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Research Article

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 M_2 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 M_1 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) M_2 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 M_2 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 M_2 generation was confirmed the occurrence of mutation in the mutant population during the mutagenesis. 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%	100	5	6	94

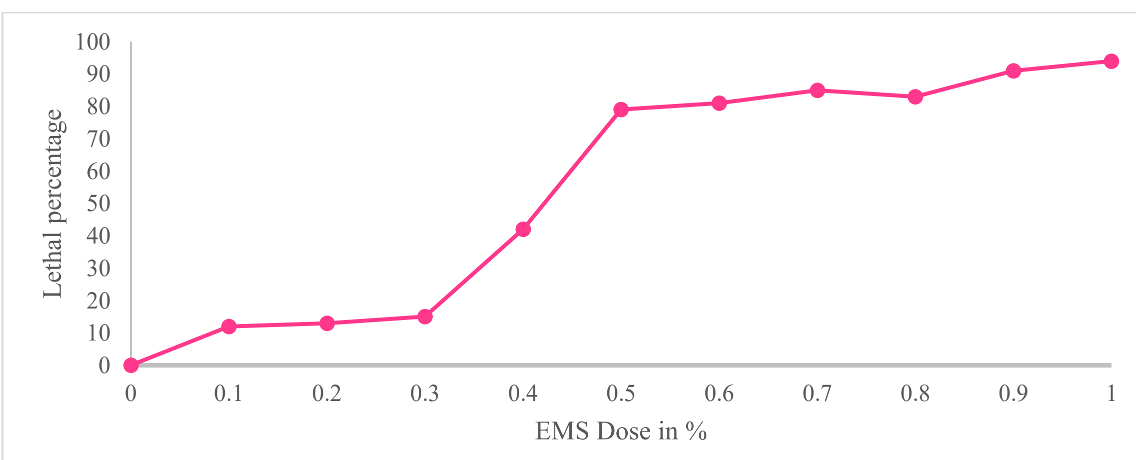

Figure 1. Effect of increasing EMS Doses on seedling mortality (Kill Curve Analysis)

(ii) Generation of M_1 plants

Along with a high dose of 0.6% of EMS, M_1 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_1 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 M_2 population and Characterization

The seeds of M_1 plants obtained from the 0.4%, 0.5%, and 0.6% EMS doses, respectively, were used to raise 700, 700, and 700 M_2 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 M_2 generation (From 10 Days Onwards)

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

(Patial 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_2 generation

Sl. No.	Genotype	Total no of plants observed	No. of chlorophyll mutants	*Albina*	*Striata*	*Xantha*	*Viridis*	*Aurea*	*Chlorina*	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 ADT43-5M_1 and ADT43-693M_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 ADT43-749M_1 and ADT43-934M_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 ADT43-1433M_1 and ADT43-1831M_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_2 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	*Albina*	175	154	113
	Striata	16	34	24
	Xantha	10	25	18
	Viridis	9	13	11
	Aurea	3	9	14
	Chlorina	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**

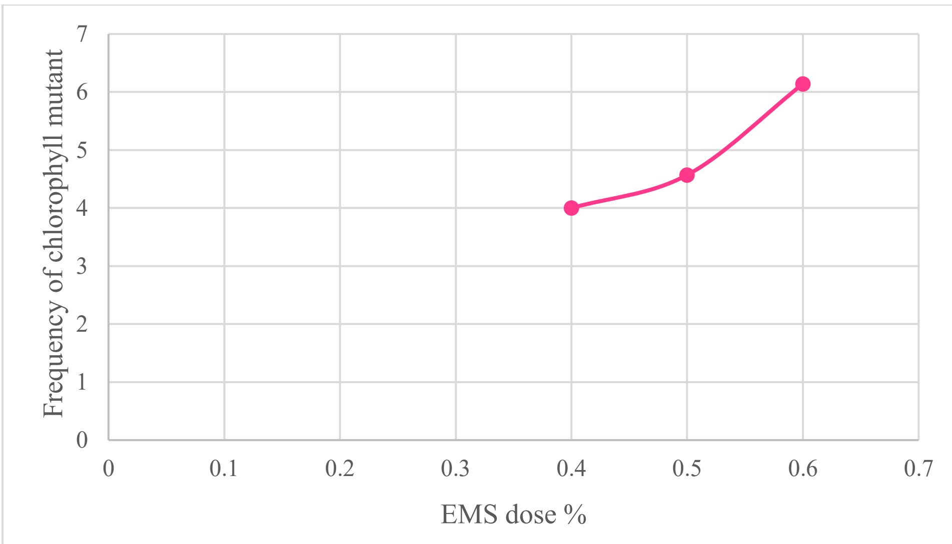

Figure 3. Effect of EMS dose on the frequency of chlorophyll mutations

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 in increases in the chlorophyll mutation frequency in the M_2 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_2 generation (Jia et al., 2019) since most of the mutations are recessive. The presence of chlorophyll mutants in the M_2 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 0.5% 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_2 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 M_2 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 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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