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.

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; Ranđelovic´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 (Džamić 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. 2014; 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 (Barut et al. 2022; Kumar and Gupta 2008), 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. Physical (X-ray, γ-ray, and neutron beam, etc.) and chemical mutagens (ethyl methane sulfonate (EMS), ethylemine (EI), nitroethyl urethane (NEU) etc.) are used in mutation breeding to create genetic variation. Chemical mutagens are an indispensable tool for creating variation in plant breeding (Kumar et al. 2019; Maluszynski 1995). Due to its effectiveness and ease of use, EMS ($CH_3OSO_2C_2H_5$) is the most widely used chemical mutagen in plants (Mir et al. 2021; Türkoğlu et al. 2023). EMS affects a very short chromosome segment that carries one or several genes and can affect the cytological, genetic, physiological, and morphological properties of plant tissues and cells (Waungh et al. 2006). A very high mutation rate consistently causes high sterility. For this reason, plant breeders aim for more mutagenic effects and less physiological damage (Konzak et al. 1965; Usharani and Amanda Kumar 2015).

Application doses of mutagens vary depending on the type of mutagen to be used and the plant material (Roychowdhury and Tah 2011). Some mutagens have lethal effects when used in high doses. Some do not produce the desired mutations at low doses. Therefore, to adjust the mutation rate, the amount of dose applied must be increased or decreased. Generally, high concentrations of mutagen cause more serious damage such as reduced seed germination, seedling damage, pollen sterility, and reduced survival of the plant during the maturation period. Therefore, to reduce the negative effects of the applied mutagen on plant parameters, more information is needed about the application time and concentrations of the mutagen (Ke et al. 2019; Khan et al. 2009).

More than 3200 mutant varieties from 200 plant species (cereals, legumes, oilseed plants, fiber plants, fruits, vegetables, ornamental plants, etc.) have been developed and made available to producers in the world. It is seen that the number of varieties developed by mutation breeding in medicinal and aromatic plants worldwide is very low compared to cereals, ornamental plants, and legumes (Kolakar et al. 2018; 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/TURKİYE).

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. 2021).

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 (1931) method. Application times were not considered in the calculation and the following formula was used (Spearman 1908; Unan et al. 2021):

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

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

Variance analysis was performed for the significance levels of the data obtained from the research, and LSD (Least Significant Difference) test was performed for significance groupings. Statistical analyzes were 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.

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}	83.30A	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.

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).

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%).

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

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

LD_{50}=1.00-[21.298/26.53]=0.197 ≅ 0.2

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

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 (0.127500 g) was determined in the 12hr application of 0.25% EMS dose. This application was followed by 0.50% EMS-12hr (0.120900 g) and 1.00% EMS-6hr (0.120620 g). Although the lowest value (0.051075 g) was obtained from the 48hr application of 0.25% EMS dose, 24hr applications of 0.50% EMS and 0.25% EMS doses (0.058695g and 0.064607 g) gave similar values and were statistically in the same group.

The highest fresh root weight (0.308785 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 (0.283880 g). The lowest value (0.053090) was measured in 48hr of application of 50% EMS dose. The highest dry root weight (0.0388700 g) was recorded in the 12hr application of 0.25% EMS dose, followed by 0.50% EMS-12hr (0.0292350 g). The lowest root dry weight (0.0058550 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.

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 (Table 4 and Table 5).

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 (Table 4 and Table 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 9hr 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 (Espino 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 (Jayakumar and Selveraj 2003; Sharma and Swamirathon 1969).

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 (Mba et al. 2010; Mullainathan et al. 2018; Tadege et al. 2008). 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 and 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 Fig. 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 (Sharamo et al. 2021; Talebi et al. 2012). 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.

Declarations

Author Contribution

B.C.S. planning, execution and statistical analysis of the study and writing of the manuscriptT.U. Conducting field trials and laboratory analyses, formal editing of the manuscriptH.D. Conducting field trials and laboratory analyzes

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Declaration of interests

 The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

 The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Tables

Tables 3 to 5 are available in the Supplementary Files section.

Figures

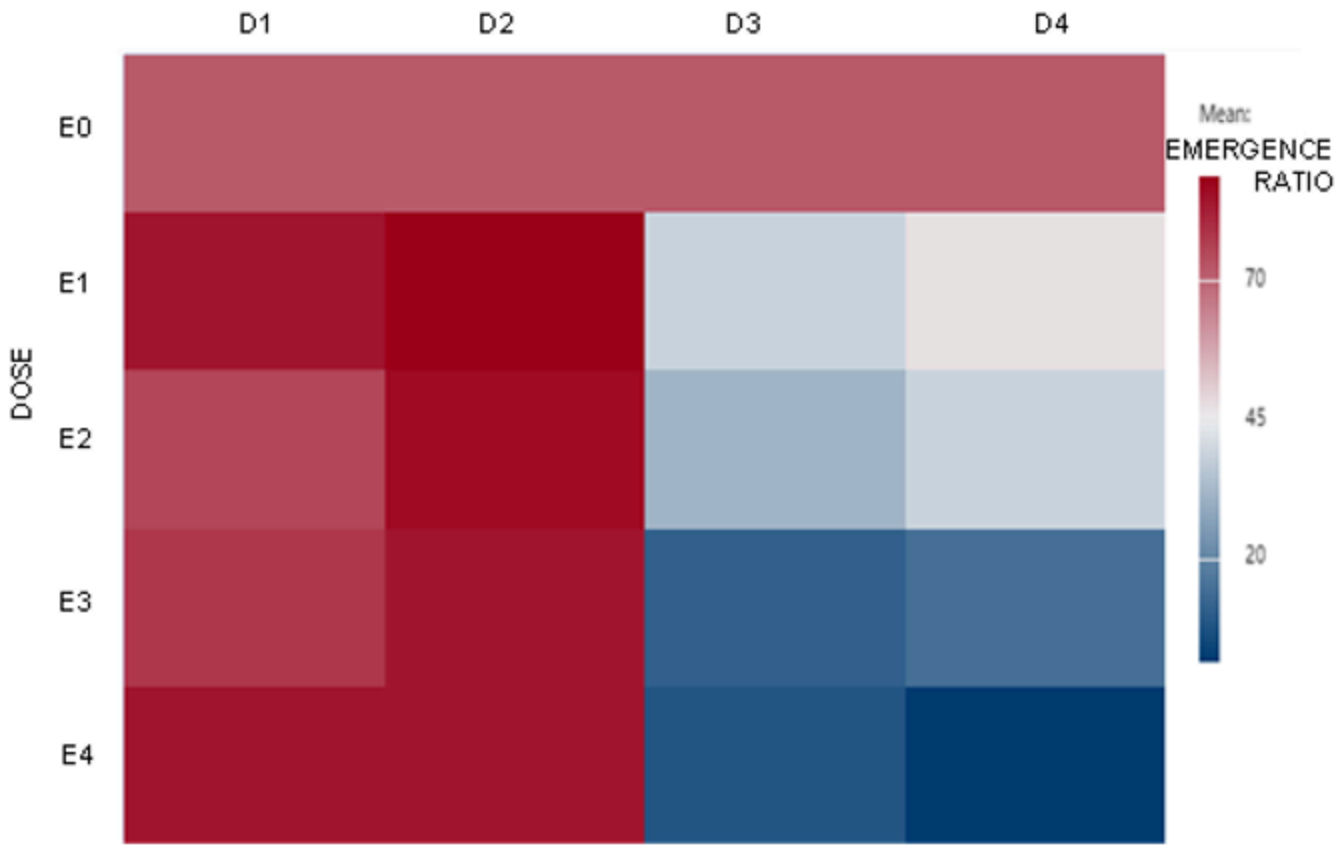

Figure 1

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]

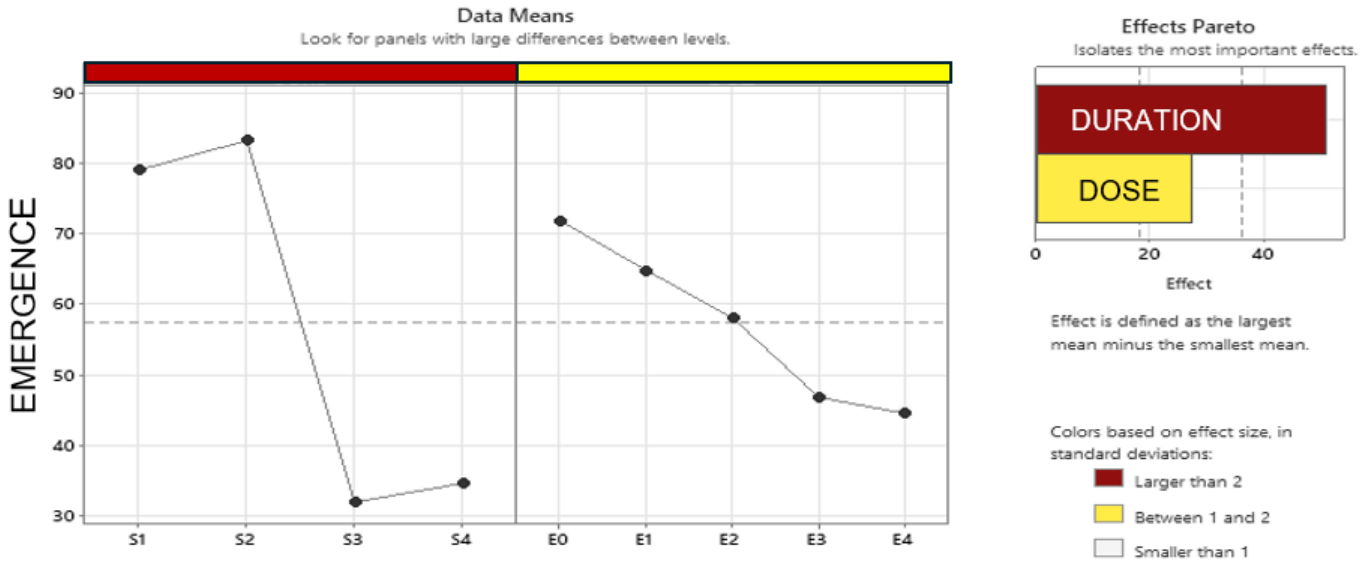

Figure 2

Effect of duration and dose of EMS on emergence ratio

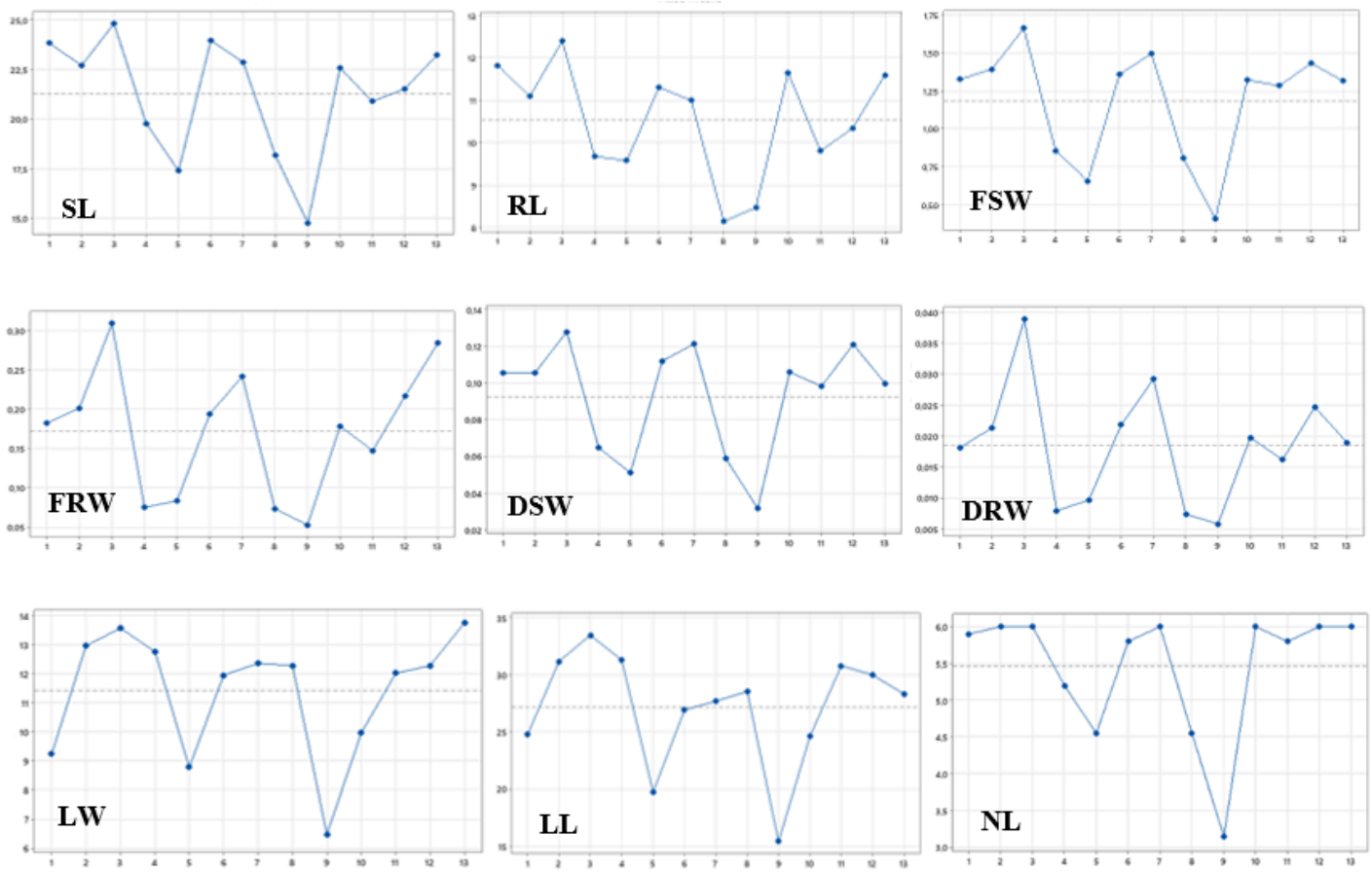

Figure 3

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)

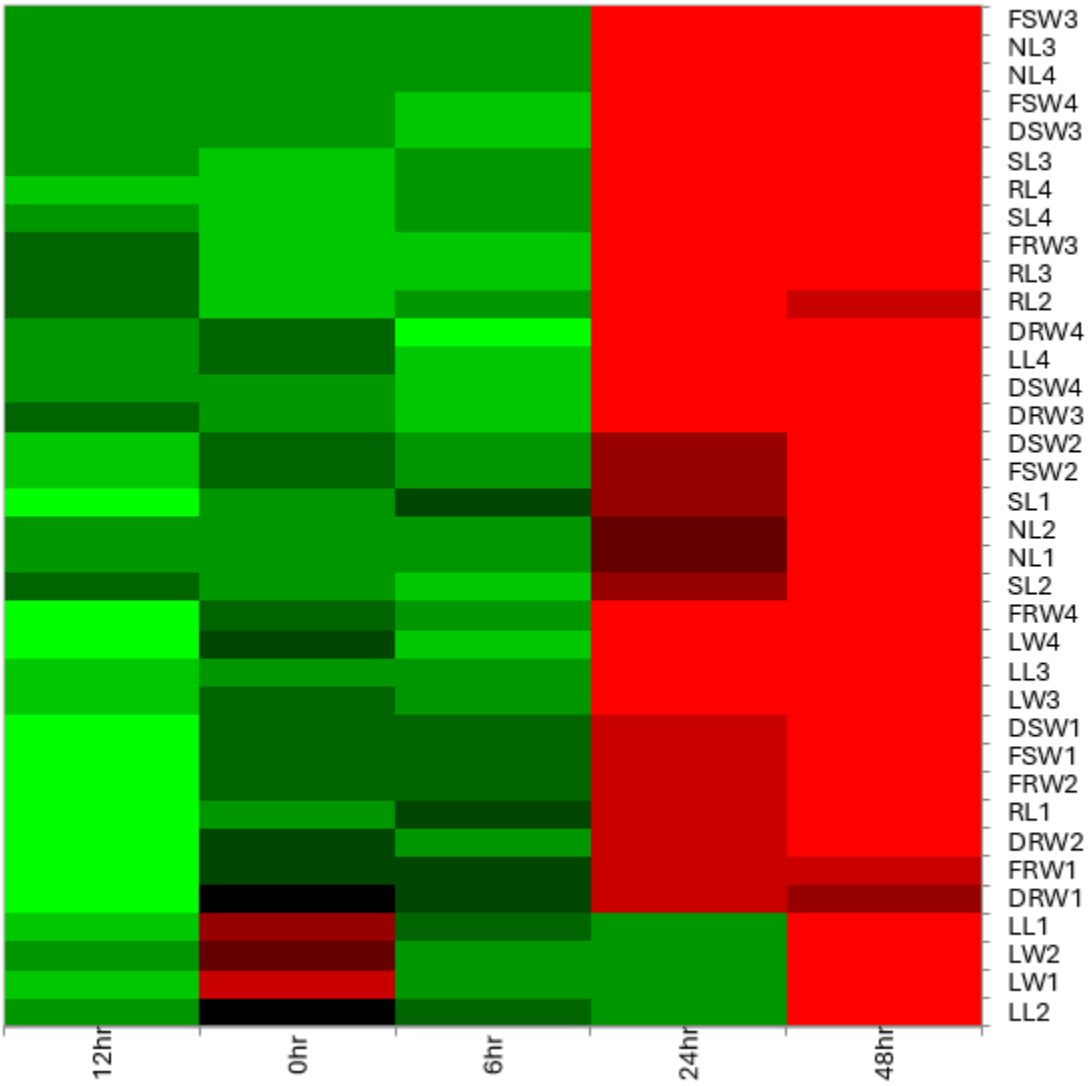

Figure 4

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)

Supplementary Files

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- Tables.docx