46.87	750
L11P1	10.42	61.42	650
L11P2	14.85	58.32	1200
L11P3	4.40	66.74	300
L11P4	14.77	63.62	800
L11P5	9.90	50.87	1100
L12P1	5.20	65.40	1200
L12P2	5.90	59.42	115
L12P4	12.40	51.72	550
L12P5	9.20	55.22	600
L13P1	12.50	55.31	750
L13P2	7.48	62.42	450
L13P3	13.96	59.32	450
L13P4	4.50	53.17	550
L13P6	12.55	55.29	100
L14P1	12.10	59.22	650
L14P3	3.00	68.56	200
L14P4	9.97	53.32	450
L14P5	5.77	46.91	300
L14P6	12.49	48.29	500
L14P7	4.40	67.61	500
Control	12.10	59.52	950
Mean	**10.62**	**55.62**	**713.89**
SD	**3.53**	**5.64**	**350.83**
SEm (±)	**0.46**	**0.74**	**46.07**

Table 4: CP, CF and other yield contributing factors of Category 2 M_2 plants

Plant No.	No. of pods	Pod length (cm)	Seed yield (g)	CP (%)	CF (%)	Forage yield (g)
L1P1	333	7.6	67.17	12.77	66.50	1050
L1P4	196	6.2	54.82	9.27	56.50	1750
L1P6	305	7.6	71.50	12.44	55.00	1350
L3P2	252	5.2	76.28	14.77	58.00	1300
L3P4	380	8.7	105.55	15.6	64.00	950
L4P3	449	8.2	107.57	12.37	55.50	1200
L4P5	495	7.8	154.84	15.05	46.50	1450
L5P2	352	8.0	89.11	10.26	52.00	1000
L6P1	312	5.8	61.76	12.42	61.00	1250
L7P1	291	7.2	77.77	10.97	54.00	1600
L13P5	180	8.5	38.75	12.25	56.5	1550
Control	370	8.1	86.17	12.56	57.5	1250
Mean	**326.25**	**7.41**	**82.61**	**12.58**	**57.08**	**1308.30**
SD	**92.84**	**1.11**	**30.12**	**1.89**	**5.30**	**246.60**
SEm (±)	**26.80**	**0.32**	**8.69**	**0.54**	**1.53**	**71.20**

Table 5: Classification of M_2 morphological putative mutants

Category	Mutant name	Characteristics
Category 1	L5P5	Non- trailing putative mutant
	L5P3, L6P2	Leaf shape putative mutant
Category 2	L3P4, L4P5	Leaf shape putative mutant
	L13P5	White pod putative mutant

CONCLUSION

A less explored aspect of genetic improvement of ricebean is mutation induction to isolate genotypes with superior fodder-related attributes. In order to improve forage-related traits in BRB-1, the present study utilized gamma irradiation. The BRB-1 seeds were treated with a specific dose of gamma ray after LD_{50} was calculated. The morphological variations in the M_1 generation were chimera formation,, anthocyanin pigmentation on the stem, more branches, light green leaves, and varying pod sizes and colours as observed visually. There were several putative mutants seen in the second generation of mutant plants. There were a few mutants that were early maturing, had high forage yields, and significantly higher crude protein, as well as fibre content, in the green leaves. M_3 and later generations of these promising genotypes will be tested in order to release improved varieties. In addition, those genotypes can serve as pre-breeding material for ricebean improvement.

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