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GAMMA-RADIATION MUTAGENESIS OF *Hibiscus cannabinus* L. V36 VARIETY: RADIOSENSITIVITY STUDY, PHENOTYPIC CHARACTERIZATION AND MULTIVARIATE ANALYSIS TO EXPLAIN VARIATION AMONG SELECTED M$_1$ PROGENIES

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ABSTRACT

Kenaf (Hibiscus cannabinus L.) is one of the world's most economically important fiber crops particularly in Asia-Pacific region. Mutation induction is a method to increase genetic divergence associated with selection, recombination, or a combination of these approaches in plant breeding. One of preliminary procedures for an execellent mutation breeding program is the radiosensitivity study to determine the optimal doses for irradiation. A total of 10 different doses of acute gamma rays (0, 100, 200, 400, 800, 1000, 1200, 1500, 1700 and 2000 Gy) from ceasium-137 source were applied to the seeds of V 36 kenaf variety. The irradiated seeds including the control were planted in trough for 30 days. The gamma irradiation effects on several parameters such as seedling survival percentages, plant height, root length, shoot fresh weight, root frest weight, shoot dry weight and root dry weight were analysed. From the radiosensitivity curve, the LD_{50} and LD_{25} values were estimated at 810 and 310 Gy, respectively. Two doses (200 Gy and 300 Gy) based on LD_{25} were chosen for evaluating the effects of gamma irradiation on morphological traits in M_1 generation. Irradiated and non-irradiated seeds were planted in the field at Beseri, Perlis. About 10 phenotypic traits of irradiated plants were observed and evaluated against the controls. Cluster analysis on M_1 progenies showed that the mutation could be classified into eight genotypic groups. The first two components from principal component analysis explained about 77.99% of variation. Number of seeds per pod, weight of seeds per plant and dry stem biomass play an important role in explaining the variation since they showed positive correlated values for the first component analysis. Morphological changes such as flower shape, flower colour, and leaf shape were also observed in M_1 generation. The findings of this study are important in determining the effectiveness of these doses in generating mutations on kenaf plant and subsequent breeding program to develop new kenaf varieties with enhanced quality traits.

Keywords: Gamma irradiation, *Hibiscus cannabinus* L., kenaf, multivariate analysis, mutation, phenotypic traits, radiosensitivity study

INTRODUCTION

Hibiscus cannabinus L. is a plant that belongs to Malvacae family. It is one of the most important sources of raw materials for industries related to fibers and papers. In 1999, Malaysia's National Economic Action Council (NEAC) has identified kenaf as a new commodity crop to be cultivated in Malaysia to replace tobacco (Basri et al., 2014). Since 2000, a number of research related to kenaf were conducted by research institutions and universities in Malaysia mainly in the identification of varieties adaptable to local climate, good agronomic practices, harvesting and mechanization as well as fiber processing and downstream application development such as animal feed and biocomposite. However, research on developing new varieties of kenaf is still lacking in Malaysia.

Development of new kenaf varieties that produce high biomass is essential for successful cultivation of kenaf. Unfortunately, the yield of kenaf in Malaysia is around 5 to 10 tonnes per hectare which is very low compared to other countries. The highest yield achieved was about 9.8 tonnes per hectare from V36 (Basri et al., 2014). Furthermore, there are lacks of kenaf variety suitable to be planted in Malaysian climate. Photoperiod insensitive and late flowering have been identified as the most essential traits required for growing kenaf plants in a tropical country like Malaysia based on previous research (Daud, 2006; Hossain et al., 2011; Wong et al., 2001). In order to overcome the problem on the lack of new varieties, development of new kenaf variety with selected target traits is crucial. Mutation induction is the best way to develop new varieties with the desirable traits in a short time compared to conventional breeding. This technique is used nowadays by plant breeders to develop new varieties by generating and utilizing genetic variability through both chemical and physical mutagenesis (Shu et al., 2012). Additionally, mutation induction becomes an important tool in plant breeding to broaden existing germplasms and improve cultivars in certain specific traits (Kurobane et al., 1979).

Lethal dose (LD) is an important measurement to determine the optimum dose for irradiation. It is a measurement that will determine the percentage of population that dies because of radiation. Usually, LD_{50} and LD_{25} are used as bench marks in radiosensitivity tests in determining the suitable doses for subsequent mutation induction of plants. LD_{50} means 50% of the irradiated samples die because of irradiation (Albokari et al., 2012) and at the same time, the other 50% survived. The same meaning applied to LD_{25} where 25% of the irradiated population dies after irradiation whilst the other 75% survived. LD_{50} normally creates high frequency of mutation while LD_{25} can be considered as well where large number of undesirable mutation can be minimized in order to determine the suitable dose for mutation induction.

Hence, the ultimate aim of this research were i) to conduct the radiosensitivity study of *Hibiscus cannabinus* L. (Variety V36) for determination of optimal dose for irradiation, ii) to characterize 10 phenotypic traits in selected M1 progenies of *Hibiscus cannabinus* L. (Variety V36) and, iii) to explain variation among the phenotypic traits in selected M1 progenies of *Hibiscus cannabinus* L. (Variety V36) using multivariate analysis.

MATERIALS AND METHODS

Plant Materials

Kenaf (*Hibiscus cannabinus* L.) variety V36 was used in this study and was provided by National Kenaf and Tobacco Board (LKTN), Perlis. Seed germination rate and moisture content were determined prior to irradiation of seeds.

Radiosensitivity Study

Kenaf V 36 variety seeds were irradiated at 10 different doses of acute gamma rays (0, 100, 200, 400, 800, 1000, 1200, 1500, 1700 and 2000 Gy) using Biobeam GM 8000 (Germany) at Malaysian Nuclear Agency. Flat method (Shu et al., 2012) was applied for sowing the seeds in trough (1 m x 3 m) filled with sand and peat. The randomized complete block design (RCBD) with 5 replications sown in different trough in the glasshouse was used. The plants were watered daily and weed control was done manually. Plant growth was monitored for 30 days in the glasshouse. After 30 days, morphological data such as: (i) seedling survival percentages; (ii) plant height; (iii) root length; (iv) shoot fresh weight; (v) root fresh weight; (vi) shoot dry weight and (vii) root dry weight were determined by measuring 10 plants per dose in each replicate and their means were used for further analysis.

Phenotypic Evaluation of Mutant Line M_1 Generations

Approximately 3 kg of kenaf seeds were treated with 3 selected dose treatments: 0 Gy, 200 Gy and 300 Gy. The irradiation process was conducted using Biobeam GM 8000 (Germany) at Malaysian Nuclear Agency. The irradiated (200 Gy and 300 Gy) and non-irradiated kenaf seeds were planted at LKTN field plot in Beseri, Perlis. The fertilizers used were NPK green at 450 kg/ha after 1 day of planting and NPK blue at 450 kg/ha after 45 days of planting. Additionally, pesticide and herbicide were also applied to control insects and weeds. The M_1 generation and its control plants were harvested at 135 days after planting. About 43 plants that differed from the control were selected and 10 phenotypic traits were recorded during harvesting period. These traits include: (i) plant height (cm); (ii) number of pod per plant; (iii) number of node per plant; (iv) length of internode (cm); (v) stem girth at bottom (cm); (vi) middle (cm); (v) top (cm); (vi) number of seed per pod; (vii) weight of seed per pod and (viii) dry stem biomass per plant (g).

Data Analysis

Morphological data collected from radiosensitivity study were subjected to Analysis of Variance (ANOVA) using SAS Version 9.2. Survival rate (%) reduction curve were constructed using a simple linear regression (Excell 2007) for determination of LD_{50} and LD_{25}. Mean, range, Coefficient of Variance (CV), and standard deviation among morphological traits of M_1 progenies were determined using SAS Version 9.2. A dendrogram was then constructed based on arithmetic average (UPGMA) and euclidian distance using NTSYS version 2. The correlation matrix of the 10 quantitative characters was used to conduct principal components analysis (PCA) using SAS version 9.2. The eigen values as a proportion of the total variance gave the relative contribution (%) of each principal component to the observed variation. Variables that had extreme high or low coefficients for each component were noted.

RESULTS

i) Radiosensitivity Study

All dose treatments including the control showed around 80% survival rate at 7 and 14 days after planting (DAP). The survival rate was further decreased after 30 days of planting especially for doses above 800 Gy (Table 1). Survival rate percentage at 30 DAP showed significant differences at $p \leq 0.05$. Survival rate (%) reduction curve for different gamma ray irradiation doses were shown in Figure 1. From this graph, the LD_{50} and LD_{25} values for *Hibiscus cannabinus* L. V36 variety were obtained at 810 Gy and 310 Gy, respectively.

Table 1: Survival rate percentage (%) of irradiated kenaf seeds at 0, 100, 200, 400, 800, 1000, 1200, 1500, 1700 and 2000 Gy after 7, 14 and 30 days after planting

	7 DAP	**14 DAP**	**30 DAP**
Dose		**Survival Rate (%)**	
0	80^{a}	80^{a}	80^{abc}
100	80^{a}	84^{a}	84^{ab}
200	84^{a}	78^{a}	74^{bc}
400	88^{a}	88^{a}	88^{a}
800	88^{a}	86^{a}	68^{c}
1000	88^{a}	90^{a}	48^{d}
1200	82^{a}	82^{a}	12^{e}
1500	88^{a}	88^{a}	6^{e}
1700	78^{a}	82^{a}	0^{e}
2000	80^{a}	88^{a}	0^{e}

Mean with different letter(s) in column are statistically different among treatment by LSD test ($p \leq 0.05$). DAP means day after planting

Plant height showed decreasing trend as doses increased after 400 Gy (Table 2). The highest plant height recorded in this study was obtained at 200 Gy with an average of 40.4 cm while the shortest value was about 4.4 cm for seeds irradiated at 1500 Gy, both recorded at the 30^{th} day after planting. Based on the survival rate, doses above 1700 Gy killed all the seedlings before the 30^{th} day and therefore no data could be recorded.

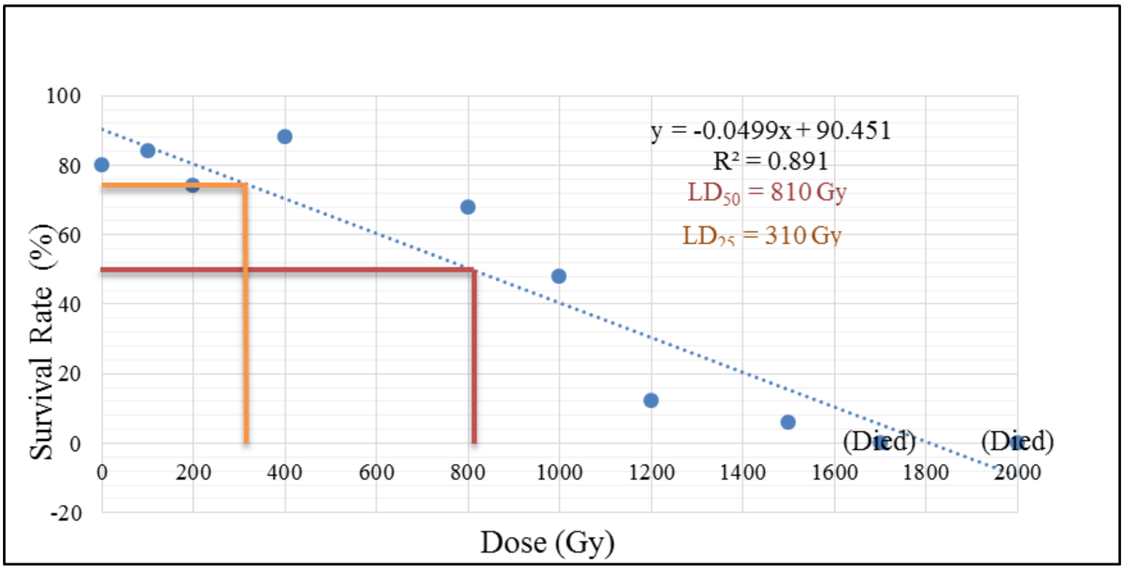


Figure 1: Radiosensitivity curves for LD $_{25}$ and LD $_{50}$ for cultivar *Hibiscus cannabinus* L. V 36 variety

Table 2: Plant height (cm) of irradiated kenaf seeds at 0, 100, 200, 400, 800, 1000, 1200, 1500, 1700 and 2000 Gy after 7, 14 and 30 days after planting

	7 DAP	**14 DAP**	**30 DAP**
Dose	**Plant Height (cm)**		
0	8.24^{a}	9.64^{c}	30.94^{c}
100	8.26^{a}	14.32^{b}	38.81^{a}
200	9.58^{a}	14.96^{ab}	40.38^{a}
400	8.98^{a}	16^{a}	35.19^{b}
800	5.22^{b}	6.42^{d}	12.39^{d}
1000	4.18^{bc}	4.68^{e}	7.69^{e}
1200	3.76^{bc}	3.94^{e}	7.31^{e}
1500	3.10^{c}	3.48^{e}	4.38^{e}
1700	3.04^{c}	3.44^{e}	0(died)^{f}
2000	3.00^{c}	3.18^{e}	0(died)^{f}

Mean with different letter(s) in column are statistically different among treatment by LSD test ($p \leq 0.05$). DAP means day after planting

Root length also showed the same trend as observed in plant height, which is inversely proportional to dose treatment. The highest root length obtained was 11.6 cm at 200 Gy while the shortest root length (5.4 cm) was observed on irradiated plants at 1500 Gy (Figure 2).

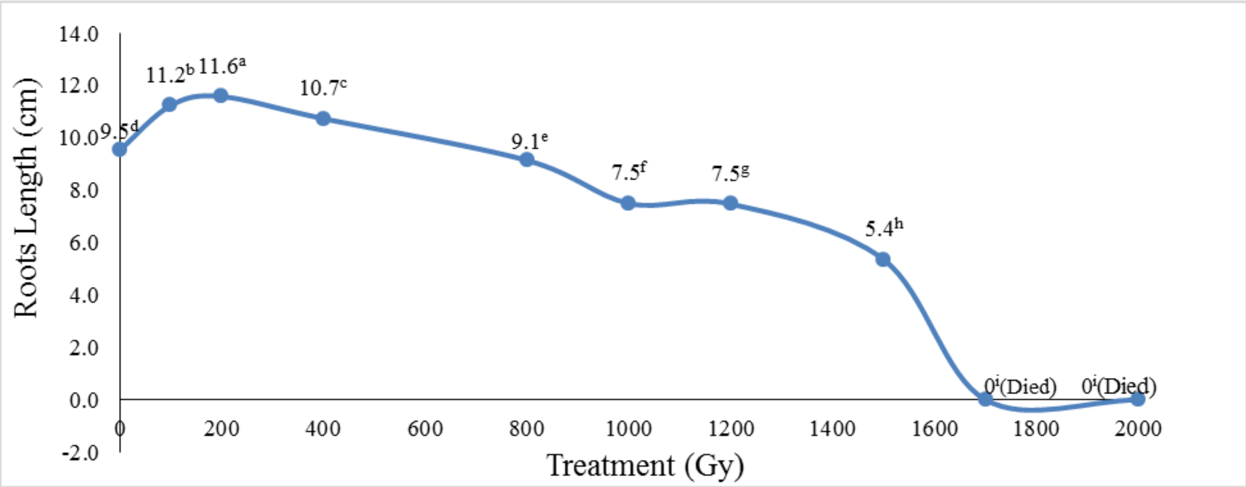
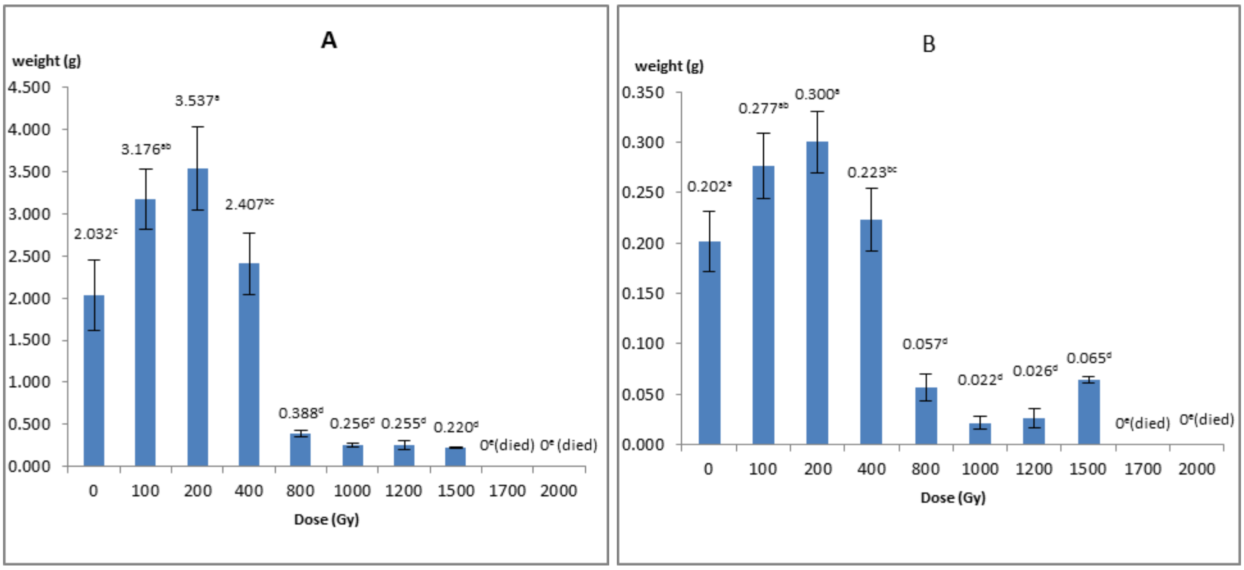


Figure 2: Root length (cm) of irradiated and non-irradiated kenaf seeds vs dose treatment (Gy) at the 30th day of planting

Note: Means with different letters column are statistically different among treatment by LSD test ($p \leq 0.05$).

Fresh weight and dry weight for stem and roots were recorded and illustrated in Figure 3. It was found that seeds treated at 200 Gy produced the highest fresh stem weight which was approximately 3.54 g and the highest average fresh root weight which was 0.30 g, as shown in Figure 3. The lowest fresh weight for stem was recorded on plant irradiated at 1500 Gy which was 0.22 g while the lowest root fresh weight was produced by plant irradiated at 1000 Gy which was 0.02 g. Meanwhile, for dry weight, the highest value for stem was recorded on sample irradiation at 100 Gy which was 0.36 g whilst the lowest was obtained from those irradiated at 1500 Gy which was 0.06 g.



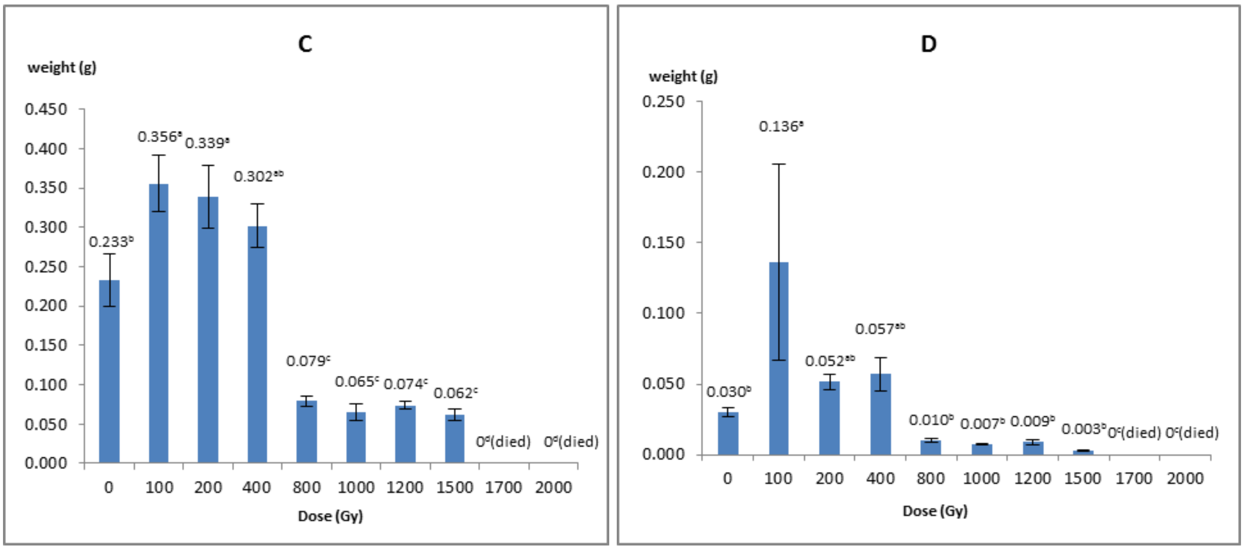


Figure 3: Fresh weight and dry weight for stem and roots of irradiated and non-irradiated kenaf at 30th day of planting. (A) Stem fresh weight (B) Root fresh weight (C) Stem dry weight (D) Root dry weight

Note: Means with different letters column are statistically different among treatment by LSD test ($p \leq 0.05$).

ii) Phenotypic Trait of Kenaf M_1 Progenies

High variability among all morphological traits was observed in M_1 progenies of kenaf (Table 3). The most widely varied morphological characters were seed weight per plant, number of pod per plant, dry stem biomass and number of seed per pod with 57.10%, 44.23%, 33.03%, and 31.32% coefficient of variance (CV), respectively.

Table 3: Mean, range, coefficient of variance (CV) and standard deviation among phenotypic traits of M_1 progenies

Traits	Mean	Range	CV (%)	Standard Deviation
Plant height	261.14	220 - 347	10.40	27.16
No of pod per plant	92.28	35 - 125	44.23	40.81
Node per plant	52.24	24 - 83	25.11	13.12
Internode length	5.17	3.0 - 8.7	24.70	1.28
Stem girth at bottom	20.35	14.4 - 30.5	16.47	3.35
Stem girth at middle	14.63	8.3 - 18.2	19.87	2.91
Stem girth at top	4.90	2.1 - 7.2	24.49	1.20
Number of seed per pod	17.42	4.6 - 48.0	31.32	5.46
Seed weight per plant	28.94	2.8 - 73.7	57.10	16.53
Dry stem biomass	162.74	86 - 383	33.03	53.76

iii) Clustering Analysis From Morphological Traits

The similarity coefficient as shown in the dendrogram varied from 0.12 to 1.73 as shown in Figure 4. All mutant progenies could be pooled into 8 genotypic groups. The mean value for morphological traits in each group was summarized in Table 4.

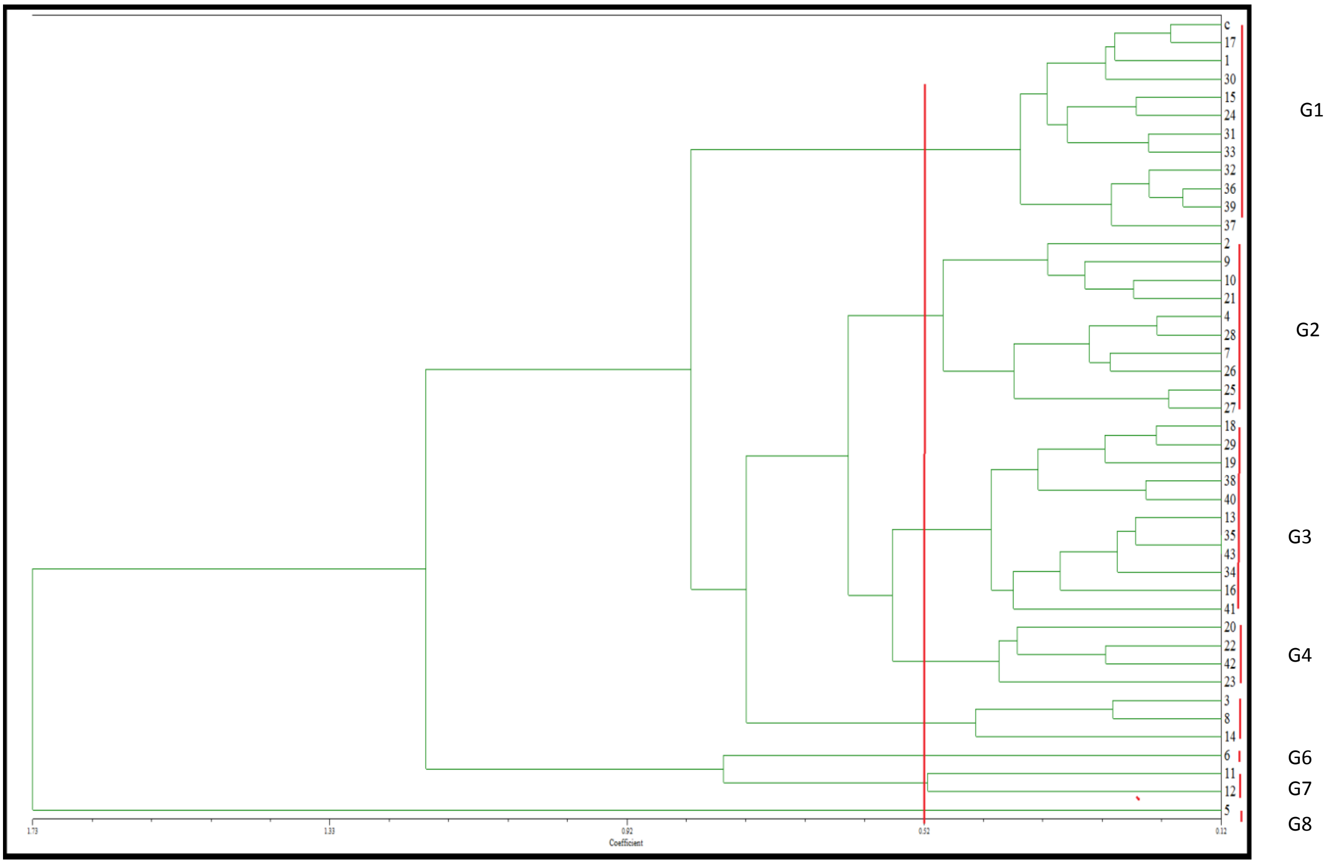


Figure 4: Cluster analysis from morphological traits of kenaf V36 variety and its M1 progenies

Table 4: The mean value for 8 genotypic groups of kenaf V36 variety and its M1 progenies

Traits	G1	G2	G3	G4	G5	G6	G7	G8
Plant height	258.65	271.20	262.36	255.50	248.50	275.00	236.50	266.00
Pod per plant	53.56	122.80	102.73	70.00	102.75	215.00	160.50	43.00
Node per plant	47.78	62.70	56.18	34.50	49.25	75.00	44.50	49.00
Internode length	5.44	4.78	5.54	4.15	5.13	3.60	4.52	7.60
Stem girth at bottom	17.54	22.86	19.18	20.20	23.39	20.26	26.85	16.40
Stem girth at middle	14.55	14.30	16.09	14.33	12.80	14.78	12.88	11.40
Stem girth at top	12.10	4.46	12.87	8.50	5.11	5.80	5.70	5.10
Number of seed per pod	12.69	18.80	17.45	20.75	20.50	18.00	21.50	20.00
Seed weight per plant	14.13	39.65	32.72	36.34	26.68	44.64	25.27	24.52
Dry stem biomass	114.30	197.50	143.00	153.25	193.75	198.00	232.00	383.00

iv) Principal Component Analysis From Morphological Traits

All morphological traits showed the highest cumulative percentage (≥ 70%) except for plant height (53.55%), number of pod per plant (66.96%), stem girth bottom (56.47%), seed weight per plant (67.98%), and dry stem biomass (53.09%) as shown in Table 5.

Table 5: Principle component analysis (PCA) explanation for 3 components of morphological traits

Traits	PC1	PC2	PC3	Final Communality	Percentage
Plant height	0.056	0.703	-0.194	0.536	53.551
Pod per plant	0.476	0.567	-0.349	0.670	66.956
Node per plant	0.258	0.794	-0.089	0.704	70.421
Internode length	0.046	-0.087	0.863	0.755	75.520
Stem girth at bottom	0.560	0.152	-0.478	0.565	56.465
Stem girth at middle	-0.312	0.810	0.352	0.877	87.655
Stem girth at top	0.635	0.464	0.460	0.830	82.963
Number of seed per pod	0.849	-0.279	0.037	0.800	80.014
Seed weight per plant	0.746	0.317	0.152	0.680	67.977
Dry stem biomass	0.678	0.210	-0.165	0.531	53.088
Eigen value	2.85	2.57	1.53		
Cumulative (%)	41.01	78.00	100.00		
Variation (%)	41.01	36.99	22.00		

DISCUSSION

Gamma induced mutation is the fast and quick method to develop a new variety which has superior genotype than its parent. Before irradiating any plant sample, radiosensitivity tests need to be done for determination of optimum dose range for irradiation. Radiosensitivity test is the relative measure that gives an indication of the quantity of recognizable effects of radiation on the irradiated objects (Morishita et al., 2003). It was varied within genotypes and cultivars. From this study, it was found that survival rates as well as the other parameters were decreased as the doses of gamma rays increased.

Previous studies also showed that some characters such as plant height and root length were decreased with the increase in the gamma ray dose treatment as seen in *Capsicum annuum* species (Omar, 2008). It is believed that irradiation might cause a block in cellular DNA and the growth rate of the irradiated plant will become slower (Mokobia and Anomohanran, 2004). The LD_{50} value was considered as an optimum dose for getting the highest mutation frequency but it may cause higher DNA damage that would result in large number of undesirable mutations (Britt, 1996). Lower dose treatment such as LD_{25} will minimize DNA damage and reduce large number of undesirable mutations and therefore chances of isolating desirable mutations will be higher. In this study, two doses (200 Gy and 300 Gy) were chosen within the LD_{25} dose range for screening and selection on desired mutants in the field. These two doses were chosen in order to reduce the number of undesirable traits and to increase the possibility of obtaining desired mutants.

Screening and selection of desired mutants are also among the critical steps before developing a new potential mutant. Assessment of important morphological traits in M1 progeny is very important to determine the effectiveness of the dose in generating mutation. Girija and Dhanavel (2013) reported role of wide variety of morphological traits in the induction of mutations in M1 generation of Cowpea. Based on the results obtained, yield component traits showed high variations among kenaf M1 progenies. The variation was seen as a manifestation of environmental response in addition to the genotypic constitution. Seed weight per plant, number of pod per plant, dry stem biomass and number of seed per pod showed the coefficient of variation more than 30% as discussed previously in Table 3. Variations in these traits are very promising for breeding purposes. The wide range observed for many of the parameters shows the complexity in describing the difference among kenaf M_1 progenies. Wide variability for morphological and agronomical characters in collections of *Hibiscus cannabinus* L. has also been reported before (Siepe et al., 1997).

The dendrogram for morphological traits was constructed based on the matrix of average taxonomic distance using the UPGMA method. Cluster analysis on morphological traits in kenaf V36 variety and its M1 progenies showed that the genotypes were classified into 8 groups. Kenaf V36 parental variety with 11 other mutant genotypes fell into group 1 and this group accounted for the highest number of genotypes. This group exhibited the lowest number of seed weight per pod and dry stem biomass with 14.13 g and 114.30 g respectively. Meanwhile, group 8 and 7 recorded the highest number of dry stem biomass with 232 g and 383 g, respectively. Seed weight per plant showed the highest value in group 6 with 44.64 g respectively. This was due to the highest number pods per plant recorded in this group. From this dendrogram, a wide genetic diversity in terms of morphological traits was obtained from M1 progenies. Ghanei et al. (2013) also discovered about 8 classified groups among single brunch Naz varieties in their M1 mutation progenies.

Principle component analysis (PCA) is another multivariate analysis for verifying the result from cluster analysis. By PCA, 10 traits were simplified into 3 principle components. A total of 78% of the total variance was explained by the first two principal components as described previously in Table 5. Thus, the variation among the 10 quantitative traits was quite high with more than 70% for first two components. However, about 66.51% of total variance was explained by the first two principal components among 16 kenaf genotypes (Faruq et al., 2013). In first principle component, all the traits showed the positive correlation except stem girth at the middle. Number of seed per pod, seed weight per plant and dry stem biomass showed the highest correlation value with 0.849, 0.746, and 0.678, respectively. Meanwhile, plant height, number of nodes per plant and stem girth at middle showed the highest correlation value at second principle component. Seed weight per plant, number pod per plant, stem girth at top and dry stem biomass have important roles to explain the variation since they showed highest and positive correlated values for each two principle component. Thus, these traits have the essential role in order to select superior kenaf mutant lines with desirable traits. Internode length recorded the lowest and negative correlated by two principle components and was therefore redundant in the variation observed. Selecting important traits which explained high variation for each principal component are needed for selecting kenaf progeny with good yield characteristics.

Kenaf has been reported to have a wider range of adaptation to environmental factors as compared to other fibre plants cultivated for commercial use (Dempsey, 1975). Thus, performance of the accessions especially in relation to the quantitative traits may be location and environment specific.

CONCLUSIONS

Increasing gamma ray doses has been found to decrease seedling survival rate, plant height, root length, shoot fresh weight, root frest weight, shoot dry weight and root dry weight. The LD_{50} and LD_{25} values for survival rate percentage were 810 Gy and 310 Gy, respectively. In this study, the most widely varied traits were seed weight per plant, number of pods per plant, dry stem biomass and number of seed per pod with 57.10%, 44.23%, 33.03%, and 31.32% coefficient of variance (CV) respectively. The 8 groups were distinguished by 10 morphological traits recorded in this study. The study on the effects of morphological trait in M_1 kenaf progeny of V36 variety explores the effectiveness of gamma irradiation in creating genetic variability which is very useful for the improvement of kenaf varieties in Malaysian tropical environment.

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