

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 for chemical mutagen ethyl-methane sulfonate (EMS) in the high-yielding rice variety ADT43. Secondly, to analyse the frequency of chlorophyll mutations in the M₂ generation.

Methods: In this study ten batches of rice seeds treated with different dosages of ethyl methane sulphonate (EMS) viz., 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, and 1.0% and kill curve analysis was carried out to determine lethal doses. To create M₁ population, the EMS doses of 0.4%, 0.5%, and with a higher dose 0.6% were used. Then, 700 (0.4% EMS), 700 (0.5% EMS), and 700 (0.6% EMS) M₂ families, were subsequently raised to record the chlorophyll mutations.

Results: The LD₅₀ value resided at 0.4% to 0.5% EMS. The lethality of the rice increased linearly with a gradual increase in the EMS dosage. Frequency of M₂ families with chlorophyll mutants recorded in the EMS doses 0.4%, 0.5%, and 0.6% were 4.00%, 4.57%, and 6.14%, respectively.

Conclusion: The presence of chlorophyll mutants in the M₂ generation confirmed the occurrence of mutations in the mutant population. Hence, the reliability of the mutant population is verified to utilise this mutant population to screen for the trait of interest further.

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 the desirable traits. 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_2 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.

Materials and Methods

(i) Estimation of Lethal Dose (LD)

For the mutagenesis study, the high-yielding variety ADT43 has been subjected. Ten batches of 100 healthy rice seeds each were soaked in distilled water for 12 hrs. The pre-soaked seeds were treated with 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% for a period of 12 hours (Unan et al., 2022) and simultaneously the control (0.0% EMS) also kept. Immediately after completion of treatment duration, 5% sodium thiosulfate solution were used to neutralize the EMS. To get rid of any remaining EMS, seeds were then carefully cleaned for six hours under running tap water (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 doses were recorded (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	83
0.90%	100	8	9	91
1.00%	100	5	6	94

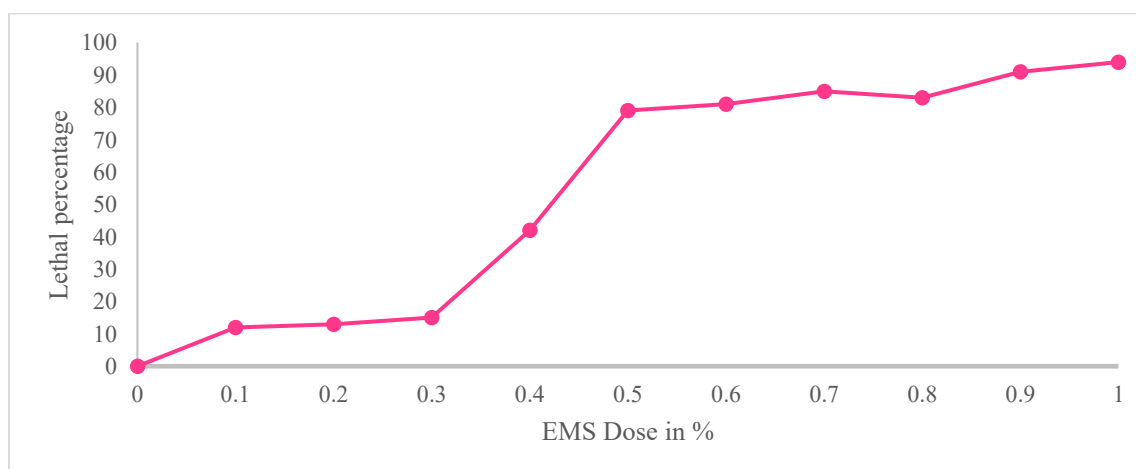


Figure 1. Effect of increasing EMS Doses on seedling mortality (Kill curve analysis)

(ii) Generation of M₁ plants

Along with a high dose of 0.6% of EMS, M₁ 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 M₁ generation was generated using the previously stated EMS doses in batches of 100 gm of seeds. 20 DAS, the seedlings were transplanted to the field. The primary panicle from the healthy-looking plants was harvested after the proper physiological maturity. Seeds from 1350 (0.6% EMS), 1240 (0.5% EMS), and 976 (0.4% EMS) plants were harvested in single plant basis.

(iii) Raising of M₂ population

The seeds of M₁ plants obtained from the 0.4%, 0.5%, and 0.6% EMS doses, respectively, were used to raise 700, 700, and 700 M₂ families. At 10 DAS, chlorophyll mutants were observed: *Albina*, *Striata*, *Xantha*, *Viridis*, *Aurea*, and *Chlorina*, and recorded as mentioned in Table 2.

Table 2. Characterization of chlorophyll mutants in M₂ 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 different types of chlorophyll mutants, viz., *Albina*, *Striata*, *Aurea*, *Xantha*, and *Chlorina* (Table 3; Figure 2).

Table 3. Characterization of chlorophyll mutants observed in M₂ 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 %
0.40%										
1	ADT43-5M ₁	55	16	13	2	1	-	-	-	29.09
2	ADT43-28M ₁	80	21	13	1	1	1	2	3	26.25
3	ADT43-47M ₁	35	1	1	-	-	-	-	-	2.86
4	ADT43-57M ₁	88	15	12	2	1	-	-	-	17.05
5	ADT43-65M ₁	55	14	11	-	1	2	-	-	25.45
6	ADT43-85M ₁	54	4	4	-	-	-	-	-	7.41
7	ADT43-92M ₁	85	24	18	2	3	1	-	-	28.24
8	ADT43-100M ₁	58	4	4	-	-	-	-	-	6.90
9	ADT43-131M ₁	38	1	1	-	-	-	-	-	2.63
10	ADT43-156M ₁	47	7	7	-	-	-	-	-	14.89
11	ADT43-264M ₁	42	1	1	-	-	-	-	-	2.38
12	ADT43-276M ₁	78	17	16	-	-	-	1	-	21.79
13	ADT43-295M ₁	45	12	12	-	-	-	-	-	26.67
14	ADT43-306M ₁	40	2	2	-	-	-	-	-	5.00
15	ADT43-326M ₁	56	2	-	2	-	-	-	-	3.57
16	ADT43-340M ₁	58	2	-	1	1	-	-	-	3.45
17	ADT43-343M ₁	58	7	5	-	-	2	-	-	12.07
18	ADT43-357M ₁	52	8	4	1	1	2	-	-	15.38
19	ADT43-361M ₁	82	3	3	-	-	-	-	-	3.66
20	ADT43-378M ₁	73	3	3	-	-	-	-	-	4.11
21	ADT43-400M ₁	71	11	11	-	-	-	-	-	15.49
22	ADT43-429M ₁	63	12	10	-	-	-	-	2	19.05
23	ADT43-432M ₁	68	10	6	4	-	-	-	-	14.71
24	ADT43-434M ₁	62	2	2	-	-	-	-	-	3.23
25	ADT43-490M ₁	64	8	6	-	1	1	-	-	12.50
26	ADT43-625M ₁	32	4	4	-	-	-	-	-	12.50
27	ADT43-690M ₁	56	6	6	-	-	-	-	-	10.71
28	ADT43-693M ₁	63	1	-	1	-	-	-	-	1.59
		1658	218	175	16	10	9	3	5	13.15
0.50%										
29	ADT43-736M ₁	36	1	1	-	-	-	-	-	2.78
30	ADT43-749M ₁	15	10	7	-	-	3	-	-	66.67

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



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 mutants 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 mutants at 66.67 and 2.33, respectively, in the 0.5% EMS dose. The highest and lowest frequencies of chlorophyll mutants 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 M₂ population at varying EMS doses based on (a) plant count and (b) family count

		0.4% EMS	0.5% EMS	0.6% EMS
a. Chlorophyll mutation frequency based on the number of plants tested				
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		218	242	186
Total number of Plants Tested		1658	1263	1400
Mutation frequency %		13.15	19.16	13.29
b. Chlorophyll mutation frequency based on the number of families tested				
Number of M ₂ families raised and tested		700	700	700
Number of M ₂ families reported with chlorophyll mutants		28	32	43
Frequency of M ₂ families with chlorophyll mutants %		4.00	4.57	6.14

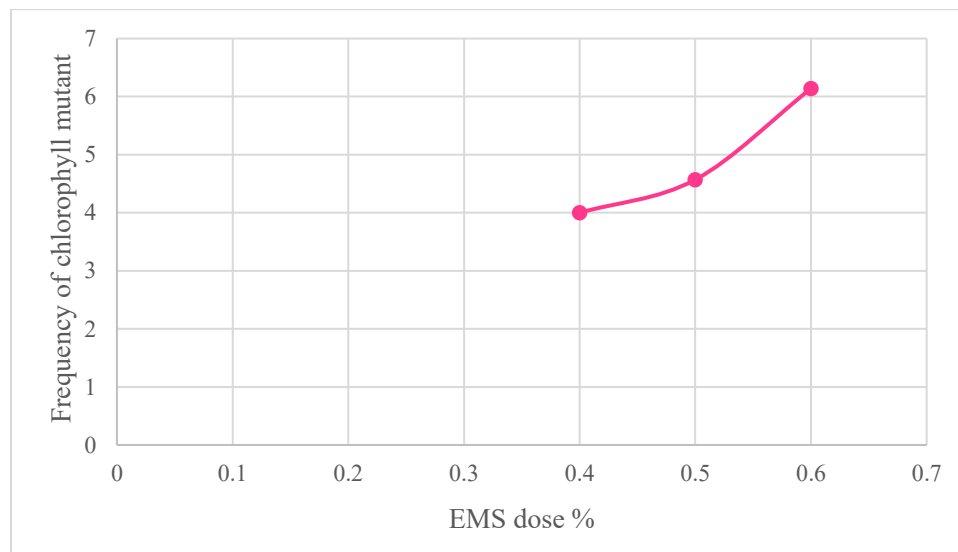


Figure 3. Effect of EMS dose on the frequency of chlorophyll mutations

In our study, 218, 242, 186 chlorophyll mutants were observed from the 1658 (0.4% EMS), 1263 (0.5%), and 1400 (0.6%) plants were subjected to the observation. Table 4a shows that the observed plants have a frequency percentage of chlorophyll mutants of 13.15 (0.4% EMS), 19.16 (0.5% EMS), and 13.29 (0.6% EMS). In addition, Table 4b shows that the frequency percentage of chlorophyll mutants in terms of number of families tested was 4.00 (0.4%), 4.57 (0.5%), and 6.14 (0.6%). This experiment showed that the increases in the EMS dose led to increases in the chlorophyll mutation frequency in the M₂ 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 M₂ generation (Jia et al., 2019) since most of the mutations are recessive. The presence of chlorophyll mutants in the M₂ 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 was estimated between 0.4% and 0.5% EMS based on kill curve analysis, suggesting that these EMS doses could provide sufficient plant recovery in rice with mutagenesis-induced mutations. In the mutation population generation, the presence of chlorophyll mutants in the M₂ generation confirms the level of accuracy in the mutagenesis treatment. An increase in the EMS dose caused a rise in the frequency of mutations in rice, as seen by the frequency of mutants in the M₂ families, which was 4.00%, 4.57% and 6.14% for the EMS doses of 0.4%, 0.5%, and 0.6% respectively. 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 and supervised the experiment. AD contributed to the identification of types of chlorophyll mutants. JM, PC, and TS edited this manuscript.

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Conflict of interest

The authors declare 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 were used for writing scientific content in the article.

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