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RESEARCH ARTICLE

REVISED Protocol for ethyl methanesulphonate (EMS) mutagenesis application in rice

[version 3; peer review: 2 approved]

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Abstract

Background: Non-transgenic chemical mutagen application, particularly ethyl methanesulfonate (EMS), is an important tool to create mutations and gain a new genetic makeup for plants. It is useful to obtain a sufficient number of mutant plants instead of working with a severe mutation in a few plants. EMS dose and exposure period have been previously studied in several crops; however, EMS used to create point mutations in presoaked rice seeds has not been sufficiently studied and there is no standard protocol for such treatment. The aim of this study is to establish a standard protocol for EMS mutagenesis application in rice.
Methods: Two studies were conducted to evaluate the effect of four durations of rice seed presoaking (0, 6, 12, and 24 hours), four EMS concentration doses (0.0%, 0.5%, 1.0%, and 2.0%), and four EMS exposure periods (6, 12, 24, and 48 hours). Germination rate, plumula and radicle length, seedling survival, LD_{50} (Lethal Dose) determination, shoot length, root length and fresh seedling weight were evaluated.
Results: Results showed that a 12-hour presoaking duration, 0.5% EMS dose, and six hours of EMS exposure were the best practices for the optimum number of mutant plants.
Conclusions: In light of both this study and the literature, a standard application protocol was established. This application protocol, detailed in this article, contains the following guidelines: (1) Presoaking: 12 hours, (2) EMS application: 0.5% dose EMS and six hours, (3) Final washing: six hours, (4) Drying: 72 hours at 38°C. A user-friendly protocol has been presented for utilization by researchers.

Keywords

EMS, dose, mutagenesis, protocol, rice

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REVISED **Amendments from Version 2**

This new version has been published after peer review. Some new literature was added and some sentences have been rewritten in this version. There is not any change in the results. The best application protocol is following that a 12-hour pre-soaking duration, 0.5% EMS dose, and six hours of EMS exposure were the best practices.

Any further responses from the reviewers can be found at the end of the article

Introduction

Rice is the staple food for nearly half of the world's population, most of whom live in developing countries. Rice is currently grown in over a hundred countries, which produce 755 million tons of paddy rice (FAO, 2019). Asian countries, including China, India, Indonesia, Bangladesh, Vietnam, Myanmar, Thailand, Philippines, Japan, Pakistan, Cambodia, South Korea, Nepal, and Sri Lanka, account for 90% of the world's total rice production. Europe, however, has several important rice producing countries such as Italy, Spain, Greece, Portugal, France, Bulgaria and Turkey. In the European Union, the rice production area is approximately 418,000 hectares, total production is close to three million tons with average yields of 6.8 tons per hectare (FAO, 2019).

Rice accounts for a third of the earth's area planted with fields crops and it supplies 35–60% calories of nutrition to the world population. People globally consumed more rice than wheat or maize, the other two staple foods. Both developed and developing nations alike grow and consume rice. Of the major staple foods of rice, wheat, and corn, rice is the most crucial food particularly for low- and middle-income nations. Rice is an essential component of complicated cereal product systems that impact issues of worldwide concern, such as food sustainability and security, poverty reduction and protection of social legacy (Chauhan *et al.*, 2017).

Rice production has some crucial problems such as irrigation scarcity, rice blast disease (*M. oryzae*), weeds and red rice. Full yield capability has not been realized due to the damage from insects and diseases, while weeds limit rice through rivalry for daylight, water, and supplements. Weed rivalry can bring about complete yield loss (Al-Khatib *et al.*, 2018; Brim-Deforest *et al.*, 2017; Espino *et al.*, 2018; Gibson *et al.*, 2002). Intensive research to solve some of these problems is being carried out supported by the European Commission. The problems of weeds and red rice is especially a problem in Europe because of their direct production system of sowing rice. The main rice area, Asia, has a production system of transplanting rice so they have no severe weed problems in their fields. Therefore, chemical companies have not been willing to develop new active ingredients for European countries. Old herbicides do not work effectively over time. The development of herbicide-tolerant rice is a more reasonable approach than developing a new active ingredient. Researchers have developed herbicide resistance systems such as Clearfield, Provisia, and Roxy Rice by mutation application (Croughan, 2015; Mankin *et al.*, 2014). Most of this research is based on plant EMS mutagenesis application.

Rice plant breeders have used point mutations in their breeding program to overcome these problems. The mutation may exist in nature besides the artificially induced mutation. Physical and chemical mutagens are used to obtain plants by mutation breeding, such as gamma rays, X-rays, fast neutrons and also ethyl methanesulphonate (EMS; $CH_3SO_3C_2H_5$), diepoxybutane (DEB, $C_4H_6O_2$) and sodium azide (NaN_3) (FAO/IAEA, 2018). The chemical mutagen EMS has been widely utilized to induce a large number of functional variations in rice. EMS alkylates guanine bases and leads to mispairing of alkylated G with T instead of C, resulting in primarily G/C- to -A/T transitions (Bhat *et al.*, 2007).

Chemical mutagen application methods have a draft protocol of presoaking, mutagen application and a final washing process. The implementation phase of these processes differs in many studies and unfortunately, there is no standard protocol for mutagenizing rice seeds. The objective of this study is to develop a standard protocol for EMS mutagenesis application in rice.

Methods

Materials

Osmancik-97 is a *Japonica* type Turkish rice variety (Unan, 2021c). The variety was released by Trakya Agricultural Research Institute, Edirne, Turkey in 1997. The parents are *Rocca* and *Europe*, which originate from Italy. The *Osmancik-97* rice variety has a plant length of 105 cm, 85 days of flowering, 135 days of maturity, a semi horizontal 16 cm panicle, 65% milling yield and 8-9 tons per hectare grain yield potential. Material samples have 14% moisture content, 98-100% germination ratio, 24 g milled 1000 grain and 34 g un-milled 1000 grain weight (Unan *et al.*, 2013).

The molecular formula of EMS (Sigma- Aldrich Inc., USA) is $C_3H_8O_3S$, molecular weight is 124.2 g, density is 1.206 g ml^{-1}, half-life is 48.5 hours at 25°C. It is a powerful mutagen for plants.

EMS mutagenesis

The experiment was carried out using a randomized parcel design with three replications for the germination experiment and four replications for the seedling experiment, and each replication used 100 seeds under a fume hood in a phytotron growth chamber. Seeds were sterilized with bleach solution (30% commercial bleach + 0.02% Triton X-100) for 15 min and washed three times with pure water. Seeds were placed in a glass container and pure water was added to a volume of 1 ml $seed^{-1}$. Seeds were presoaked for 0, 6, 12 or 24 hours at 20°C. Afterwards, the water was decanted and again 1 ml $seed^{-1}$ of 0.0%, 0.5%, 1%, or 2% concentrations of EMS (v/v) in water was added. Seeds were incubated for six, 12, 24, or 48 hours in different concentrations of EMS solution at 20°C under the fume hood. Subsequently, EMS-treated seeds were washed

with pure water five times for five minutes (total 25 minutes) (Talebi *et al.*, 2012). The seeds were washed again with running tap water for six hours (Sagel *et al.*, 2017). EMS is a mutagenic chemical and it is important to reduce harmful effects of EMS on the ecology and person health subject to appropriate storage, correct using, suitable disposal and transportation.

Seedling survival rate is the ratio of surviving seedlings 21 days after sowing of seeds (Evangelina *et al.*, 2010). In the seedling experiment, seedling survival of rice seeds with each of the four presoaking durations, four EMS doses and four exposure periods was determined as the percentage of seedlings that survived 21 days after seeding in the phytotron chamber.

Seedling survival (%) = (survived rice seedlings / sowed rice seeds) × 100

Imbibition rate was calculated as the percentage of water intake of seeds hourly. 100g of seeds which had 14% water content were incubated in pure water at 20°C and the weight noted each hour for 48 hours with three replications. The seeds were removed from the water, drained for one minute and dried with blotting paper for 30 seconds and then measured with an analytical balance (AS 3Y, Radwag Wagi Elektroniczne, Poland). Imbibition rate was calculated using the following formula:

Imbibition rate (%) = (last weight - first weight) × 100 / first weight

Experiment 1: Germination experiment

The experiment was carried out using a randomized block design with three replications for germination. Experiment factors were four presoaking durations, four EMS doses, and four EMS exposure periods. 100 EMS-treated seeds were used for each treatment besides 100 untreated control seeds on filter paper soaked in 30ml of pure water in petri dishes. Untreated control seeds were managed under the same conditions except EMS exposure. The seeds were then put in the phytotron at 25°C and 30°C with 12-hour cycles of light and dark conditions for seven days. After seven days, the number of seeds that germinated, with 5 mm plumula being accepted as germinated (Cruz & Milach, 2004), under these conditions was recorded. Seedling length of the plants were measured using a digital caliper (Insize standart calipper, Germany). The roots were scanned using an Epson 11000XL scanner at a resolution of 600 dpi. Root traits were obtained using WinRHIZO 2009 Pro software (Regent Instruments). The equation to calculate germination percentage was (seeds germinated / total seeds) × 100 (IRRI, 2002).

Experiment 2: Seedling experiment

The experiment was carried out using a randomized block design with four replications. Experiment factors are four presoaking durations, four EMS doses, four EMS exposure periods, and their controls. Twenty seeds for each presoaking duration, EMS-treatment and EMS exposure duration seeds and their controls were sown in a plastic plant tray. Control seeds included no-presoaked seeds and no-EMS exposure seeds. Sterilized soil was used in the experiment. The 28-cell plant tray had a diameter of 7 cm and a depth of 7.4 cm. The plant trays were then put in the phytotron at 25°C and 30°C with 12-hour dark and 12-hour light cycles for 21 days, respectively. After 21 days the surviving seedlings' length, root length and fresh plant weight were measured (IRRI, 2002). The fresh plant weight measurement equipment used for analytical weighing was manufactured by Radwag Wagi Elektroniczne, Poland (Radwag, AS 3Y analytical balances). The length of the plants was measured using a digital caliper (Insize standart calipper, Germany). The roots were scanned using an Epson 11000XL scanner at a resolution of 600 dpi. Root traits were obtained using WinRHIZO 2009 Pro software (Regent Instruments).

EMS LD50 Determination

The calculations are based on the following formula according to Spearman - Karger (1931) method:

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

LD_{50} = Arithmetic means of dose that half of the plant's dead; D_h = highest dose for plants; a = half the sum of the plants reacting with two consecutive doses; b = Mean mortality of the plants between two consecutive doses; m = number of died plants in each group.

Factsheet and flowchart of protocol for EMS mutagenesis application in rice

A one-page user protocol might be useful in laboratory studies. Hence, a single page user protocol has been created. The materials used in the protocol are simply defined in the factsheet. Protocol application stages and durations are given for presoaking, EMS application, final washing, and drying. In addition, a flowchart is supplied for users. This flowchart shows a schematic illustration for how to utilize the protocol. The factsheet and flowchart of the protocol for EMS mutagenesis application in rice are supplied as *Extended data* (Unan, 2021b).

Statistical analysis

Three-way analysis of variance was used in order to detect any statistically significant differences between presoaking duration, EMS dose, and EMS exposure period. Significant differences between the averages were evaluated using the Tukey least significant difference (LSD) test at p-value <0.01. LSD tested the differences in observed averages of all tested parameters between treatment and non-treatment seeds. Statistical analysis was conducted using JMP 7.0 software.

Results

Imbibition rate

The imbibition rate was calculated for *Osmancik-97* rice at the start of the experiment. The increase in seed weight happening over the imbibition time period hourly and every six hours at 20°C in the phytotron was determined (Figure 1 and Figure 2). Initial moisture content was 14%. The seeds with 14% moisture were considered to have 0% water intake; water intake was calculated as a percentage increase in moisture content. Rapid increases of water uptake were calculated in first hour as more than 10%. Subsequently, the rapid rising

proceeded up to 25% in the first 12 hours. Finally, the increase reached 30% in the first 24 hours. No significant increase was seen after 24 hours. The seeds weight reached equilibrium as around 30% in the pure water. During the 0, 6, 12 and 24 hours presoaking (imbibition) stage, the seeds had 0%, 19.1%, 24.1%, and 29.5% water intake, respectively (Unan, 2021a).

Germination experiment

Germination is a crucial factor for EMS mutagenesis experiments. The analysis of variance revealed significant ($P < 0.01$) differences in germination percentage between each EMS dose, exposure period and presoaking and their combinations. Germination was ranked from 0% to 100% in this study. When evaluated in terms of the EMS dose, the lowest average germination observed was 33.4% for the 2% EMS dose. The highest mean germination observed was 98.8% for the control plot (Table 1). As per Table 1, the outcomes acquired show that a decrease in seed germination occurred with a corresponding increase in EMS dose ($P < 0.01$). Considering EMS exposure period compared to all controls (0.0%), mean germination percentage was 91.0%, 75.9%, 13.9%, and 0.0% for six, 12, 24, and 48-hour exposure periods, respectively. When evaluated in terms of presoaking, the lowest mean germination (49.1%) for zero hours (dry seed) presoaking. The highest mean germination observed was 71.8% at 12 hours

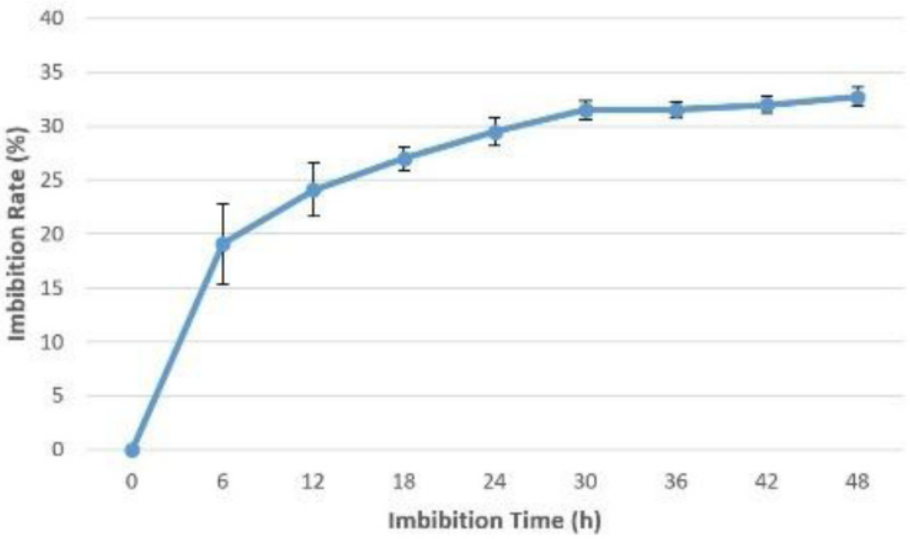


Figure 1. Water uptake measurement compared to imbibition time interval at six hours in *Osmancik-97* rice variety.

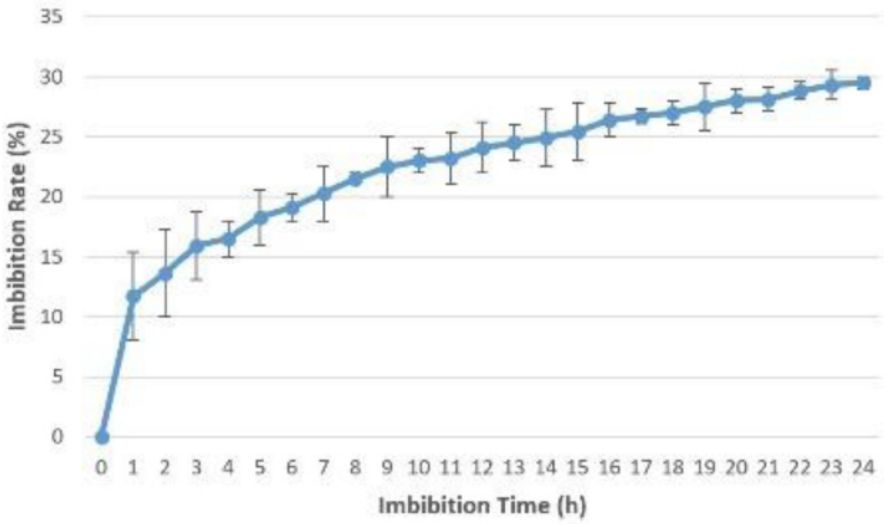


Figure 2. Water uptake measurement compared to imbibition time hourly in *Osmancik-97* rice variety.

presoaking. Higher dosages EMS application without presoaking prevented germination and all 48-hour durations prevented germination. It should be emphasized that the chemical reduces the germination ability of dry seeds and also EMS application for more than 24 hours prevents germination to a high extent. Most combinations resulted in 100% germination. However, six hours application, 0.5% EMS dose, and 12 hours presoaking interaction might be preferred for maximum germination of mutant seeds.

Plumula length is another indicator factor used in EMS mutagenesis experiments. There are significant ($P < 0.01$) differences in plumula length with each EMS dose, exposure period, presoaking, and their combinations according to the analysis of variance. Plumula length ranged from 0 mm to 62.0 mm in the germination experiment. In terms of the EMS dose, the lowest mean plumula length measured was 20.0 mm for the 2% EMS dose plot. The highest mean plumula length measured was 52.6 mm for the control plot (Table 2). Statistical analysis on plumula length showed an attendant decrease in plumula length with applied increases in the concentration of EMS. As per Table 2, the outcomes acquired show that a decrease in plumula length was observed with a corresponding increase in EMS dose ($P < 0.01$). When evaluated in terms of the

Table 1. Effect of EMS application dose, EMS exposure period and presoaking duration on germination in rice (%).

EMS application dose (%)	EMS exposure period (h)	Presoaking duration (hours)				Mean
		0	6	12	24	
0.0%	6	100a	100a	95b	95b	97.5c
	12	100a	100a	100a	100a	100.0a
	24	100a	95b	100a	100a	98.8b
	48	100a	100	95b	100a	98.8b
Mean		*100a*	*98.8b*	*97.5c*	*98.8b*	98.8A
0.5%	6	100a	100a	100a	100a	100.0a
	12	75e	95b	100a	100a	92.5d
	24	80d	100a	100a	55g	83.8f
	48	0l	0l	0l	0l	0.0k
Mean		*63.g*	*73.8e*	*75.0d*	*63.8g*	69.1B
1%	6	60f	100a	100a	100a	90.0e
	12	35i	100a	100a	100a	83.8f
	24	0l	75e	50h	15j	35.0i
	48	0l	0l	0l	0l	0.0k
Mean		*23.8m*	*68.8f*	*62.5h*	*53.8i*	52.2C
2%	6	35i	100a	100a	85c	80.0g
	12	0l	15j	100a	80d	48.8h
	24	0l	10k	10k	0l	5.0j
	48	0l	0l	0l	0l	0.0k
Mean		*8.8n*	*31.3l*	*52.5j*	*41.3k*	33.4D
General Average		49.1D	68.1B	71.8A	64.4C	63
**	**	**				

**: significant at the 1% level; NS: no significant differences. Values followed by the same letter are not statistically significantly different. A: Presoaking duration; A: EMS application dose; a: EMS application dose × EMS exposure period interaction; a: EMS application dose × Presoaking duration; a: Three-way interaction which EMS application Dose × EMS exposure period × Presoaking duration. LSD_{dose} = 0,09; $LSD_{Duration}$ = 0.09; $LSD_{Presoaking}$ = 0.11; $LSD_{Dose\times Duration}$=0.22; $LSD_{Dose\times Presoaking}$=0.22; $LSD_{Duration\times Presoaking}$=0.22; $LSD_{Dose\times EMSduration\times Presoaking}$ = 0,45; CV (%) = 4.44. CV, coefficient of variation; EMS, ethyl methanesulfonate; LSD, least significant difference.

Table 2. Effect of EMS application dose, EMS exposure period and presoaking duration on plumula length in rice (mm).

EMS application dose (%)	EMS exposure period (h)	Presoaking duration (hours)				Mean
		0	6	12	24	
0.0%	6	62.2ab	60.1ac	49.7hl	52.9ej	56.2a
	12	50.6fl	50.5fl	51.1fl	51.8fk	51.0b
	24	47.5ln	52.9ej	49.5hl	54.6df	51.1b
	48	58.1bd	63.0a	54.0dg	32.9t	52.0b
Mean		*54.6a*	*56.6a*	*51.1b*	*48.1c*	52.6A
0.5%	6	42.4oq	53.4ei	49.4hl	57.1ce	50.6b
	12	31.3t	51.2fl	50.6fl	52.2fk	46.3c
	24	38.1rs	47.0ln	49.8gl	38.6qs	43.4d
	48	0.0w	0.0w	0.0w	0.0w	0.0h
Mean		*27.9f*	*37.9d*	*37.5d*	*36.9d*	35.1B
1%	6	18.0u	49.1jm	54.1df	53.6eh	43.7d
	12	40.3pr	44.9mo	47.3ln	43.4np	43.9d
	24	0.0w	42.6oq	34.8st	7.6v	21.2f
	48	0.0w	0.0w	0.0w	0.0w	0.0h
Mean		*14.6h*	*34.1e*	*34.0e*	*26.2f*	27.2C
2%	6	44.9mo	59.8ac	53.3ei	48.1km	51.5b
	12	0.0w	15.3u	49.3il	39.5pr	26.0e
	24	0.0w	4.3v	6.8v	0.0w	2.8g
	48	0.0w	0.0w	0.0w	0.0w	0.0h
Mean		*11.2i*	*19.9g*	*27.3f*	*21.9g*	20.0D
General Average		27.1C	37.1A	37.5A	33.3B	33.7
**	**	**				

**: significant at the 1% level; NS: no significant differences. Values followed by the same letter are not statistically significantly different. A: Presoaking duration; A: EMS application dose; a: EMS application dose × EMS exposure period interaction; a: EMS application dose × Presoaking duration; a: Three-way interaction which EMS application Dose × EMS exposure period × Presoaking duration. LSD_{dose} = 1.0; $LSD_{Duration}$= 1.0; $LSD_{Presoaking}$ = 1.0; $LSD_{Dose\times Duration}$=2.1; $LSD_{Dose\times Presoaking}$=2.1; $LSD_{Duration\times Presoaking}$=2.1; $LSD_{Dose\times EMSduration\times Presoaking}$ = 4.2; CV (%) = 7.7. CV, coefficient of variation; EMS, ethyl methanesulfonate; LSD, least significant difference.

exposure period, the mean plumula length was 50.1, 41.8, 29.6, and 13.0 mm for the six, 12, 24, and 48 hours exposure periods, respectively. Regarding presoaking, the lowest mean plumula length observed was 27.1 mm for the zero hours (dry seed) presoaking plot. The highest mean plumula length observed was 37.5 mm for the 12 hours presoaking plot. EMS application without presoaking and 48 hours of EMS application and their combinations nearly forestalled plumula length. It should be underlined that the EMS harms the plumula length capacity of dry seeds and furthermore EMS application for over 24 hours with EMS application doses of 1–2% forestalls plumula length. Many of the combinations had a 5 mm plumula length. However, six hours application, 0.5% EMS dose, and 24 hours presoaking showed the best results except for the 0% (control) EMS dose application.

Radicle length ranged from 0.0 to 47.8 mm in this study. The analysis of variance showed significant ($P < 0.01$) differences in radicle length with each presoaking duration, EMS dose, exposure period, and their combinations. When evaluated in

terms of the EMS dose, the lowest and highest mean radicle length observed was 10.7 mm and 33.7 mm for the 2% EMS dose plot and control plot, respectively (Table 3). Increasing EMS doses caused shortening of the radicle length. Considering each exposure period, the mean radicle length was 33.7, 19.8, 14.9, and 10.7 mm for the six, 12, 24, and 48 hours exposure periods, respectively. When evaluated in terms of presoaking, the lowest and highest mean radicle length observed was 14.9 mm and 23.8 mm for the zero hours (dry seed) and 12 hours presoaking plot, respectively. Many of the combinations resulted in 20 mm radicle length, which is the optimum radicle length. However, 12 hours application, 0.5% EMS dose, and 24 hours presoaking combinations showed the best results except for the 0% (control) EMS dose application.

Seedling experiment

Germinated seeds might lose their vitality over time at the seedling stage. Hence, seedling survival is a crucial factor for mutation experiments. In this study, the germination rate was 98.8% in the germination experiment and survival seedling rate was determined as 90.2% in the seedling experiment in the control plots. Although all conditions and applications are the same, a loss of 8.6% was experienced. This illustrates the importance of seedling trials in addition to germination trials in mutation experiments.

Seedling survival decreased substantially with increasing EMS dose (Table 4). To investigate the reasons behind this dramatic decrease in seedling survival with increasing EMS dose, the level of seedling damage by EMS exposure period in presoaked and dry seeds before sowing was examined. The presoaking of seeds before sowing has a strong effect on seedling survival rate. This may suggest that presoaked seeds could tolerate EMS exposure periods up to 24 hours, as they tolerate high EMS doses during the seedling stage.

A significant interaction was also observed between the EMS exposure period and EMS dose. This is might be a result of EMS concentration in seeds increasing with increasing exposure time, particularly when the seeds are incubated in EMS solution for longer.

The analysis of variance revealed significant ($P < 0.01$) differences in surviving seedlings with each EMS dose, exposure period, presoaking period, and their interactions. The surviving rate was ranked from 0% to 100% in this study. When evaluated in terms of the EMS dose, the lowest survival rate observed was 14.8% for the 2% EMS dose plot. The highest surviving rate observed was 90.2% for the control plot (Table 4). Statistical analysis on survival rate showed an attendant decrease in germination with applied increases in the concentration of EMS. As per Table 4, the outcomes acquired show that a decrease in seed germination occurred with a corresponding increase in EMS dose ($P < 0.01$). Considering each exposure period, the mean germination percentage was 44.5%, 47.3%, 52.3%, and 25.0% for 6-, 12-, 24-, and 48-hour exposure periods, respectively. When evaluated in terms of presoaking, the lowest mean survival rate observed was 30.1% for the zero hours (dry seed) presoaking plot. The highest mean survival rate observed was 52.7% for the 12 hours presoaking plot. EMS application without presoaking and 48 hours of EMS application and their combinations almost prevented seedling survival. It should be emphasized that the chemical reduces the germination ability of dry seeds and also EMS application for 48 hours inhibit germination to a high extent. Correspondingly, the survival rate also decreased. In addition, there was a difference between germination rate and survival rate up to 8.6%. It could be reasoned that seedlings that germinated weakly after the mutation application were unable to survive.

There were significant effects of presoaking duration, EMS exposure period, EMS dose, and some of the combinations provided a 100% survival rate. Survival rates were similar for 24-hour exposure period, 0.5% EMS dose and 12 hours presoaking plots compared with control plots.

Seedling shoot length is an important feature showing the development of seedlings after mutation application. Seedling shoot length was significantly ($P < 0.01$) affected by presoaking duration, EMS dose, EMS exposure period, and their combinations. Seedling shoot length varied between 0.0-36.3 mm and the experiment average was 16.0 mm. The highest mean shoot length was measured 30.6 mm on the control plot (Table 5). The consequences of the seedling experiment indicated that increasing EMS doses caused a significant decrease in seedling shoot development (Table 5). A significant decrease was observed of over 50% when EMS dose was 0.5% and higher. As EMS exposure period increased, a significant decrease in seedling shoot length occurred, especially for the 24-hour EMS exposure period. At a dose of 0.5% EMS, the lowest EMS dose, an exposure period of 48 hours resulted in a significant decrease (no growth of shoots) in seedling shoot length compared with the control. The results indicated that no presoaking caused a significant decrease in seedling shoot development. A significant decrease was observed of approximately 50% in the non-presoaked plot. In terms of the interaction between presoaking duration and EMS exposure period, a significant decrease in seedling shoot length occurred, especially for no presoaking and 48-hour EMS exposure period. The longest seedling shoots were observed for the 12 hours presoaking duration, 0.5% EMS dose and 12-hour exposure period conditions when compared to the other combinations except for 0% EMS dose.

Seedling root length is another important character of seedling stage development in rice. Seedling root length was significantly ($P < 0.01$) affected by EMS dose, EMS exposure period, presoaking duration, and their combinations. Seedling root length varied between 0.0–5.1 cm and the experiment average were 2.2 cm. The highest mean root length was measured at 3.5 cm for the 0% EMS dose control plot (Table 6). The result of the seedling experiment indicated that increasing EMS doses caused a significant decrease in seedling root development. A significant decrease was observed of over 50% when EMS

Table 3. Effect of EMS application dose, EMS exposure period and presoaking duration on radicle length in rice (mm).

EMS application dose (%)	EMS exposure period(hour)	Presoaking duration (hours)				Mean
		0	6	12	24	
0.0%	6	25.7jk	31.7fg	43c	46.2ab	36.7b
	12	42.8c	43.9bc	47.8a	33.3eg	41.9a
	24	30.8gh	32.1eh	38.4d	29.6hi	32.7c
	48	32.5eh	29.5hi	21.4lm	11.1o	23.6f
Mean		*32.9b*	*34.3b*	*37.7a*	*30.1c*	33.7A
0.5%	6	13.6no	27.5ij	34.ef	35.1e	27.5e
	12	15.2n	29.6hi	42.0c	46.ab3	33.3c
	24	19.0m	19.6m	15.1n	12.9no	16.7h
	48	0.0r	0.0r	0.0r	0.0r	0.0k
Mean		*11.9ij*	*19.2f*	*22.7d*	*23.5d*	19.8B
1%	6	10.7o	15.4n	32.0fg	29.7hi	21.9g
	12	23.kl	34.2ef	32.3eh	19.6m	27.3e
	24	0.0r	20.0m	18.8m	3.7pq	10.6j
	48	0.0r	0.0r	0.0r	0.0r	0.0k
Mean		*8.4k*	*17.4g*	*20.8e*	*13.2hi*	14.9C
2%	6	25.1jk	34.7ef	34.1ef	23.1kl	29.3d
	12	0.0r	6.7p	18.6m	24.3kl	12.4i
	24	0.0r	1.7qr	3.5q	0.0r	1.3k
	48	0.0r	0.0r	0.0r	0.0r	0.0k
Mean		*6.3l*	*10.7j*	*14.1h*	*11.9ij*	10.7D
General average		14.9C	20.4B	23.8A	19.9B	19.7
**	**	**				

**: significant at the 1% level; NS: no significant differences. Values followed by the same letter are not statistically significantly different. A: Presoaking duration; A: EMS application dose; a: EMS application dose × EMS exposure period interaction; a: EMS application dose × Presoaking duration; a: Three-way interaction which EMS application Dose × EMS exposure period × Presoaking duration. LSD_{dose} = 0.7; $LSD_{Duration}$= 0.7; $LSD_{Presoaking}$ = 0.7; $LSD_{Dose\times Duration}$=1.5; $LSD_{Dose\times Presoaking}$=1.5; $LSD_{Duration\times Presoaking}$=1.5; $LSD_{Dose\times EMSduration\times Presoaking}$ = 3.0; CV (%) = 9.4. CV, coefficient of variation; EMS, ethyl methanesulfonate; LSD, least significant difference.

dose was 1% and higher. As EMS exposure period increased, a significant decrease in seedling root occurred, especially for the 24-hour EMS exposure period. At doses of 0.5% EMS and higher, an exposure period of 48 hours resulted in a significant decrease (no growth of roots) in seedling root length compared with the control. The results indicated that no presoaking caused a significant decrease in seedling root development. A significant decrease was observed of approximately 50% when seeds were not presoaked. In terms of presoaking duration, a significant decrease in seedling root length occurred especially for the 48-hour EMS exposure period. The longest seedling roots were obtained for the 12 hours presoaking duration, 2% EMS dose and six-hour exposure period conditions when compared to the other combinations.

Fresh seedling weight is another notable parameter that indicates seedling development after mutation. Fresh seedling weight was significantly ($P < 0.01$) affected by presoaking duration, EMS dose, EMS exposure period, and their combinations. Fresh seedling weight varied between 0.0-195.9 mg and the experiment average was 99.5 mg. The conclusion of the seedling experiment indicated that increasing EMS doses caused a

Table 4. Effect of EMS application dose, EMS exposure period and presoaking duration on surviving seedling in rice seedling experiment (%).

EMS application dose (%)	EMS exposure period (hours)	Presoaking duration (hours)				Mean
		0	6	12	24	
0.0%	6	68.8bc	93.8a	56.3ce	31.3fh	62.5c
	12	100.0a	93.8a	100.0a	100.0a	98.4a
	24	100.0a	100.0a	100.0a	100.0a	100.0a
	48	100.0a	100.0a	100.0a	100.0a	100.0a
Mean		*92.1ab*	*96.9a*	*89.1ab*	*82.8b*	90.2A
0.5%	6	18.8hj	31.3fh	37.5eh	25.0gi	28.1f
	12	6.3ij	18.8hj	56.3ce	56.3ce	34.4ef
	24	62.5bd	93.8a	100.0a	62.5bd	79.7b
	48	0.0j	0.0j	0.0j	0.0j	0.0i
Mean		*21.9fg*	*35.9d*	*48.4c*	*35.9d*	35.5B
1%	6	0.0j	43.8dg	81.3ab	62.5bd	46.9d
	12	0.0j	68.8bc	50.0cf	56.3ce	43.8de
	24	0.0j	25.0gi	68.8bc	0.0j	23.4fg
	48	0.0j	0.0j	0.0j	0.0j	0.0i
Mean		*0.0i*	*34.4de*	*50.0c*	*29.7df*	28.5C
2%	6	25.0gi	43.8dg	50.0cf	43.8dg	40.6de
	12	0.0j	0.0j	25.0gi	25.0gi	12.5gh
	24	0.0j	6.3ij	18.8hj	0.0j	6.25hi
	48	0.0j	0.0j	0.0j	0.0j	0.0I
Mean		*6.25hi*	*12.5gh*	*23.4eg*	*17.2gh*	14.8D
General average		30.1C	44.9B	52.7A	41.4B	42.3
**	**	**				

**: significant at the 1% level; NS: no significant differences. Values followed by the same letter are not statistically significantly different. A: Presoaking duration; A: EMS application dose; a: EMS application dose × EMS exposure period interaction; a: EMS application dose × Presoaking duration; a: Three-way interaction which EMS application Dose × EMS exposure period × Presoaking duration. LSD_{dose} = 5.7; $LSD_{Duration}$ = 5.7; $LSD_{Presoaking}$ = 5.7; $LSD_{Dose\times Duration}$=11.42; $LSD_{Dose\times Presoaking}$=11.42; $LSD_{Duration\times Presoaking}$=11.42; $LSD_{Dose\times EMSduration\times Presoaking}$ = 23.0; CV (%) = 3.9. CV, coefficient of variation; EMS, ethyl methanesulfonate; LSD, least significant difference.

significant decrease in fresh seedling weight. In terms of EMS dose, the highest fresh seedling weight measured was 170.2 mg for the control plot (Table 7). A significant decrease was observed of approximately 50% when the EMS dose was 0.5% and higher. There was a significant decrease in fresh seedling weight with the increase in EMS application time, especially for the 48-hour EMS application time. It was determined that the seedlings did not develop and fresh weight was not obtained for plots with 48 hours of EMS exposure combined with EMS doses of 0.5%, 1%, and 2%. In addition, it was observed that the fresh seedling weight was dramatically decreased in the plots without presoaking. A significant decrease was observed of more than 30% when non-presoaked. In terms of presoaking duration, a significant decrease in fresh seedling weight occurred especially for the 48-hour EMS exposure period. The highest fresh seedling weight was calculated for the 12 hours presoaking duration, 0.5% EMS dose and six-hour exposure period conditions when compared to the other combinations.

EMS LD_{50} Determination

In terms of the LD_{50} dose determination study, four EMS doses were considered and other applications such as EMS exposure

Table 5. Effect of EMS application dose, EMS exposure period and presoaking duration on shoot length in rice seedling experiment (mm).

EMS application dose (%)	EMS exposure period (h)	Presoaking duration (h)				Mean
		0	6	12	24	
0.0%	6	31.9ae	30.5af	26.5bj	19.3im	27.0b
	12	33.1ac	31.5af	33.1ac	31.8af	32.4a
	24	32.6ae	31.7af	33.9ab	30.2ag	32.1a
	48	21.4hl	36.3a	33.7ab	33.1ad	31.1ab
Mean		*29.7a*	*32.5a*	*31.8a*	*28.6a*	30.6A
0.5%	6	18.8jm	17.4ko	24.9ck	18.9jm	19.9c
	12	4.3pq	12.4mp	30.0ag	26.2bj	18.2cd
	24	20.7hm	19.6im	24.6ek	18.2jn	20.8c
	48	0.0q	0.0q	0.0q	0.0q	0.0f
Mean		*10.9de*	*12.3ce*	*19.9b*	*15.8bc*	14.7B
1%	6	0.0q	22.0gl	27.6bi	26.3bj	18.9cd
	12	0.0q	23.5fl	18.2jn	20.3hm	15.5d
	24	0.0q	9.9np	17.6kn	0.0q	6.9e
	48	0.0q	0.0q	0.0q	0.0q	0.0f
Mean		*0.0f*	*13.8cd*	*15.8bc*	*11.6ce*	10.3C
2%	6	10.3np	28.3ah	16.0lo	24.6dk	19.8c
	12	0.0q	0.0q	9.1op	25.5bk	8.6e
	24	0.0q	4.3pq	16.0lo	0.0q	5.1e
	48	0.0q	0.0q	0.0q	0.0q	0.0f
Mean		*2.6f*	*8.1e*	*10.3de*	*12.5cd*	8.4C
General average		10.8C	16.7B	19.4A	17.1B	16.2
**	**	**				

**: significant at the 1% level; NS: no significant differences. Values followed by the same letter are not statistically significantly different. A: Presoaking duration; A: EMS application dose; a: EMS application dose × EMS exposure period interaction; *a*: EMS application dose × Presoaking duration; a: Three-way interaction which EMS application Dose × EMS exposure period × Presoaking duration. LSD_{dose} = 2.1; $LSD_{Duration}$ = 2.1; $LSD_{Presoaking}$ = 2.1; $LSD_{Dose\times Duration}$=4.1; $LSD_{Dose\times Presoaking}$=4.1; $LSD_{Duration\times Presoaking}$=4.1; $LSD_{Dose\times EMSduration\times Presoaking}$ = 8.2; CV (%) = 37.5. CV, coefficient of variation; EMS, ethyl methanesulfonate; LSD, least significant difference.

period and presoaking duration were ignored. EMS doses of 0%, 0.5%, 1%, and 2% were utilized in the study. In this research, LD_{50} dose was determined of surviving rates rather than germination rate. Survival rates were lower than germination rates. The Spearman-Karger method, which was introduced by Spearman in 1908 and modified by Karger in 1931, was utilized. The Spearman-Karger equation evaluated based on the dose increase rates and the number of died plants in Table 8. The highest EMS dose was 2%, and the highest EMS dose mortality rate was 85.2%. At the end of the study, 0.5% EMS dose was determined as the LD_{50} dose. When other subjects were excluded, the LD_{50} dose recommended by most researchers for mutation was 0.5% EMS dose. In this article, in which the optimum conditions of EMS application Dose, EMS exposure period, and Presoaking duration are tried to be determined, 0.5 EMS dose is recommended when evaluated together with other variables.

Discussion

The experimental results of both the germination experiment and the seedling experiment revealed that the presoaking duration, EMS dose, EMS exposure period, and their interactions

Table 6. Effect of EMS application dose, EMS exposure period and presoaking duration on root length in rice seedling experiment (cm).

EMS application dose (%)	EMS exposure period (h)	Presoaking duration (h)				Mean
		0	6	12	24	
0.0%	6	4.8ab	3.9ae	3.7af	1.4il	3.5ac
	12	3.5ag	3.1ci	3.1ci	4.1ac	3.4ac
	24	4.0ad	4.1ac	3.3bh	3.2bh	3.6ab
	48	2.3ej	3.9ae	4.2ac	3.3bh	3.4ac
Mean		*3.7ab*	*3.7a*	*3.6ab*	*3.0ac*	3.5A
0.5%	6	3.3bh	2.4dj	3.8af	3.2bh	3.1bd
	12	0.8jl	1.5il	3.3bh	3.8af	2.3de
	24	3.5ag	2.9ci	4.4ac	2.7ci	3.4ac
	48	0.0l	0.0l	0.0l	0.0l	0.0i
Mean		*1.9eg*	*1.7eg*	*2.9bd*	*2.4ce*	2.2B
1%	6	6.66E-16	3.3bh	3.9ae	3.5ag	2.7ce
	12	1.80E-16	3.4bh	2.9ci	2.8ci	2.3ef
	24	0.0l	2.0gk	2.3ej	0.0l	1.1gh
	48	0.0l	0.0l	0.0l	0.0l	0.0i
Mean		*0.0h*	*2.2dg*	*2.3cf*	*1.6fg*	1.5C
2%	6	2.1fk	5.1a	5.1a	4.1ac	4.1a
	12	0.0l	0.0l	2.0gk	3.8af	1.4fg
	24	0.0l	0.5kl	1.8hk	0.0l	0.6hi
	48	0.0l	0.0l	0.0l	0.0l	0.0i
Mean		*0.5h*	*1.4g*	*2.2cg*	*1.9eg*	1.5C
General average		1.5C	2.3B	2.7A	2.2B	2.2
**	**	**				

**: significant at the 1% level; NS: no significant differences. Values followed by the same letter are not statistically significantly different. A: Presoaking duration; A: EMS application dose; a: EMS application dose × EMS exposure period interaction; *a*: EMS application dose × Presoaking duration; a: Three-way interaction which EMS application Dose × EMS exposure period × Presoaking duration. LSD_{dose} = 0.4; $LSD_{Duration}$ = 0.4; $LSD_{Presoaking}$ = 0.4; $LSD_{Dose\times Duration}$=0.82; $LSD_{Dose\times Presoaking}$=0.82; $LSD_{Duration\times Presoaking}$=0.82; $LSD_{Dose\times EMSduration\times Presoaking}$ = 1.65; CV (%) = 54.5. CV, coefficient of variation; EMS, ethyl methanesulfonate; LSD, least significant difference.

were significant. The result of the experiment was similar to study results of Talebi *et al.* (2012), and Ramchander *et al.* (2014). Slight variations appeared in terms the most suitable combination of factors. However, results were obtained that could be used to make a standard protocol. Presoaking is an important stage for the EMS solution to diffuse into the seed and optimum presoaking duration is expressed as the presoak duration when the seed reaches full saturation. Although from previous experiments it was recommended that maximum water intake of the seeds is reached for EMS mutation application, it was determined to be useful water intake level at around 25% in this research. Rice reached this water intake level at 12 hours. The results illustrated that the rice seed reached full saturation after 24 hours presoaking. However, when the presoaking duration was evaluated on its own and with other conditions, it was determined that the 12-hour presoaking duration was the most suitable time for EMS application. In addition, EMS exposure periods of more than six hours might be

Table 7. Effect of EMS application dose, EMS exposure period and presoaking duration on fresh seedling weight in rice seedling experiment (mg).

EMS application dose (%)	EMS exposure period (h)	Presoaking duration (hours)				Mean
		0	6	12	24	
0.0%	6	192.5ab	168.4ag	143.6ai	121.5el	156.5bd
	12	195.9a	178.0ad	187.1ab	176.0ae	184.3a
	24	188.5ab	168.9ag	186.1ab	163.2ai	176.7ab
	48	150.2ai	183.4ac	165.8ah	153.2ai	163.1ac
Mean		*181.8a*	*174.7ab*	*170.7ab*	*153.5b*	170.2A
0.5%	6	123.3dk	110.9hm	177.5ad	118.1gm	132.4df
	12	33.8no	81.5jn	170.7ag	175.2af	115.3eg
	24	148.0ai	142.9ai	142.5ai	108.3im	135.4ce
	48	0.0o	0.0o	0.0o	0.0o	0.0i
Mean		*76.3df*	*83.8de*	*122.7c*	*100.4cd*	95.8B
1%	6	0.0o	128.4cj	147.4ai	145.7ai	105.4fg
	12	0.0o	150.4ai	120.6fl	138.2bi	102.3g
	24	0.0o	68.5kn	116.4gm	0.0o	46.2h
	48	0.0o	0.0o	0.0o	0.0o	0.0i
Mean		*0.0g*	*86.8de*	*96.1ce*	*70.9ef*	63.5C
2%	6	63.0mn	169.7ag	108.3im	139.3bi	120.1eg
	12	0.0o	0.0o	66.6ln	156.0ai	55.6h
	24	0.0o	30.0no	109.5im	0.0o	34.9h
	48	0.0o	0.0o	0.0o	0.0o	0.0i
Mean		*15.8g*	*49.9f*	*71.1ef*	*73.8df*	52.6C
General average		68.4C	98.8B	115.1A	99.7B	95.5
**	**	**				

**: significant at the 1% level; NS: no significant differences. Values followed by the same letter are not statistically significantly different. A: Presoaking duration; a: EMS application dose; *a*: EMS application dose × EMS exposure period interaction; a: EMS application dose × Presoaking duration; *a*: Three-way interaction which EMS application Dose × EMS exposure period × Presoaking duration. LSD_{dose} = 13.8; $LSD_{Duration}$ = 13.8; $LSD_{Presoaking}$ = 13.8; $LSD_{Dose\times Duration}$=27.7; $LSD_{Dose\times Presoaking}$=27.7; $LSD_{Duration\times Presoaking}$=27.7; $LSD_{Dose\times EMSduration\times Presoaking}$ = 55.3; CV (%) = 40.8. CV, coefficient of variation; EMS, ethyl methanesulfonate; LSD, least significant difference.

Table 8. Estimating LD_{50} lethal concentrations of EMS doses according to Spearman-Karger Method.

EMS Dose (%)	Dose Difference (a)	No of Dead (m)	Mean Mortality (b)	Product (a x b)
0	-	9.8	-	-
0.5	0.5	64.5	37.2	18.6
1	0.5	71.5	68.0	34.0
2(D_h)	1.0	85.2	78.4	78.4
			Sum of Product	131.0
			LD_{50}	0.5

LD_{50} = Arithmetic means of dose that half the sum of the plants reacting with two consecutive doses; Dh = highest dose for plants a= Half the sum of the plants reacting with two consecutive doses; b= Mean mortality of the plants between two consecutive doses; a × b = Product; m = number of died plants in each group; Sum of Product = Sum of all product; LD_{50} = Dh – [$\sum$ (a × b) / m]. LD_{50}= 2 - (131.0 / 85.2) = 0.5

damaging to the seed. The seeds might tolerate a long exposure period of 12 hours or 24 hours. However, 48 hours of application caused the seed to irreversibly lose its germination ability. EMS application doses of 0.5%, 1%, and 2% reduced surviving seeds by roughly 50%, 60%, and 80%, compared to the 0% EMS dose. The LD_{50} was determined as 0.5% EMS dose. Furthermore, the mutated seeds can be stored for three to four weeks after drying and retain more than 85% of their germination ability; the result of Tonthong *et al.* (2018) also supported this process. In this study, 12 hours presoaking duration, a six-hours EMS exposure period, and 0.5% EMS dose were determined to be the most appropriate combination. The EMS application protocol might be successfully utilized in rice mutation research.

Conclusion

The most suitable EMS application practice was determined to be 12 hours presoaking, 0.5% EMS dose, and six hours EMS exposure for rice. The protocol includes the following: (1) Presoaking: 12 hours, (2) EMS application: 0.5% dose EMS and six hours, (3). Final washing: six hours, (4) Drying: 72 hours at 38°C. In addition, the protocol sheets are presented as a user-friendly protocol as *Extended data* (Unan, 2021b).

Data availability

Underlying data

Zenodo: Dataset related paper "protocol for ems mutagenesis application in rice". https://doi.org/10.5281/zenodo.4549457 (Unan, 2021a).

This project contains the following underlying data:

- Protocol_for_EMS_application_in_rice_data.xlsx

Extended data

Zenodo: Factsheet related paper "protocol for ems mutagenesis application in rice".

https://doi.org/10.5281/zenodo.4587383 (Unan, 2021b).

This project contains the following extended data:

- Factsheet of Protocol for EMS mutagenesis application in rice.pdf
- Flow Chart of Protocol for EMS Mutagenesis Application in Rice.pdf

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

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I am satisfied with the revisions except for the title of the research article. I am still not convinced with the explanation given by the author for generalizing it as 'Protocol for ethyl methanesulphonate (EMS) mutagenesis application in rice', instead of naming the specific rice variety. But that's just a suggestion and it's up to the author whether or not to go through with it. I am satisfied with the revisions overall and 'APPROVE' the manuscript in its current form.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 15 November 2021

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I could not find the manuscript version in Microsoft word format with line numbers listed. So, I will

try to detail and list the paragraphs and sections I had issues with.

The manuscript overall reads well. There is a proper structure and flow to the contents of the manuscript. But, there are some incomplete references in the introduction, inaccurate observations in the results section and incomplete discussion section where the observations from the results section have been shortened and duplicated as discussion.

1. My first concern is regarding the title of the manuscript. It says 'Protocol for ethyl methanesulphonate (EMS) mutagenesis application in rice'. There are thousands of rice varieties and the study was conducted only on *Osmancik-97.* This protocol may or may not produce similar outcomes in other rice varieties, and other rice varieties were not studied in this research. So, making the title more specific by changing it to Japonica/*Osmancik-97* rather than generalizing it plainly as 'rice' would be more appropriate or else would be a mischaracterization in my opinion.

2. In paragraphs 3 and 4 in the introduction and in materials section, there are several statements such as, 'The main rice area, Asia, has a production system of transplanting rice so they have no severe weed problems in their fields', 'Old herbicides do not work effectively over time', 'Researchers have developed herbicide resistance systems such as Clearfield, Provisia, and Roxy Rice by mutation application', 'Rice plant breeders have used point mutations in their breeding program to overcome these problems', 'mutations exist in nature besides the artificially induced mutation', 'Physical and chemical mutagens are used to obtain plants by mutation breeding, such as gamma rays, X-rays, fast neutrons and also ethyl methanesulphonate (EMS; CH3SO3C2H5), diepoxybutane (DEB, C4H6O2) and sodium azide (NaN3)', 'The chemical mutagen EMS has been widely utilized to induce a large number of functional variations in rice', 'Osmancik-97 is a Japonica type Turkish rice variety. The variety was released by Trakya Agricultural Research Institute, Edirne, Turkey in 1997', 'The parents are Rocca and Europe, which originate from Italy'. All these statements are used in this manuscript without any references. Please cite proper references.

3. In the methods section, under 'EMS mutagenesis', it would be great if the authors could add a couple of sentences addressing how the unused and the leftover EMS solutions were neutralized and discarded, since EMS is a carcinogen. That would greatly help the future researchers that are going to follow and cite this protocol. (Just a suggestion).

4. In the results section, under the 'Imbibition rate', In figures 1 and 2, it would be more informative to add the standard deviation/standard error bars to show case significant differences between treatments.

5. In the results section, under the 'Germination experiment', in the first paragraph it says 'Considering EMS exposure period, mean germination percentage was 91.9%, 81.3%, 55.6%, and 24.7% for six, 12, 24, and 48-hour exposure periods, respectively'. The author is getting these mean germination values after taking into account the mean of the controls (0.0% EMS) along with the rest of the EMS treatments. By doing this, the author is masking the effect of EMS treatment on germination percentage. The proper way to do this is by considering controls (0.0% EMS) as one group and the rest of the EMS treatments as the other group. This way, the authors can properly compare the mean germination values between the controls and EMS-treatments. Next sentence says 'Most combinations resulted in 100% germination'. This observation doesn’t seem accurate. Barring 0.0% EMS treatment

(controls), 17 of the 48 combinations showed 100% germination whereas 31 of the 48 did not. In the next paragraph, it says 'EMS application for over 24 hours with EMS application doses of 1–2% forestalls plumula length' This observation seems inaccurate. Instead of stating EMS application for over 24 hours with EMS application, 'EMS application for 48h with EMS application doses of 1–2% forestalls plumula length', is more accurate based on the data in table 2.

6. In the results section, under the 'Seedling experiment', In the fourth paragraph it says 'It should be emphasized that the chemical reduces the germination ability of dry seeds and also EMS application for more than 24 hours inhibit germination to a high extent'. Characterizing it as more than 24 hours is inaccurate. Since the authors have not tested any other pre-soaking times between 24 H and 48H. Calling it as 'more than 24 hours' might imply that pre-soaking for 25H or 26H or 30H might inhibit germination, which may or may not be true since the authors haven't tested that. The authors tested '48H'. So, it would be logical to report it as 'EMS application for 48 hours inhibited germination to a high extent'.

7. In the results section, under the 'EMS LD50 Determination', it says 'In this article, in which the optimum conditions for three-way are tried to be determined, 0.5 EMS dose is recommended when evaluated together with other variables.' Three-way of what? This needs more clarity.

8. In the Discussion section, it says 'The result of the experiment was similar to study of Talebi *et al.* (2012), and Ramchander *et al.* (2014).' Please indicate how the results of this study are similar to the studies reported by Talebi *et al.* (2012), and Ramchander *et al.* (2014). The next sentence says 'Slight variations appeared in terms of the most suitable combination of factors' Combination of factors for? The sentence should either be simplified or more clarity needed. In the same paragraph, it says 'Although from previous experiments it was recommended that maximum water intake of the seeds is reached for EMS mutation application, water intake level at around 25%, reached in 12 hours, was determined to be useful in this research.' This sentence doesn't make sense. Please rephrase it or break it down into two sentences to convey exactly what the author is trying to say. In the next line, it says 'it was determined that the 12-hour period was the most suitable time for EMS application'.12H period of pre-soaking or EMS exposure?

9. In the discussion section, no discussion/explanation was given for why and how the increase in EMS concentrations, exposure time and pre-soaking is effecting the different factors studied in this study. What might be the physical, physiological and biochemical components of the rice seed affected by EMS-treatment/pre-soaking and how that manifests into the differences observed in terms of germination percentages, root length, shoot length etc. And if those observations are in line or not in line with other EMS studies published in rice, wheat and other food crops.

Thank you

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plant biology, plant physiology, biochemistry, molecular biology, molecular physiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 29 October 2021

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R. Kleynhans
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No further comments

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
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Are sufficient details of methods and analysis provided to allow replication by others?

Yes

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Are all the source data underlying the results available to ensure full reproducibility?
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Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 18 August 2021

https://doi.org/10.21956/openreseurope.14387.r27304

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The experiment was well designed and are testing valid factors for the induction of mutations. Data is presented with relevant factors that is usually measured when determining optimum conditions for the generation of mutations. My major concern however is that there is no indication of the actual assessment of mutations that would be present or not. Normally LD_{50} values are calculated from the measurements taken as an indication of the best doses and durations for the generation of mutations.The authors however choose a best protocol based on the best measurements for the various treatments that might not result in mutations or very low levels of mutations. I think that the data presented can easily be reworked to establish LD_{50} values that would better indicate the possible presence of mutations, especially since there is no indication of possible mutation observations given from the plants grown.

There is also a lot of variation present in different values given for the 0% EMS control data in some of the tables, that might indicate natural variation, that is not necessarily due to treatment effects, making it imperative to rather work with LD_{50} or even LD_{25} values. Then data is presented as a percentage of the control treatments.

The three-way interactions observed is always difficult to work with and in this case makes some of the data in the tables difficult to interpret. The indication of significant values in the tables with upper case, lower case and italic letters could be explained better. I was not always sure what is compared with what – especially with the values inside the various pre-soaking blocks. A clear explanation will help to interpret the tables with more ease. Some of the letter are also confusing – e.g. table 2 there is things like ej (letters normally follow - ef). I do not know what is compared with what when looking at all the values followed by lower case letters. Does ej actually indicate e to j (efghj)? Explaining this below the tables would make it easier to understand as well.

Rice is one of the crops with the most induced mutations and many different protocols have been used in this regard (see review by Viana *et al* 2019). EMS has been used extensively in the past and indications are that specific doses and durations of EMS application will vary depending on cultivar used (see review by Viana *et al* 2019). Selecting a general protocol is thus difficult as this might not be applicable to various cultivars or varieties. To compare results with previously published articles it is thus important to use LD_{50} values and indicate that the suggested protocol is based on these values and applicable to the tested variety. Discussion to compare data with published research can thus be improved.

I did not pick up any other data that include the pre-soaking treatment and this could certainly be novel as there is clear indications that the pre-soaking treatment can to some extent protect the seed from the damage caused by the EMS treatment. The question still remains if this better growth values will then still result in mutations.

The data has merit but would add better value if LD_{50} and LD_{25} values could be calculated.
Further clarifications and questions:

- Page 4 - “Imbibition rate (%) = (last weight - first weight) × 100 / first weight”: If this was used to describe the percentage imbibition rate - then the graphs should also reflect the % imbibition rate on the y-axis and not indicate weight in gram per 100 seed.

- Page 4: “Statistical analysis on germination showed an attendant decrease in germination with applied increases in the concentration of EMS.” I think the whole sentence can be removed as the next sentence state the same thing and is fine with statistical proof as presented in the table.

- Page 5, para 1: Lower EMS concentrations without pre-soaking still resulted in germination. Only the higher dosages without presoaking prevented germination and all 48 hour durations prevented germination. Just correct the sentence.

- Page 6: If I read the table correctly and you are here referring to overall pre-soaking data - the lowest value is 27.1 and not 11.2.

- Page 6: “ EMS application for over 24 hours forestalls plumula length.” Only for 1 and 2% application. 24 hours at 0.5% still resulted in plumula growing.

- Page 7, Table 2: If I assume correctly, this material was soaked in water to replace the EMS exposure time. There is a lot of variation in these controls with significant differences - making it difficult to interpret results further on. Is the results observed really due to the

treatment or part of the natural variation? Long soaking in water (presoaking + 0% exposure also significantly changes the plumula length. These changes are however not consistent in the three-way interaction. making interpretation of other results difficult. Maybe stick to the two way interactions and single factor data.

Below is a list of corrections relating to the article:

- Intro para 1: please correct “rice producer countries” to “rice producing countries”.
- Intro para 2: please correct “particularly low and middle income nations” to “particularly for low…”
- EMS mutagenesis para 1: Please correct “using a randomized parcel design” to “using a randomized block” (correct throughout article) and “Seeds are sterilized” to “Seeds were sterilized”.
- EMS mutagenesis para 2: Please replace “Seedling survival rate is the ratio of survive seedlings” to “the ratio of surviving seedlings”.
- Factsheet and flowchart of protocol for EMS mutagenesis application in rice: Please correct ‘This flowchart shows a schematic for how..” to “This flowchart shows a schematic illustration…”
- Experiment 2: Seedling experiment – para 1: I do not think viol is the correct word in this paragraph I would rather use plant tray in all descriptions where the word viol is used.
- Experiment 2: Seedling experiment – para 1: Please replace ‘for’ with ‘with’ in the statement “The plant viols were then put in the Fitotron at 25°C and 30°C for 12-hour dark and 12-hour light cycles for 21 days”.
- Page 5, para 1: Rewrite as “ When evaluated in terms of presoaking, the lowest mean germination (49.1%) for zero hours (dry seed) presoaking.
- Table 5: Please correct “duration on shoot” to “duration on shoot length”.
- Table 6: Please correct “duration on root” to “duration on root length”.

References

1. Viana VE, Pegoraro C, Busanello C, Costa de Oliveira A: Mutagenesis in Rice: The Basis for Breeding a New Super Plant.*Front Plant Sci*. 2019; **10**: 1326 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and does the work have academic merit?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Comments on this article

Version 2

Author Response 07 Feb 2022

Rasim Unan

Thanks for your review of this article's second version (V2). I accepted most of your recommendations except for the manuscript title for this reason the article need to be released new version (V3). I have discussed your review below.

1. There are many varieties of rice in the world. It is difficult to make separate studies for each variety. This study was conducted to determine a standard practice in rice in overall. The results might be giving an idea for the applications to be made in other rice varieties. Writing only the `Osmancik-97 variety` in the title may reduce the effectiveness of the article. The reader may be reluctant to interpret that it is applicable only in one form and use it for their work. Since the visibility and impact of the article is higher, it is more appropriate to keep it as a 'rice'. I prefer to keep the title the same.

2. Appropriate references are given for most of the mentioned statements. The references given was also added to the reference list.

3. I congratulate you for your consideration to public health. A sentence has been added to the EMS mutagenesis section to reduce its harmful effects.

4. The standard error bars were added in figures 1 and 2 in the results section.

5. This section has been re-evaluated to remove the masking the effect of EMS treatment on germination percentage effect. The results were as follows. `Considering EMS exposure period compared to all controls (0.0%), mean germination percentage was 91.0%, 75.9%, 13.9%, and 0.0% for six, 12, 24, and 48-hour exposure periods, respectively.`

6. In the results section, it was rewritten that 'EMS application for 48 hours inhibited germination to a high extent.'

7. Three way means EMS application Dose, EMS exposure period, and Presoaking duration. A descriptive sentence has been added in the result section.

8. In the discussion section, some sentences were reorganized according to your review. It was rewritten that `it was determined to be useful water intake level at around 25% in this research. Rice reached this water intake level at 12 hours.`

9. Physical, physiological, and biochemical components of the rice seed were not examined in this trial. Therefore, no comment has been discussed about the component. Thanks.

Competing Interests: No competing interests were disclosed.