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RESPONSE OF SEEDLING DEVELOPMENT OF CLARY SAGE (*SALVIA SCLAREA* L.) TO ETHYL METHANE SULFONATE (EMS) APPLICATIONS

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Abstract

This study was carried out to evaluate the effects of ethyl methane sulfonate (EMS) on the emergence rate and seedling characteristics of clary sage (*Salvia sclarea*) and to lay the foundation for future breeding studies. *S. sclarea* seeds were kept at 4 different EMS doses (0.25%, 0.50%, 0.75% and 1%) for 4 different durations (6, 12, 24 and 48 hours). Seedlings were grown in the greenhouse. 0.25% EMS concentration was determined as the LD_{50} dose for *S. sclarea* used in the research. Among the EMS doses, the highest emergence value (72.00%) was taken from the control application and the lowest value (46.94%) was taken from the 1.00 EMS application. Among the application durations, the highest value (83.30%) gave 12hr, while the lowest value (32.15%) gave 24hr. Considering the Dose x Application Duration interaction, the highest (88.75%) and lowest (1.25%) values were recorded in 0.25% EMS-12hr and 1.00%EMS-48hr applications, respectively. Among the seedling characteristics examined, the highest values for seedling and root length, fresh and dry seedling weight, fresh root weight and leaf length were obtained from the 0.25% EMS-12hr application. The findings obtained from our study showed that as the application time and dose increased, the emergence was negatively affected, and that the EMS application time was more effective than the EMS application dose.

Key words: *Salvia sclarea*; Ethyl methane sulfonate; Emergence; Seedling length; Root length

Introduction

Türkiye is an important gene center of the *Salvia* genus. 99 species of this genus, 51 of which are endemic, are naturally distributed in the Flora of Türkiye (Güner *et al.*, 2012). *Salvia sclarea* L. (Clary sage), one of these species and widely seen in Türkiye, is a biennial or perennial plant that can grow up to 140 cm, with a thick, erect stem branched at the top. It has lilac, white or pale blue flowers. The seeds are brown, round and triangular. Its leaves are stalked, heart-shaped, and have gray glandular hairs on the plant (Koul *et al.*, 2017; Aćimović *et al.*, 2018; Ranđelović *et al.*, 2022). The plant develops rosette leaves the first year and blooms the following year. Usually flowering begins in May and continues until the end of August. Those whose ripening period is less than 170 days are considered early, those whose ripening period is between 170 and 200 days are considered medium early, and those whose ripening period is more than 200 days are considered late (Aćimović *et al.*, 2018).

Clary sage is grown commercially largely in Russia, Bulgaria, France, and Morocco, and approximately 150 tons of essential oil is produced annually in these countries (Dzamic *et al.*, 2008; Hristova *et al.*, 2013; Yaseen *et al.*, 2015; Tuttolomondo *et al.*, 2020). Clary sage contains a valuable essential oil that is widely used in perfumery industries as a source of fragrance with a refreshing and long-lasting note (Yaseen *et al.*, 2015; Sharmeen *et al.*, 2021).

One of the most important needs in the production of medicinal and aromatic plants, which are important sources of plant secondary metabolites with a wide range of uses around the world (Kumar & Gupta, 2008; Barut *et al.*, 2022), is the development of varieties with high yield and quality. Breeding studies continue based on variations caused by genetic and environmental factors both between species and between individuals of the same species.

With mutation breeding, a wide range of genetic variability can be created in the plant material studied. Ionizing radiation (X-rays, gamma rays, neutron beams) and chemical agents (such as ethyl methanesulfonate, ethylemine, and nitroethyl urethane) are employed in mutation breeding to induce genetic diversity. Chemical mutagens are essential for generating genetic variability in plant breeding programs. (Maluszynski *et al.*, 1995; Kumar *et al.*, 2019). EMS ($CH_3OSO_2C_2H_5$) is the most common chemical mutagen used in plant breeding due to its efficacy and ease of application (Saima Mir *et al.*, 2021; Türkoğlu *et al.*, 2023). Plant tissues and cells' cytological, genetic, physiological, and morphological characteristics can all be impacted by EMS, which impacts a very brief chromosomal region that contains one or more genes. EMS may alter the cytological, genetic, physiological, and morphological characteristics of plant tissues and cells by affecting a relatively brief chromosomal section that contains one or more genes (Waugh *et al.*, 2006). High sterility is regularly caused by an extremely high mutation rate. Plant breeders therefore strive for less physiological harm and greater mutagenic consequences (Konzak *et al.*, 1965; Usharani & Ananda Kumar, 2015).

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Mutagen doses vary based on the mutagen type and plant species (Roychowdhury & Tah, 2011). Excessive mutagen dosages can result in lethal effects, whereas insufficient dosages may fail to induce the desired mutations. Consequently, the dosage of the mutagen must be titrated to achieve the desired mutation rate. High concentrations of mutagens can induce severe deleterious effects, such as reduced seed germination, seedling viability, pollen sterility, and plant survival. To mitigate the adverse effects of mutagens on plant parameters, further research is required to optimize application timing and concentrations (Siddiqui *et al.*, 2009; Ke *et al.*, 2019).

To date, more than 3200 mutant varieties derived from 200 plant species have been released for commercial cultivation. Nevertheless, the application of mutation breeding to medicinal and aromatic plants remains relatively limited compared to cereals, ornamental plants, and legumes (Kolakar *et al.*, 2018; Saima Mir *et al.*, 2021).

In this study, it was aimed to determine the optimum dose and duration by examining the effects of different doses and durations of EMS on seedling development of clary sage. There is no clary sage variety in Türkiye. This study is a preliminary study of the clary sage mutation breeding program. At the same time, this study is the first report in the field of mutation breeding in clary sage.

Materials and Methods

This research was conducted in the greenhouse located at Yozgat Bozok University, Faculty of Agriculture (Yozgat/Türkiye).

Material: Seeds obtained from *Salvia sclarea* plants (germination value average 80%) grown in the collection plots at Yozgat Bozok University Agricultural Application and Research Center were used as plant material in the research.

Ethyl methane sulfonate (EMS) application: EMS to be used as chemical mutagen was calculated as 1 mL of solution for each seed, and the pH of the solution was adjusted to 7.5 with phosphate buffer solution. For each application group, the seeds were kept in the EMS solution prepared separately in glass conical flasks in a magnetic stirrer at room temperature for 6, 12, 24 and 48 hours without pre-soaking. Four different EMS concentrations (0.25%, 0.50%, 0.75% and 1%) were applied, excluding the control. Distilled water was used as control. At the end of the application, the seeds were washed 20 times with 40 mL of pure water (Kim *et al.*, 2006; Unan *et al.*, 2022).

Preparation of phosphate buffer solution:

1) 100 mL 1 M K_2HPO_4 and 100 mL 1 M KH_2PO_4 were prepared,
2) 70 mL of 1 M K_2HPO_4 is taken into a separate container, 20 mL of 1 M KH_2PO_4 is added to it and the pH is fixed by adding 1 M KH_2PO_4 to 7.5 and
3) 1 M phosphate buffer solution was diluted with distilled water until it reached 100 mM (Kim *et al*., 2006).

The experiment was conducted according to the random parcels with 4 replications. Seeds treated with EMS were sown directly in vials containing 4:1 peat: perlite in the greenhouse (14.04.2020) and routine irrigation was applied. Values for emergence rate (%), seedling length (SL) (mm), root length (RL) (mm), fresh seedling weight (RSW) (g), dry seedling weight (DSW) (g), number of leaves (NL) (piece/plant), leaf width (LW) (mm) and leaf length (LL) (mm) were determined 28 days after sowing (11.05.2020). 10 seedlings randomly selected from each repetition of each dose were used in the measurements. Since not enough seedlings could be obtained from 24hr and 48hr applications of 0.75% and 1.0% EMS doses, they were not evaluated.

EMS LD_{50} value was calculated based on the seed emergence values based on the Spearman-Karger method. Application times were not considered in the calculation and the following formula was used (Spearman, 1908; Unan *et al.*, 2022):

$$LD50 = Dh - [\Sigma\,(a \times b) / m]$$

Dh = Highest dose for plants
a = Half of the total number of plants reacting with two consecutive doses
b = Average mortality of plants between two consecutive doses
m = Number of dead plants in each group.

In the formula, the number of seeds that did not emerge was used instead of the number of dead plants.

Statistical analysis

The study was established according to the completely randomized design with two factors (1. Factor: dose and 2. Factor: duration) and four replications. Variance analysis (ANOVA) was performed for the significance levels of the data obtained from the research, and LSD (Least Significant Difference) test was performed for significance groupings ($p \leq 0.05$), and correlation analysis was performed for pairwise relationships between data. Mean and standard deviation were expressed as mean± SD. Statistical analysis was carried out using the MINITAB package program (Evans, 2009).

Results

Seed emergence and LD_{50} value: According to the variance analysis results of the emergence values of EMS-treated seeds, statistical significance was recorded at the 1% level in dose, duration, and dose x duration interaction. Mean values for seed emergence are summarized in Table 1.

Considering the doses, the highest seed emergence (72.00%) was taken from the control application, followed by 0.25%, 0.50%, 0.75% and 1.00% EMS doses, respectively. Among the four-application duration, the highest seed emergence was recorded in 6hr and 12hr applications (79.15% and 83.30%, respectively). The average emergence value was determined as 34.13% in applications made for 24hr and 48hr. According to dose x duration interaction, the highest seed emergence (88.75%) was obtained from the 12hr application of 0.25% EMS dose. Values very close to this rate were recorded at 0.50%, 0.75% and 1.00% EMS doses of the same duration and 0.25% and 1.00% EMS doses of 6hr (Fig. 1). These applications were statistically in the same group (Table 1). It was determined that the effect of application duration on the seed emergence rate was higher (Fig. 2).

Table 1. Mean values and difference grouping of emergence rates of *S. sclarea* seeds applied with EMS (%).

EMS (%)	Application duration				
	6hr	12hr	24hr	48hr	Mean
Control	72.00 ± 7.87^{b1}	72.00 ± 7.87^{b}	72.00 ± 7.87^{a}	72.00 ± 7.87^{a}	72.00^{A}
0.25	84.75 ± 6.5^{a}	88.75 ± 2.22^{a}	39.00 ± 5.77^{b}	47.25 ± 9.81^{b}	64.31^{B}
0.50	75.75 ± 9.18^{ab}	86.75 ± 4.35^{a}	31.25 ± 14.31^{b}	39.00 ± 15.53^{b}	58.19^{C}
0.75	78.50 ± 7.68^{ab}	84.25 ± 3.86^{a}	10.50 ± 8.35^{c}	14.50 ± 12.92^{c}	47.67^{D}
1.00	84.75 ± 7.32^{a}	84.75 ± 5.91^{a}	8.00 ± 4.16^{c}	1.25 ± 1.26^{d}	46.94^{D}
Mean	79.15^{A}	83.30^{A}	32.15^{B}	36.11^{B}	

LSD $_{(DOSE\ 0.05)}$ = 5.946 LSD $_{(DURATION\ 0.05)}$ = 5.319 LSD $_{(DOSEXDURATION\ 0.05)}$ = 11.893

[1]The difference between means indicated with the same letter is statistically insignificant

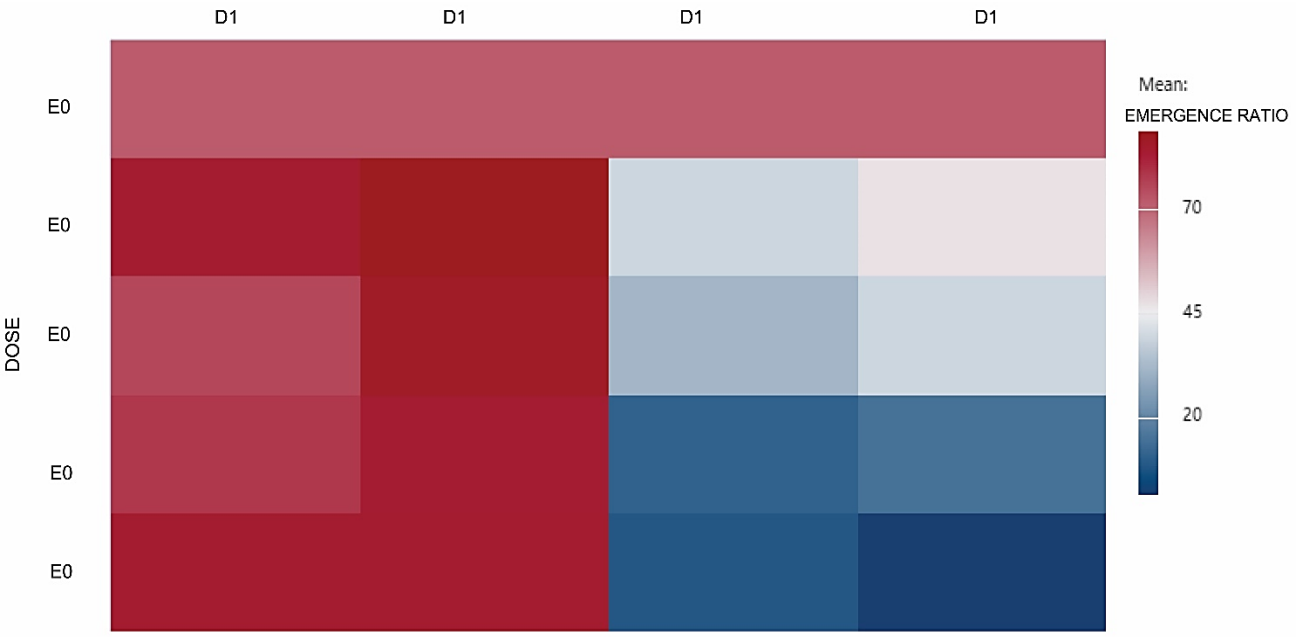

Fig. 1. Change in emergence values according to doses and durations of EMS application.
E0: Control, E1: % 0.25 EMS, E2: % 0.50 EMS, E3: % 0.75EMS, E4: % 1.0 EMS- D1:6 hr, D2: 12 hr, D3: 24 hr, D4: 48 hr]

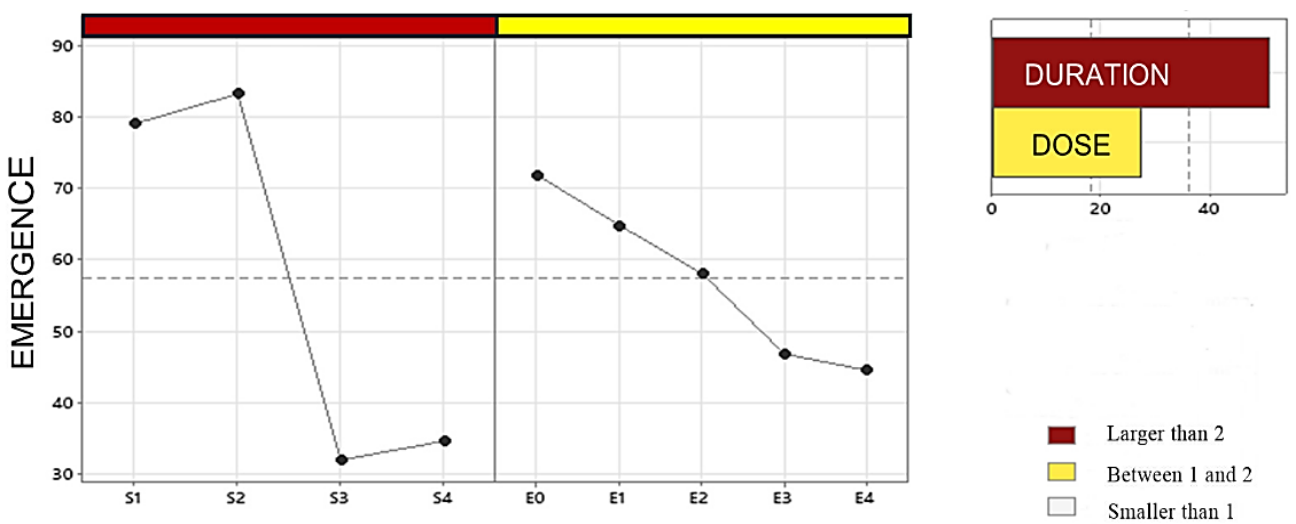

Fig. 2. Effect of duration and dose of EMS on emergence ratio.

LD_{50} lethal concentration estimate for EMS: In determining the LD_{50} dose, EMS doses were considered, ignoring the application times used in the study. Control (0.0%), 0.25%, 0.50%, 0.75% and 1.0% EMS doses were used in the study. LD_{50} dose was determined according to the proportion of seeds that did not emerge. The evaluation made using the Spearman-Karger equation is summarized in Table 2. The lowest emergence was recorded at the highest EMS dose (1.00%).

As a result of the calculations, 0.25% EMS concentration was determined as the LD_{50} dose for *S. sclarea* used in our study.

Table 2. LD_{50} value of EMS doses according to the Spearman-Karger Method.

EMS Dose (%)	Dose difference (a)	Number of seeds that do not show emergence (m)	Average number of seeds showing no emergence (b)	Product (axb)
0	-	14	-	
0.25	0.25	17.84	15.92	3.98
0.50	0.25	20.91	19.38	4.845
0.75	0.25	26.17	23.54	5.885
1.00 (Dh)	0.25	26.53	26.35	6.588
			Total product	21.298
			LD50	0.25

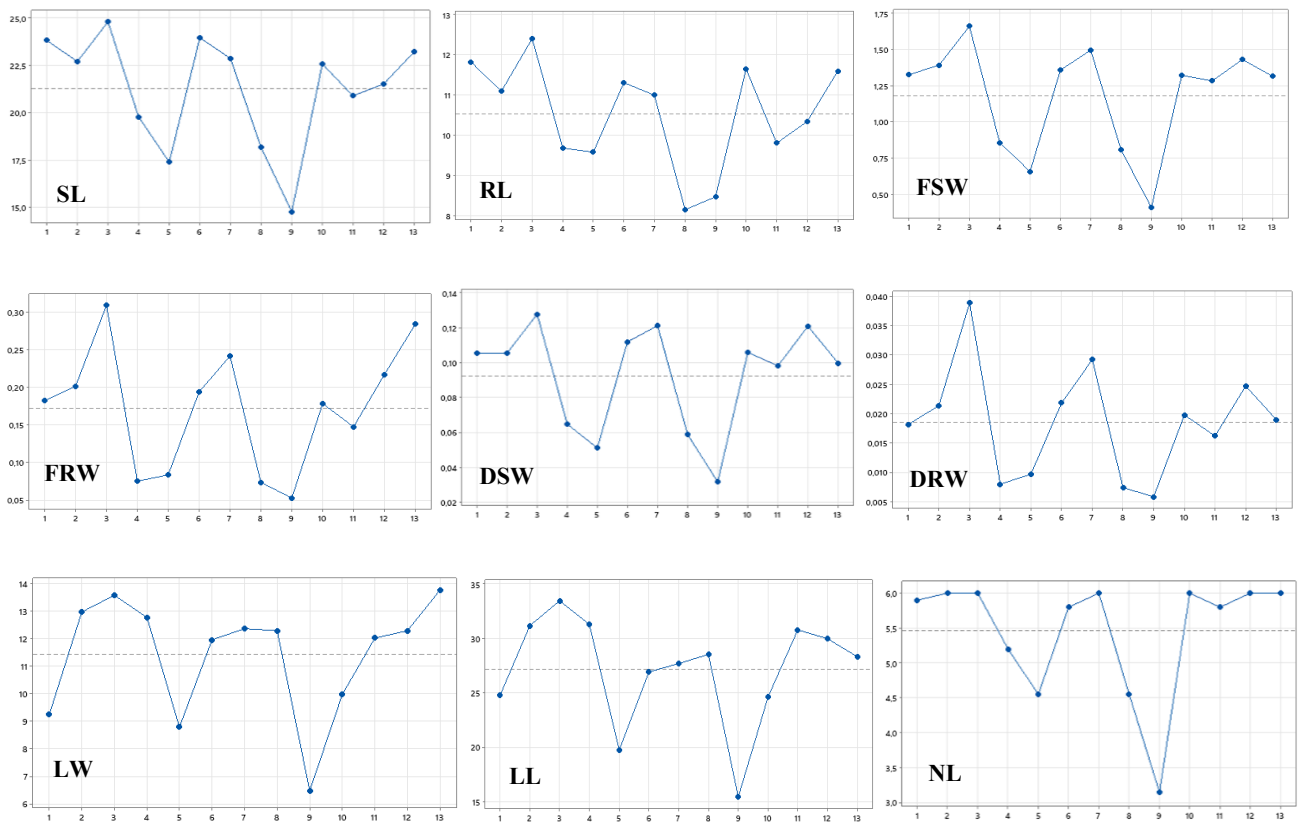

Fig. 3. Impacts of EMS applications on the investigated seedling characteristics.
(Letters from 1 to 13 on the horizontal axis in the graphs indicate EMS applications. 1-Control, 2-0.25% EMS-6hr, 3-0.25% EMS-12hr, 4-0.25% EMS-24hr, 5-0.25% EMS-48hr, 6-0.50% EMS-6hr, 7-0.50% EMS-12hr, 8-0.50% EMS-24hr, 9-0.50% EMS-48hr, 10-0.75% EMS-6hr, 11-0.75% EMS-12hr, 12-1.00% EMS-6hr, 13-1.00% EMS-12hr)

Seedling characteristics: The average values of seedling characteristics obtained according to the applications in the research are presented in Table 3. Since there was not sufficient emergence in the 24hr and 48hr periods of the 0.75% EMS and 1.00% EMS doses, they were excluded from evaluation. The change caused by EMS applications in the investigated seedling characteristics is presented in Fig. 3.

The longest seedling length (24.81 mm) was recorded in the 12hr application of 0.25% EMS dose. This value was followed by 6hr application of 0.50% EMS dose and 12hr application of control and 1.00% EMS dose, respectively. The root lengths of the seedlings took values between 9.81 mm (0.75% EMS-12hr) and 12.40 mm (0.25% EMS12-hr).

The highest fresh seedling weight was recorded in the 12hr application of 0.25% EMS dose and the 6hr application of 1.00% EMS dose. These values were followed by 0.25%-6hr, 0.50%-6hr, control, 0.75%-6hr, 1.00%-12hr, and 0.75%-12hr applications. These applications were statistically in the same group. The lowest fresh seedling weight was recorded in the 48hr application of 0.50% EMS dose. The highest dry seedling weight (0.1275 g) was determined in the 12hr application of 0.25% EMS dose. This application was followed by 0.50% EMS-12hr (0.1209 g) and 1.00% EMS-6hr (0.1206 g). Although the lowest value (0.0510 g) was obtained from the 48hr application of 0.25% EMS dose, 24hr applications of 0.50% EMS and 0.25% EMS doses (0.0586 g and 0.0646 g) gave similar values and were statistically in the same group.

The highest fresh root weight (0.3088 g) was recorded in the 12hr application of 0.25% EMS dose. This application was followed by a 12hr application of 1.00% EMS dose (0.2839 g). The lowest value (0.0531) was measured in 48hr of application of 50% EMS dose. The highest dry root weight (0.0389 g) was recorded in the 12hr application of 0.25% EMS dose, followed by 0.50% EMS-12hr (0.0292 g). The lowest root dry weight (0.0059 g) was determined in the 48hr application of 0.50% EMS dose.

The highest number of leaves in the seedlings, 6 pieces, was obtained from the application of 0.25% and 1.00% EMS doses for 6 and 12hr, 0.50% EMS for 6hr and 0.75% EMS for 12hr. These applications were followed by control, 0.50% EMS-6hr and 0.50% EMS 24 hours. The lowest value, with 3.15 pieces, was recorded in 0.75% EMS-6hr and 0.50% EMS-48hr applications. The longest leaf width (13.7665 mm) was taken from the 12hr application of 1.00% EMS dose. This application was followed by 0.25%-12hr (13.5815 mm), 0.25%-6hr (12.9750 mm) and 0.25%-24hr (12.7775 mm) applications, respectively. These four applications were statistically in the same group. The shortest leaf width (6.4792 mm) was obtained from the 6hr application of 0.75% EMS dose. The longest leaf length (33.4350 mm) was measured in 12hr application of 0.25% EMS dose. This application was followed by 0.25% EMS-24hr (31.2710 mm), 0.25% EMS-6hr (31.1593 mm), 0.75% EMS-24hr (30.7775 mm) and 1.00% EMS-6hr (29.9600 mm), respectively. These four applications were statistically in the same group. The shortest leaf length (19.7400 mm) was recorded in the 48hr application of % 0.25 EMS dose.

The effects of the EMS application dose and duration used within the scope of the study on the examined characters are presented in Fig. 4.

$$LD_{50} = Dh - [\Sigma (a \times b) / m]$$

$$LD_{50}=1.00-[21.298/26.53]=0.197 \cong 0.2$$

In our study, when EMS applications were compared with the control group, it was observed that some of the characters examined changed their binary relationships with each other (Tables 4 and 5).

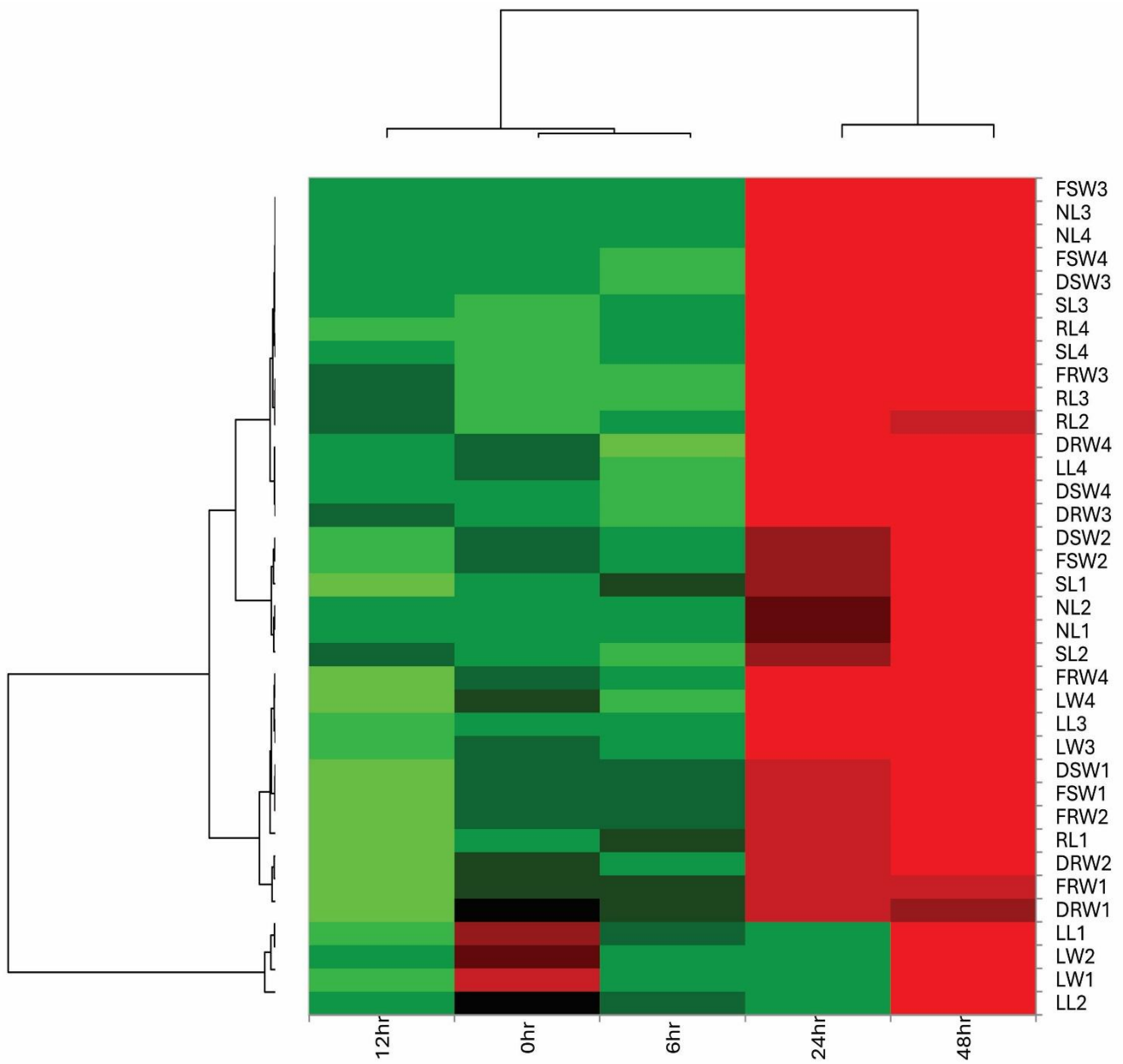

Fig. 4. Change in seedling characters according to doses and durations of EMS application.
(Color scale: Red to green through black. 0 hr control application on horizontal axis. 1, 2, 3, and 4 next to the characters indicate the doses of 0.25% EMS, 0.50% EMS, 0.75% EMS and 1.0% EMS, respectively)

Table 4. The observed correlation coefficients among the characters examined in control group plants[1].

	SL	RL	FSW	NL	FRW	DSW	DRW	LW
RL	0,995							
FSW	0,734	0,711						
NL	-0,684	-0,675	-0,037					
FRW	-0,241	-0,262	-0,749	-0,534				
DSW	0,402	0,428	0,812	0,375	-0,983			
DRW	0,387	0,369	-0,277	-0,934	0,8	-0,681		
LW	0,429	0,4	-0,196	-0,934	0,772	-0,655	0,992	
LL	0,386	0,381	-0,311	-0,937	0,784	-0,658	0,991	0,969

[1]Correlation Coefficient: 0.00-0.10 negligible correlation, 0.10-0.39 weak correlation, 0.40-0.69 moderate correlation, 0.70-0.89 strong correlation, 0.90-1.00 very strong correlation (Schober *et al*., 2018).

Table 5. The observed correlation coefficients among the characters examined in plants treated with EMS[1].

	SL	RL	FSW	NL	FRW	DSW	DRW	LW
RL	0,913							
FSW	0,955	0,848						
NL	0,935	0,812	0,939					
FRW	0,887	0,888	0,904	0,8				
DSW	0,939	0,837	0,992	0,928	0,89			
DRW	0,844	0,84	0,908	0,745	0,912	0,909		
LW	0,759	0,509	0,729	0,778	0,643	0,677	0,541	
LL	0,711	0,454	0,727	0,762	0,547	0,677	0,545	0,942

[1]Correlation Coefficient: 0.00-0.10 negligible correlation, 0.10-0.39 weak correlation, 0.40-0.69 moderate correlation, 0.70-0.89 strong correlation, 0.90-1.00 very strong correlation (Schober *et al*., 2018).

To determine the relationships between the characters examined in the study, correlation analysis separately was carried out with the data obtained from the plants in the control group and treated with EMS (Tables 4 and 5). In control group plants, a positive relationship was determined between SL and RL (very strong) and FSW (strong). A negative relationship was observed between SL and NL. It was recorded positive relationships between SW with DSW (strong), and FRW with DRW, LW, LL (strong), and DRW with LW, LL (very strong), and LW with LL (very strong). A negative correlation was found between NL and all other features except DSW. Especially a very strong negative relationship was determined between NL and DRW, LW and LL (Table 4).

Mostly strong-very strong and positive correlations were detected in the relationships between the examined traits in EMS-applied plants (Table 5).

Discussion

Sudden hereditary changes that occur in the genotype of an organism are called mutations, and an organism with such hereditary changes is called a mutant. Such genetic changes may occur naturally at a very low rate. However, it can also be induced experimentally with the help of various physical and chemical mutagens (Mba, 2013; Zakir, 2018).

In plant breeding programs, induced mutagens have contributed greatly by creating mutant varieties with improved and desired genetic changes in agronomically important traits of plants (Mba, 2013).

The basic principle is to determine the most appropriate mutagen dose and duration for mutagens used in mutation breeding studies. As the applied mutagen dose increases, the mutation frequency also increases. However, it increases in physiological damages. For this reason, it is of great importance to determine the mutagen application dose, which varies according to species and varieties, and the dose that reduces growth by 50% (LD_{50}) before starting breeding studies (Durga Devi *et al*., 2021; Raina *et al*., 2022). Regarding EMS doses, the term low or high dose is relative, and the optimum dose may differ depending on the genotypes used (Shamshad *et al*., 2023).

In a study examining different doses and durations of EMS in Chia (*Salvia hispanica* L.); 12hr of 0.37% EMS and 6hr of 0.76% EMS applications reduced the germination rate of seeds by 50%. It was determined that 3hr and 6hr applications of the highest concentration, 1.6% EMS, reduced the germination rate by 10% and 27%, respectively. For this reason, it has been stated that a concentration of 0.37-0.76% EMS by volume for a period of 9-12hr was found to be appropriate to induce mutation in this species (Thaboran *et al*., 2020).

In the study carried out to examine the mutagenic effects of EMS on a local fenugreek (*Trigonella foenum-graecum* L.) variety; EMS concentrations of 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8% and 0.9% were applied to fenugreek seeds. The germination percentage of the seeds was calculated, and 0.4% EMS dose was determined as the LD_{50} value (Kavina *et al*., 2020). In a different study, the highest decrease in the germination rate of Vernonia (*Centrapalus pauciflorus*) seeds (10%) was recorded at the highest EMS dose (1.1%) and the longest application time (2hr) (Hadebe *et al.*, 2017). Similarly, there was 0% germination in soybeans (*Glycine max*) treated with 0.9% EMS for 24hr, and 70% germination was achieved with the lowest application time of 12hr- 0.3% EMS (Espina *et al.*, 2018). It has been reported that the decrease in germination percentage may be due to the degradation of meristematic tissue at the cellular level (Sharma & Swaminathan, 1969; Jayakumar & Selvaraj, 2003).

Considering the doses in our study, the highest emergence value (72.00%) was taken from the control application. Among the four application periods, the highest emergence values were recorded in the 6hr (79.15%) and 12hr (83.30%) applications. The average emergence value was determined as 34.13% in applications made for 24hr and 48hr. When doses and durations were evaluated together, the highest emergence value (88.75%) was obtained from the 12hr application of 0.25% EMS dose. Values very close to this rate were recorded at 0.50%, 0.75% and 1.00% EMS doses of the same period and 0.25% and 1.00% EMS doses of 6hr, and 0.25% EMS concentration was determined as the LD_{50} dose for *S. sclarea*.

Seedling development in the M1 generation is generally used as a tool to decide the natural effects of different physical and chemical mutagens (Tadege *et al.*, 2008; Mba *et al.*, 2010; Aviya, 2018). As it is known, EMS affects the growth and development of the plant to which it is applied. Increasing EMS application time and dose reduces the survival rate of seedlings (Muñoz-Miranda *et al.*, 2019). In *Coriandrum sativum*, the survival rate decreased by 42.84% at the highest EMS dose (0.5%) and application time (5h) (Kumar & Pandey, 2019). A similar result was recorded by (Sharamo *et al.*, 2021), who studied barley (*Hordeum vulgare*). It is stated that this may be related to the fact that seeds left exposed for shorter periods of time absorb lower amounts of mutagen and this causes less harmful effects compared to those exposed for a longer time (Kulkarni, 2011). As seen in Figure 4, similar results were found in our research.

It has been emphasized that seedling characteristics such as seedling height, root length, root and shoot biomass decrease proportionally with increasing EMS dose and application time (Talebi *et al.*, 2012; Sharamo *et al.*, 2021). On the other hand, it has been observed that the effect of application time on seedlings is more effective than the dose (Gerami *et al.*, 2017). In our study, the lowest values in the seedling characters of *S. sclarea* were obtained from the 48hr application of 0.50% EMS dose. Positive effects of low dose and short application time on seedling development were observed. The highest values in SL, RL, FSW, FRW, DSW, and DRW properties were taken from 0.25% EMS-12hr application. It was observed that the findings we obtained from the research were compatible with the literature data.

Conclusion

The effectiveness of mutagen in mutation breeding studies varies depending on the plant species and plant material studied, the dose and duration of mutagen applied. Therefore, it is necessary to determine the effective mutagen dose and duration at the beginning of mutation breeding studies. In our study, 0.25% EMS concentration was determined as the LD_{50} dose for *S. sclarea* used in the research. Research findings have shown that EMS application duration is more effective than EMS application dose. In mutagen studies, the duration of application administration must also be considered when determining the appropriate dose. Since it is the first mutation study conducted in *Salvia sclarea*, we think that the findings will make significant contributions to the literature.

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