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Short Communication

Characterization of Ethyl Methanesulfonate (EMS)-Induced Mutants in Durum Wheat: Dose-Response Analysis and Mutagenic Effects Assessment

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Abstract

This study aimed to identify the optimal dosage of ethyl methanesulfonate to achieve at least 50% seed germination and a 30% survival rate in durum wheat, and to investigate phenotypic variations in subsequent mutant generations. The study employed response surface methodology to optimize EMS mutagenesis conditions, analyzing three variables: imbibition time, ethyl methanesulfonate concentration, and ethyl methanesulfonate exposure duration, each at three distinct levels. The optimal conditions were determined to be a 4-hour imbibition period followed by an 18-hour exposure to a 0.7% ethyl methanesulfonate solution. Under these conditions, 10% of the M_1 generation and 3% of the M_2 generation exhibited phenotypic variations. Common morphological abnormalities observed included altered spike morphology, abnormal leaf coloration, and irregular leaf morphology. These findings contribute to the practical methodologies used in the field of plant genetics and crop improvement, particularly for generating genetic variability in cereal crops. Additionally, it provided valuable insights into the optimal conditions for inducing mutagenesis in durum wheat, which can be applied in Targeting Induced Local Lesions IN Genomes (TILLING) and eco-TILLING experiments.

Keywords: Chemical mutagenesis, Genetic variation, TILLING, Mutation induction, Phenotypic diversity

Introduction

Mutagenesis has been used to generate genetic variation in various cereal crops, making it an effective and popular tool for crop improvement. Numerous varieties have been released using different mutation breeding techniques (Ahloowalia *et al.* 2004; Suzuki *et al.* 2008; Xin *et al.* 2008; Caldwell *et al.* 2004; Slade *et al.* 2005).

Ethiopia is recognized as one of the primary centers of origin and diversity for durum wheat (*Triticum turgidum* L. var. durum), with a wide range of landraces and traditional varieties cultivated across diverse agro-ecological zones. This rich genetic diversity is attributed to the country's varied topography, climate, and traditional farming practices, which have collectively contributed to the

development and maintenance of a broad genetic base. The genetic pool in Ethiopian durum wheat includes a variety of traits related to biotic and abiotic stress resistance, quality characteristics, and adaptability to different environmental conditions (Tesfaye, 2009; Mengistu and Pe. 2016).

Despite this high genetic diversity, targeted mutagenesis using ethyl methanesulfonate (EMS) remains a valuable tool for enhancing specific traits and accelerating breeding programs. Mutation breeding is particularly useful for identifying novel alleles that may not be present in the existing genetic pool or for creating new genetic combinations that can confer advantageous traits such as improved yield, disease resistance, and stress tolerance. Moreover, recent advancements in DNA screening methods, such as TILLING (Targeting Induced Local Lesions IN Genomes) and eco-TILLING, allow for the efficient identification and utilization of these induced mutations in breeding programs.

The aim of this study was to optimize the conditions for EMS mutagenesis in durum wheat and to characterize the resulting phenotypic variations in the M_1 and M_2 generations. By optimizing parameters such as EMS concentration, exposure time, and seed imbibition time, we seek to enhance the efficiency of mutation induction and to provide a valuable resource for durum wheat improvement.

Materials and Methods

Description of experimental materials

Durum Wheat (*Triticum Turgidum* L. var. "Denbi") seeds, originally collected from Ethiopia, were used to determine optimal mutagenesis conditions and evaluate phenotypic variants. This variety was chosen for its local adaptation and importance in Ethiopian agriculture. The variety was released in 2009 by Debrezeit Agricultural Research Center (DZARC) and its pedigree /selection history/ is AJAIAIBAUSHEN… CSS98IY00025-0MXI-3QK-4DZR.

Development of mutagenic seeds under the optimized condition

Pre-soaking

To enhance the absorption of the EMS solution into the embryo, seeds were soaked in an ample amount of distilled water for 4 hours with gentle shaking at room temperature. The water was changed every 2-3 hours. After the imbibition period, excess water was removed from the seeds by draining them using filter papers.

Buffer solution preparation

Phosphate buffer was used to dilute the EMS solution. A 0.1M sodium phosphate buffer was prepared by mixing solutions of 0.5M monobasic sodium phosphate

and 0.5M dibasic sodium phosphate. The pH of the buffer solution was maintained at 7.2 by adding a small amount of dilute HCl.

EMS treatment

The mutagenesis was conducted inside the fume hood. 7.0 mL of EMS was added to a new flask and filled with sodium phosphate solution up to the 100 mL mark. The solution was filtered using a double 0.2 μm chrome disk (inside the fume hood). Following this, the solution was thoroughly agitated for 10 minutes. Then, the seeds were classified into groups, each weighing 10 grams, and covered with a thin sheath. The seeds were immersed in the EMS solution for 18 hours with gentle shaking while they were in the stocking. After 18 hours of EMS treatment, the seeds were washed with sodium thiosulphate (10% w/v or 100 mM) to inactivate the effect of the EMS prior to sowing. The inactivation process was conducted in the fume hood by soaking the seeds in the solution for 5 hours. Again, the seeds were washed 10 times using distilled water. Then, the seeds were allowed to dry at room temperature for 2 hours before being planted in a well-prepared seedbed for a grow-out test.

In the subsequent growing season, the M_1 generation plants were self-fertilized to produce the M_2 generation. Over 1000 M_2 generation plants were generated from M_1 seeds. The M_2 seeds were also planted again in a plant-row fashion to produce the next (M_3) generation.

Phenotypic examination

The overall work flow of mutant population development is presented in Figure 1. In brief, wild type seeds (M_0) were treated with 0.7 % EMS solution for 18 hours. The M_1 seeds were planted in the glasshouse of Chonbuk National University in 2019. The M_1 plants were harvested and the seeds were sown next year to grow the M_2 generation. Similarly, M_3 generation plants were harvested from M_2 plants. The presence of mutant phenotypes was examined throughout all growth stages in all generations.

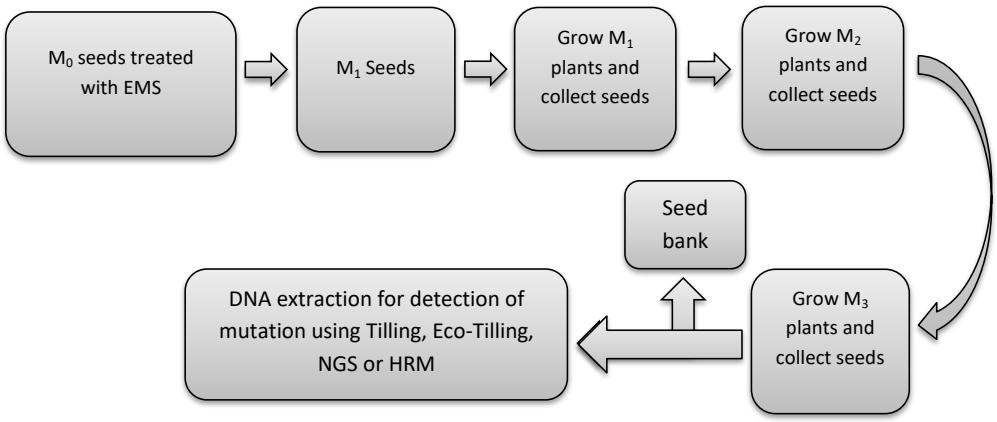

Figure 1. A flow chart representing the activities performed to develop EMS-induced mutations in durum wheat. M_0 seeds were treated with EMS mutagen to produce the M_1 population, from which M_2 seeds were harvested. M_3 seeds were harvested from M_2 plants, and some of the seeds are stored for future use. DNA samples were collected from M_3 plants for the detection of mutation.

Experimental design for lethal dose optimization

Response Surface Methodology (RSM) modeled and optimized EMS conditions using Central Composite Design (CCD). Design-Expert software was used to analyze the data. EMS concentration (%, v/v), EMS exposure time (hours), and imbibition time (hours) were used as independent variables, whereas the response variables were germination percentage, viability and survival rate (%). The experimental variables used in the coded form is given in Table 2.

The experimental results were analyzed using RSM to fit a second-order polynomial equation.

r = the response,
β_o *= constant term,*
β_i *= regression coefficient of the linear terms,*
β_{ii} *= regression coefficient of quadratic terms,*
β_{ij} *= regression coefficient of interaction terms,*
X_i, X_j *= coded variables,*
n = number of independent variables.

Results and Discussion

Optimization experiment

An effective and economical method for inducing mutations is vital for crop improvement and generating variations. Ethyl methanesulfonate (EMS) concentration, imbibition time, and EMS treatment time are critical factors in the mutagenic process. Initially, the optimal EMS conditions were determined using

dose-response curve methods. Various EMS concentrations (0.30% to 0.90%) were tested while maintaining a constant imbibition time (5 hours) and treatment time (18 hours). This method effectively predicted the EMS dose needed to achieve at least 50% germination. However, it did not consider the interactions between different factors over time. Thus, Response Surface Methodology (RSM) based on Central Composite Design (CCD) was employed to identify the best combination of EMS concentration, imbibition time, and treatment time for optimal survival rate.

The germination rate of plants treated with a 0.75% EMS dose closely matched the targeted rate achieved through RSM (i.e 0.70%.). Consequently, the 0.70% EMS concentration was chosen for developing the TILLING population. The graphical presentations of the response factors as affected by the independent variables are presented in Figure 2 and Figure 3. The contour plots represent the interaction effects of the independent factors. Optimal mutagenesis conditions for a 50-60% germination rate, derived from response surface analysis, were an EMS concentration of 0.7%, an EMS treatment time of 18 hours, and an imbibition time of 4 hours.

Increasing EMS concentration beyond 0.7% resulted in reduced seed germination. Longer EMS treatment and imbibition times increased the absorbance of the mutagen solution into the seeds, enhancing the mutagenesis rate. Bahar and Akkaya (2009) reported similar findings, supporting the results of this study.

All statistical parameters, including the highly significant coefficient of multiple determination (R-squared = 0.80) and the non-significant lack of fit test, indicated a good fit between the trial data and the model. The ANOVA model was significant at the 0.05 significance level (Table 1). Based on the adjusted R-squared and predicted R-squared values, the highest-order polynomial model was selected. The resulting linear model in terms of coded variables for the germination rate is as follows:

Germination rate =59.62-0.7023x1-2.92x2-8.57x3+0.7500x1x2+0.500x1x3+
1.00x2x3+0.6185x12-1.15x22+10.16x32

The optimized conditions in this study are consistent with those reported by Ndou *et al.* (2013), who achieved a 50-60% germination rate in seeds exposed to a 0.7% EMS concentration for 2 hours. Their study also noted the negative effects of EMS on plant height and seed viability.

High doses of EMS resulted in reduced seed germination. Increasing the EMS dose, imbibition time, and exposure time beyond the optimal level negatively affected all traits considered in the study. Similar findings were reported by Rupinder and Kole (2005) and Chen *et al.* (2012). Singh and Kole (2005) noted

that higher EMS concentrations reduced germination, likely due to differences in water potential outside the seed, preventing sufficient water uptake for proper germination.

Phenotypic response of seedlings to the identified optimum EMS concentration was observed starting from the date of germination. Variation in leaf size and leaf shape were observed. Some of the seedlings shriveled and started to die soon after transplanting. Less than 40 % of the seedlings survived and reached maturity. Various mutant phenotypes were observed and recorded throughout the plant growth stages. Gradual increase in EMS concentration beyond 0.7 %, at a constant EMS treatment time of 18 hours, was lethal and led to poor survival rate. The survived seedlings were all capable of setting seeds.

Phenotypic response of seedlings

Phenotypic responses to the optimal EMS concentration were observed from germination onwards. Variations in leaf size and shape were noted. Some seedlings shriveled and died shortly after transplanting, with less than 40% surviving to maturity. Various mutant phenotypes were recorded throughout the plant growth stages. Increasing EMS concentration beyond 0.7%, with an EMS treatment time of 18 hours, was lethal and led to a poor survival rate. The surviving seedlings were capable of setting seeds.

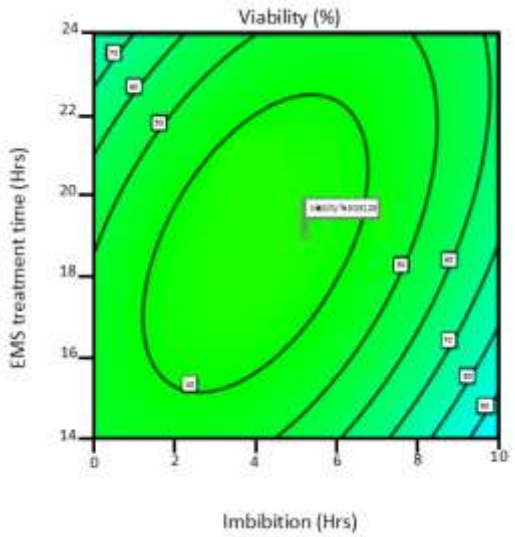

Figure 2: A response surface plot showing the effect of imbibition time and EMS treatment time on viability of durum wheat seeds at an EMS concentration of 0.7 %.

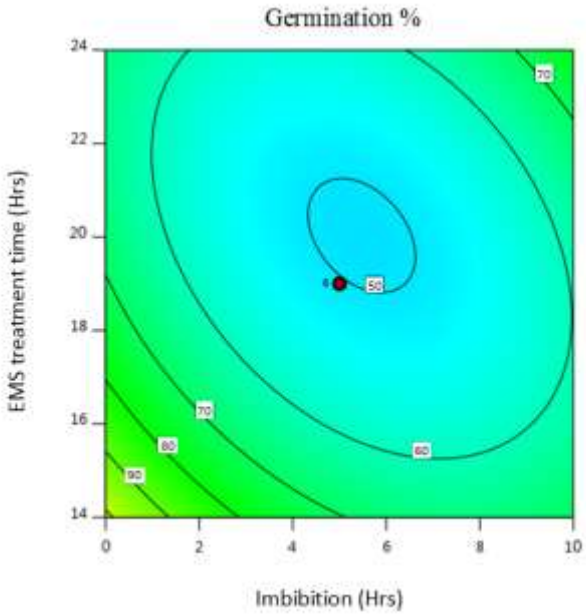

Figure 3: A response surface plot showing the effect of imbibition time and EMS treatment time on germination rate of durum wheat seeds at an EMS concentration of 0.7 %.

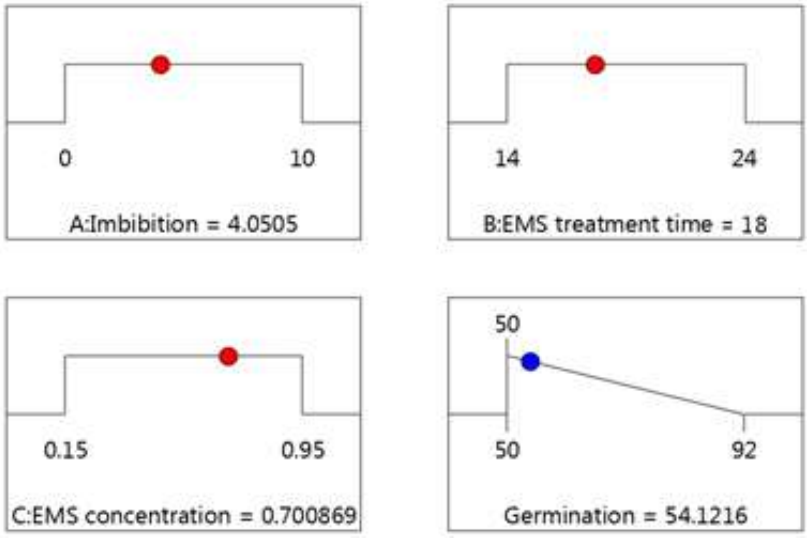

Figure 4: A graphical presentation of the optimized EMS conditions to achieve a 50 % germination rate in durum wheat seeds.

Analysis of variance (ANOVA)

The coefficient of determination (R-squared) value from the regression line was 80. This indicates that the fitness of the linear model was satisfactory and can

explain 80% of the variations. The model p-value was also found to be less than 0.05, which indicates that the conditions in the model are significant.

The degrees of freedom for the numerator (df from the Model row) were 9, and for the denominator (df from the Residual row) were 10. At a 0.05 significance level, the critical F-value was determined to be 2.70. The model's F-value was 4.18, indicating a statistically significant difference between groups or conditions when considering the entire model.

Except for EMS concentration (Factor C), other factors (imbibition, EMS treatment time) and interaction terms ((AB, AC, BC, A2, B2, and C2)) did not show a statistically significant effect on the response variable. The residual row represents the error term, indicating unexplained variation in the model.

Table 1. ANOVA for quadratic model (Germination %)

Source	Sum of squares	df	Mean Square	F-value
Model	2698.01	9	299.78	4.18
A-Imbibition	6.74	1	6.74	0.0939
B-EMS treatment time	116.36	1	116.36	1.62
C-EMS concentration	1003.13	1	1003.13	13.99
AB	4.50	1	4.50	0.0627
AC	2.00	1	2.00	0.0279
BC	8.00	1	8.00	0.1115
A2	5.51	1	5.51	0.0769
B2	19.04	1	19.04	0.2654
C2	1488.91	1	1488.91	20.76
Residual	717.19	10	71.72	

Table 2. Experimental design for optimization of variables using Central Composite Design

Standard order[a]	Run order[b]	Uncoded Imbibition time (hr)	Uncoded EMS treatment (hr)	EMS concentration	Response (Germination %)
1	11	5	14	0.55	90
2	10	10	19	0.55	87
3	13	5	19	0.15	50
4	19	5	19	0.55	68
5	9	5	19	0.55	70
6	1	0	14	0.15	80
7	8	10	24	0.95	53
8	15	5	19	0.55	49
9	20	5	19	0.55	58
10	4	10	24	0.15	55
11	6	10	14	0.95	65
12	2	10	14	0.15	58
13	7	0	24	0.95	70
14	12	5	24	0.55	75
15	16	5	19	0.55	45
16	3	0	24	0.15	58
17	17	5	19	0.55	60
18	5	0	14	0.95	80
19	18	5	19	0.55	77
20	14	5	19	1.5	72

[a]Non randomized
[b]Randomized

Phenotypic characterization of the mutants

Based on the optimization experiment, treating seeds with a 0.7% EMS concentration for 18 hours was chosen and applied to approximately 2500 seeds (~120g) in different batches. This treatment resulted in 1215 mutant M_1 lines for phenotypic investigation. Both mutated and control seeds were planted under similar conditions for comparison. The mutant generations were maintained using the ear-to-row method for evaluating individual mutant lines.

Phenotypic characteristics of M_1 and M_2 mutant populations were recorded. Untreated normal seeds were used as a control. Out of the total 1215 M_1 plants, 126 plants exhibited abnormal phenotypic characteristics. Only 430 plants out of 1215 M_1 plants produced fertile M_2 seeds. Low fertility is common in chemically induced mutant plants (Girija *et al.* 2013; Kim *et al.* 2006). In the M_1 generation, 46 out of the 126 mutant phenotypes were chimeral leaf mutants. Mutant phenotypes were observed in less than 3% of the M_2 mutant population. Generally, altered phenotypes were observed in leaf structure, pigmentation, seed morphology, and developmental characteristics such as maturity time. Higher numbers of altered mutant phenotypes were recorded in the area of pigmentation, followed by morphological structures, and then developmental features. Overall characteristics of the M_1 and M_2 mutagenized populations are summarized in Table 3.

Table 3. Observed aberrant phenotypes in M_1 and M_2 generation population

Category of mutant phenotypes	Specific mutant characteristics
Leaf chimeras (Necrotic Mutants)	Yellow-green (pale green) leaves, yellow colored leaves in the left/right side of the midrib, chimeric mutant seedling having patches of pale green tissue parts, small to medium chlorotic and necrotic lesions leading to early senescence of lower leaves, unique developmental pattern: green leaves having yellow midrib and yellow leaves having green midrib in the same plant
Seedling and leaf morphology	Narrow stiff and erect leaves, distortion caused by adherence of first two leaves, seedling distortion caused by tightly rolled first three leaves adhering at leaf tips, shiny and smooth leaves, tightly rolled first leaf encasing emerging second leaf, wrinkled leaf blades, irregular first leaves and tightly rolled (hook-like rolls) second leaves, prostrate growth habit
Spike and Kernel morphology	Small kernel, infertile kernel, abnormal spike morphology, variation in seed size, variation in spike color
plant height	Shortened internodes, dwarf and semi-dwarf
Plant growth habit	Highly variable dates of maturity, multiple tillers, infertile tillers
Lethal effect	Withering

Out of 1215 M_1 plants, 126 exhibited abnormal phenotypes, and only 430 produced fertile M_2 seeds, consistent with previous reports of low fertility in chemically induced mutants (Girija *et al.* 2013; Kim *et al.* 2006). In the M_1 generation, 46 out of 126 mutant phenotypes were chimeral leaf mutants. Less than 3% of the M_2 mutant population exhibited mutant phenotypes, including

variations in leaf structure, pigmentation, seed morphology, and developmental traits. Higher numbers of altered phenotypes were observed in pigmentation, followed by morphological and developmental features.

Compared to the wild-type (M_0) seeds, most M_1 and M_2 families showed delayed maturity, reduced tiller number, shorter plant height, and abnormalities in leaf coloration and spike characteristics.

Subcategories of mutants representing some of the visual phenotypes observed in the M_1 and M_2 generation population are shown in Figure 5.

Figure 5. Visual representation of various mutant phenotypes observed at M_2 generation of durum wheat (Var. Denbi). This includes stunted seedling growth habit (A), variable maturity dates (B), shortened internodes and yellow spikes (C), abnormal leaf morphology (D, E), and necrotic or chlorophyll mutants (F, G, H). [original photo taken by Daniel H.].

Conclusion and Recommendation

This study successfully optimized the conditions for EMS mutagenesis in durum wheat using Response Surface Methodology (RSM). Our findings reveal that an EMS concentration of 0.7% for an 18-hour treatment period yields the optimal germination rate of 50%. The dose-response analysis confirmed that higher EMS concentrations and longer treatment durations adversely affect plant germination rates.

The implementation of this optimized EMS protocol allowed us to generate 1,215 mutagenized M_1 lines, which exhibited a broad spectrum of chlorophyll and

morphological mutations. These mutants are poised to enhance research in durum wheat genetics, breeding, and functional genomics.

Furthermore, the developed EMS mutagenesis protocol proves to be highly effective in minimizing negative impacts on seed viability and fertility. This makes it an excellent tool for TILLING (Targeting Induced Local Lesions IN Genomes) and ecoTILLING applications. Overall, this optimized protocol offers significant potential for advancing durum wheat research and contributing to crop improvement initiatives, thus providing a valuable resource for the scientific community and agricultural development.

Acknowledgements

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