Bulletin of the National Research Centre

RESEARCH Open Access

Genetic Amelioration in lentil (*Lens culinaris* L.) using Different Doses of Ethyl Methane Sulphonate

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Abstract

Background: Lentil is an important source of food not only locally but globally. The importance of this legume crop is highlighted in light of the transport of strategic food sources such as wheat, maize, and rice due to environmental challenges. So, the process of genetic improvement of this crop has become imperative.

Results: The final results confirmed that the three lentil cultivars Giza 9 (G1), Giza 29 (G2), and Giza 51 (G3) were exhibited unparalleled superiority for all growth, germination, and other traits under study when exposed to the three doses of ethyl methane sulfonate (EMS) compared to the control. Moreover, the three doses of this mutagen exceeded on the control experiment and this superiority were most severe especially at the third dose. The higher limit of positive percentages values of induced mutagenesis in M2 lentil generation (the second mutant generation) was observed in all studied traits especially at the third dose of EMS (0.3). This fact confirmed the success of the genetic improvement process in all lentil traits mentioned above.

Conclusion: The three lentil cultivars (Giza 9, Giza 29, and Giza 51) were recorded highly mean values of all studied in M2 generation for the three doses of EMS. The third dose of mutagen (0.3) was come in the first rank followed by the second dose (0.2) then the first dose in this regard.

Keywords: Lentil, Ethyl methane sulfonate, SDS-PAGE electrophoresis, Isozyme analysis, Germination and growth traits, Genetic improvement

Background

Lentils (*Lens culinaris L.*) are considering one of the most famous legumes in the world and it is one of the annual crops. Lentils contain little calories, food fibers useful for the digestive system, and also contain carbohydrates. Also, lentils are not containing harmful cholesterol and included in the composition of lentil's fatty acids, amino acids, a good rate, and a high percentage of water. The aim of this investigation is genetic improving for the Egyptian lentil cultivar traits and at the forefront of these characters come germination, growth, and the final crop traits which were a fertile field for research and studies. Breeding by using mutation is considering one of the most important methods of genetic improvement which plays an important role in the recent period in bringing about a positive genetic change that would have improved the characteristics of a large number of crops especially lentils. This is what we will deal with in some detail in this regard. Ali and Shaikh (2007) showed the genetic exploitation in lentil by induced mutations and revealed that mutant line AEL 49/20 recorded the highest rank of grain yield in regional trials proceeded under various agro-climatic regions in Sindh province. Four various kinds of chlorophyll mutants' viz Albino, Viridis, Xantha, and Chlorina were discovered in the treated population of maize by Gnanamurthy et al. (2011) when seeds were treated with various levels of

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mutagenesis namely, ethyl methane sulfonate (EMS), diethyl sulfate, and sodium azide. Results confirmed that EMS was found to very impact and revealed high level of genetic improvement in maize plants higher than the rest mutagenesis for manufacturing useful mutants in M1 and M2 generations. Ahirwar et al. (2014) studied induced mutations using gamma rays and EMS in lentil and detected that the combination of each mutagen was succeeded for exhibited highly significant differences for all agro-morphological traits under study in M1 and M2 generations. Induced genetic variability and heritability in macrosperma and microsperma using EMS in lentil were evaluated by Rana and Solanki (2015). Results confirmed that higher values of the genetic parameters, variance, heritability in the broad sense, and genetic advance were showed in the macrosperma cv., LH90-54 than the microsperma cv., LH89-48 making it quite clear that macrosperma accessions are more mutable than the microsperma varieties. Appreciation of genetic rebuttal and trait assembly for yield and its components in the lentil population improved by using chemical mutagenesis were revealed by Amin et al. (2015). Results revealed the importance of MMS for enhancing and improving lentil traits through breeding program mutations. Also, concentrated on the fruitful role for the interaction within the medium limits of MMS and DMSO for decreasing biological risks besides increasing highly limits of useful mutants responsible for intension the final output of the bulk for the population feeding on lentils. Morphology of lentil plants characterized with high output than its wild type is changed by the enormous ambit of macro mutations are called high yielding mutant at 0.25% dose of caffeine (Shahwar et al. 2017). Tabti et al. (2018) studied the definition of desirable mutants in quantitative characters in the second mutant generation of lentil and revealed that a lot of induced mutations were observed in M2 generation, for example, chlorophyll mutation with percentage (2.76%) stunted growth (1.14%) and dwarf mutants (0.35%), respectively. Shahwar et al. (2019) studied the induction of phenotypic diversity in mutagenized lentil plants through using heavy metals and detected that effective and fruitful mutations in the second mutant generation lentil populations were obtained at the lowest level of each of heavy metals with highest mutation frequency in cadmium than lead nitrate. Successive exposure of plants for different stresses factors in the environment such as salinity, drought, heat, and high light leading to increased accumulation of reactive oxygen species (ROS) (Carvalho 2008; Suzuki et al. 2012). Subsequently, ROS affected on most plant cell functions by damaging of protein, DNA, and lipids (Kai et al. 2012; Geng et al. 2019). Generally, plant cells use two mechanisms to detoxify ROS, enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR), and dehydroascorbate reductase (DHAR) and non-enzymatic low molecular weight antioxidants such as ascorbate, α-tocopherol, carotenoids, and glutathione (Mittler 2017). After all, it has been presented and clarified with some detail about the results of some scientists in the strategy of genetic improvement of lentil cultivars using different types of mutagens. It can be said that the aim of this investigation can be summarized as follows:

1) Genetic improvement and creation of beneficial mutations in lentil plants using different doses of EMS
2) Shedding light on the optimum and safe dose of this mutagen through evaluating some important morphological traits responsible for the development of lentil cultivars
3) Production of abundant food, healthy, and security in light of the scarcity of food resources needed for the vast majority of people through the use of modern scientific systems such as SDS protein electrophoresis, isozymes electrophoresis besides tradition breeding methods

Materials and methods

Plant materials

This investigation used three Egyptian lentil cultivars namely, Giza 9, Giza 29, and Giza 51. The previous three cultivars were performed from leguminous Crops Research Department, Field Crops Research Institute, Agricultural Research Center (ARC), Giza, Egypt. These varieties were known to be widely famous as genetic material highly tolerant for biotic and abiotic stresses. Therefore, the genetic improvement may give super new variety traits.

Accordingly, the EMS was used in this investigation to cause the process of mutagenesis. Highly positive and significant limit of genetic improvement was the right decision after reviewing a large number of papers included a lot of mutagenesis as it was of great importance, feasibility, and effectiveness in this regard.

Methods

One thousand pure seeds of the three lentil cultivars were placed in a 500-ml flask for each genotype and ultrapure water was added to about 5 cm level above the seeds (~ 100 ml). Seeds were soaked overnight at room temperature for 20 h. Subsequently, the water was decanted and 50 ml of 0.1%, 0.2%, and 0.3% concentrations of EMS (v/v) in water was added. Seeds were incubated for 12 at room temperature followed by decantation of the EMS and rinsing with 100 ml of ultrapure water (5 times, 4 min each) and 200 ml of ultrapure water (4 times, 15 min each). Seeds were then rinsing under

running tap water for 4 h planting in petri dishes and when planting stage started immediately transfer to pots.

Preparing of ethyl methane sulfonate (EMS) stocks

1) The first dose 0.1%: 1 g EMS was dissolved in 1000 ml distilled water.
2) The second dose 0.2%: 2 g EMS was dissolved in 1000 ml distilled water.
3) The third dose 0.3%: 3 g EMS was dissolved in 1000 ml distilled water.

Sowing

After mutagenesis, each genotype was grown under the four treatment conditions, the control besides the three doses of EMS (0.1, 0.2, and 0.3%) in half of November 2017 in pots experiment. Each treatment was replicated 5 times. Each replicate consisted of 10 pots to produce the first mutagenic generation (M1). The seeds of the three lentil varieties for the four treatments were grown in half of November 2018 in pots. Each treatment was replicated 5 times where each replicate consisted of 10 pots to get and produce the second mutagenic generation (M2) in 2018. All germination, morphological, and growth traits were calculated for M_2 generation in 2018.

Studied traits

The following traits in M2 generation were recorded.

1- Germination percentage was calculated by counting only normal seedlings 8 days after planting according to ISTA (1985).
2- Seedling fresh weight (g) was determined by weighting normal seedlings according to Krishnasamy and Seshu (1990).
3- Seedling dry weight (g) was determined using 10 normal seedlings dried in a hot-air oven at 110 °C for 17 h to obtain the seedling dry weight according to Krishnasamy and Seshu (1990).
4- Shoot length
5- Root length
6- Seedling length (cm) was measured as an average of ten normal seedlings 8 days after sowing.
7- Germination energy percentage was calculated according to the method of Ruan et al. (2002).

8 and 9- Seedling vigor indices 1 and 2 were measured according to Krishnasamy and Seshu (1990).

10:- Electrical conductivity

Note: seedling vigor index 1 and seedling vigor index 2 were performed according to the method of Abdul-Baki et al. (1973) and electrical conductivity was conducted according to the method of Thomas (1960) and Matthews and Bradnock (1967) and modified methods by ISTA (2006) and Matthews and Powell (1981), respectively.

All data were statistically analyzed as a factorial experiment design as the technique of analysis of variance (ANOVA) as described by Gomez and Gomez (1984) and SAS program version (1985).

Improvement or positive and negative value percentage of induced mutagenesis (M2 generation) in lentil (*Lens culinaris L.*) seedling is as follows: it was assessment by yield under stress (the value of trait for EMS treatment)—(the value of trait under control)/(the value of trait under control) × 100.

Correlation coefficients: simple phenotypic correlation coefficients among all studied traits for the control and the three EMS treatments were estimated using the formula suggested by Miller et al. (1958).

Molecular and biochemical studies

2,2′-azino-di-(3-ethyl-benzothiazole-6-sulfonic acid) (ABTS), hydrogen peroxide of analytical grade (30% w/v), 1-chloro-2, 4-dinitrobenzene (CDNB), and reduced glutathione (GSH) are products of Sigma-Aldrich Co. The buffers and other chemical reagents used in this study were of laboratory grade.

Protein determination: protein concentration was measured by the Bio-Rad assay using bovine serum albumin as a standard (Bradford 1976). Measurements were done on the shimadzu UV spectrophotometer at 595 nm.

SDS-protein electrophoresis: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to study the protein banding patterns of the three lentil genotypes under the control and treatments. Protein fractionation was performed according to the method of Laemmli (1970) as modified by Studier (1973).

Preparation of crude homogenate: fresh weight sample (1 g) was frozen, then homogenized in 10 ml of 50 mM cold Tris-HCl buffer, pH 7 containing 2 mM β-mercaptoethanol. The homogenates were centrifuged at 10,000 rpm for 20 min and the supernatant was used as a crude material for enzymatic assays.

Enzyme assays

Peroxidase (POX): peroxidase activity was determined according to the method of Childs and Bardsley (1975), with slight modifications using ABTS as a reducing substrate, in a reaction mixture (1 ml) containing 2 mM H_2O_2, 0.36 mM ABTS, and 100 mM sodium phosphate buffer (pH 6) and peroxidase concentration which gave linear response for of 3 min. The change in absorbance at 414 nm was followed at 1 min intervals. One unit of POX activity was defined as the amount of enzyme that oxidized 1 mmol ABTS per minute at 25 °C under the assay conditions.

Glutathione *S*-transferase (GST): the GST activity was determined spectrophotometrically with aromatic substrate (CDNB) by monitoring the change at 340 nm due

to thioether formation at 25 °C as described by Habig et al. (1974). The assay mixture contained in a total volume of 1 ml, 0.1 M potassium phosphate buffer, pH 6.5, and 1 mM (CDNB) in ethanol 1 mM GSH and the enzyme solution. One unit of glutathione *S*-transferase activity is defined as the amount of enzyme which catalyzes the formation of 1.0 μmole of thioether per minute at 25 °C under the assay conditions.

Isozymes electrophoresis

Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted according to Stegemann et al. (1985) to identify isozyme variations between controls and EMS treatments of three lentil genotypes. Two isozymes systems, POX and PPO, were analyzed. After electrophoresis, gels were stained according to their enzyme systems with the appropriate substrate and chemical solutions and then incubated at room temperature in dark for complete staining. For POX activity, 0.125 g of benzidine-dihydrochloride HCl as a substrate, 2 ml glacial acetic acid and was completed with distilled water up to 50 ml. The gel was placed into this solution and 5 drops of hydrogen peroxide were added and incubated at room temperature until bands appeared. The gel was incubated at room temperature until bands appear (Brown 1978). For PPO activity, 15 mg cathecol and 50 mg sulfanilic acid, as substrates, were dissolved in 100 ml of 0.1 M sodium phosphate buffer, pH 6.8. The gel was placed into this solution and incubated at 30 °C for 30 min until bands appeared (Manchenko 2002).

Results

Mean performances

In general, we note that the average data for the third dose of ethyl methane sulfonate (EMS) (0.3) was superior in all traits under study compared to the standard experiment and also outperformed on the rest doses of EMS (0.1 and 0.2) for the three lentil genotypes. This is what we will discuss in detail in this regard (Table 1 and Fig. 1). For germination percentage trait, Giza 51 was recorded highly mean values for the third level of EMS compared to the control followed by Giza 29 and then followed by Giza 9. As well as this dose was the best and ideal level responsible for mutagenesis which gave positive results that reflect the desired genetic improvement process in the three lentil cultivars where this dose of EMS (0.3) was recorded 96.03% for Giza 51 followed by 95.47% for Giza 29 and followed by 93.14% for Giza 9, respectively. Also, the second dose (0.2) followed by the first dose (0.1) of EMS were showed actual superiority compared to the control for the three lentil genotypes for germination percentage, respectively. With respect to seedling fresh weight trait, the third dose of EMS was exhibited the best trend in this regard followed by the second dose and then followed by the first dose for the three genotypes where the third dose (0.3) was (0.680) in Giza 29 followed by (0.675) in Giza 9 and then followed by (0.590) for Giza 51, respectively. Concerning seedling dry weight, the third dose of EMS in Giza 51 (0.037) followed by Giza 9 (0.034) followed by Giza 29 (0.026) was recorded the best trend for genetic improvement in M2 or the second mutant generation of lentil seedling besides the second dose followed by the first dose were also superior in this regard for the three lentil cultivars mentioned above. The other remaining traits namely, shoot length, root length, seedling length, germination energy percentage, seedling vigor index 1, and seedling vigor index 2 were had reached and achieved the same results for the above-described traits where the third dose of mutagen using EMS was the most influential and superior in showing highly size of the genetic change and improvement for the three lentil varieties. The second dose of EMS comes next in terms of excellence in causing genetic change and then the first dose comes in the back terms for the order of excellence compared to the control treatment. For electrical conductivity trait, results were came slightly different where the first dose of EMS (0.1) was recorded (0.22) in Giza 9 and considered the best dose for genetic improvement because it proved that the mutagen had an essential role for reducing the level of electrical conductivity compared to the control experiment followed by the first dose of EMS also in Giza 29 (0.16) and then followed by the third level of EMS in Giza 51 (0.07), respectively. In the end, the mean mutagenesis proved that M2 generation mutagenesis for the three lentil genotypes (Giza 9, Giza 29, and Giza51) were a good example of genetic improvement based on sweeping superiority in all traits under study as well as that the three mutagen doses (0.1, 0.2, and 0.3%) used in this investigation were all superior compared to the control treatment especially the third dose.

Positive and negative percentages of induced mutagenesis in M2 generation

The percentages of positive and negative values of induced mutagenesis M2 in lentil seedling through using various doses of EMS are shown in Table 2. Data confirmed that the third dose (0.3%) of EMS exhibited the highest rank of positive values of induced mutagenesis compared to the control in all traits under study for the three lentil varieties, then the second dose comes in the second rank in some traits and the first dose comes in the last rank in the rest traits and that is will also be listed and clarified in the next context. The third dose was recorded the highest superiority values of positive induced mutagenesis over the two remaining doses compared to the experiment treatment in all traits studied in

Table 1 Average for lentil M2 seedling traits under control and ethyl methane sulfonate (EMS)

Treatments	Traits	Germination %	Seedling Fresh weight (g)	Seedling dry weight (g)	Shoot length (cm)	Root length (cm)	Seedling length (cm)	Germination energy %	Seedling vigor index 1	Seedling vigor index 2	Electrical conductivity
A. Level of trait											
Low vigor		68.73	0.478	0.017	10.39	7.22	12.95	69.87	962.44	1.25	0.07
High vigor		96.03	0.680	0.037	18.24	14.37	25.14	96.0	2341.53	3.55	0.26
***F* test**		**	**	**	**	**	**	**	**	**	**
B. EMS doses											
Giza 9	**Control**	**68.73**	**0.517**	**0.021**	**12.80**	**7.22**	**17.12**	**71.04**	**1176.65**	**1.44**	**0.26**
	Level (I) of EMS	77.33	0.598	0.027	15.32	10.06	19.08	89.59	1475.45	2.08	**0.22**
	Level (II) of EMS	91.50	0.622	0.029	18.03	12.55	24.42	94.26	2234.43	2.65	0.23
	Level (III) of EMS	**93.14**	0.675	0.034	18.24	13.04	25.14	96.0	2341.53	3.16	0.24
	Mean of mutagenesis	87.32	0.631	0.03	17.19	11.88	22.88	93.28	2017.13	2.63	0.23
	***F* test**	**	**	**	**	**	**	**	**	**	**
	LSD at 0.05	1.28	1.74	1.53	1.68	2.06	2.54	1.39	4.19	1.05	0.36
Giza 29	**Control**	**73.82**	**0.534**	**0.017**	**10.39**	**8.14**	**14.32**	**73.41**	**1057.10**	**1.25**	**0.19**
	Level (I) of EMS	84.13	0.611	0.019	12.50	9.54	14.67	90.84	1234.18	1.59	**0.16**
	Level (II) of EMS	92.16	0.645	0.022	14.03	10.05	15.75	91.97	1451.52	2.02	0.20
	Level (III) of EMS	**95.47**	0.680	0.026	14.56	10.16	16.38	93.22	1563.79	2.48	0.18
	Mean of mutagenesis	90.58	0.645	0.022	13.69	9.91	15.60	92.01	1416.49	2.03	0.18
	***F* test**	**	**	**	**	**	**	**	**	**	**
	LSD at 0.05	1.46	1.37	1.44	1.15	1.78	3.08	1.52	3.72	2.54	0.15
Giza 51	**Control**	**74.32**	**0.478**	**0.025**	**13.46**	**10.02**	**12.95**	**69.87**	**962.44**	**1.85**	**0.09**
	Level (I) of EMS	83.51	0.513	0.029	14.89	11.69	14.02	83.50	1170.81	2.42	0.08
	Level (II) of EMS	91.78	0.576	0.034	16.80	13.08	17.47	91.28	1603.39	3.12	0.11
	Level (III) of EMS	**96.03**	0.590	0.037	17.04	14.37	18.03	94.70	1731.42	3.55	**0.07**
	Mean of mutagenesis	90.44	0.559	0.033	16.24	13.04	16.50	89.82	1501.87	3.03	0.086
	***F* test**	**	**	**	**	**	**	**	**	**	**
	LSD at 0.05	1.71	1.54	1.16	2.18	2.11	1.57	1.06	3.15	1.82	0.027

**Highly significant differences at level 1%

Giza 9 where the values were 35.51% for germination, 30.56 g for seedling fresh weight, 61.90 g for seedling dry weight, 42.50 cm for shoot length, 80.60 cm for root length, 46.84 cm for seedling length, 35.13% for germination energy, 98.99 for seedling vigor index 1, and 119.44 for seedling vigor index 2, respectively. In the same context, Giza 29 lentil accession comes in the second rank for exhibiting positive values of induced mutagenesis for the same dose in the traits: germination percentage, fresh weight, seedling dry weight, shoot length, and seedling vigor index 2. While Giza 51 comes in the second rank for the third dose in the traits: root length, seedling length, germination energy percentage, and seedling vigor index 1. With respect to electrical conductivity trait, negative values were considered the ideal and superior values. Because they indicated that decreasing the level of soil salinity where the ideal and optimum values were (– 22.22) in Giza 51 for the third

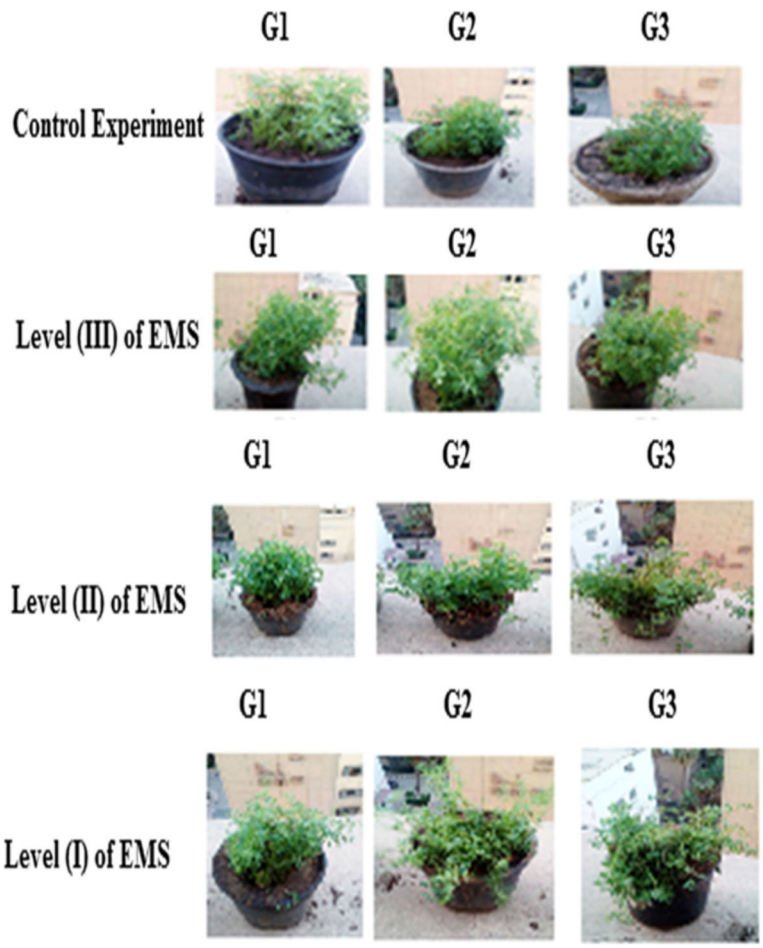

Fig. 1 Impact of the four ethyl methane sulfonate (EMS) (0, 0.1, 0.2, and 0.3) treatments for the three lentil cultivars G1 (Giza 9), G2 (Giza 29), and G3 (Giza 51), respectively

dose of EMS (0.3) followed by the first dose in Giza 29 (– 15.78) then followed by the first dose in Giza 9 (– 15.38) for recording induced mutagenesis in M2 generation, respectively. After all that has been presented and clarified, it can be said that these excellent values, whether were positive in all traits and negative for electrical conductivity trait would be representing the size of the genetic improvement obtained by using this important mutagen (EMS) to bring about this positive change in the M2 generation for the three lentil genotypes.

Correlation coefficients

Results obtained in Table 3 revealed that significant and highly significant positively correlated coefficients were observed among germination percentage trait and the rest traits except electrical conductivity trait where it exhibited significant and highly negative significant correlation coefficients among all traits under investigating for the control experiment. On the same track, the other three treatments of EMS (0.1, 0.2, and 0.3%) showed in Tables 4, 5, and 6 were recorded the same results obtained in the control experiment, respectively.

Molecular and biochemical studies

Protein electrophoretic pattern

Table 7 and Fig. 2 show the electrophoretic pattern of equal concentration (20 μg) for protein extracted from Giza 9 and Giza 29 lentil cultivars under EMS doses (0, 0.1, 0.2, and 0.3). Band with MW about 110 KDa was detected in control and all treatments of Giza 9 and Giza 29 lentil cultivars with a gradual increase in intensity toward higher doses of EMS. A sharp band with about MW of 60 KDa was detected in controls and all treatments except 0.3 EMS dose of Giza 29 lentil cultivar in which the protein intensity slightly decreased.

Table 2 Percentages of positive and negative values for lentil M2 seedling traits under control and ethyl methane sulfonate (EMS)

Treatments	Traits / Doses of EMS	Germination %	Seedling fresh weight (g)	Seedling dry weight (g)	Shoot length (cm)	Root length (cm)	Seedling length (cm)	Germination energy %	Seedling vigor index 1	Seedling vigor index 2	Electrical conductivity
Giza 9	**Level (I) of EMS**	12.51	15.66	28.57	19.68	39.33	11.44	26.11	25.39	44.44	**-15.38**
	Level (II) of EMS	33.12	20.30	38.09	40.85	73.82	42.64	32.68	89.89	84.02	-11.53
	Level (III) of EMS	**35.51**	**30.56**	**61.90**	**42.50**	**80.60**	**46.84**	**35.13**	**98.99**	**119.44**	-7.69
Mean of improvement		27.04	22.17	42.85	34.34	64.58	33.64	31.30	71.42	82.63	-11.53
Giza 29	**Level (I) of EMS**	13.96	14.41	11.76	20.30	17.19	2.44	23.74	16.75	27.20	**-15.78**
	Level (II) of EMS	24.84	20.78	29.41	35.03	23.46	9.98	25.28	37.31	61.60	5.26
	Level (III) of EMS	29.32	27.34	52.94	40.13	24.81	14.38	26.98	47.93	98.40	-5.26
Mean of improvement		22.70	20.84	31.37	31.82	21.82	8.93	25.33	33.99	62.40	-5.26
Giza 51	**Level (I) of EMS**	12.36	7.32	16.0	10.62	16.66	8.26	19.50	21.65	30.81	-11.11
	Level (II) of EMS	23.49	20.50	36.0	24.81	30.53	34.90	30.64	66.59	68.64	22.22
	Level (III) of EMS	29.21	23.43	48.0	26.59	43.41	39.22	35.53	79.89	91.89	**-22.22**
Mean of improvement		21.68	17.08	33.33	20.67	30.20	27.46	28.55	56.04	63.78	-3.70

Furthermore, in Giza 9 lentil cultivar, bands with MWs of 48, 40, and 30 KDa, respectively were detected in the controls and their intensities were increased by increasing EMS dose. While in Giza 29 cultivar, the intensity of these bands was gradually decreased with a complete loss of the band with MW of 40 KDa for 0.2 and 0.3 EMS doses. Table 7 and Fig. 3 show the changes through Giza 51 lentil cultivar under control and treatment with different EMS doses. A new protein band with MW about 110 KDa appeared for 0.2 and 0.3 EMS doses which were not seen in both control or 0.1 EMS dose. Besides, bands having MWs of 60, 48, 40, and 30 KDa were gradually disappeared under treatment with different EMS doses.

Antioxidant enzymes activity

Table 8 shows a gradual increase in POX activity by increasing EMS dose in comparison with untreated genotypes. For GST activity, there is a clear difference between treatments and controls, but it is almost equal for all treatments. A drastic increase (74 folds) was noticed in POX activity of 0.3 DME dose for the Giza 51 lentil cultivar. Therefore, the increased POX activity protects plant against toxic effects of H_2O_2.

Catalase and glutathione reductase cannot be detected under our assay conditions.

Antioxidant isozymes electrophoresis

Peroxidase (POX) isozymes

The electrophoretic patterns of POX isozymes of the treatments differ from their controls (Table 9 and Fig. 4). For Giza 9 lentil cultivar, one band of POX enzyme appeared and its activity increased gradually by increasing the EMS doses. For Giza 29 lentil cultivar, one POX band also appeared and its activity increased in 0.2 and 0.3 doses of EMS. However, four POX isozymes were detected in 0.2 and 0.3 doses of EMS for Giza 51 lentil cultivar in comparison to the control as shown in Fig. 4 and Table 9. This observation may be due to the higher dose of EMS which in turn induces POX to reach their maximum activity for neutralization of the free radicals emitted under stress conditions.

Table 3 Correlation coefficients among M_2 lentil seedling traits under control

Traits	Germination %	Seedling fresh weight (g)	Seedling dry weight (g)	Shoot length (cm)	Root length (cm)	Seedling length (cm)	Germination energy %	Seedling vigor index 1	Seedling vigor index 2	Electrical conductivity
Germination %	**1.0**									
Seedling fresh weight (g)	**0.88****	**1.0**								
Seedling dry weight (g)	**0.79****	**0.74****	**1.0**							
Shoot length (cm)	**0.70****	**0.81****	**0.76****	**1.0**						
Root length (cm)	**0.53***	**0.75****	**0.78****	**0.61****	**1.0**					
Seedling length (cm)	**0.62****	**0.89****	**0.87****	**0.93****	**0.59***	**1.0**				
Germination energy %	**0.71****	**0.85****	**0.92****	**0.80****	**0.74****	**0.90****	**1.0**			
Seedling vigor index 1	**0.80****	**0.73****	**0.61****	**0.77****	**0.96****	**0.86****	**0.57***	**1.0**		
Seedling vigor index 2	**0.93****	**0.69****	**0.97****	**0.87****	**0.89****	**0.64****	**0.86****	**0.77****	**1.0**	
Electrical conductivity	**− 0.58***	**− 0.77****	**− 0.74****	**− 0.85****	**− 0.65****	**− 0.71****	**− 0.66****	**− 0.83****	**− 0.52***	**1.0**

*Highly significant differences at level 5%
**Highly significant differences at level 1%

Table 4 Correlation coefficients among M_2 lentil seedling traits under 0.1% ethyl methane sulfonate (EMS)

Traits	Germination %	Seedling fresh weight (g)	Seedling dry weight (g)	Shoot length (cm)	Root length (cm)	Seedling length (cm)	Germination energy %	Seedling vigor index 1	Seedling vigor index 2	Electrical conductivity
Germination %	**1.0**									
Seedling Fresh weight (g)	**0.75****	**1.0**								
Seedling dry weight (g)	**0.69****	**0.94****	**1.0**							
Shoot length (cm)	**0.72****	**0.87****	**0.96****	**1.0**						
Root length (cm)	**0.78****	**0.84****	**0.89****	**0.70****	**1.0**					
Seedling length (cm)	**0.67****	**0.65****	**0.71****	**0.84****	**0.69****	**1.0**				
Germination energy %	**0.92***	**0.73****	**0.85****	**0.90****	**0.87****	**0.86****	**1.0**			
Seedling vigor index 1	**0.97****	**0.94****	**0.66****	**0.57***	**0.92****	**0.75****	**0.84****	**1.0**		
Seedling vigor index 2	**0.80****	**0.58***	**0.97****	**0.87****	**0.76****	**0.93****	**0.91****	**0.69****	**1.0**	
Electrical conductivity	**− 0.66****	**− 0.74****	**− 0.50****	**− 0.64****	**− 0.83****	**− 0.82 ****	**− 0.81****	**− 0.78****	**− 0.58***	**1.0**

*Highly significant differences at level 5%
**Highly significant differences at level 1%

Table 5 Correlation coefficients among M2 lentil seedling traits under 0.2% ethyl methane sulfonate (EMS)

Traits	Germination %	Seedling fresh weight (g)	Seedling dry weight (g)	Shoot length (cm)	Root length (cm)	Seedling length (cm)	Germination energy %	Seedling vigor index 1	Seedling vigor index 2	Electrical conductivity
Germination %	**1.0**									
Seedling Fresh weight (g)	**0.86****	**1.0**								
Seedling dry weight (g)	**0.76****	**0.83****	**1.0**							
Shoot length (cm)	**0.91****	**0.77****	**0.97****	**1.0**						
Root length (cm)	**0.54***	**0.87****	**0.73****	**0.81****	**1.0**					
Seedling length (cm)	**0.68****	**0.82****	**0.74****	**0.88****	**0.58***	**1.0**				
Germination energy %	**0.60****	**0.57***	**0.76****	**0.75****	**0.93****	**0.91****	**1.0**			
Seedling vigor index 1	**0.92****	**0.72****	**0.53***	**0.86****	**0.73****	**0.79****	**0.82****	**1.0**		
Seedling vigor index 2	**0.83****	**0.69****	**0.96****	**0.92****	**0.90****	**0.55***	**0.88****	**0.70****	**1.0**	
Electrical conductivity	**− 0.64****	**− 0.83****	**− 0.50***	**− 0.50***	**− 0.71****	**− 0.79 ****	**− 0.69****	**− 0.86****	**− 0.66****	**1.0**

*Highly significant differences at level 5%
**Highly significant differences at level 1%

Table 6 Correlation coefficients among M_2 lentil seedling traits under 0.3% ethyl methane sulfonate (EMS)

Traits	Germination %	Seedling fresh weight (g)	Seedling dry weight (g)	Shoot length (cm)	Root length (cm)	Seedling length (cm)	Germination energy %	Seedling vigor index 1	Seedling vigor index 2	Electrical conductivity
Germination %	**1.0**									
Seedling Fresh weight (g)	**0.96****	**1.0**								
Seedling dry weight (g)	**0.87****	**0.71****	**1.0**							
Shoot length (cm)	**0.90****	**0.76****	**0.84****	**1.0**						
Root length (cm)	**0.68****	**0.82****	**0.52***	**0.66****	**1.0**					
Seedling length (cm)	**0.98****	**0.92****	**0.73****	**0.79****	**0.80****	**1.0**				
Germination energy %	**0.72****	**0.80****	**0.96****	**0.78****	**0.60****	**0.82****	**1.0**			
Seedling vigor index 1	**0.59***	**0.63****	**0.75****	**0.81****	**0.78****	**0.56***	**0.93****	**1.0**		
Seedling vigor index 2	**0.86****	**0.77****	**0.55***	**0.69****	**0.82****	**0.70****	**0.79****	**0.83****	**1.0**	
Electrical conductivity	**− 0.64****	**− 0.67****	**− 0.53***	**− 0.70****	**− 0.55***	**− 0.83****	**− 0.51***	**− 0.67****	**− 0.92****	**1.0**

*Highly significant differences at level 5%
**Highly significant differences at level 1%

Table 7 Electrophoretic pattern of protein extracted from G1, G2, and G3 cultivars under control and different EMS treatment

Band no.	MW	G1				G2				G3			
		Control	Dose 1	Dose 2	Dose 3	Control	Dose 1	Dose 2	Dose 3	Control	Dose 1	Dose 2	Dose 3
1	30	+	+	++	+++	+	+	++	++	+	+	−	−
2	40	+	+	+	+	+	+	−	−	++	+	−	−
3	48	+	+	++	++	+	+	++	++	+	−	−	−
4	60	++	++	+++	+++	+++	+++	++	+	+++	++	++	+
5	110	+	+	++	+++	+	+	++	++	−	−	+	+

Polyphenol oxidase (PPO) isozymes

One band only with different intensities can be detected for polyphenol oxidase (PPO) in all lentil genotypes under different EMS doses (Table 10 and Fig. 5). This band appeared in the control and all treatments with different densities and intensities.

Discussion

There is no doubt that this investigation sheds light and intensity on the use of a method of plant breeding and genetic improvement is plant breeding using mutations and in particular the use of a safe mutagen and has a great reputation in this context is ethyl methane sulfonate. This technique is considering very important and fruitful for obtaining beneficial genetic differences and variations to bring about a genetic change in the botanical generation under genetic improvement. Results are shown in Table 1 showed that the three doses of EMS (0.1, 0.2, and 0.3%) were all superior compared to the standard treatment given the flawed measurements of all traits under study for the three lentil cultivars (Giza 9, Giza 29, and Giza 51). Among these doses, the third dose of EMS comes at the forefront in terms of excellence for causing genetic changes that directly reflect on the efficiency and quality of the genetic improvement process in the second mutant generation (M2) for the three lentil varieties followed by the second dose then followed by the first dose in this regard. The M2 mutant generation of the three lentil cultivars were superior in all traits under investigation where Giza 9 was excelling in some traits whereas the other two cultivars (Giza 29 and Giza 51) as superior in the rest traits for the three EMS doses. In the end, this study was able to provide a good vision in reaching excellent genetic variations and changes in the second mutant generation of the three lentil varieties in all tested traits. Accordingly, it can be said that the three doses of EMS were safe, excellent, and important in bringing about this change of the genetic traits in the positive direction and that continuing to cultivate them with the simple selection in segregation

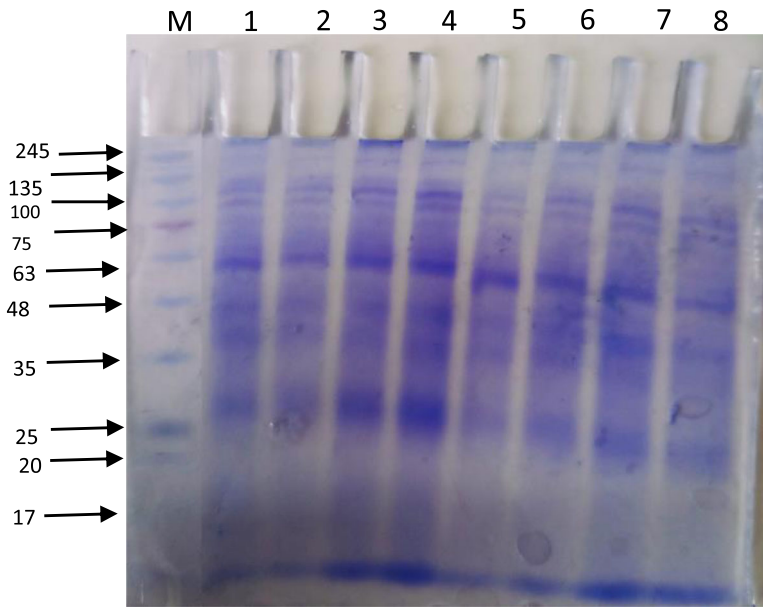

Fig. 2 SDS-PAGE of protein extracted from G1 and G2 cultivars under control and treatment with different EMS doses. M marker, lane 1 = G1 (control), lane 2 = G1 (0.1 EMS), lane 3 = G1 (0.2 EMS), lane 4 = G1 (0.3 EMS), lane 5 = G2 (control), and lanes 6, 7, and 8 for G2 with 0.1, 0.2, and 0.3 EMS dose, respectively

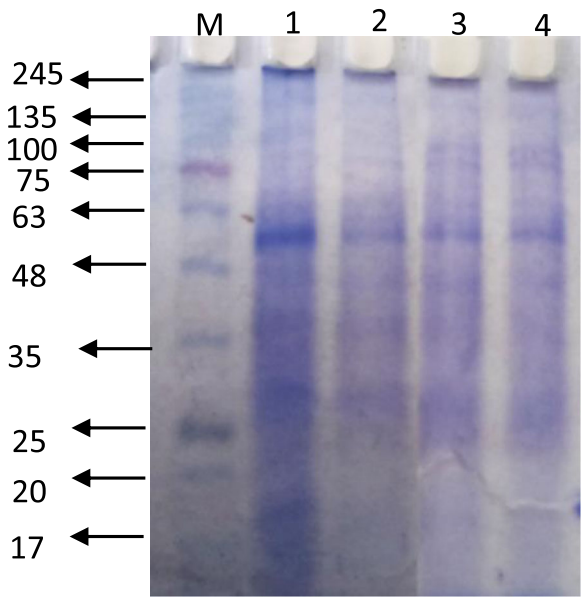

Fig. 3 SDS-PAGE of protein extracted for G3 cultivar under control and treatment with different EMS doses. M marker, lane 1 = G3 (control), lane 2 = G3 (0.1 EMS), lane 3 = G3 (0.2 EMS), lane 4 = G3 (0.3 EMS)

generations will inevitably lead to reaching genetically improved and excellent lentil lines in all traits of germination and growth as well as high yielding. Thus, the process of genetic improvement will be achieved its goals. These results were in agreement with those obtained by Ali and Shaikh (2007), Khursheed et al. (2009), Shah et al. (2011), Gnanamurthy et al. (2011), Singh (2012), Ahirwar et al. (2014), Amin et al. (2015), Rana and Solanki (2015), Heiba et al. (2016), El-Demardash et al. (2017), Kishk et al. (2017), Shahwar et al. (2017), Tabti et al. (2018), and Shahwar et al. (2019).

Table 8 Changes in peroxidase (POX) and glutathione *S*-transferase (GST) activities of three lentil cultivars using three doses of ethyl methane sulfonate (0.1, 0.2, and 0.3%)

Sample	POX specific activity*	GST specific activity*
G1 (control)	0.006 ± 0.001	0.08 ± 0.003
G1 (0.1 EMS)	0.011 ± 0.001	0.17 ± 0.005
G1 (0.2 EMS)	0.017 ± 0.001	0.17 ± 0.010
G1 (0.3 EMS)	0.060 ± 0.002	0.17 ± 0.005
G2 (control)	0.006 ± 0.001	0.10 ± 0.004
G2 (0.1 EMS)	0.011 ± 0.002	0.14 ± 0.007
G2 (0.2 EMS)	0.025 ± 0.002	0.15 ± 0.005
G2 (0.3 EMS)	0.080 ± 0.004	0.15 ± 0.020
G3 (control)	0.005 ± 0.001	0.10 ± 0.004
G3 (0.1 EMS)	0.020 ± 0.003	0.16 ± 0.015
G3 (0.2 EMS)	0.050 ± 0.007	0.17 ± 0
G3 (0.3 EMS)	**0.370 ± 0.030**	**0.24 ± 0.005**

*Specific activity was expressed in unit/mg protein

This study was very successful in detecting safely and influencer doses of ethyl methane sulfonate which had the greatest impact for inducing a significant, important, and positive genetic change in three lentil cultivars reaching to the second mutant generation (M2) and this was done after reviewing, listing, and analyzing data obtained from all germination and growth traits under study (Table 2). The three doses of this mutagen gave excellent results in all studied traits compared to the standard treatment. While the impact of these three doses for induction this genetic and positive change for creating beneficial mutations was not equal where the third dose came in the foreground in terms of excellence for induction this effect on all studied traits with significant form and was remarkably followed by the second dose and finally the first dose came in the last place in this context. Thus, it is possible to recommend the use of these three doses, especially the third dose of EMS (0.3) because it was not only excellent and superior in creating beneficial and positive mutations in addition, recorded mean values higher than the rest doses, but also they represent safe and secure boundaries which do not negatively affect on the physiological and biochemical processes of the stages of growth and germination. These results were in agreement with those reported by Gnanamurthy et al. (2011), Shah et al. (2011), Ahirwar et al. (2014) Rana and Solanki (2015), Heiba et al. (2016), El-Demardash et al. (2017), Kishk et al. (2017), Tabti et al. (2018), and Shahwar et al. (2019).

All the results obtained from the correlation coefficient test for the three treatments of EMS besides the control showed a significant and highly significant positively correlated among all traits under study excluding electrical conductivity trait where it showed the same previous results, but in the opposite direction. This fact confirms the extent of genetic integration between all traits for improvement growth, germination, and vitality traits in the three genotypes of lentil seedlings (Tables 3, 4, 5, and 6). The third dose of EMS (0.3) was the most positive and effective treatment for improving all the previous traits in M2 generation of lentil seedlings followed by the second dose (0.2) and then followed by the first dose (0.1) compared to the control. This indicates the importance of positive correlation among all traits mentioned above in reaching the best level of genetic improvement for lentil traits and also confirms the extent of achieved success resulting from the significant genetic change thanks to the use of the most important mutagenic in this regard EMS solution with the three different doses comes in the forefront. These results were in agreement with those reported by El-Mouhamady (2003), Malik et al. (2007), Tyagi and Khan

Table 9 Effects of different ethyl methane sulfonate (EMS) doses on peroxidase (POX) isozymes of lentil cultivars

Band no.	G1				G2				G3			
	Control	Dose 1	Dose 2	Dose 3	Control	Dose 1	Dose 2	Dose 3	Control	Dose 1	Dose 2	Dose 3
1	–	–	–	–	–	–	–	–	–	–	+	++
2	++	+++	+++	+++	++	+	+++	++	++	++	++++	+++
3	–	–	–	–	–	–	–	–	–	–	+	+
4	–	–	–	–	–	–	–	–	–	–	+	+
Total	1	1	1	1	1	1	1	1	1	1	4	4

(2011), Sarutayophat (2012), Amin et al. (2015), Heiba et al. (2016), El-Demardash et al. (2017), Kishk et al. (2017), Shree et al. (2018), and Vu et al. (2019).

Results obtained in Table 7 and Fig. 2 agree with Singh and Datta (2010) who observed an increase in protein intensity of irradiated wheat seeds accompanied by qualitative changes in their protein profiles. On the same track, results observed in Table 7 and Fig. 2 are also in agreement with Kiong et al. (2008) and Maity et al. (2009) who noted a general decrease in total protein content of soybean seeds under higher doses of radiation and Celik et al. (2014) who found that protein content of irradiated soybean seeds was decreased with increasing radiation time. The presence of some bands in the treatments and their absence from the control results (Table 7 and Fig. 3) could be referring to the activation of some genes related to EMS exposure. This activation in gene expression is due to the few conservative genes found in plants. These results are in agreement with Yang et al. (2006) who found that gene expression pattern is changed upon exposure to high temperature.

Results present in Table 8 are agreed with Ali et al. (2003) who found a higher POX activity in leaves and roots of radish might in order to survive under metals stress. In the present work, higher activity of GST may be attributed to direct binding of glutathione with protein to accommodate EMS effect which is similar to the study of Reddy et al. (2005) on radish under lead stress. Results illustrated in Table 9 and Fig. 4 related to POX isozymes are in agreement with Geng et al. (2019) who study the effect of higher doses of gamma radiation on seeds of sweet osmanthus before germination. On the contrary, band number 2 appeared with different intensities and densities in all treatments as compared to control which shows the varying enzyme status under stress conditions. These results are in opposite to those found by Grouch et al. (1983) and Roy and Mandal (2005) who stated that the decrease in POX activity after roasting may be due to the protein denaturation. It is well known that peroxidase isozymes and protein patterns are used as potent biomarkers for plants under stress conditions (Radotic et al. 2000; El-Beltagi and Mohamed 2010). In

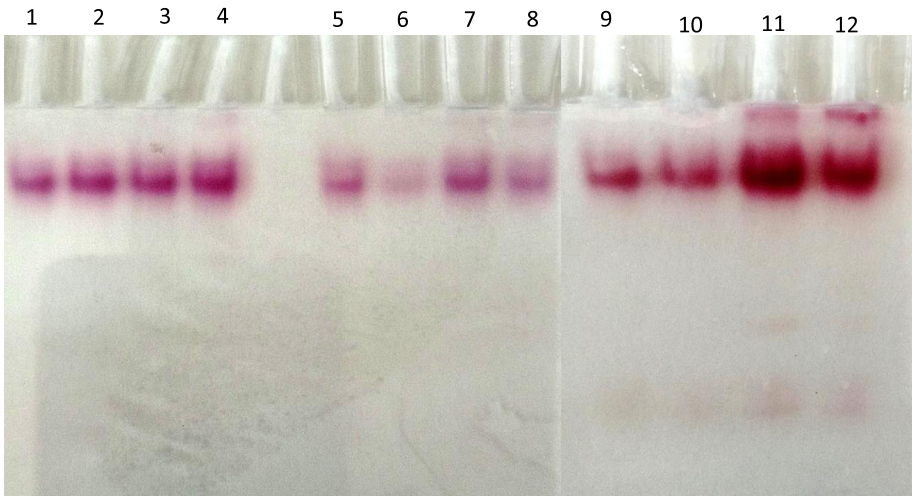

Fig. 4 Electrophoretic patterns of the peroxidase isozymes for the three lentil cultivars (G1, G2, G3) under control and treatment with different EMS doses. Lane 1 = G1 (control), lane 2 = G1 (0.1 EMS), lane 3 = G1 (0.2 EMS), lane 4 = G1 (0.3 EMS), lane 5 = G2 (control), and lanes 6, 7, and 8 = G2 with 0.1, 0.2, and 0.3 EMS, respectively. Lane 9 = G3 (control) and lanes 10, 11, and 12 = G3 with 0.1, 0.2, and 0.3 EMS doses, respectively

Table 10 Effects of different ethyl methane sulfonate (EMS) doses on polyphenol oxidase (PPO) isozymes of lentil cultivars

Band no.	G1				G2				G3			
	Control	Dose 1	Dose 2	Dose 3	Control	Dose 1	Dose 2	Dose 3	Control	Dose 1	Dose 2	Dose 3
1	++	+++	++	+	+	+	++	+++	+	+++	+	++
Total	1	1	1	1	1	1	1	1	1	1	1	1

another study on cucumber plant under salt stress conditions, El-Baz et al. (2003) found that isoperoxidase profile was modified with the induction of new proteins compared to control plant and this behavior may be returned to changes in gene expression under the effect of salt stress (Razzaque et al. 2019).

These differences found in Table 10 and Fig. 5 related to PPO may be due to the induction of new compounds during EMS treatments that regulate the activity of the defense enzymes of lentil cultivars. It was found that mushroom and coffee PPO activity was decreased on roasting treatment (Gautam et al. 1998). El-Beltagi et al. (2010) returned the decrease in PPO activity after roasting to protein denaturation. Overall, the induction or suppression of stress isozymes is related to ROS level with quantitative changes in enzyme levels which observed in intensities and number of isozyme bands during EMS treatment. This observation is in agreement with Nagesh and Devaraj (2008) who reported that isozyme activity decline due to their gradual degradation or structural modification under increased iron toxicity doses.

At the end of the context, it was find that molecular and biochemical marker studies have already succeeded in standing on the biochemical evidence at the molecular level, which had the greatest credit for proving the occurrence of genetic change and improvement required by using this safe mutagen (EMS). Hence, the process of genetic improvement in the second mutant generation of the three lentil varieties has reached its desired goal of producing new, safe, and beneficial lentil mutations in terms of health in this context.

Conclusion

This study succeeded in discussing the vital and effective role of ethyl methane sulfonate (EMS) for creating beneficial mutations in lentil plants. This investigation also focused on clarifying the impact related to the different doses of this mutagen and determining the most superior and safe dose at the same time in causing this required genetic improvement. This study used three locally lentil cultivars (Giza 9, Giza 29, and Giza 51) which were known to have a superior and distinct character in many morphological and physiological traits in addition, its higher yield compared to the rest of the cultivars. Some important traits of growth and germination were chosen to be evaluated under three different doses of EMS besides the control experiment for all treatments. SDS-PAGE electrophoresis and isozymes analysis were also an effective stage and a very important aspect in this research to determine the physiological and biochemical impacts that lead to the creation and demonstration of beneficial positive mutations in M2 generation of lentil. Ultimately, this study recommended and confirmed that the third dose of EMS (0.3) was not only the most superior dose in all studied traits compared to the control, but it was also safe and largely achieved for the largest level of genetic improvement for growth and germination traits in lentil. Then, followed

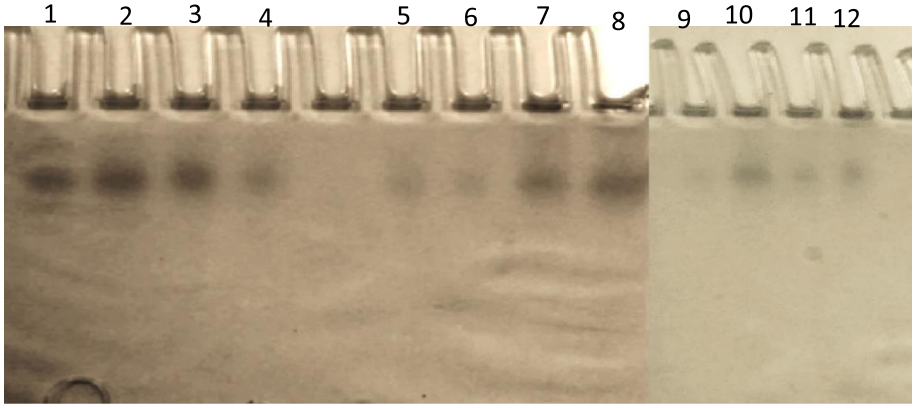

Fig. 5 Electrophoretic patterns of polyphenol oxidase isozymes for three lentil cultivars (G1, G2, G3) under control and treatment with different EMS doses. Lane1 = G1 (control), lane 2 = G1 (0.1 EMS), lane 3 = G1 (0.2 EMS), lane 4 = G1 (0.3 EMS), lane 5 = G2 (control), and lanes 6, 7, and 8 = G2 with 0.1, 0.2, and 0.3 EMS, respectively. Lane 9 = G3/(control) and lanes 10, 11, and 12 = G3 with 0.1, 0.2, and 0.3 EMS doses, respectively

by the second and first dose, respectively which were also superior compared to standard experiment.

Abbreviations

EMS: Ethyl methane sulfonate; *: Significant at 5%; **: Significant at 1%; LSD at 5%: List significant differences at 5%; Level (I) of EMS: First level of ethyl methane sulfonate (0.1%); Level (II) of EMS: Second level of ethyl methane sulfonate (0.2%); Level (III) of EMS: Third level of ethyl methane sulfonate (0.3%); M2 generation: The second mutant generation; G1: Genotype one (Giza 9); G2: Genotype two (Giza 29); G3: Genotype three (Giza 51)

Acknowledgements

Not applicable.

Authors' contributions

ABAE: done the part of plant breeding, statistical analysis, and written this part. AMG: done the part of protein electrophoretic pattern and written this part. GSAA: done the part of antioxidant enzyme activity and written this part. All authors read and approved the final manuscript.

Funding

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 3 March 2020 Accepted: 14 April 2020
Published online: 05 June 2020

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