Induction of Useful Mutation in Mulberry (Morus) Variety S_{54} by Gamma Irradiation in M_{1} Generation

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Authors' contributions

Present research work was carried out in collaboration between all authors. Author HLR conducted complete analysis of the study, wrote the protocol and drafted of the manuscript. Author VNYM managed literature survey and computer word processing. Author MR designed the experiment and supervised overall experiment. All authors read and approved the final manuscript.

ABSTRACT

Aims: Plentiful mulberry varieties available in nature, they lack one or the other important economic trait required for silkworm Bombyx mori L. as food. Efforts have been made to induce phytomorphological variability in mulberry variety S_{54} using gamma rays.

Experimental Design: RBD Method with three replications/treatment was followed.

Place and Duration of Study: Mulberry garden, Department of Sericulture, Jnana Bharathi, Bangalore University and Mist chamber, Indian Institute of Horticultural Research (IIHR), Bangalore, Karnataka, India between 2006-2011.

Methodology: Gamma ray (1kR-10Kr) was used to induce variability in juvenile twigs of mulberry for various agro-botanical characters viz., sprouting, rooting, internodal distance, leaf area, plant height etc. and leaves were subjected to biochemical analysis.

Results: Mulberry variety S_{54} showed linear decrease in growth parameters with the increased gamma ray dosage and plants exhibited variability with increased rooting.

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(81.33%), plant height (147.86cm) and leaf area (146.22cm²) when compared to control in M₁ generation at 7kR. Mutants showing favourable characters were grown for M₂ generation which exhibited marked improvement in growth and yield parameters. Biochemical constituents in S₅₄ mutant leaves recorded at 7kR showed increased proteins, carbohydrate, chlorophyll a and b.

**Conclusion:** Mulberry cuttings irradiated with gamma ray (7kR) exhibited favourable traits in rooting, plant height and leaf area over the control in M₁ generation and mutants were grown for M₂ generation and marked improvement in growth, yield and bio-chemical parameters were observed.

**Keywords:** Mulberry; gamma; irradiation; agrobotanical; yield; proteins; total sugars; chlorophyll.

1. **INTRODUCTION**

Mulberry is a fast growing, sub arboreal deciduous plant found in tropical, subtropical and temperate climates of northern hemisphere and is capable of thriving under wide range of agro-climatic conditions. Mulberry exhibits plasticity and is a versatile plant. Successful exploitation of various mutagenic agents for inducing aberration has become one of the most important lines of contemporary research. S₅₄ mulberry variety leaves are well suited for rearing young age (Chawki) silkworms. Mutation induction in mulberry started towards the end of 1950’s in Japan [1,2,3]. Mutation induction techniques such as radiation or chemical mutagens are good tools for increasing variability in crop species because spontaneous mutations occur with an extremely low frequency. Mutation techniques have significantly contributed to plant improvement worldwide and have made an outstanding impact on the productivity and economic value of some crops. Mutation breeding has been widely employed in recent times for improving vegetatively propagated crop plants and gamma rays have been proved to be highly potent in inducing variability in mulberry plant [4]. Radiation is a tool for inducing variability in crop plants [5]. Investigation pertaining to the radiation effect was reviewed [6]. Radiation such as x-rays and gamma rays found to affect biological events such as survival percentage, seed germination, growth and yield of the plant [7,8,9]. Many workers used physical mutagens for induction of variability in mulberry [10,11,12]. Exposure to gamma radiation is known to produce morphological mutants, physiological and biochemical mutants [13]. Mutation breeding make use of the possibility of altering the genes by exposing different parts of mulberry plants to physical mutagens [14,15]. Present investigation aims at improving morpho and phytochemical traits of already existing mulberry cultivar S₅₄.

2. **MATERIALS AND METHODS**

2.1 Study Area

Mulberry variety S₅₄ was procured from mulberry germplasm bank maintained at Jnana Bharathi Campus, Bangalore University, Bangalore. The field experiment was conducted at mulberry germplasm bank maintained at Jnana Bharathi Campus and laboratory experiments were done at Indian Institute of Horticultural Research (IIHR), Bangalore and Moriculture Laboratory in the Department of Sericulture, Bangalore University.
2.2 Sampling and Sample Analysis

Juvenile twigs of S₅₄ mulberry genotype were used for cuttings preparation, only middle part of the twigs were taken. Newly prepared juvenile cuttings were irradiated with different doses of gamma rays (1kR to 10kR) from Co⁶⁰ gamma unit installed at the Indian Institute of Horticulture Research (IIHR), Hesaraghatta, Bangalore-560088. Irradiation was conducted during summer months and replications were maintained for the calculation of mean values of all the parameters studied. Irradiated cuttings were planted in earthen pots which were filled with a mixture of well dried pulverized garden soil, fine sand and well decomposed farmyard manure in the proportion of 1:1:1 with three replications having ten cuttings each maintained for six months before transplanting them in to the main field. Transplanted twigs were planted in randomized block design (RBD) with 90cm x 90cm spacing. Necessary cultural operations such as timely irrigation, weeding, intercultivation, manuring, protection against desiccation, diseases and pests were ensured. Suitable controls were maintained in similar conditions for comparative studies. At M₁ and M₂ generations, data related to growth responses such as sprouting, rooting, survivability, internodal distance, branching pattern, leaf area and pollen fertility were recorded [16,17,18].

2.3 Statistical Analysis

Data collected on various parameters were tabulated using “Method of Analysis of Variance” appropriate to the experimental design [19,20].

3. RESULTS AND DISCUSSION

Sprouting percentage in the irradiated population of S₅₄ mulberry variety ranged from 78.49% to 36.28% when compared to control plants (92.48%). Sprouting percentage decreases with the increase in doses of gamma rays (Table 1). Control plants sprouted 5ᵗʰ-6ᵗʰ day after planting and plants treated at 1kR-5kR gamma rays took 11-13 days to sprout. Higher doses (6kR-10kR) took 15 days to sprout. Drastic reduction in the sprouting percentage was observed in cuttings irradiated with 7kR-9kR. At 10kR, though the irradiated cuttings sprouted initially, they failed to grow further exhibiting complete lethality. Present results are in conformity with the findings of other workers [21,22]. Sprouting is adversely affected by higher doses of gamma rays. Sensitivity of plant material depends on the genetic constitution, DNA amount, dose employed, replication at initial stages, stage of development and genotype. Gamma rays are highly penetrating in nature, might have developed cells which are undergoing meiotic division in bud region [23]. Decrease in sprouting percentage with the increase in gamma ray dosage is due to partial cell death and also due to destruction of auxin or due to inhibition of auxin synthesis [24,25,26]. Reduction in sprouting and survival percentage of vegetatively propagated crops was reported by several workers [27,28]. Rooting percentage revealed that, control plants of S₅₄ mulberry variety showed 88.33% of rooting. The irradiated hardwood stem cuttings of this taxa exhibited varied responses to different doses of gamma rays. Rooting was not affected much at 1kR and 2kR. However, rooting percentage was decreased from 3kR-9kR. It is interesting to note that at 7kR 81.33% of rooting was observed. Rooting behaviour of a variety is purely a genetic character [29]. Spontaneous mutants of KNG variety produced low percentage of rooting (16%-32%) when compared to control (96%) [30]. Survivability in S₅₄ mulberry cultivar was recorded 88%. In treated population, considerable decrease in survivability percentage was observed. Data recorded in the present investigation revealed that, in lower doses (1kR and 2kR), treated population exhibited better survival rate and at higher doses considerable
decrease in survivability was noticed. At 8kR and 9kR, plants showed stunted growth, weak and feeble branches. Survivability percentage was maximum at 1kR (81.49%) and minimum at 8kR (35.18%). Destruction of auxin in treated plants may be the reason for decrease in survival percentage after radiation [31]. Series of events occurring at cellular level affect the vital macromolecules and results in physiological imbalance [32]. Low doses of gamma ray irradiation could be used as safe as well as effective method in mulberry and survivability depends on the disturbances caused at the physico-chemical level in cells or acute chromosomal damage or due to combined effect of both [33]. Retardation of root growth is one of the most common responses of plant subjected to ionizing radiation. 10kR exposure induced 100% inhibition of growth in Pinus [34]. Fasciation induced by gamma rays was observed in Gerbera jamesonii and this may be due to the triggering of gene responsible for hormonal action of cytokinins production due to point mutation [35].

Gamma rays known to influence plant growth and development by inducing cytological, genetical, biochemical, physiological and morphogenetical changes in cells and tissues. Reduction in plant height may be attributed to a drop in auxin level, inhibition of auxin synthesis or decline in assimilation mechanisms [36]. Growth parameters such as height of the plants in the treated population ranged from 33.32cm to 147.86cm. Control plants revealed a mean height of 138.98cm. In general, height decreased in the plants irradiated at 5kR-9kR. However, at 7kR slightly increased height was recorded (147.86cm) than in control. At 10kR though the irradiated cuttings sprouted initially, they did not grow further. Branching pattern of treated saplings was varied depending upon the doses of gamma rays administered. Maximum branching was observed at 6kR and 7kR and it was adversely decreased at higher doses (8kR and 9kR). In the treated population, internodal distance was not affected much. However, a marginal decrease in internodal distance was noticed at 2kR (2.75cm). Leaf area was found highest at 7kR (146.22cm²) and least at 9kR (72.67cm²). In control plants leaf area was 144.26cm². Decreased leaf area was observed at higher doses (8kR and 9kR). At 8kR, mutants having boat shaped leaves with wrinkled texture were observed. At 9kR, mutants with yellow sectored leaves noticed. Petiole length remained more or less unaltered in treated populations except in the populations irradiated with 1kR (3.06cm) and 5kR (3.01cm). Similarly, inflorescence length was found more or less unaltered. Number of flowers per inflorescence showed some variations with 13.33 at 5kR and 18.0 at 6kR compared to control 26.28. Deformity in inflorescence shape was observed in one of the populations irradiated at 7kR. Pollen fertility decreased with increase in gamma rays doses. Among the plants treated at 7kR beneficial mutants were selected for rising M₂ generation. Various propagation and growth parameters were recorded for M₂ generation along with control. Growth parameter like sprouting recorded an increase by 1.02% over control plants. Cuttings took 11 days to sprout. Rooting percentage was increased by 2.03% over control. Increase in survivability percentage was observed to the extent of 1.01%. Marginal increase in height and number of branches of the mutant plant was noticed. These mutants also exhibited shortened internodal distance and increased leaf area when compared to control. This accounted for an increase in yield by 13.09% than control. Plant height is a quantitative trait and is mainly controlled by polygene and each gene contributes small effects, which is called genetic additive effect [37]. Mutations are not stable and undergo recombination during meiosis [38]. Multicellular organisms have the ability to recover from sub lethal doses of ionizing radiations and selecting the desired mutant is controlled by a single gene [39]. Reduction in plant height due to an increasing production of active radicals that are responsible for lethality or due to increasing radiation induced gross structural chromosomal changes [40]. Gamma ray inhibition of growth may be due to distraction or damage to apical meristem [41]. Survival of plants at maturity and reduction in plant height depends on the nature and extent of chromosomal damage [42,43]. In M₁ generation, significant increase in
plant height at 2.5kRD and stunted growth of plant at 5kRD was observed. Slow growth in higher doses of gamma irradiation and increase/decrease in plant height at lower and higher doses respectively was due to auxin synthesis. At higher doses, delayed and stunted growth was observed in dahlia [44]. Irradiation of lower doses of gamma rays significantly improved vegetative traits while higher doses of gamma rays proved depressing for same parameters [45]. Formation of new shoots is decreased due to increased dosage of gamma rays. Gamma rays are more potent and highly penetrating, might have developed cells that were undergoing meiotic division in the bud-region. Increased gamma-ray dosage has direct negative effect on plant tissue and mutation can be lethal. Primary injuries are due to the retardation or inhibition of cell division. Cell death affects the growth habit and changes in plant morphology. If the dose is too low, there will not be enough mutation because of low mutation frequency and results in small mutated sector [46]. Internodal distance was found to be affected by cell number and length or both in barely [47]. Similar findings were observed in gamma irradiated Mysore local mulberry variety [48]. Somatic mutation occurs when mutant cells continue to divide, individual cell contain a patch of tissue with genotype different from rest of the body cells and also due to karyotype changes, point mutations, somatic crossing over and gene arrangement, changes in DNA amplification and segregation of pre-existing chimera tissue variation in leaves occur [49]. It was also attributed to the disturbances in phytochromes, chromosomal aberrations, mitotic inhibition, disrupted auxin synthesis, disturbances in DNA synthesis etc. [50,51,52,53]. Radiation induced pollen sterility has been reported by various researchers in crop plants [54,55].

Biochemical parameters like proteins, total soluble sugars, amino acids, phenolic content, chlorophyll-a, chlorophyll-b, total chlorophyll and moisture content were studied in both mutant and control plants of S_{54} variety. Analysis was carried out in tender, medium and coarse leaves separately. Amount of proteins found to be 10.04%, 9.49% and 9.23% respectively in tender, medium and coarse leaves of mutants when compared to control (tender 9.68%; medium 9.29%; coarse 8.87%). Medium leaves of mutant showed 9.49% of protein when compared to 9.29% of control. Whereas in case of coarse leaves of control, protein content were 8.87% much lesser than treated plants (9.23%). Total soluble sugars present in tender (8.58%) and coarse (8.17%) leaves of mutants were higher compared to control (8.34% in tender and 7.92% in coarse leaves). But in case of medium leaves, total soluble sugars were slightly lesser in mutants (8.01%) than in control (8.04%). Amino acid content was found higher in all the orders of leaves. In control variety, 64.62\mu\text{mole/gf.wt.}, 60.49\mu\text{mole/gf.wt.} and 53.29\mu\text{mole/gf.wt.} in tender, medium and coarse leaves respectively compared to 62,57\mu\text{mole/gf.wt.} 58.61\mu\text{mole/gf.wt.} and 52.74\mu\text{mole/gf.wt.} of mutant population. Phenolic content in tender and coarse leaves of the control plants were higher (98mg/gf.wt., 8.02mg/gf.wt.) compared to mutant (6.78mg/gf.wt., 7.98mg/gf.wt.). However, it was higher in medium leaves of mutant (7.43mg/gf.wt.) compared to the control (7.29mg/gf.wt.). With respect to chlorophyll-b, higher amount was present in tender leaves of control (1.04mg/gf.wt) compared to mutant (0.98mg/gf.wt). In medium leaves of mutant plants it was higher (1.43mg/gf.wt) compared to control (1.29mg/gf.wt). Chlorophyll-a was higher in tender, medium and coarse leaves of mutant (2.70, 3.98, 4.01mg/gf.wt respectively) compared to 2.67, 3.52 and 3.98mg/gf.wt of control plant. Total chlorophyll was 4.01, 4.09, 5.02mg/gf.wt in control leaves population compared to three different orders of the mutant leaves such as 3.98mg/gf.wt, 5.14mg/gf.wt and 5.28mg/gf.wt. Regarding moisture content, it was higher in all three grades of leaves in mutant plants (69.4%, 65.2% and 63.4%) compared to 68.7%, 64.2% and 62.3% in tender, medium and coarse leaves respectively (Table 2).
### Table 1. Effect of gamma irradiation on propagation and growth attributes of S₅₄ mulberry variety at M₁ generation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sprouting (%)</th>
<th>Rooting (%)</th>
<th>Survival (%)</th>
<th>Plant height (cm)</th>
<th>Number of branches</th>
<th>Internodal distance (cm)</th>
<th>Leaf area (cm²)</th>
<th>Petiole length (cm)</th>
<th>No. of flowers/inflorescence</th>
<th>Inflorescence length (cm)</th>
<th>Pollen fertility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92.48</td>
<td>88.00</td>
<td>88.00</td>
<td>138.98</td>
<td>5.88</td>
<td>3.81</td>
<td>144.26</td>
<td>3.29</td>
<td>26.28</td>
<td>3.01</td>
<td>88.29</td>
</tr>
<tr>
<td>1kR</td>
<td>72.19</td>
<td>79.10</td>
<td>81.49</td>
<td>131.14</td>
<td>6.01</td>
<td>3.85</td>
<td>129.31</td>
<td>3.05</td>
<td>22.09</td>
<td>2.81</td>
<td>81.46</td>
</tr>
<tr>
<td>2kR</td>
<td>78.49</td>
<td>81.27</td>
<td>79.48</td>
<td>128.78</td>
<td>6.14</td>
<td>2.75</td>
<td>134.27</td>
<td>3.21</td>
<td>25.18</td>
<td>2.83</td>
<td>83.29</td>
</tr>
<tr>
<td>3kR</td>
<td>67.20</td>
<td>73.14</td>
<td>68.17</td>
<td>114.57</td>
<td>5.78</td>
<td>3.98</td>
<td>131.13</td>
<td>3.24</td>
<td>24.26</td>
<td>2.79</td>
<td>76.16</td>
</tr>
<tr>
<td>4kR</td>
<td>53.18</td>
<td>61.18</td>
<td>54.33</td>
<td>101.68</td>
<td>5.97</td>
<td>3.78</td>
<td>128.29</td>
<td>3.18</td>
<td>23.23</td>
<td>2.66</td>
<td>65.24</td>
</tr>
<tr>
<td>5kR</td>
<td>49.28</td>
<td>59.78</td>
<td>47.08</td>
<td>94.18</td>
<td>6.18</td>
<td>3.81</td>
<td>101.01</td>
<td>3.01</td>
<td>13.33</td>
<td>2.69</td>
<td>61.17</td>
</tr>
<tr>
<td>6kR</td>
<td>51.06</td>
<td>51.39</td>
<td>49.98</td>
<td>88.68</td>
<td>6.73</td>
<td>3.68</td>
<td>98.47</td>
<td>3.29</td>
<td>18.00</td>
<td>2.74</td>
<td>63.28</td>
</tr>
<tr>
<td>7kR</td>
<td>44.19</td>
<td>81.33</td>
<td>40.50</td>
<td>147.86</td>
<td>6.71</td>
<td>2.99</td>
<td>146.22</td>
<td>3.21</td>
<td>20.18</td>
<td>2.52</td>
<td>56.29</td>
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<tr>
<td>8kR</td>
<td>36.28</td>
<td>42.78</td>
<td>35.18</td>
<td>34.18</td>
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<td>2.87</td>
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<td>18.29</td>
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<tr>
<td>9kR</td>
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<td>29.16</td>
<td>38.24</td>
<td>33.32</td>
<td>4.09</td>
<td>2.80</td>
<td>72.67</td>
<td>3.11</td>
<td>19.14</td>
<td>2.51</td>
<td>27.39</td>
</tr>
<tr>
<td>10kR</td>
<td>--</td>
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</tr>
<tr>
<td>SEM</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>2.48</td>
<td>0.86</td>
<td>0.47</td>
<td>7.29</td>
<td>0.67</td>
<td>3.04</td>
<td>0.29</td>
<td>8.24</td>
</tr>
<tr>
<td>CD @ 5%</td>
<td>5.1</td>
<td>4.3</td>
<td>4.3</td>
<td>3.57</td>
<td>1.91</td>
<td>1.01</td>
<td>8.76</td>
<td>1.04</td>
<td>4.79</td>
<td>0.78</td>
<td>10.98</td>
</tr>
</tbody>
</table>

### Table 2. Biochemical constituents in the leaves of S₅₄ mulberry mutant recovered at 7kR gamma irradiation at M₂ generation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leaf maturity</th>
<th>Proteins (%)</th>
<th>Total soluble sugars (%)</th>
<th>Amino acids (µmole/gf.wt.)</th>
<th>Phenols (mg/gf.wt.)</th>
<th>Chlorophyll-a (mg/gf.wt.)</th>
<th>Chlorophyll-b (mg/gf.wt.)</th>
<th>Total Chlorophyll (mg/gf.wt.)</th>
<th>Moisture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Tender</td>
<td>9.68</td>
<td>8.34</td>
<td>64.82</td>
<td>6.98</td>
<td>2.67</td>
<td>1.04</td>
<td>4.01</td>
<td>68.7</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>9.29</td>
<td>8.04</td>
<td>60.49</td>
<td>7.29</td>
<td>3.52</td>
<td>1.29</td>
<td>4.09</td>
<td>64.2</td>
</tr>
<tr>
<td></td>
<td>Coarse</td>
<td>8.87</td>
<td>7.92</td>
<td>53.29</td>
<td>8.02</td>
<td>3.98</td>
<td>1.69</td>
<td>5.02</td>
<td>62.3</td>
</tr>
<tr>
<td>Mutant</td>
<td>Tender</td>
<td>10.04</td>
<td>8.56</td>
<td>62.57</td>
<td>6.78</td>
<td>2.70</td>
<td>0.98</td>
<td>3.89</td>
<td>69.4</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>9.49</td>
<td>8.01</td>
<td>58.61</td>
<td>7.43</td>
<td>3.98</td>
<td>1.43</td>
<td>5.14</td>
<td>65.2</td>
</tr>
<tr>
<td></td>
<td>Coarse</td>
<td>9.23</td>
<td>8.17</td>
<td>52.74</td>
<td>7.98</td>
<td>4.01</td>
<td>1.47</td>
<td>5.28</td>
<td>63.4</td>
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<tr>
<td>SEM</td>
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<td>--</td>
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<td>--</td>
</tr>
<tr>
<td>CD @ 5%</td>
<td>--</td>
<td>1.04</td>
<td>NS</td>
<td>1.58</td>
<td>0.96</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>1.68</td>
</tr>
</tbody>
</table>
Leaf quality is influenced by number of factors such as variety, cultivation practices, incidence of pests and diseases, method of harvesting and preservation of leaves [56]. Number of workers have reported different traits such as protein, amino acid, carbohydrate, nitrogen, chlorophyll contents and leaf moisture are responsible for mulberry leaf quality [57,58] and one single variety consists of all the nutrients at the highest level [59,60].

4. CONCLUSION

Mulberry is highly versatile and polygenic plant exhibits considerable plasticity. Juvenile twigs of S$_{66}$ treated with varying doses of gamma ray (1kR-10kR). Plants irradiated at 7kR showed considerable variation in M$_1$ generation compared to control plants with respect to rooting, plant height and yield parameters. Cuttings of mutants were grown in M$_2$ generation and plants recovered indicated remarkable beneficial agro botanical parameters like rooting, survivability, plant height, yield and biochemical constituents over plants grown in M$_1$ generation. However, further systematic yield trials and evaluation of these variants over a period will establish their potentiality as cultivars.

ACKNOWLEDGEMENTS

We profoundly thank Dr. S.B. Angadi, Senior Scientist, 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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

10. Aliev MO. Use of chemical mutagens combined with hybridization of mulberry forms differing in ploidy. Shelk. 1977;1:7-8


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