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RESEARCH ARTICLE

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Investigating the impact of ethyl methanesulfonate (EMS) on in vitro germination indices of sorghum with Taguchi design

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ABSTRACT: Sorghum (*Sorghum bicolor* L.) is a major edible cereal and forage crop, cultivated all over the world, and can grow on low-fertility lands. However, the development of new cultivars with desired traits is highly desirable and can be achieved by using chemical mutagens. The seeds were treated with ethyl methane sulfonate (EMS) at the rates of 1.0%, 2.0%, and 3.0%. Surface-sterilized seeds were treated for 2h, 4h, and 6h, followed by culturing the seeds on Murashige and Skoog (MS) medium for germination. Data regarding germination were calculated after 2, 4, 6, and 8 days, followed by analysis using the GerminaR package, with different germination metrics. Data generated using GerminaR was analyzed by analysis of variance (ANOVA) and Taguchi Design (TD) analysis for multifactorial optimization. Results illustrated that seed treatment with.0% EMS for 2 hours enhanced germination performance with low mean germination time (MGT), uncertainty (UNC), germination speed, and synchrony. Whereas application of 3.0% EMS for longer durations negatively impacted germination metrics, indicating stress-induced delays and variability. The results of the TD model highlighted that EMS concentration primarily influences germination percentage (mgp) and synchrony (SYN). Whereas treatment time influenced temporal parameters such as mean germination time (MGT) and germination variance. The study underscores the utility of germination indices and Taguchi optimization in assessing and refining EMS-induced mutagenesis protocols for crop improvement.

KEYWORDS: Ethyl methane sulfonate (EMS), Germination Indices, Sorghum, Taguchi Design

INTRODUCTION

Sorghum (*Sorghum bicolor* L.) is an edible forage crop of C4 plants [1], cultivated across the globe due to its high adaptability under different climatic conditions [2, 3]. Sorghum ranks fifth among cereals and grains and is considered a staple food due to its high nutritional values [4, 5] in Africa and Asia. Its survival under adverse conditions [6] increases its significance for food security at the international level [7]. However, researchers are seeking new cultivars with high nutrition for both humans and forage, and employing multiple techniques ranging from lassial breeding to modern biotechnological approaches [8, 9]. Chemical-induced mutation is one of the prominent techniques for improving traits, but optimizing the dose with minimum damage to plants is a prerequisite for the whole process.

Germination is a complex biological and physiological process regulated by genetic and environmental factors [10]. It is the first step for seedling establishment after germination and controlling the whole growth phases of plant life. During germination, seed behavior and response to external stimuli are highly significant and can be analyzed by quantitative parameters [10, 11]. These Quantitative parameters, also known as germination indices or germination indexes (GI), give an understanding of the germination process in response to different climatic or induced conditions like stresses or treatments. Researchers have reported different germination indices to understand the process to make plans accordingly up to harvesting. The mathematical expressions for different germination indices are helpful to understand the germination process from different angles and aspects [11, 12]. In this study, EMS was used as a chemical agent to investigate its impact on seed germination indices. EMS is a powerful chemical mutagen for inducing mutagenesis in plants [13], and it also regulates the germination process [14, 15].

Seed treatment with chemical agents under in vitro conditions offers to visualize the whole germination process easily, and can be employed for different types of stressors (biotic or abiotic) [16]. However, the optimization of different factors like stressor type or concentration is also significant. The analysis of data with traditional ANOVA analysis often fails to present the actual impact of these stressors and requires other statistical or mathematical

models to understand the complex biological processes, like germination [17]. Design of experiment (DOE) is a powerful tool for optimizing multiple factors [18]. Two main models used in DOE are response surface methodology and Taguchi design (TD). The TD is a powerful tool to optimize the data by using signal-to-noise (S/N) ratio. The S/N ratio of all factors generates the delta, and the system ranks the input factors based on the high delta score of all output or dependent variables [19, 20]. In this study, sorghum seeds were treated with different concentrations of EMS with different exposure times. The results were analyzed by generating different germination indices, followed by analyzing data using traditional statistical analysis and Taguchi design.

MATERIAL AND METHODS

In vitro EMS Treatment

The seeds of the sorghum genotype used as forage were selected for EMS treatment. After surface sterilization, seeds were primed with different concentrations (1.0%, 2.0%, and 3.0%) of EMS for 2h, 4h, and 6h under in vitro conditions. The stock solution of EMS was filter sterilized and stored at 4 ºC. After priming, 10 seeds were placed in each jar for germination, kept in the automated growth chamber (24±2 ºC; 50.0% R.H., and 16 h light photoperiod). The MS medium was prepared according to a standard protocol for in vitro germination studies.

Germination Indices

Germination was controlled after every two days for up to 8 days to analyze germination indices. For this purpose, the GerminaR package [21] was used.

Germination percentage (GRP): The germination percentage (GRP) refers to the percentage of seeds that completed the germination process.

$$grp = \frac{\sum_{i=1}^{k} n_i}{N} \times 100$$

where n_i is the number of seeds germinated in the i^{th} time; N represents the total number of seeds in each experimental unit; k refers to the last day of germination evaluation.

Mean germination time (MGT): The MGT denotes the number of seeds germinated with respect to the number of seeds germinated at the time of evaluation.

$$mgt = \frac{\sum_{i=1}^{k} n_i t_i}{\sum_{i=1}^{k} n_i}$$

t_i is the time from the beginning of the experiment to the i^{th} observation.

Germination speed coefficient (GSP): The GSP refers to how quickly seed germination occurs, and a higher GSP means faster germination.

$$gsp = \frac{\sum_{i=1}^{k} G_i}{\sum_{i=1}^{k} G_i X_i} \times 100$$

G_i is the number of seeds germinated in the i^{th} time, and X_i represents the number of days from sowing.

Uncertainty (UNC): The germination uncertainty (UNC) is used to measure how evenly germination is spread out over time. When UNC values are low, it usually means that more seeds are germinating at the same time. In other words, low uncertainty indicates that germination is more concentrated. This index captures how spread out or clustered the germination is.

$$unc = -\sum_{i=1}^{k} f_i \log_2 f_i \text{ with } f_i = \frac{n_i}{\sum_{i=1}^{k} n_i}$$

Synchronization (SYN): The germination synchrony (SYN) was originally developed to estimate the degree of overlapping of flowering among individuals in a population. Later, it was also used as a germination index, and the value of SYN = 1 expresses the germination of entire seeds at the same time, and SYN = 0 expresses the germination of at least two seeds, one at a time. More importantly, SYN only produces a non-zero value if and only if two seeds complete the seed germination process at the same time.

$$syn = \frac{\sum_{i=1}^{k} C_{n_i,2}}{C_{\sum n_i,2}} \text{ with } C_{n_i,2} = \frac{n_i(n_i-1)}{2}$$

Germination variance (VGT): The index of germination variance refers to the germination time of the seeds that vary within a population. The high VGT refers to the high variability and vice versa.

$$vgt = \frac{\sum_{i=1}^{k} n_i \left(t_i - \bar{t}\right)^2}{\sum_{i=1}^{k} n_i - 1}$$

Germination coefficient of variation (CVG): The germination coefficient of variation is a statistical technique used for assessing the uniformity of seed germination. High CVG refers to high variability, and vice versa, low CVG refers to uniform germination over time.

$$cvg = \left(\frac{\sqrt{vgt}}{mgt}\right) \times 100$$

Statistical Analysis

The germination data were recorded at an interval of 2 days. The generated data regarding germination using GerminaR was subjected to ANOVA analysis, and differences between means were tested with Tukey's. The data were also analyzed with the Taguchi Method to find out

the robustness of the model using the S/N ratio. For both statistical analyses, the Minitab 21.0 program was employed.

RESULTS AND DISCUSSION

ANOVA analysis of gemination indices

Germination indices or germination index refer to understanding the germination process using different statistical or mathematical tools [22]. These tools are highly efficient in understanding the seed viability, uniformity, speed, and robustness under varying conditions, making them more reliable indicators, especially vigor or performance under stress conditions [17]. These indices have been documented to understand seed germination against different stressors like aging, pollutants, drought, and salt stress, etc [23]. Therefore, these indices are highly effective and serve as sensitive, multifactorial metrics that offer deeper insight and aid in seed selection, breeding, and environmental monitoring [11, 24]. In this study, seeds exposed to EMS concentration for different treatment times were analyzed with two different techniques using classical ANOVA and a modern statistical model to find out the most effective factor regulating the germination process. The results of germination indices are presented in Table 2. Results revealed that the maximum GRP was recorded for treating seeds with 1.0% EMS for 6 h. Whereas seed treatment with 1.0% EMS for 2 h was found to be optimum and resulted in the lowest MGT, UNC, and VGT. and high GSP, and SYN index. Treating seeds with 3.0% EMS for 2,4, and 6 h yielded a relatively high but similar GRP score of 62.50%. Whereas values for other parameters were very close to each other, and recorded as 3.88-4.79 for MGT, 1.42-1.48 for GSP, 1.42-1.48 for UNC, 0.29-0.32 for SYN, and 57.86-60.49 for CVG. In general, seed treatment with 3.0% EMS for 4 h was most optimal. Whereas, the least effective combination was recorded as 3.0% EMS for 6 h based on gemination index.

Table 1. ANOVA analysis of germination indices of sorghum

EMS (%)	Time (h)	Mean	StDev	Mean	StDev	Mean	StDev
		GRP (%)**		MGT (d) ^{ns}		GSP (%)^{ns}	
0	0	55.00ab	5.77	4.133	0.625	0.246	0.039
1	2	50.00ab	11.55	3.625	0.725	0.283	0.050
1	4	45.00ab	10.00	4.625	0.854	0.222	0.045
1	6	65.00a	5.77	4.345	0.901	0.239	0.062
2	2	55.00ab	5.77	4.183	1.243	0.258	0.086
2	4	52.50ab	5.00	4.750	0.526	0.212	0.025
2	6	55.00ab	5.77	4.167	0.467	0.242	0.027
3	2	62.50a	5.00	3.929	0.992	0.268	0.076
3	4	62.50a	5.00	3.881	0.887	0.268	0.062
3	6	62.50a	5.00	4.786	0.362	0.209	0.016
EMS (%)	Time (h)	MGR (d^{-1})^{ns}		UNC (bit)^{ns}		SYN^{ns}	
0	0	24.64	3.96	1.639	0.338	0.216	0.145
1	2	28.33	5.02	1.172	0.358	0.350	0.159
1	4	22.24	4.53	1.605	0.209	0.158	0.016
1	6	23.98	6.25	1.678	0.454	0.216	0.124
2	2	25.81	8.67	1.366	0.417	0.316	0.159
2	4	21.27	2.57	1.683	0.280	0.183	0.088
2	6	24.23	2.79	1.522	0.221	0.250	0.100
3	2	26.88	7.63	1.466	0.601	0.317	0.252
3	4	26.82	6.24	1.424	0.332	0.290	0.097
3	6	20.98	1.62	1.483	0.480	0.297	0.160
EMS (%)	Time (h)	VGT (d^2)^{ns}		SDG (d)^{ns}		CVG (%)^{ns}	
0	0	6.36	2.12	2.495	0.429	60.25	2.58
1	2	5.15	1.42	2.254	0.302	62.75	3.84
1	4	8.07	2.48	2.814	0.448	61.09	2.21
1	6	6.83	2.57	2.569	0.560	58.99	1.54
2	2	6.80	3.42	2.530	0.728	60.58	3.51
2	4	8.08	1.43	2.835	0.263	59.77	1.41
2	6	6.07	1.32	2.454	0.270	58.91	1.45
3	2	5.72	2.58	2.343	0.559	59.89	2.50
3	4	5.87	2.62	2.372	0.568	60.94	2.97
3	6	7.66	0.90	2.765	0.164	57.86	2.23

**significant at p0.01; ^{ns}non-significant, d:days, StDev: standard deviation

Table 2. The Taguchi Design-based S/N ratio results of germination indices of sorghum

	GRP (%)**		MGT (d) ns		GSP (%)ns	
Level	**EMS (%)**	**Time (h)**	**EMS (%)**	**Time (h)**	**EMS (%)**	**Time (h)**
1	34.70	34.70	-12.40	-12.40	27.60	27.60
2	34.11	34.67	-12.54	-12.04	27.46	27.96
3	34.57	34.29	-12.91	-12.98	27.09	27.02
4	35.86	35.58	-12.55	-12.98	27.45	27.02
Delta	1.75	1.29	0.51	0.95	0.51	0.95
Rank	1	2	2	1	2	1
	MGR (d^{-1})ns		**UNC (bit)ns**		**SYNns**	
Level	**EMS (%)**	**Time (h)**	**EMS (%)**	**Time (h)**	**EMS (%)**	**Time (h)**
1	-12.40	-12.40	-4.426	-4.426	-17.41	-17.41
2	-12.54	-12.04	-3.521	-2.838	-14.40	-13.80
3	-12.91	-12.98	-3.777	-4.006	-14.91	-14.87
4	-12.55	-12.98	-3.611	-4.064	-13.43	-14.08
Delta	0.51	0.95	0.905	1.588	3.98	3.61
Rank	2	1	2	1	1	2
	VGT (d^2)ns		**SDG (d)ns**		**CVG (%)ns**	
Level	**EMS (%)**	**Time (h)**	**EMS (%)**	**Time (h)**	**EMS (%)**	**Time (h)**
1	-16.42	-16.42	7,638	7,638	-35.61	-35.61
2	-16.68	-15.88	7,722	6,893	-35.70	-35.72
3	-17.16	-17.55	7,875	8,171	-35.53	-35.65
4	-16.49	-16.90	7,476	8,009	-35.50	-35.36
Delta	0.74	1.67	0,399	1,278	0.20	0.37
Rank	2	1	2	1	2	1

EMS: Level 1:control, Level 2= 1.0 % EMS, Level 3= 2.0 % EMS, Level 3= 4.0 % EMS; Time: Level 1 = 0 h, Level 2 = 2 h, Level 3 = 4 h, Level 4 = 6 h

Taguchi design analysis

The Taguchi design is an efficient statistical tool for optimizing multiple factors by ranking them based on the S/N ratio. For germination studies, the model offers researchers to investigate the impact of external factors [25, 26]. Furthermore, the TD model also offers sensitivity analysis to identify the most critical factors [27]. The results of TD analysis are presented in Table 2, and a graphical presentation of the data is presented in Figure 1a-i. For GRP, EMS was the most superior factor compared to time, with a delta score of 1.75 and 1.29, respectively. Whereas the S/N ratio score was recorded 34.11-35.86 for EMS and 34.29-35.58 for treatment time. The results of MGT exhibited the negative S/N ratio for both EMS concentration and treatment time, and ranged from -12.40 to -12.91 (EMS) and -12.04 to -12.98 for treatment time. The result of the delta score ranked treatment time first and EMS second. The S/N ratio score of GSP was observed to be 27.06-27.60 for EMS and 27.02-27.96 for treatment time. The delta score followed the pattern of MGT and ranked treatment time as the more significant factor compared to EMS. The results of MGR also revealed a negative S/N ratio score like MGT, and recorded -12.40 to -12.91 for EMS and -12.04 to -12.98 for treatment time.

The results of the delta score ranked treatment time and EMS as first and second. The UNC results were also negative for the S/N ratio and ranged from -3.611 to -4.426 for EMS and -2.838 to -4.426 for treatment time. The delta score of the S/N ratio revealed time as a more significant factor than EMS. Likewise, the S/N ratio of SYN was also negative and ranged from -13.43 to -17.41 (EMS) and -13.80 to -17.41 (treatment time), with EMS ranked 1st. The results of the S/N ratio of VGT revealed a high delta score of treatment time (1.67) compared to the EMS (0.74). However, the S/N ratio was also negative and ranged from -16.49 to -17.16 for EMS and -15.88 to 16.90 for treatment time. The S/N ratio of CVG metric was recorded as -35.50 to -35.70 (EMS) and -35.36 to -35.72 (treatment time), with treatment time having a higher delta score (0.37) than EMS (0.20).

The TD analysis provided a robust framework for investigating the impact of EMS concentration and treatment time on different germination indices. Results revealed the trait-specific dominance, and EMS was more significant for GRP and SYN indices. On the contrary, treatment time was more critical for temporal parameters like MGT, MGR, and UNC. These trends reflect the biological nature of parameters as EMS regulates the physiological and genetic mutations,

which in turn regulate the germination process [14, 28]. Whereas treatment time modulates the depth and duration of those biological factors. It was evident from the results that negative S/N ratios for most of the traits underline the stressful nature of EMS. Results revealed that MGT and MGR exhibited negative S/N ratios, indicating variability and the possibility of physiological stress. Treatment time exhibited a higher delta score for both traits, showing that longer exposure to EMS may lead to delayed or reduced germination due to cellular stress or toxicity. Results revealed that effective mutagenesis without compromising seedling vigor is a big challenge.

On the contrary, a positive but narrow S/N ratio gap for GSP revealed that both factors can be co-optimized for maximum speed. Although the use of Taguchi design is limited in plant sciences, especially for germination, studies on investigating germination in response to ZnO nanoparticles in ghaf seeds [25], optimizing factors regulating seed germination in barley [24], and the impact of priming techniques [29] using Taguchi design have been documented. Optimization of multiple input factors using statistical models can be employed followed by employing machine learning models for analyzing prediction and optimization.

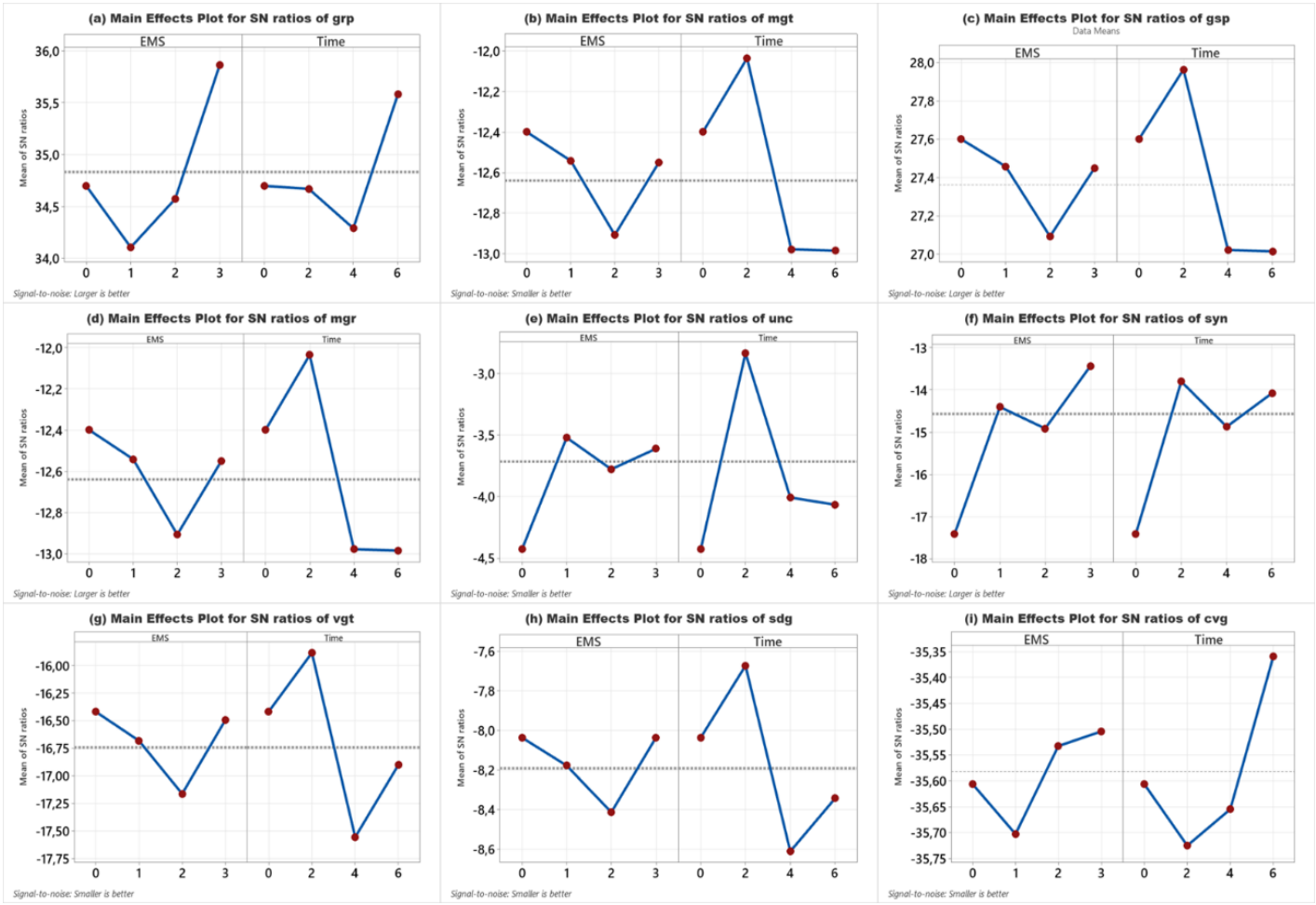

Figure 1: Signal-to-noise (S/N) ratio analysis of different germination indices using Taguchi design (a) GRP, (b) MGT, (c) GSP, (d) MGR, (e) UNC, (f) SYN, (g) VGT, (h) SDG, and (i) CVG

CONCLUSION

The study presents the impact of EMS on in vitro germination performance of sorghum by adopting two different statistical approaches of ANOVA and Taguchi design. Both statistical models revealed the significant impact of EMS concentration and treatment time on germination indices. It was concluded that low EMS concentration for a shorter time can induce mutation without affecting seed vigor or viability. The TD model excels in the relevance of EMS on germination traits. The study highlights the significance of combining germination indices with

advanced statistical tools to better understand the complex biological responses to chemical mutagens.

DECLARATION

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The study was conducted by the first author under the supervision of the second author as a part of PhD thesis research work.

Authorship Contributions

Concept: M.A., Design: M.A., Data Collection or Processing: A.K., Analysis or Interpretation: M.A., A.K., Literature Search: A.K., Writing: M.A., A.K.

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Competing interests

The authors declared that there is no conflict of interest.

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