

# Analysis of chlorophyll mutations in EMS-induced mutant population of rice (*Oryza sativa* L.)

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**Background:** The purpose of this study was to determine the lethal dose given by the chemical mutagen ethyl-methane sulfonate (EMS) in the high-yielding rice variety ADT43. The frequency of chlorophyll mutations has been analysed in the M<sub>2</sub> generation.

**Methods:** A pilot study that consisted of treating ten batches of rice seeds using different dosages of ethyl methane sulphonate (EMS) and conducting kill curve analysis was used to determine lethal doses. The M<sub>1</sub> population was then divided into three batches, each with EMS dosages of 0.4%, 0.5%, and 0.6%. Three batches of the M<sub>2</sub> population, involving the 700, 700, and 700 M<sub>2</sub> families, were subsequently exposed to chlorophyll mutation in the subsequent cropping and studied.

**Result:** The range of the LD50 value is 0.4% to 0.6% EMS. The lethality of the rice increased linearly with a gradual increase in the EMS dosage. At EMS dosages of 0.4%, 0.5%, and 0.6%, the rates of the chlorophyll mutation were 4, 4.57, and 6.14, respectively.

**Conclusion:** Since the presence of chlorophyll mutants in the M<sub>2</sub> generation is confirmed, the reliability of the mutant population is verified to utilise this mutant population to screen for the trait of interest.

**Keywords:** chlorophyll mutant, EMS, mutation breeding, rice

## Introduction

Almost half of the world's population depends on rice as one of their primary staple foods. The primary goal of the crop breeding program is to develop new varieties in order to improve characteristics. A general method for producing new alleles in rice is induced mutagenesis (Jadhav et al., 2023). Functional mutations could be induced through mutagenesis and characterized by both forward (Gurunathan et al., 2019) and reverse (da Luz et al., 2021) genetics approaches. The improved genetic architecture in the rice has been generated through physical and chemical mutagenic agents (Kumawat et al., 2022). Because of its ability to create novel genetic differences among crop plants, EMS is one of the

most commonly used mutagens to create a mutant population in rice (Hameed et al., 2019; Jia et al., 2019). Successful plant breeding programs depend on an understanding of the biological impacts of mutagens (Jankowicz-Cieslak et al., 2021). As defined by Phillips & Rines (2009), a mutation is a change to an organism's genetic material that does not occur by regular recombination and segregation. It provides an introduction to crop plant genetic enhancement (Adamu & Aliyu 2007). Stadler (1948) discovered the first intentional mutation in a barley plant and established that radiation treatment may change one tiller without changing the others. Various types of chlorophyll deficiency, including white, yellow, or virescent seedlings, have been observed as a result of mutation induction. Moreover, if one is to acquire the right mutant population in crops with high rates of mutation and achieve the goals of breeding programs, precision in adhering to the mutagenesis protocol is also crucial. In a mutagenesis technique, the existence of chlorophyll mutants in the M<sub>2</sub> generation is the best indicator of mutation rate. This study generated a mutant population in the rice variety ADT43 using the EMS mutagen, documented the different types of chlorophyll mutants, and evaluated the frequency of chlorophyll mutations at several EMS dosages.

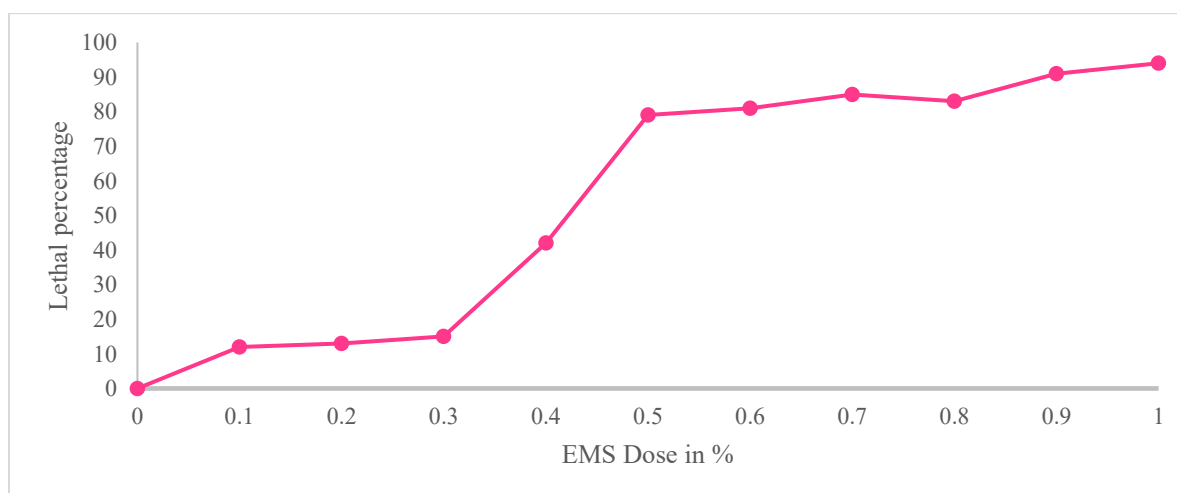
## Materials and Methods

### (i) Estimation of Lethal Dose (LD)

For the mutagenesis study, the high-yielding variety ADT43 has been selected. For one whole day, ten batches of 100 healthy rice seeds each were soaked in distilled water. Ethyl Methane Sulphonate (EMS) at concentrations of 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, and 1.0% was applied to pre-soaked seed batches for a period of 12 hours (Unan et al., 2022). In addition, the control batch that was not treated by EMS was also exposed. The 5% sodium thiosulfate solution was given to the treated seed batches to neutralize the EMS as soon as the EMS treatment incubation period was over, following the decantation of the EMS liquid. To get rid of any remaining EMS, seeds were then carefully cleaned for six hours under running tap (Sagel et al., 2017). In the raised bed nursery, the seeds were planted in field conditions. At 14 DAS, observations of the seedlings' lethality and survival for each of the EMS dosages were taken (Table 1). To diagrammatically represent the lethality, a kill curve analysis was done (Figure 1).

**Table 1. Survival rate of rice seedlings under different EMS doses**

EMS dose	Total seed sowing	No of germination	Survival %	Lethal % to EMS doses
Control	100	96	100	0
0.10%	100	85	88	12
0.20%	100	84	87	13
0.30%	100	82	85	15
0.40%	100	56	58	42
0.50%	100	20	21	79
0.60%	100	18	19	81
0.70%	100	14	15	85
0.80%	100	11	12	88
0.90%	100	8	9	91
1%	100	5	6	94



**Figure 1. Effect of increasing EMS Doses on seedling mortality (Kill Curve Analysis)**

## (ii) Production of M<sub>1</sub> Seeds and Establishment of the Population

In combination with a high dose of 0.6% of EMS, M<sub>1</sub> generation is generated with these two doses because the LD value has been shown to be between 0.4% and 0.5% of the EMS dose. So, the M<sub>1</sub> generation was generated using the previously stated EMS doses in batches of 100 g of seeds. Twenty days after being sown, the seedlings were transplanted to the field. Three healthy plants were used as a single plant for harvesting the seeds. These include seeds from 1350 (0.6% EMS), 1240 (0.5% EMS), and 976 (0.4% EMS) plants.

## (iii) Production of M<sub>2</sub> population

The seeds of M<sub>1</sub> plants obtained from the 0.4%, 0.5%, and 0.6% EMS doses, respectively, were used to raise 700, 700, and 700 M<sub>2</sub> families from the aforementioned doses. At 10 DAS, a number of chlorophyll mutants were observed: *Albina*, *Striata*, *Xantha*, *Viridis*, *Aurea*, and *Chlorina*, as mentioned in Table 2.

**Table 2. Characterization of chlorophyll mutants in M<sub>2</sub> generation (From 10 Days Onwards)**

Mutant Type	Description
<i>Albina</i>	The seedling had a whitish leaf, survived for 15 days
<i>Xantha</i>	Seedlings' leaves were pale yellow in colour
<i>Viridis</i>	Light green colour in the early stages of growth
<i>Chlorina</i>	Seedlings' leaves were yellowish-green in colour.
<i>Aurea</i>	Seedlings' leaves were yellow
<i>Striata</i>	characterized by the presence of longitudinal stripes on the leaves that were either white or yellow in color, and it was a viable one.

(Patil et al., 2017), (Sellapillai et al., 2022), (Ramchander et al., 2014)

## Results

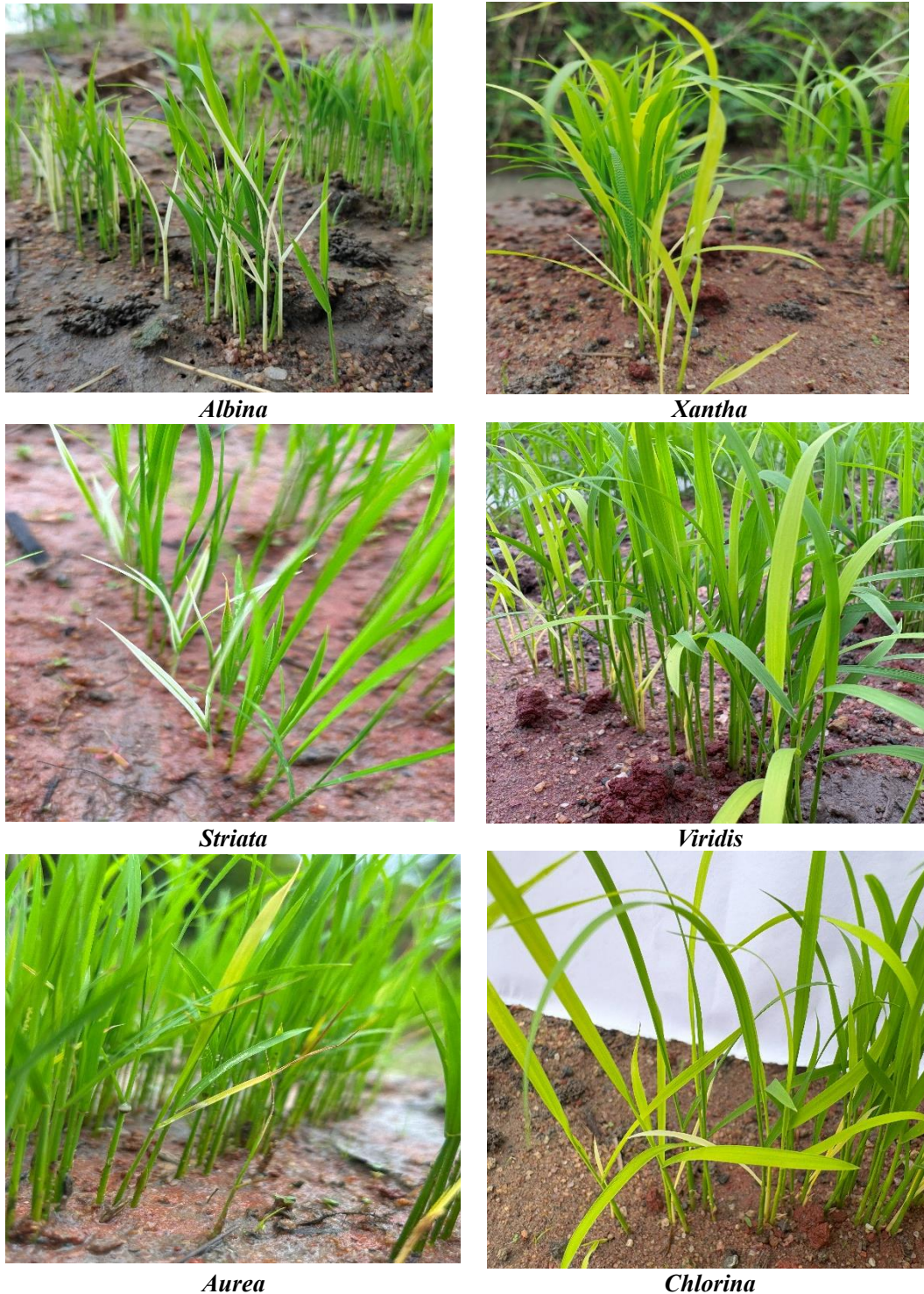
Our experiment reported with different types of chlorophyll mutants, viz., *Albina*, *Striata*, *Aurea*, *Xantha*, and *Chlorina* (Table 3; Figure 2).

**Table 3. characterization of chlorophyll mutants observed in M<sub>2</sub> generation**

Sl. No.	Genotype	Total no of plants observed	No. of chlorophyll mutants	<i>Albina</i>	<i>Striata</i>	<i>Xantha</i>	<i>Viridis</i>	<i>Aurea</i>	<i>Chlorina</i>	Frequency %
<b>0.40%</b>										
1	ADT43-5M <sub>1</sub>	55	16	13	2	1	-	-	-	29.09
2	ADT43-28M <sub>1</sub>	80	21	13	1	1	1	2	3	26.25
3	ADT43-47M <sub>1</sub>	35	1	1	-	-	-	-	-	2.86
4	ADT43-57M <sub>1</sub>	88	15	12	2	1	-	-	-	17.05
5	ADT43-65M <sub>1</sub>	55	14	11	-	1	2	-	-	25.45
6	ADT43-85M <sub>1</sub>	54	4	4	-	-	-	-	-	7.41
7	ADT43-92M <sub>1</sub>	85	24	18	2	3	1	-	-	28.24
8	ADT43-100M <sub>1</sub>	58	4	4	-	-	-	-	-	6.90
9	ADT43-131M <sub>1</sub>	38	1	1	-	-	-	-	-	2.63
10	ADT43-156M <sub>1</sub>	47	7	7	-	-	-	-	-	14.89
11	ADT43-264M <sub>1</sub>	42	1	1	-	-	-	-	-	2.38
12	ADT43-276M <sub>1</sub>	78	17	16	-	-	-	1	-	21.79
13	ADT43-295M <sub>1</sub>	45	12	12	-	-	-	-	-	26.67
14	ADT43-306M <sub>1</sub>	40	2	2	-	-	-	-	-	5.00
15	ADT43-326M <sub>1</sub>	56	2	-	2	-	-	-	-	3.57
16	ADT43-340M <sub>1</sub>	58	2	-	1	1	-	-	-	3.45
17	ADT43-343M <sub>1</sub>	58	7	5	-	-	2	-	-	12.07
18	ADT43-357M <sub>1</sub>	52	8	4	1	1	2	-	-	15.38
19	ADT43-361M <sub>1</sub>	82	3	3	-	-	-	-	-	3.66
20	ADT43-378M <sub>1</sub>	73	3	3	-	-	-	-	-	4.11
21	ADT43-400M <sub>1</sub>	71	11	11	-	-	-	-	-	15.49
22	ADT43-429M <sub>1</sub>	63	12	10	-	-	-	-	2	19.05
23	ADT43-432M <sub>1</sub>	68	10	6	4	-	-	-	-	14.71
24	ADT43-434M <sub>1</sub>	62	2	2	-	-	-	-	-	3.23
25	ADT43-490M <sub>1</sub>	64	8	6	-	1	1	-	-	12.50
26	ADT43-625M <sub>1</sub>	32	4	4	-	-	-	-	-	12.50
27	ADT43-690M <sub>1</sub>	56	6	6	-	-	-	-	-	10.71
28	ADT43-693M <sub>1</sub>	63	1	-	1	-	-	-	-	1.59
		<b>1658</b>	<b>218</b>	<b>175</b>	<b>16</b>	<b>10</b>	<b>9</b>	<b>3</b>	<b>5</b>	<b>13.15</b>
<b>0.50%</b>										
29	ADT43-736M <sub>1</sub>	36	1	1	-	-	-	-	-	2.78
30	ADT43-749M <sub>1</sub>	15	10	7	-	-	3	-	-	66.67

31	ADT43-759M <sub>1</sub>	30	15	9	1	2	2	1	-	50.00
32	ADT43-769M <sub>1</sub>	34	11	11	-	-	-	-	-	32.35
33	ADT43-778M <sub>1</sub>	24	3	3	-	-	-	-	-	12.50
34	ADT43-800M <sub>1</sub>	52	17	13	-	-	-	-	4	32.69
35	ADT43-828M <sub>1</sub>	34	11	7	1	1	-	2	-	32.35
36	ADT43-829M <sub>1</sub>	48	14	8	-	4	2	-	-	29.17
37	ADT43-830M <sub>1</sub>	25	6	6	-	-	-	-	-	24.00
38	ADT43-842M <sub>1</sub>	38	2	2	-	-	-	-	-	5.26
39	ADT43-869M <sub>1</sub>	53	2	2	-	-	-	-	-	3.77
40	ADT43-882M <sub>1</sub>	39	6	5	-	-	1	-	-	15.38
41	ADT43-916M <sub>1</sub>	43	10	4	-	4	1	1	-	23.26
42	ADT43-931M <sub>1</sub>	39	7	7	-	-	-	-	-	17.95
43	ADT43-934M <sub>1</sub>	43	1	1	-	-	-	-	-	2.33
44	ADT43-941M <sub>1</sub>	51	5	5	-	-	-	-	-	9.80
45	ADT43-977M <sub>1</sub>	42	11	9	2	-	-	-	-	26.19
46	ADT43-1054M <sub>1</sub>	40	7	4	3	-	-	-	-	17.50
47	ADT43-1109M <sub>1</sub>	42	5	5	-	-	-	-	-	11.90
48	ADT43-1144M <sub>1</sub>	68	16	-	10	3	3	-	-	23.53
49	ADT43-1175M <sub>1</sub>	53	7	2	3	2	-	-	-	13.21
50	ADT43-1202M <sub>1</sub>	38	10	9	1	-	-	-	-	26.32
51	ADT43-1234M <sub>1</sub>	59	15	5	4	2	1	1	2	25.42
52	ADT43-1238M <sub>1</sub>	52	4	4	-	-	-	-	-	7.69
53	ADT43-1254M <sub>1</sub>	25	8	3	2	3	-	-	-	32.00
54	ADT43-1275M <sub>1</sub>	36	2	2	-	-	-	-	-	5.56
55	ADT43-1282M <sub>1</sub>	36	2	2	-	-	-	-	-	5.56
56	ADT43-1283M <sub>1</sub>	39	3	3	-	-	-	-	-	7.69
57	ADT43-1317M <sub>1</sub>	36	18	7	2	4	-	4	1	50.00
58	ADT43-1331M <sub>1</sub>	21	5	2	3	-	-	-	-	23.81
59	ADT43-1351M <sub>1</sub>	36	4	2	2	-	-	-	-	11.11
60	ADT43-1398M <sub>1</sub>	36	4	4	-	-	-	-	-	11.11
		<b>1263</b>	<b>242</b>	<b>154</b>	<b>34</b>	<b>25</b>	<b>13</b>	<b>9</b>	<b>7</b>	<b>19.16</b>
<b>0.60%</b>										
61	ADT43-1421M <sub>1</sub>	28	3	3	-	-	-	-	-	10.71
62	ADT43-1433M <sub>1</sub>	25	13	11	1	1	-	-	-	52.00
63	ADT43-1436M <sub>1</sub>	22	1	1	-	-	-	-	-	4.55
64	ADT43-1519M <sub>1</sub>	4	2	2	-	-	-	-	-	50.00
65	ADT43-1522M <sub>1</sub>	20	6	4	1	1	-	-	-	30.00
66	ADT43-1528M <sub>1</sub>	14	4	4	-	-	-	-	-	28.57
67	ADT43-1541M <sub>1</sub>	18	1	1	-	-	-	-	-	5.56
68	ADT43-1572M <sub>1</sub>	17	3	-	3	-	-	-	-	17.65
69	ADT43-1663M <sub>1</sub>	32	2	-	1	1	-	-	-	6.25
70	ADT43-1665M <sub>1</sub>	48	4	-	4	-	-	-	-	8.33
71	ADT43-1687M <sub>1</sub>	36	3	2	-	-	1	-	-	8.33
72	ADT43-1670M <sub>1</sub>	38	4	-	-	-	-	4	-	10.53
73	ADT43-1720M <sub>1</sub>	24	1	1	-	-	-	-	-	4.17
74	ADT43-1725M <sub>1</sub>	35	6	-	3	-	2	1	-	17.14
75	ADT43-1734M <sub>1</sub>	42	3	3	-	-	-	-	-	7.14
76	ADT43-1736M <sub>1</sub>	29	2	2	-	-	-	-	-	6.90
77	ADT43-1741M <sub>1</sub>	18	1	1	-	-	-	-	-	5.56
78	ADT43-1742M <sub>1</sub>	34	4	1	2	-	-	-	1	11.76
79	ADT43-1745M <sub>1</sub>	40	2	1	-	-	1	-	-	5.00
80	ADT43-1800M <sub>1</sub>	20	5	5	-	-	-	-	-	25.00
81	ADT43-1801M <sub>1</sub>	52	7	6	1	-	-	-	-	13.46
82	ADT43-1808M <sub>1</sub>	23	5	2	-	-	-	1	2	21.74
83	ADT43-1812M <sub>1</sub>	33	2	2	-	-	-	-	-	6.06
84	ADT43-1819M <sub>1</sub>	27	3	-	-	3	-	-	-	11.11
85	ADT43-1831M <sub>1</sub>	43	1	-	-	-	1	-	-	2.33
86	ADT43-1845M <sub>1</sub>	38	1	-	-	-	-	1	-	2.63
87	ADT43-1872M <sub>1</sub>	32	2	2	-	-	-	-	-	6.25
88	ADT43-1919M <sub>1</sub>	28	6	3	-	-	2	-	1	21.43
89	ADT43-1924M <sub>1</sub>	40	8	4	2	1	-	1	-	20.00
90	ADT43-1947M <sub>1</sub>	38	6	3	-	3	-	-	-	15.79
91	ADT43-1950M <sub>1</sub>	52	11	10	1	-	-	-	-	21.15
92	ADT43-1963M <sub>1</sub>	29	9	3	2	1	-	3	-	31.03
93	ADT43-1971M <sub>1</sub>	55	6	-	2	1	3	-	-	10.91
94	ADT43-1974M <sub>1</sub>	40	4	4	-	-	-	-	-	10.00
95	ADT43-1989M <sub>1</sub>	39	3	3	-	-	-	-	-	7.69
96	ADT43-2026M <sub>1</sub>	39	4	4	-	-	-	-	-	10.26
97	ADT43-2029M <sub>1</sub>	31	9	9	-	-	-	-	-	29.03
98	ADT43-2047M <sub>1</sub>	35	2	2	-	-	-	-	-	5.71
99	ADT43-2048M <sub>1</sub>	43	2	2	-	-	-	-	-	4.65
100	ADT43-2052M <sub>1</sub>	39	4	3	1	-	-	-	-	10.26
101	ADT43-2058M <sub>1</sub>	30	10	-	-	5	1	3	1	33.33
102	ADT43-2066M <sub>1</sub>	30	1	1	-	-	-	-	-	3.33
103	ADT43-2079M <sub>1</sub>	40	10	8	-	1	-	-	1	25.00
<b>Total</b>		<b>1400</b>	<b>186</b>	<b>113</b>	<b>24</b>	<b>18</b>	<b>11</b>	<b>14</b>	<b>6</b>	<b>13.29</b>



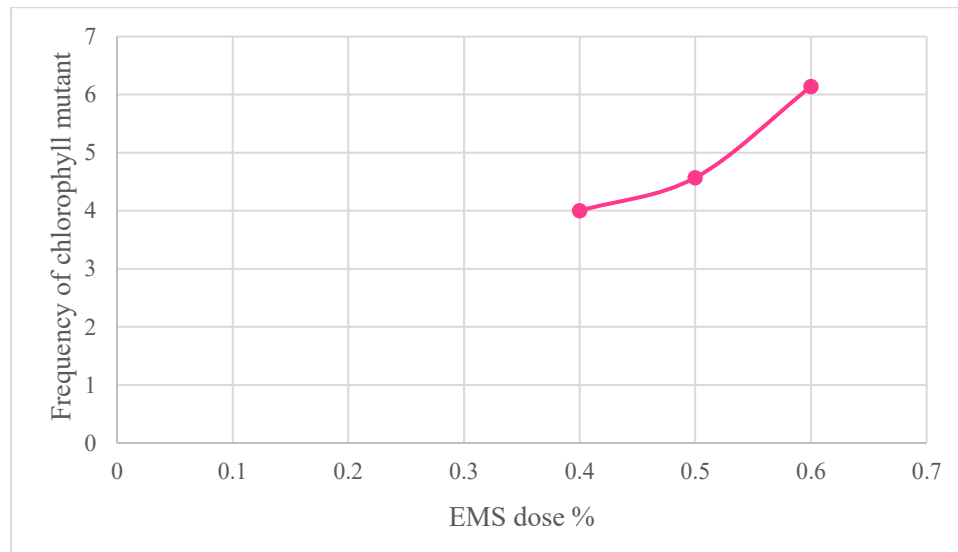


**Figure 2. Chlorophyll-deficient mutants observed in  $M_2$  generation families**

The  $M_2$  families ADT43-5 $M_1$  and ADT43-693 $M_1$  had the highest and lowest frequencies of chlorophyll mutation at 29.09 and 1.59, respectively, in the 0.4% EMS dose. The  $M_2$  families ADT43-749 $M_1$  and ADT43-934 $M_1$  had the highest and lowest frequencies of chlorophyll mutation at 66.67 and 2.33, respectively, in the 0.5% EMS dose. The highest and lowest frequencies of chlorophyll mutation in the  $M_2$  families ADT43-1433 $M_1$  and ADT43-1831 $M_1$  were found to be 52.0 and 2.33, respectively, in the 0.6% EMS dose.

**Table 4. Chlorophyll mutation frequency in the M<sub>2</sub> population at varying EMS doses based on (a) plant count and (b) family count**

		0.4% EMS	0.5% EMS	0.6% EMS
<b>a. Chlorophyll mutation frequency based on the number of plants tested</b>				
chlorophyll mutant	<i>Albina</i>	175	154	113
	<i>Striata</i>	16	34	24
	<i>Xantha</i>	10	25	18
	<i>Viridis</i>	9	13	11
	<i>Aurea</i>	3	9	14
	<i>Chlorina</i>	2	7	6
Total number of chlorophyll mutants		<b>218</b>	<b>242</b>	<b>186</b>
Total number of Plants Tested		<b>1658</b>	<b>1263</b>	<b>1400</b>
Mutation frequency %		<b>13.15</b>	<b>19.16</b>	<b>13.29</b>
<b>b. Chlorophyll mutation frequency based on the number of families tested</b>				
Number of M <sub>2</sub> families raised and tested		700	700	700
Number of M <sub>2</sub> families reported with chlorophyll mutants		28	32	43
Frequency of M <sub>2</sub> families with chlorophyll mutants %		<b>4</b>	<b>4.57</b>	<b>6.14</b>



**Figure 3. Effect of EMS dose on the frequency of chlorophyll mutations**

The doses of 0.4% EMS, 0.5% EMS, and 0.6% EMS, respectively, led to the identification of 218, 242, 186, and chlorophyll mutants. Table 4a shows that the observed plants have a frequency of chlorophyll mutations of 13.15 (0.4%), 19.16 (0.5% EMS), and 13.29 (0.6% EMS). In addition, Table 4b shows that the frequency of chlorophyll mutants in terms of family volume numbers tested was 4 (0.4%), 4.57 (0.5%), and 6.14 (0.6%). The increases in the EMS dose lead in increases in the chlorophyll mutation frequency in the M<sub>2</sub> families (Figure 3).

## Discussion

The mutagenic efficiency depends on the dose of the mutagen, and it should be determined to get the optimum number of plant recovery with a higher number of mutations (Pratap & Kumar, 2011). The germination and survival rates progressively declined with increasing EMS concentrations (Table 1), which is consistent with prior findings (Hernández Muñoz et al., 2019). Visible changes due to mutations induced by EMS could be expressed mostly in the M<sub>2</sub> generation (Jia et al., 2019) since most of the mutations are recessive. The presence of chlorophyll mutants in the M<sub>2</sub> progenies is an indicator for the occurrence of mutations in the mutant population (Prasannakumari et al., 2024). Lalitha et al. (2020) supported that the presence of mutations in the chlorophyll synthesis genes leads to the generation of different types of chlorophyll mutants. According to Prasannakumari et al. (2024), increasing the EMS and Gamma doses in rice increases the frequency of chlorophyll mutation.

## Conclusion

In order to create genetic variation for crop development, this research aimed to determine the mutagenic effects of ethyl-methane-sulfonate (EMS) on the rice variety ADT43. As the concentration of EMS increased, the rates of germination and survival gradually decreased. The LD50 fell between the 0.4% to 0.6% EMS doses, suggesting that these EMS doses could provide sufficient plant recovery in rice with mutagenesis mutations. In the mutation population generation, the presence of chlorophyll mutants in the M<sub>2</sub> generation confirms the level of accuracy in the research. An increase in the EMS dose causes a rise in the frequency of mutations in rice, as seen by the frequency of mutation in the M<sub>2</sub> families, which was 4, 4.57 and 6.14 for the EMS doses of 0.4%, 0.5%, and 0.6%. In conclusion, the produced mutant populations are highly reliable for evaluating several desirable traits.

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## Author contributions

BIH conducted the experiments. SG designed this experiment and guided. AD contributed to the identification of types of chlorophyll mutants. JM, PC, and TS edited this manuscript.

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NIL.

## Conflict of interest

The author declares no conflict of interest. The manuscript has not been submitted for publication in any other journal.

## Ethics approval

Not applicable.

## AI tool usage declaration

The authors declare that no AI and associated tools are used for writing scientific content in the article.

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