American Journal of Plant Sciences, 2021, 12, 1945-1962
https://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Gamma Irradiation Enhanced Leaf Bioactive Components and Bioassay Parameters in M_5 Mulberry (*Morus* Sp.) Mutant

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How to cite this paper: Ramesh, H.L., Shivashankarappa, L.H. and Yogananda-murthy, V.N. (2021) Gamma Irradiation Enhanced Leaf Bioactive Components and Bioassay Parameters in M_5 Mulberry (*Morus* Sp.) Mutant. *American Journal of Plant Sciences*, **12**, 1945-1962.
https://doi.org/10.4236/ajps.2021.1212134

Received: May 30, 2021
Accepted: December 26, 2021
Published: December 29, 2021

Abstract

Gamma radiation is an effective tool for inducing genetic variation in plant characters. In the present experiment, M_5 mulberry variety juvenile twigs were subjected to source Co^{60} gamma irradiation (1 kR - 10 kR) and mutants grown in triplicates in randomized block design to raise M_1 and M_2 generation. In M_2 generation plants were subjected to phytochemical and bioassay tests. Silkworm rearing parameters and commercial characters of cocoons were recorded by feeding cross breed silkworms. Results show that M_5 mutant leaves revealed significant variations in phytochemical constituents and moisture content. Bioassay tests recorded significant differences compared to control in M_2 generation. Commercial characters like cocoon weight (1.41 g), shell weight (0.24 g), shell percentage (16.29 %), filament length (821.00 mts), renditta (8.2), denier (2.24) and effective rate of rearing (92.14 %) were increased. It is concluded that, gamma rays treatment enhances the mulberry plants leaf bioactive components, silkworm rearing and cocoon parameters and shows beneficial variants in M_2 generation.

Keywords

Bioassay, Cocoon, Commercial Characters, Gamma Rays, Genotype, Irradiation, Mulberry, M_5, Phytochemical

1. Introduction

Mulberry plant (*Morus alba* L.) is an imperative food crop for silkworms [1],

extensively cultivated throughout tropical, subtropical and temperate areas in the world, and exhibits substantial plasticity due to its heterozygous nature. India being a vast country with complex agro climatic zones, has a wide scope for cultivating a number of elite mulberry varieties in different areas [2]. Cross pollination is the rule rather than exception in mulberry and this provides a high degree of heterozygosity and genetic diversity in the mulberry gene pool. Mutations can be induced through irradiations, both ionizing (X-rays, β-rays, gamma rays and fast neutrons) and non-ionizing (ultraviolet rays) radiations [3]. Mutation breeding has been widely employed in recent times for improving vegetatively propagated crop plants. The fresh and nutritious quality of mulberry leaf plays a significant role in growth and development of silkworms alleviating the cocoon production and silk efficiency [4]. Mutation breeding has the potency to induce variability and causes vagaries in the genetic constitution of DNA through addition or deletion or frame shift mutation. Mutation induction in mulberry started towards the end of 1950's in Japan [5] [6]. Mutation breeding has been widely employed in recent times for improving vegetatively propagated plants and gamma rays have proved to be highly effective in inducing variability in mulberry plant [7] [8].

Gamma irradiation is extensively used as an alternative agent for improving genetic diversity in agriculture due to its high diffusion ability [9]. Efforts were made to stimulate plants development by exposing seeds or growing plants to low doses of ionizing radiation, dating back to 1960s [10]. Low and high ionizing radiations were used extensively in breeding programs as they cause mutations beyond wide spectrum [11]. Gamma rays are the most energetic form of electromagnetic radiation and possess an energy level from 10 keV (kilo electron volts) to several hundred keV and are considered as the utmost penetrating radiations compared to alpha and beta rays [12]. The indications often witnessed in irradiated plants were enhancement of germination, seedling growth and other biological responses [13]. The exposure to gamma irradiations can have stimulatory effects on specific morphological parameters and can increase the plants yield in terms of growth and to withstand water shortage ability [14]. It was witnessed that low doses of gamma rays stimulated cell division, growth and plants development [15].

The nutritional quality of leaves is determined by its major components, such as water, dietary fiber, carbohydrates, proteins, micronutrients, fats, amino acids and vitamins [16] [17] [18]. The cultigen, harvesting time and degree of mulberry leaves maturity influence the functional and nutrient components significantly [19] [20]. Tender leaves contain more active substances than mature leaves [21]. Nutritional quality of leaves fluctuates from variety to variety and season. Quality is highly influenced by cultivation practices, leaf preservation techniques, age and position of leaves [22]. Moisture content of leaves has direct bearing on the growth and development of silkworms by favoring ingestion, assimilation and excretion of nutrients. Quality of mulberry leaves directly influ-

ences the commercial characters of cocoon viz., cocoon weight, shell weight, filament length, denier and renditta [23]. Bioenergetics mainly focuses on the energy transformation and rate of ingestion and percentage of digestibility in larvae play a crucial role in the biosynthesis of raw silk production. Present investigation aims at evolving mulberry mutant through gamma irradiation to ascertain the superiority of dominant mutant of the genotype by subjecting the leaves to phytochemical analysis and bioassay tests.

2. Materials and Methods

Juvenile twigs of M_5 mulberry genotype were used for cuttings and only middle parts of the twigs were taken in order to maintain the optimum moisture and desired carbon-nitrogen ratio [24]. Each cutting with 15 cm length contains 2 - 3 active vegetative buds free from pathogen and pests were prepared. During cuttings preparation care was taken to avoid damage to the buds and cut ends [25] [26]. Cuttings were irradiated with different doses of gamma rays (1 kR - 10 kR) from Co^{60} gamma unit at Indian Institute of Horticulture Research, Hessaraghatta, Bangalore. Irradiated cuttings were planted in earthen pots filled with dried pulverized garden soil, fine sand and decomposed farmyard manure mixture in 1:1:1 proportion and maintained with consistent care [2] [27]. Three replications with 25 cuttings each were maintained for six months before transplanting them in to main garden and transplanted saplings were maintained in randomized block design with 90 cm × 90 cm spacing. Necessary cultural operations like timely irrigation, weeding, intercultivation, manuring, protection against desiccation, diseases and pests were ensured [28] [29]. Suitable controls were also maintained in similar conditions for comparative studies. Data related to phytochemical analysis and bioassay tests were recorded at M_2 generation.

Phytochemical analysis

Freshly harvested leaves were washed in distilled water to remove adhering particles and blotted with tissue paper. 10 g leaf sample was taken with 100 ml of distilled water in a clean mortar and pestle and ground well. The homogenate was centrifuged at 3000 RPM for 20 min, the supernatant was collected and dried, used for further analysis.

Estimation of Proteins

Protein contents were estimated according to Lowry's method [30]. In this process, 10 mg of leaf extract was dissolved in 10 ml of water, from this 0.1 ml, 0.2 ml and 0.3 ml (100, 200 and 300 µg) was taken, to this 5 ml of alkaline copper reagent was added and incubated at room temperature for 10 minutes. Then 0.5 ml of folin-ciocalteau reagent was added and incubated for 30 minutes at room temperature in Dark. The optical density was measured at 660 nm against a blank in systronics colorimeter (105 MK). Bovine Serum Albumin was taken as the standard and the concentration was expressed in percentage.

Estimation of Total Soluble Sugars

Total soluble sugars were assessed following the procedure using anthrone

reagent [31]. In this process, 100 mg of leaf extract was taken in a test tube and hydrolysis by adding the 5 ml of 2.5 M HCL and boiled for three hours and then neutralise with sodium carbonate until the effervesce ceases and then filter the solution. Filtrate was dissolved in 100 ml of water, from this 0.1 ml, 0.2 ml and 0.3 ml (100, 200 and 300 μg) was taken and 4 ml of anthrone reagent was added from the side walls of the test tube and shaken gently. The mixture was incubated in a boiling water bath at 90˚C for 10 minutes for colour development and it was cooled to room temperature. Optical density was measured at 625 nm against a blank using systronics colorimeter. The amount of sugar present in the extract was measured using a standard curve prepared using the anhydrous glucose and the concentration were expressed in percentage.

Estimation of Free Amino Acids

Free amino acids were analysed by adopting modified ninhydrin method [32]. 100 mg of leaf extract was hydrolysed by 1 ml of 0.2 M HCL and add 10 ml of 80% ethanol. Filter the sample and filtrate is made up to 100 ml. From this 0.1 ml, 0.2 ml and 0.3 ml (100, 200 and 300 μg) was taken and 0.2 ml of methyl ninhydrin solution was added. The mixture was incubated in a boiling water bath for 15 min and cooled to room temperature. The volume was then made up to 5 ml with 60% ethanol. Optical density was measured at 570 nm against a blank in a systronics colorimeter. The amount of amino acids present in the extract was calculated by using a standard curve prepared from glycine and the concentration was expressed in μ mole/gm.

Estimation of Phenols

Estimation of phenols was done according to folin-ciocalteau method [33]. In this process, 100 mg of the leaf extract was taken in 80% ethanol and centrifuged at 10,000 rpm for 10 min, supernatant was taken, dried and dissolved in 100 ml water. From this 0.1 ml, 0.2 ml and 0.3 ml (100, 200 and 300 μg) was taken, 1 ml of FC reagent and 2 ml of sodium carbonate were added in a test tube, agitated gently and heated for 1 min in boiling water bath. It was cooled to room temperature and the volume was made up to 5 ml with distilled water. Optical density was measured at 650 nm against a blank in the systronic colorimeter. The amount of phenols present in the extract was calculated by using a standard curve prepared from gallic acid and the concentration was expressed in mg/gm.

Estimation of Chlorophyll Contents

The extraction of chlorophyll from fresh mulberry leaves without maceration was carried out according to the procedure [34]. 50 mg of leaf bits were taken in a glass vial and 7 ml of dimethyl sulphoxide (DMSO) was added to it and kept for 3 hours in an electric oven at 65˚C. Finally the volume of the extract was made up to 10 ml with DMSO and assayed immediately. Chlorophyll-a, chlorophyll-b and total chlorophyll contents were estimated according to procedure [35]. 3 ml of chlorophyll extract was transferred to a cuvette and optical densities of chlorophyll-a and chlorophyll-b were measured at 645 nm and 663 nm respectively by using systronics 105 (MK 1) spectrometer against a DMSO blank. Chlorophyll contents were calculated using following equations.

$$\text{Total chlorophyll} = \frac{20.2D_{645} + 8.02D_{663} \times 1000 \times \text{weight of leaf (g)}}{V}$$

$$= \text{mg of total chlorophyll per gram fresh weight}$$

$$\text{Chlorophyll-a} = \frac{(12.7 \times D_{663}) - (2.67 \times D_{645}) \times 1000 \times \text{weight of leaf (g)}}{V}$$

$$= \text{mg of chlorophyll-a per gram fresh weight}$$

$$\text{Chlorophyll-b} = \frac{(22.9 \times D_{645}) - (4.68 \times D_{663}) \times 1000 \times \text{weight of leaf (g)}}{V}$$

$$= \text{mg of chlorophyll-b per gram fresh weight}$$

where V = Volume of the extract, D = Optical density.

Determination of Leaf Moisture Content

Leaf moisture content was determined on fresh weight basis [36]. For each maturity, 25 leaves/replication/variety were harvested separately from a longest shoot and leaves were wiped with a muslin cloth to remove dust particles and fresh weight was recorded immediately. Then leaves were dried in hot air oven at 80˚C for 48 hours till constant weight was attained and dry weight was recorded. Leaf moisture content of tender, medium and coarse leaves was calculated separately by using following formula and expressed in percentage (%).

$$\text{Leaf moisture content (\%)} = \frac{\text{Fresh weight of leaves} - \text{Dry weight of leaves}}{\text{Fresh weight of leaves}} \times 100$$

Silkworm Rearing Analysis

Plants showing beneficial characters in M_2 generation were subjected to bioassay studies to ascertain the feeding efficiency. Disease free crossbreed (PM × NB_4D_2) silkworm's layings (DFL's) were procured from National Silkworm Seed Project (NSSP), Bangalore. Totally 100 larvae were reared and three replications were maintained in each experiment. Appropriate cellular rearing techniques were conducted with congenial atmosphere for the growth and development of silkworm larvae [37] [38]. Cocoons were harvested on 5th day of mounting and assessed for commercial parameters viz., hatching percentage, larval weight, effective rate of rearing (ERR), cocoon weight, shell weight, shell percentage, total filament length, renditta and denier to evaluate the rearing performance and standard methods were employed for the cocoon quality assessment [39].

Statistical Analysis

The experiment was conducted in triplicates and the results were statistically analyzed using one-way anova in Graph PadPrism. All the standards were calculated by regression correlation values above 0.99 and Pearson's correlation is less than 0.05.

3. Results

Phytochemical Studies

M_5 mutant mulberry leaves were used for the phytochemical tests and their nutritional values were tabulated in **Table 1** and the graphical appraisal was de-

fined for easy understanding (Figure 1). Phytochemical profile of the gamma irradiated mutant and control leaves were escalated in the order tender > medium > coarse leaves. It is pertinent to note that protein percentage was better in leaves with low gamma dose (4 kR) than control leaves. It was observed that protein increased over control plants in tender (7.5%) leaves. Medium (10.14%) and coarse (8.94%) leaves fared better over control and indicated the values of medium (8.29%) and coarse leaves (7.87%) respectively (Figure 1(a)). Tender leaves with high protein is voraciously consumed by first and second instar silkworms for their growth and development compared to medium and coarse leaves consumed by third, fourth and fifth instar silkworms for qualitative cocoons.

Irradiated plants in M_2 generation showed significant rise in total soluble sugars in coarse (6.39%) < medium (7.58%) < tender (7.94%) leaves compared to control coarse (6.28%) < medium (6.28%) < tender (7.78%) leaves respectively (Figure 1(b)). It was observed that accumulation of amino acid appeared in the order of tender (57.29 µmole/gm) > medium (53.69 µmole/gm) > coarse (51.21 µmole/gm) in control compared to treated tender (56.31 µmole/gm) > medium (54.72 µmole/gm) > coarse (52.48 µmole/gm) leaves (Figure 1(c)). Phenols are secondary metabolites boost the immunity in mulberry plants and no significant results were observed. Phenolic compounds gradually decrease from tender-medium-coarse leaves in both the control and treated plants. Control plants showed better results over the counter parts and analyzed 6.79 µmole/gm, 7.97 µmole/gm and 7.48 µmole/gm compared to 6.82 µmole/gm, 6.99 µmole/gm and 7.01 µmole/gm respectively (Figure 1(d)).

No significant results were observed in Chlo-a (Figure 1(e)) and Chlo-b (Figure 1(f)) but total chlorophyll content yielded fruitful results. Coarse leaves (5.69 mg/gf·wt) showed better results over control coarse leaves (4.58 mg/gf·wt).

Table 1. Phytochemical constituents of M_5 mulberry mutant leaves recovered at 4 kR gamma irradiation (M_2 generation).

Treatment	Leaf maturity	Proteins (%)	Total soluble sugars (%)	Amino acids (µmole/gm)	Phenols (mg/gm.)	Chlo-a (mg/gm.)	Chlo-b (mg/gm)	Total Chlorophyll (mg/gm)	Moisture content (%)
Control	Tender	09.04	7.78	57.29	6.79	3.04	1.05	3.80	69.07
	Medium	08.29	7.47	53.69	7.97	4.17	1.49	4.98	66.15
	Coarse	07.87	6.28	51.21	7.48	4.23	1.35	4.58	61.21
Mutant	Tender	12.18	7.94	56.31	6.82	3.18	1.15	4.01	72.01
	Medium	10.14	7.58	54.72	6.99	4.06	1.52	5.02	62.19
	Coarse	08.94	6.39	52.48	7.01	4.39	1.39	5.69	56.28
SEM	-	01.38	0.78	00.57	-	-	-	0.08	02.97
CD at 5%	-	02.27	0.91	00.98	NS	NS	NS	0.29	03.76

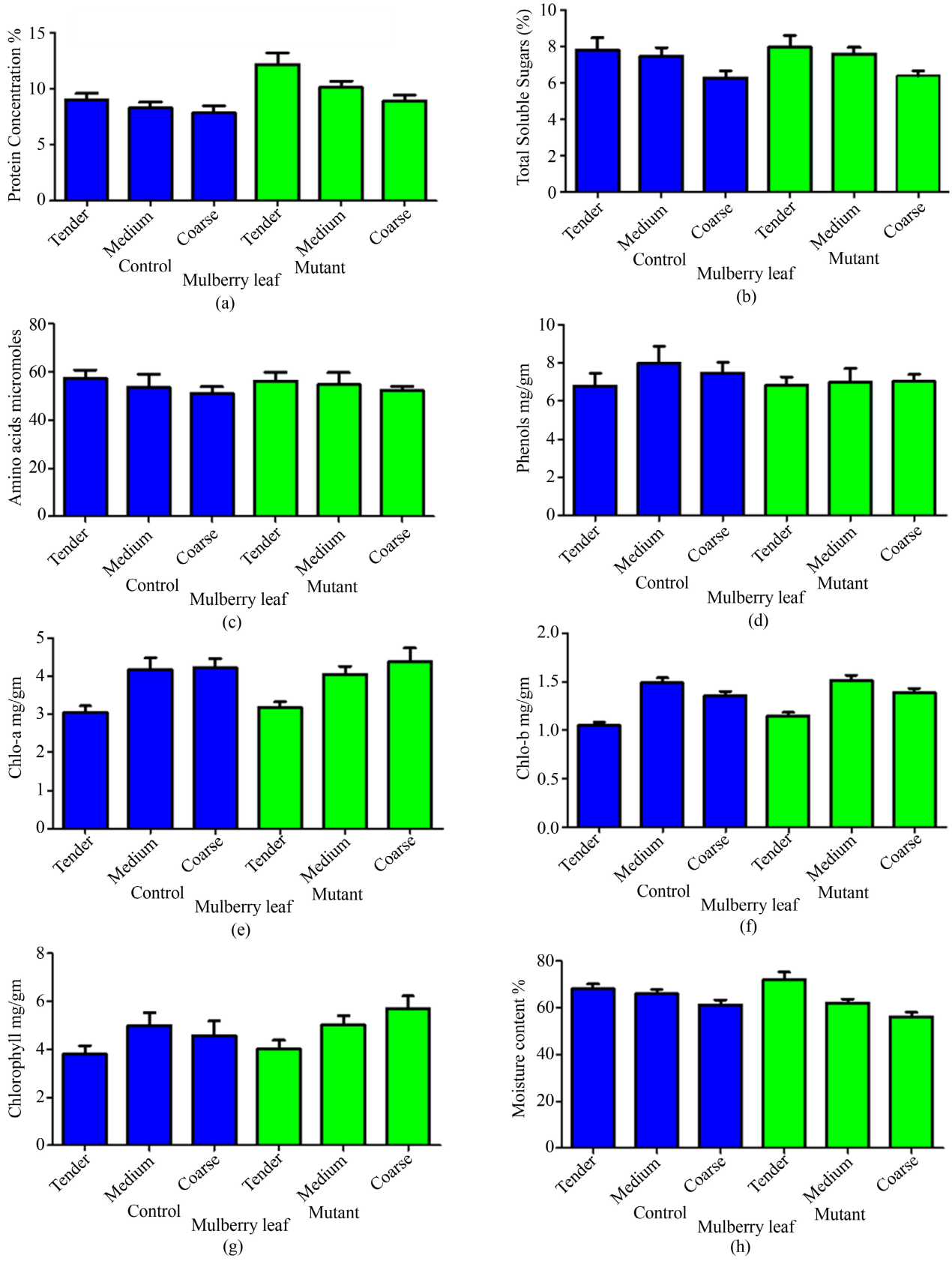

Figure 1. Different phytochemical constituents analysis of M_5 mutant mulberry leaves in M_2 generation.

Total chlorophyll content in medium and tender leaves also markedly vary compared to same order leaves in the control counterpart (Figure 1(g)). Significant increase in moisture content was observed in tender leaves (72.01%) of treated population than control (69.07%). In medium leaves of control plants (66.15%) showed better accumulation of water over treated plants (62.19%). Coarse leaves demonstrated 61.21% and 56.28% in control and irradiated plants respectively (Figure 1(h)).

Bioassay Studies

Bioassay parameters of silkworms reared on M_5 mutant mulberry leaves were evaluated and documented in Table 2 and the graphical assessment (Figures 2(a)-(i)) was well-defined for easy understanding. Due to the presence of β-sito sterol, morin, tannin as secondary metabolites in mulberry leaves, they

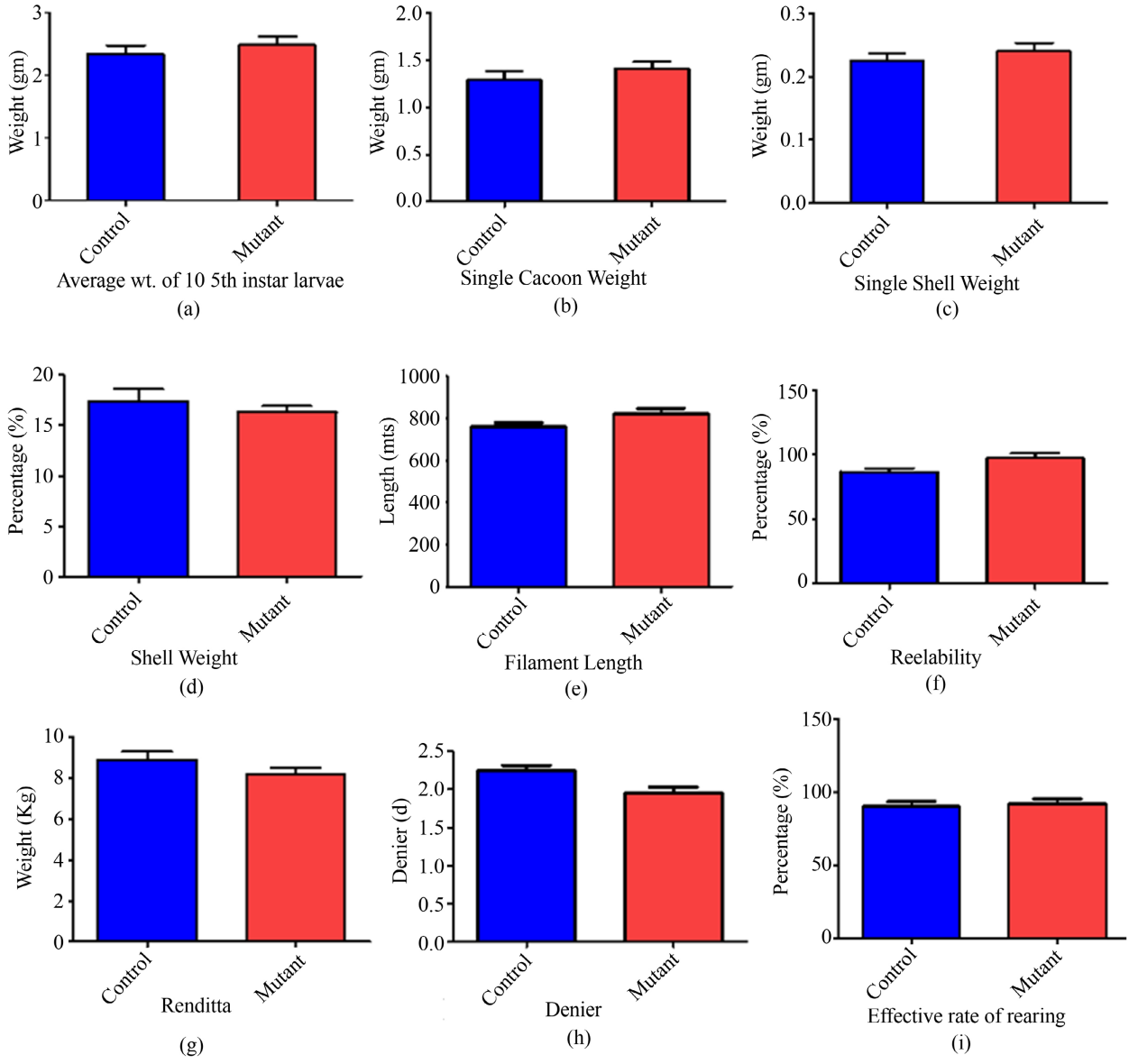

Figure 2. Analysis of silkworms reared on M_5 mutant mulberry leaves and commercial characters of cocoons in M_2 generation.

Table 2. Silkworm rearing performance of cross breed (PM × NB_4D_2) race fed with M_5 mulberry mutant leaves (M_2 generation).

Treatment	Average wt. of 10 5th instar larvae (g)	Single cocoon weight (g)	Single shell weight (g)	Shell weight (%)	Filament length (mts)	Reelability (%)	Renditta (kg)	Denier (d)	Effective rate of rearing (%)
Control	2.35	1.32	0.21	15.90	748.00	85.66	8.90	2.24	88.91
Mutant	2.49	1.41	0.24	17.02	821.00	97.28	8.20	1.95	92.14
SEM	0.12	0.08	-	0.78	42.00	9.81	0.04	0.58	1.78
CD @ 5%	0.18	0.10	NS	1.04	68.20	11.04	0.09	0.94	2.91

Hatching percentage: 95%.

constitutes the only food for silkworms *Bombyx mori* L. The mulberry leaves obtained in M_2 generation were fed to cross breed (PM × CSR_2) silkworms (**Figure 3**) to test the quality of irradiated leaves for economic characters of the cocoons (**Figure 4**). Results revealed the significant variations in cocoon weight (1.41 g), filament length (821.0 mts), renditta (8.2), denier (1.95) and effective rate of rearing (92.14%) in cocoons obtained from silkworms fed with irradiated leaves compared to cocoon wt. (1.32 g), filament length (758.0 mts), renditta (8.9), denier (2.24) and effective rate of rearing (89.91%) in control lot respectively.

4. Discussion

Gamma rays are a class of ionizing radiations intermingles with atoms or molecules to produce free radicals in cells. These radicals can adapt significant components of plant cells and subsequently affect different morphological, anatomical, biochemical and physiological characters of plants. These changes are predominately depending on the strength and duration of the gamma irradiation level [40]. Low dose irradiation brings in hormonal signaling network in plant cells or by increasing the antioxidant capacity due to fluctuating light and temperature on the growth and development of plants and accumulation of water. It was anticipated that these mutational effects might have induced variations in cellular structure and metabolism like dilation of thylakoid membranes, alteration in photosynthesis and pigment accumulate [41] [42].

Fenugreek plants exposed to higher dose of irradiation (400 Gy) showed significant decrease in soluble protein content. Results obtained by other authors also showed that total proteins reduced with increased gamma ray dosage caused by higher metabolism and hydrolyzing enzyme activities in germinating seeds [43]. Protein degradation and recycling are essential response of the plants to irradiation since the breakdown of proteins generate free amino acids required for de novo proteins synthesis [44]. Lower values of protein content observed at the highest dose of gamma irradiation (400 Gy) compared with lower doses and control plants. Gamma irradiation formed Disulphide Bridge between polypeptide chain that may affect the aggregation and conformation of low molecular

Figure 3. Silkworms reared on M_5 mutant mulberry leaves in M_2 generation.

Figure 4. Cocoons recovered from silkworms reared on M_5 mutant mulberry leaves in M_2 generation.

weight protein [45]. Mulberry leaves are rich source of proteins, carbohydrates, total chlorophyll and total carotenoids, ascorbic acid and mineral elements [46] [47]. Deficiency of certain nutrients or an imbalance of nutrients in leaves cause changes in the composition or metabolic activity of silkworms [48] [49]. Carbohydrates, proteins and lipids play an important role in the biochemical process underlying growth and development of insects [50] [51]. Total soluble sugars exist in the form of water soluble carbohydrate and are vital for carbohydrate metabolism. Sugar content in mulberry leaves in closely related to silkworms health. Mulberry leaves with high sugar content field's good rearing results [52] [53] [54]. Total soluble sugars exert a positive role in the alleviation of imposed stress via osmotic adjustment in plants [55]. Gamma irradiation induces several changes and cells in order to prevent structural proteins and to maintain membrane phospholipids in liquid crystalline phase, soluble sugar accumulate in the stressed cell [56]. Due to lower dosage of gamma irradiation, total soluble sugars significantly changed and the behaviour denotes increasing osmoprotectants may come as defensive mechanism caused by the pre exposing gamma rays

leading to more enzymes involved in the anabolism [57]. It was noticed in Chamomile that irradiation with 0.0 - 10 krad showed gradual increase in total soluble sugars and enhancement of carbohydrate content [58]. In *Eruca vesicaria* pre sowing gamma irradiation treatment at 20 Gy dose enhanced the total soluble sugars. It is decided that lower doses of gamma rays is effective in synthesis of carbohydrate in plants [59].

Amino acids acts as intermediates in metabolism and results depicted that control leaves performed better compared to irradiated leaves. The changes in amino acid induced by irradiation could probably due to free radicals that might be formed in association with splitting of peptide bonds, deamination and decarboxylation reaction of amino acid followed by chain of chemical reactions forming new radicals. Ecological conditions are known to markedly influence the total nitrogen that in turn affects the relative proportion of essential amino acids [60]. Amino acids increased significantly after irradiation and lysine, histidine and arginine decreased with increased irradiation doses. Change in the amino acid content would be considered as a result of biological effect of gamma irradiation [61]. Phytochemical property such as phenol has indirect effect on fodder quality. Biochemical analysis indicated that status of phenol content may change due to irradiation treatment that may cause oxidative damage and make the leaves to lose flavor. Gamma irradiation decreased the total phenol content of M_2 mutant extracts compared to control in almond hull [62] [63]. The effect of ionization on phenolic compounds is dose dependent, they act as defense against pathogen and gamma irradiation may fluctuate phenolics of plant metabolites in soybean samples [64]. Gamma irradiation destructive process of oxidation was capable of breaking chemical bonds of polyphenols, liberating soluble phenols of low molecular weight [65]. Fresh leaf analysis demonstrated enhancement of total chlorophyll content. Biochemical variation observed in chlorophyll contents of irradiated and non-irradiated leaves after four weeks of study. Irradiation with gamma rays may damage the chlorophyll pigments with simultaneous loss of photosynthetic ability [66]. Low intensities of gamma ray are more effective in producing chlorophyll mutation [67].

Plant pre-treated with lower dose (20 Gy) of gamma irradiation induced changes in chloroplasts [68]. Plants irradiated with low dose gamma rays (20 Gy) improved photosynthesis, increased water and chlorophyll content of soybean [69]. Plants irradiated with gamma rays (20 Gy) induce amplified photosynthetic activity and leaves lose less water [70]. In mulberry leaves, moisture content plays a vital role in improving nutrition levels that in turn advance the palatability and digestibility of leaves by silkworms as well as normal growth and development of silkworms and cocoons quality [71]. It is a genetic character and influenced by available soil moisture and root proliferation nature of mulberry variety [72]. Availability of moisture content in mulberry leaves enhances silkworm feeding efficiency, in turn increases growth rate [73]. Importance of dietary moisture content in relation to silkworm growth was emphasized that, decrease in leaf

moisture content influenced different energetic parameters such as assimilation and conversion efficiency of food that decreases with reducing dietary moisture content of leaf. It is a recognised fact that, moisture content of mulberry leaves declined gradually with an increase in leaf growth and varieties [74].

Different doses of gamma irradiation produce unrestricted radicals that may induce unfavorable or beneficial characters in plant cells. Higher dosages damage the cell and lower exposure stimulates the various biological activities [75]. Lower dosage of gamma rays may improve biochemical component of leaves consumed to improve the economic traits [76]. Growth and development of silkworm *Bombyx mori* L. is known to fluctuate depending on the mulberry leaf quality and quantity used as food that in turn designated by commercial characters of cocoons [77]-[82]. It was reported that S_{41} variety with higher protein and lower sugar content exhilarated higher larval weight [83].

5. Conclusion

Attempt has been made to induce variation for important morpho-economic traits in mulberry using gamma source. Utility of gamma irradiation helps in gene expression of antioxidant enzymes in the production of reactive oxygen species and induces oxidative stress in cells. It was concluded from the present studies that lower dose (4 kR) of gamma irradiation brought vagaries of characters in propagation and growth attributes. Promising results recorded in plant height, rooting, intermodal distance, number of leaves and leaf area in M_2 mutants, because mutations induced by gamma irradiation are mainly recessive and can only be selected in advanced generations. M_5 mulberry variants grown in M_2 generation provided beneficial results in chemo assay and bioassay study. It is a primary analysis and requires several generations of propagation with multilocational trials for bioactive components and bioassay parameters to confirm the dominance of the mutant.

Acknowledgements

Authors are thankful to Indian Institute of Horticulture Research, Govt. of India, Bangalore for providing technical assistance and irradiation of mulberry cuttings in Co^{60} gamma chamber located in the Institute.

Conflicts of Interest

Authors have declared no potential conflict of interest regarding the publication of this paper.

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