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EMS-mutagenesis, *In vitro* Selection for Drought (PEG) Tolerance and Molecular Characterization of Mutants in Rice (*Oryza sativa* L.) Employing qRT-PCR and ISSR Markers

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DROUGHT is a major agronomic problem requiring immediate efforts to solve, to detect its effects on crop productivity. Tissue culture and mutagenic potentiality of EMS (ethyl methane sulfonate) were used to develop rice-promising drought-tolerant lines. Mature embryos of two genotypes (Sakha 101 and Giza 177) of rice were used for developing callus. Three callus induction strategies were investigated by applying EMS as a mutagen to evaluate and determine the best procedure for selection of drought-tolerant rice cell lines. For screening of calli response to PEG (polyethylene glycol), two-months-old well-proliferated treated calli of three protocols were sub-cultured for two weeks with 5% and 7% PEG-6000. Molecular response to PEG was evaluated by detecting gene expression of rice dehydrin, phytoglobin 1, 2, and 5 genes via qRT-PCR (quantitative real time-polymerase chain reaction). The relative gene expression of all genes was significantly increased in the PEG-treated calli compared with the control in both genotypes and Giza 177 showed higher expression levels than Sakha 101. Genetic variations were assessed among three putative mutants arising *in vitro*, their mother plants, and a drought-tolerant genotype (IET 1444) using ISSR fingerprinting. Similarity coefficients reflected the genetic relationship between rice regenerants and their mother plants. Cluster analysis showed regenerated Sakha 101 mutant line and IET 1444 were grouped together at a dissimilarity distance of 1.00. *In vitro* screening of EMS mutants with the creation of chemical drought using PEG-6000 to assess tolerance could be a good track to developing drought-tolerant rice lines.

Keywords: Drought stress, Ethyl methane sulfonate, ISSR fingerprinting, *Oryza sativa*, qRT-PCR, Somaclonal variation.

Introduction

Rice (*Oryza sativa L.*) is one of the world's most crucial food security crops. It is essential to the human diet and nourishes more than half of the world's population. Stresses resulting from physiological and environmental factors seriously reduce its yield and quality. Drought is one of the major abiotic stresses in the past decades. It is still the major limited factor of rice production worldwide and will still be in the possible future (Lobell & Tebaldi, 2014; Lesk et al., 2016). Due to the increasing water supply constraints, it became a goal in Egypt and many other places to produce rice with the least amount of irrigation (Elshafei et al., 2019). The production of rice strains resistant to drought has been quite low so far, making it impossible to improve rice production on a national scale. Several efforts have been made to obtain improved cultivars with high yield and drought tolerance. Several mutant collections have been established in rice with various genetic backgrounds (Wang et al., 2013; Li et al., 2017). Water scarcity in Egypt has hampered horizontal growth of farmed land. Employing traditional breeding methods to boost rice yield has become insufficient since Egyptian rice cultivars have reached a plateau and further improvements are

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impossible to obtain. As a result, employing novel technology like a tissue culture technique alone or in combination with mutational breeding would be more effective.

The biotechnological tools have been developed to address the critical problems to improve crops for a sustainable agriculture. Amongst these tools, the in-vitro culture alone or combined with mutagenesis, induced with physical, chemical or biological agents, by using (PEG) as a drought stimulating agent can be exploited to increase genetic variability and mutants, as a potential source of new commercial lines. There are more studies on finding the varieties of rice tolerant to drought stress using biotechnology to reduce the danger of abiotic stress, especially drought stress on agriculture and rice production (Kadhimi et al., 2014). The makeup of culture media and subculture cycles frequently causes somaclonal variation. Somaclonal variation is the term for the genetic and phenotypic variation that caused by tissue culture among regenerated plants (Bairu et al., 2011). Assuming that there is a correlation between cellular and in vitro plant responses, in vitro culture has been employed to produce drought-tolerant plants. It is crucial to note that tissue culture allows for the fast cloning of selected variants and handles large populations, increasing the effectiveness of mutagenic treatments (Predieri, 2001).

A mutation is defined as any alteration to an organism's DNA that is not the result of normal recombination and segregation (Phillip & Rines, 2009). In addition to conventional plant breeding methods, direct utilization of mutation is a very valuable strategy. The key benefit of this method is that it takes less time to breed a crop with better characters than the hybridization approach does to get the same results. There are three ways to induce mutations: biologically through the use of transposons and T-DNA, physically through the use of fast neutron, UV, and x-ray radiation, or chemically through the use of N-methyl-N-nitrosourea (MNU), 1,2,3,4-diepoxybutane (DEB), or ethyl methane sulfonate (EMS). Among these chemicals, EMS has emerged as one of the most efficient, dependable, potent, and widely applied chemical mutagens in plants (Rime et al., 2019).

It is now possible to examine the expression of rice genes in specific subsets or globally (Jain et al., 2006) thanks to the entire sequencing of the rice genome. Drought resistance is a quantitative trait, implying several genes, involved in activation of a complicated regulatory mechanism for the enhancement of drought resistance (Cramer et al., 2011). These genes are known as Candidate genes, for example *OsNAC5* (Zhou et al., 2013), and Dehydrin Gene, *OsDhn1* (Kumar et al., 2014). phytoglobins genes are also involved in drought stress mitigation in rice (Shankar et al., 2018).

Many publications have successfully used the ISSR technique to confirm genetic diversity and discover somaclonal variations of many plant species, including tea (Devarumath et al., 2002), date palm (Ahmed et al., 2012), *Picorriza kurroa* (Rawat et al., 2013), *Artemisia absinthium* (Kour et al., 2014), sugarcane (Thorat et al., 2017; Shingote et al., 2019) and *Vicia* (El-Badan et al., 2022). ISSR has also been used to detect polymorphism produced by EMS in tissue culture grown plants (Perera et al., 2015). The present investigation is conducted to use tissue culture techniques and mutagenic potentiality of EMS to develop rice promising drought-tolerant lines, depending upon mutagenesis and somaclonal variation, assess the role of plant hemoglobin, dehydrin and other genes as candidate genes for drought tolerance and detect the genetic variation between mutant lines and their corresponding parents applying molecular markers.

Materials and Methods

Determination of optimal EMS concentration and mutagenic treatment

Three rice genotypes; IET 1444 (drought tolerant), and Giza 177 and Sakha 101 (drought sensitive) were obtained as grains from the Rice Research and Training Center, Sakha Research Station, Kafrelsheikh Governorate, Agriculture Research Center, Egypt. Healthy and mature dehulled rice grains of the two sensitive genotypes (Giza 177 and Sakha 101) were soaked in water overnight at room temperature for 24h. The soaked grains were treated with 0 % as control, 0.5%, 1% and 1.5% (v/v) aqueous EMS for 6h at room temperature (25°C). Finally, the treated grains were rinsed in distilled water several times to remove the residual EMS. Then grains were sterilized using 0.1% freshly prepared mercuric chloride ($HgCl_2$) for 20min. Sterilized grains were rinsed with sterile distilled water several times to remove all remaining sterilizing agents and finally air-dried on sterile filter paper for 3min sterilized laminar air flow cabinet.

Rice grains were inoculated on MS (Murashige and Skoog) medium supplemented with 2, 4-D and Kinetin with concentration of 2 mg/l and 0.5 mg/l respectively. Cultures were incubated in growth chamber at 25 ± 2ºC in the dark for four weeks for callus induction. The germination percentage, callus fresh weight and the callus induction rate (CIP) were quantified to determine the lethal dose 50%. CIP was determined according to formula (Arzani & Mirodjagh, 1999); CIP= (number of explants formed calli/ number of cultured explants) × 100

Mutagenesis of rice calli using EMS and in vitro selection of drought-tolerant lines

As a control experiment, sterilized grains were cultured on MS callus induction medium. Cultures were incubated in growth chamber at 25±2ºC in the dark for four weeks, then the roots and the residual of the grain were removed from calli. The induced calli were transferred to fresh media of the same composition for proliferation one month again. Three *in vitro* callus induction strategies were investigated applying EMS as a mutagen to evaluate and determine the best procedure for selection of drought tolerant rice cell lines. These procedures could be summarized in the following protocols and Fig. 1 , Protocol 1: Two months-old well proliferated embryogenic calli of both Giza 177 and Sakha 101 were cut into small pieces and treated with 0.4 % sterile aqueous EMS (made in sterilized distilled water and membrane filtered) for 18h according to Chen et al. (2013).

Protocol 2; The mature dehusked rice grains of both genotypes were treated with 1 % (v/ v) aqueous EMS , inoculated on callus induction medium and cultures were incubated in growth chamber at 25± 2ºC in the dark for four weeks. Protocol 3; The Mature dehusked rice grains of two genotypes were treated with 1% (v/v) aqueous EMS. The treated grains were inoculated on MS callus induction medium supplemented with 5% and 7% PEG for four weeks. The roots and the residual of the grain were removed from calli of protocol 2 and 3 and they were transferred to MS medium of the same composition for proliferation one month again.

For evaluation of calli response to PEG, the two months-old well proliferated treated calli of three strategies were transferred onto callus induction medium supplemented with 5% and 7% PEG for two weeks. The mean fresh weight of callus was recorded before and after proliferation, the callus induction rate (CIP) was calculated after one month according to formula (Arzani & Mirodjagh, 1999): CIP= (number of explants formed calli/ number of cultured explants) × 100. Growth Index (GI) of callus tissue or increasing value of callus fresh weight was calculated after PEG treatment according to Sahraroo et al. (2014): GI= (W1 -W0)/ W0, where W0 was the weight of callus tissue before treatment and W1 the final weight of callus after culture period of PEG treatment.

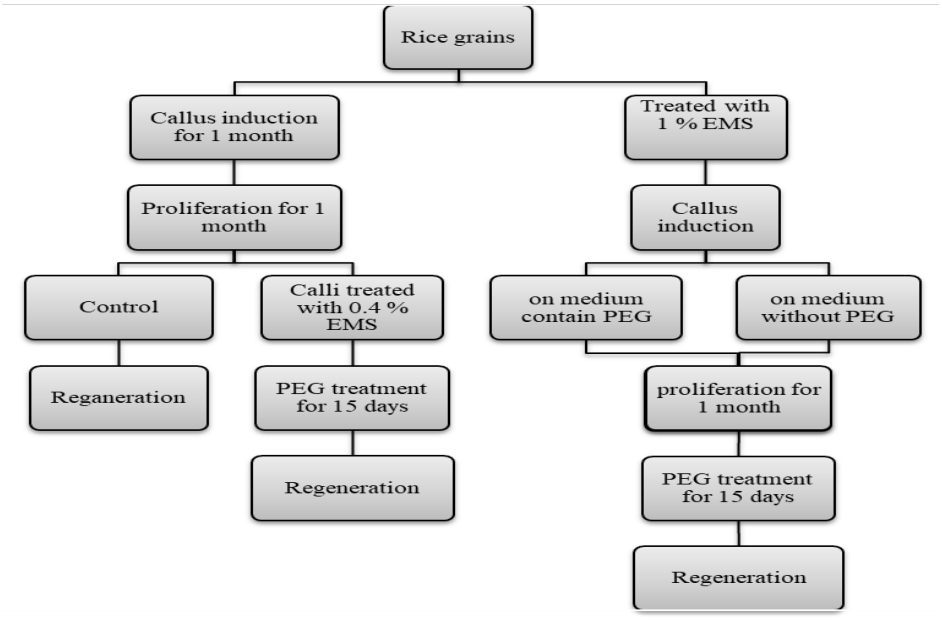

Fig. 1. Flowchart summarized three protocols for in vitro callus induction investigation applying EMS as a mutagen to evaluate and determine the best one for selection of drought tolerant rice cell lines

Regeneration

All treated and controlled calli were transferred to MS medium supplemented with 2mg/L Kinetin and 0.5mg/L NAA) for regeneration. Cultures were incubated in growth chamber at 25 ± 2ºC in continuous light tell the regenerated plantlets appear with transferring to fresh medium monthly. The results of each treatment were obtained from at least three independent experiments. The experiments were completely randomized designed. Each one contained three replicates and each replicate consisted of a jar cultured with five explants.

Quantitative RT-PCR

To evaluate molecular response to PEG, rice dehydrin, hemoglobin 1, 2 and 5 gene expressions were detected by qRT-PCR. Total RNA was extracted from rice calli after two weeks of 7% PEG treatment using Trizol reagent (Life Technologies Invitrogen, USA). 1µg of total RNA was reverse-transcribed into single-stranded complementary DNA by using QuantiTects Reverse Transcription Kit (Qiagen, USA). Relative gene expression of target genes was measured using the rice actin RAc1 as a house-keeping gene for the normalization of expression of target genes, (RAc1-forward, 5'-CATGCTATCCCTCGTCTCGACCT-3'; RAc1-reverse, 5'-CGCACTTCATGATGGAGTTGTAT-3') (McElroy et al., 1990). C-DNA amplicons were amplified via Thermo Scientific Maximas SYBR® Green/Fluorescein qPCR Master Mix (2X) through specific primers (Table 1) with thermal cycling conditions of 95ºC for 5min for initial denaturation, followed by 40 cycles of denaturation for 1min at 95ºC , annealing for (30-60) sec at (55-65)ºC according to primer used (Table 1), an extension of 1min at 72ºC , and final extension at 72ºC for 7min. The relative expression levels of target gene were normalized to the expression level of reference gene using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Real-time PCR analyses involved three biological replicates of amplification for each sample and for each biological replicate, two technical replicates were analyzed.

DNA extraction and PCR amplification

To determine any molecular variations among rice regenerants from the mentioned protocols as compared to their mother plants. The total genomic DNA was isolated from 100mg of fresh leaves of regenerated rice seedlings and mother plants by methods of Clarke (2009). The DNA was quantified using a nano-drop ND-100 P330 spectrophotometer (IMPLN) Germany. The 260/280 absorption ratio ranged from 1.7 to 1.8, which indicates DNA purity. For the genetic characterization of the studied varieties, thirteen ISSR primers were used (Table 2). The PCR amplification was performed in a final volume of 20µL reaction volume containing 10µL of Dream Taq ® Green PCR Master Mix (Thermo Fisher Scientific, UK), 2µL of 50ng/µL genomic DNA template, 1µL of ISSR primer, and 7µL of free nuclease water. The amplification was programmed for conditions with an initial denaturation at 94°C for 5min, 35 cycles of denaturation for 30sec at 94°C, annealing for 45sec at 42°C, extension for 45sec at 72°C, and 10min extension at 72°C. DNA amplification was performed using a primus 25 advanced ® cycler machine.

TABLE 1. primer sequence of Dehydrin (OsDhn1) and Phytoglobin (OsPgb) 1, 2 and 5 genes

Target gene	Sequence	Annealing temperature	References
Dehydrin (OsDhn1)	5'-AGCTCAAACAAGTCAAGAGC-3' 5'-AAGCACCAAACTAACACACG-3'	56°C for 1min	Kumar et al. (2014)
Phytoglobin 1 (OsPgb 1)	5'-TAAACCAGCTGTCAGGAAGCA-3' 5'-AGCAGCT-AGCATGCCTGTCGA-3'	65°C for 30sec	Arredondo-Peter et al. (1997)
Phytoglobin 2 (OsPgb 2)	5'-AGGAATCAAATCGAAGCAGCC3' 5'-GGAGGTGGAGCAGTATATATA-3	60°C for 30sec	Arredondo-Peter et al. (1997)
Phytoglobin 5 (OsPgb 5)	5' -ATGGGGTTCAGCGAGACGC-3' 5' -TTAGGCAGCCTTCTTCAT-3'	55°C for 30sec	Garrocho-Villegas et al. (2008)

TABLE 2. list of ISSR primers codes and sequence 5'-3'

ISSR primer code	Sequence 5'-3'
UBC 812	GAGAGAGAGAGAGAGAA
UBC 814	CTCTCTCTCTCTCTCTA
UBC 818	CACACACACACACACAG
UBC 836	AGAGAGAGAGAGAGAGYA
UBC 840	GAGAGAGAGAGAGAGAYT
UBC 842	GAGAGAGAGAGAGAGAYG
UBC 843	CTCTCTCTCTCTCTCTRA
UBC 848	CACACACACACACACARG
UBC 852	TCTCTCTCTCTCTCTCRA
UBC 857	ACACACACACACACACYG
HB9	GTGTGTGTGTGTGG
HB12	CACCACCACGC
HB14	CTCCTCCTCGC

Following the completion of polymerase chain reaction (PCR), a 10µL sample of the PCR-amplified products was electrophoresed using 1.2% agarose gel containing 0.05µg/mL ethidium bromide in a Cleaver multiSUB® electrophoresis system in Tris–borate ethylenediaminetetraacetic acid buffer, using a constant voltage of 100V for 1h. Visualization of the gels was carried out under UV light, and images were captured using a high-quality gel documentation system (WiseDoc®, WGD-30, DATHAN Scientific, Co., Ltd.). Each reaction was conducted at least twice, and only reliable products were considered. To determine the molecular weight of the amplified DNA fragments, Thermo Scientific Gene-Ruler DNA ladders of 100bp in length (100ng/µL) were utilized as molecular size markers.

Data analysis

The data were analyzed by one-way or two ways analysis of variance (ANOVA) using IBM/SPSS version 20 Statistical Analysis Software. Significant differences among genotypes were calculated by Duncan's multiple range tests. A significant level of $P< 0.05$ was used for all statistical procedures. Results are shown as mean ± SD. The genetic relationships among examined cell lines and their mother plants were estimated based on ISSR data. Bands were scored in binary matrices, as 1 for presence and 0 for absence in the profile of each population. Dendrograms were constructed from the similarity matrix data of ISSR cluster analyses by the use of the unweighted pair group method with arithmetic average (UPGMA) based on Euclidean distance (Sokal & Mickener, 1958). The data were analyzed by the NTSYS-pc software version 2.02 (Rohlf, 2002).

Results

Sensitivity assay of EMS for determination of lethal dose 50

Determination of the right dose of EMS is critical for mutagenic treatment. Hence, based on the median lethal dose, the optimal EMS dose concentration was determined for further processing of rice calli for mutagenic treatment. For this purpose, Sakha 101 and Giza 177 grains were subjected to different EMS concentrations, (Fig. 2). It was observed that influence of different EMS treatments on the rice calli revealed that each of callus induction percentage, germination percentage and callus fresh weight decreased significantly as the EMS concentration increase.

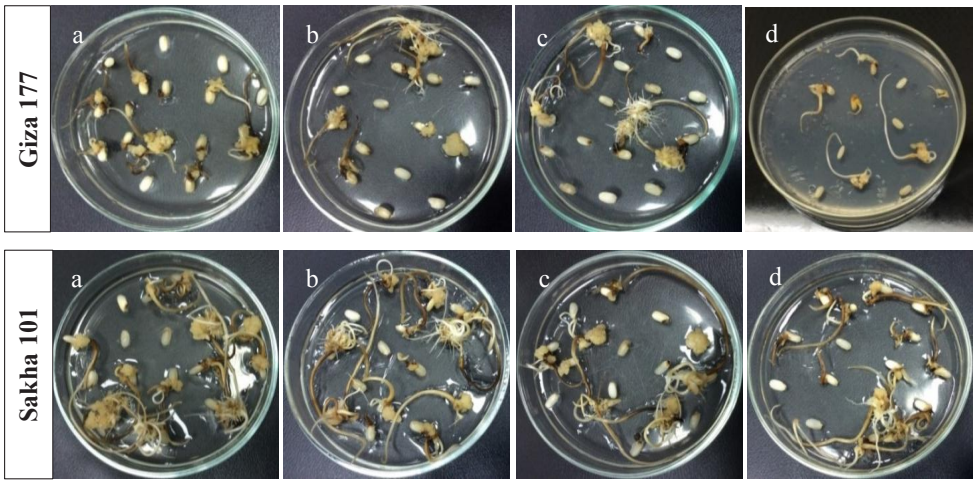

Fig. 2. The response of Giza 177 and Sakha 101 genotypes to different concentrations of EMS, a (0%), b (0.5%), c (1%) and d (1.5%) for determination lethal dose 50

The percentage of callus induction in Sakha 101 ranged from 41.91 % to 71.25 % but in Giza 177 ranged from 30.72 % to 67.08 % as shown in Table 3. The highest value was recorded in control where the lowest value was recorded in EMS dose 1.5%. In Sakha 101, the EMS doses 0.5% and 1% approximately record the same percentage of callus induction. Callus fresh weight in Sakha 101 ranged from 0.264g (EMS dose 1.5%) to 1.190g (control), while in Giza 177 ranged from 0.221g (EMS dose 1.5 %) to 0.743g (control) as shown in Table 3. The germination percentage ranged from 25.81% to 86.00% and from 43.64% to 77.25% in Sakha 101 and Giza 177 respectively (Table 3). The highest percentage was recorded in the control treatment and the lowest was recorded in EMS dose 1.5%. Moreover, the ability of Sakha 101 to withstand EMS hazard was found to be stronger than Giza 177, as shown in Fig. 3 and Table 3. Based on the median lethal dose, the selection criteria determined for mutagenic treatment of Sakha 101 and Giza 177 calli was 1% concentration of EMS solution.

Effect of various concentrations of EMS and PEG-6000 stimulated stress on calli differentiation

Callus induction from mature embryos was assessed. Currently, three protocols were investigated applying EMS as a mutagen to evaluate and determine the best procedure for selection of drought tolerant rice cell lines. For evaluation of calli response to PEG, two months-old well proliferated treated calli of three strategies were transferred onto callus induction medium supplemented with 5% and 7% PEG for two weeks Fig. 4 and Fig. 5 (a, b).

TABLE 3. The effect of EMS treatments on callus induction percentage, germination percentage and callus fresh weight

EMS conc. (%)	Callus induction %		Germination %		Callus fresh weight (g)	
	Sakha 101	Giza 177	Sakha 101	Giza 177	Sakha 101	Giza 177
Control	71.25±5.59^{c}	67.08±2.72^{d}	86.00±5.04^{d}	77.25±3.11^{d}	1.190±0.110^{d}	0.743±0.088^{c}
0.5%	56.86±5.06^{b}	60.25±2.56^{c}	71.33±4.31^{c}	65.40±2.25^{c}	0.839±0.099^{c}	0.614±0.029^{b}
1.0%	55.52±3.89^{b}	53.07±2.89^{b}	58.29±7.08^{b}	56.92±5.56^{b}	0.640±0.086^{b}	0.559±0.046^{b}
1.5%	41.91±4.25^{a}	30.72±5.06^{a}	25.81±7.41^{a}	43.64±7.61^{a}	0.264±0.031^{a}	0.221±0.039^{a}
F value	31.95***	104.02***	88.31***	38.74***	98.709***	81.548***

*: P ≤ 0.05, **: P ≤ 0.01, ***: P ≤ 0.001.

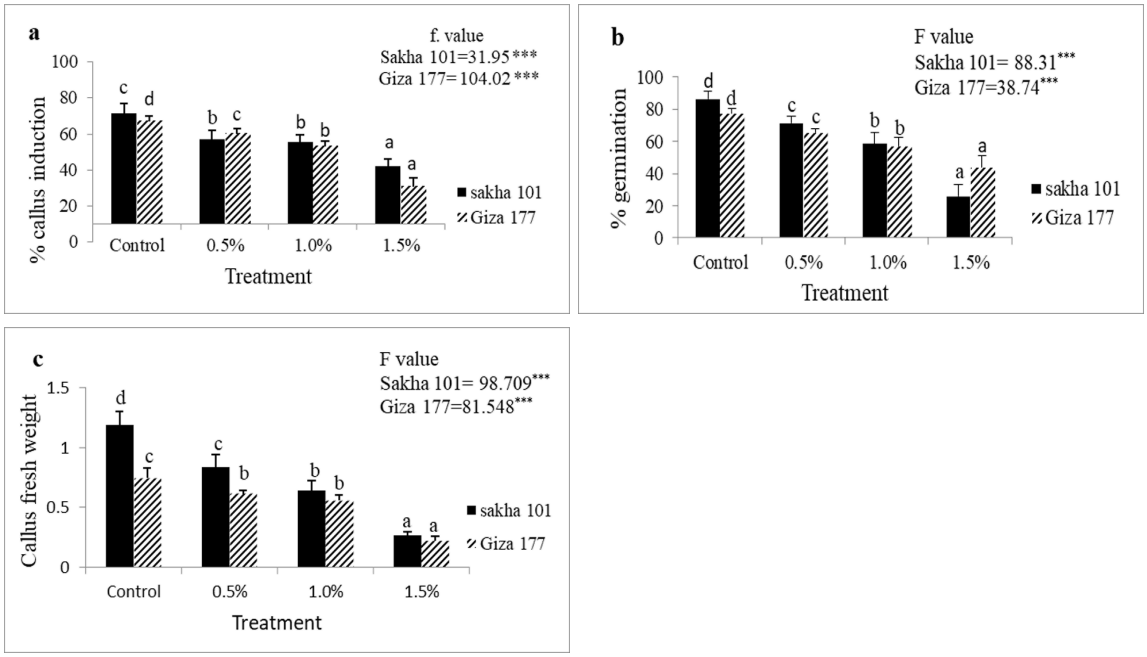

Fig. 3. Histogram showing the effect of different concentrations of EMS on % callus induction (a), % germination (b) and callus fresh weight (c)

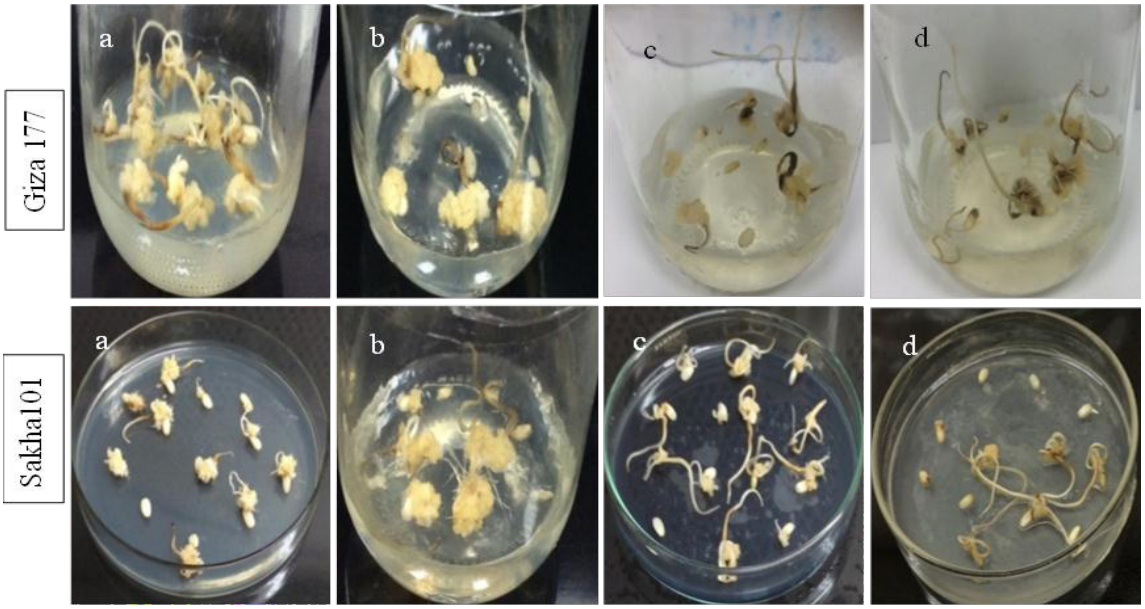

Fig. 4. callus induction of rice grains (Giza 177 and Sakha 101) treated with EMS different protocols, control/ protocol 1 (a), protocol 2 (b) and protocol 3 (c,d), 5 % and 7 % PEG respectively

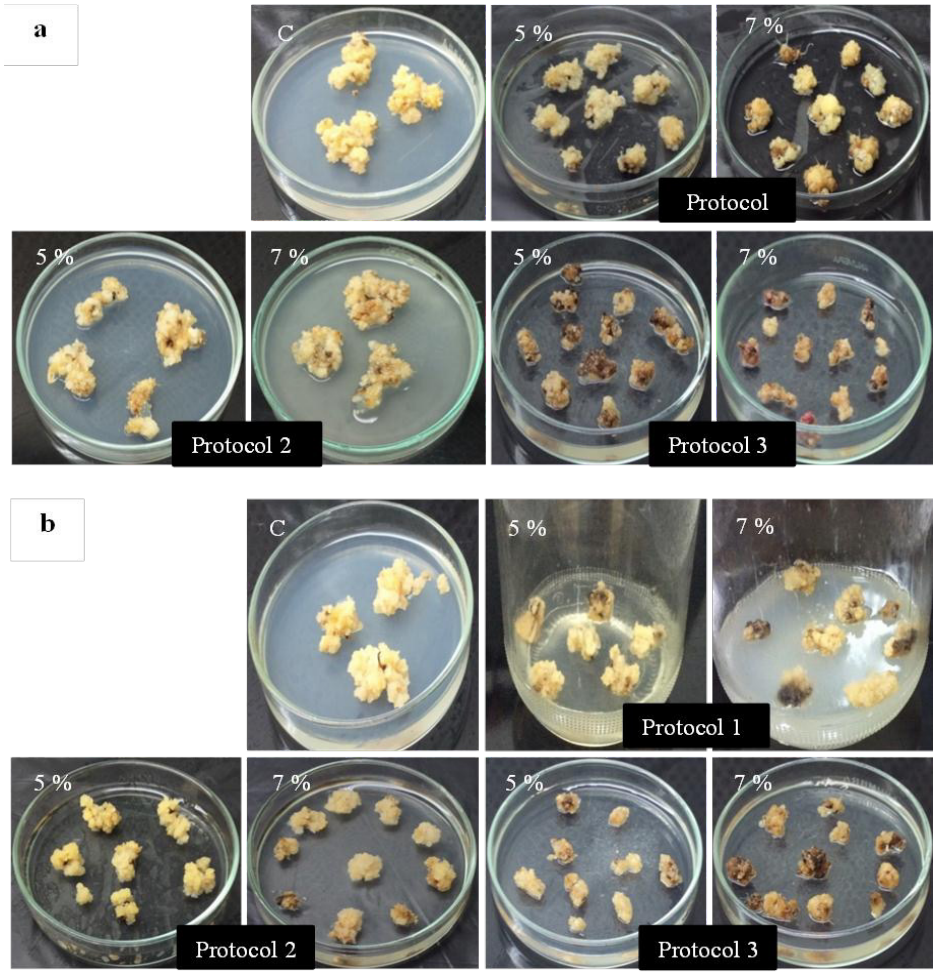

Fig. 5. The response of Giza 177 (a) and Sakha 101(b) calli of different protocols to PEG treatment for 15 days

All calli showed normal growth during the first week after initiating treatment. After 2 weeks, browning areas formed on all calli, including the control. Callus induction % (CI) recorded values of 88.7% and 86.1% for Sakha 101 and Giza 177 respectively in control. It consistently declined to 82.8 and 63.8 for Sakha 101 and Giza 177 respectively in Protocol 2. Protocol 3 (5% and 7% PEG treatment) showed 78.9 and 44.4 respectively for Sakha 101 while 61.8 and 56.1 respectively for Giza 177. Based on data in Table 4 and Fig. 6 (a), it was shown that protocol 2 is best procedure for selection of drought tolerant rice cell lines as it showed an increase in studied parameters compared to other protocols. A significant difference in callus fresh weight (FW) was found among different treatments (Table 4, Fig. 6 b). Callus fresh weight of Sakha 101 recorded values of 1.77, 0.72, 0.54 and 0.22g in control, protocol 2 and protocol 3 with 5% and 7% PEG treatment respectively, while the same parameter in Giza 177 recorded values of 1.07, 0.61, 0.46 and 0.38g in control, protocol 2 and protocol 3 with 5% and 7% PEG treatment respectively.

Growth index was significantly differed among different EMS and PEG treatments Table 4, Fig. 6 (c). This parameter recorded the highest value in control treatment, 1.24 in Sakha 101 and 1.81 in Giza 177. In Protocol1, Sakha 101 and Giza 177 were reduced to 0.38 and 0.82 respectively in 5% PEG treatment and were 0.192 and 0.484 in 7% PEG treatment. On the other hand, protocol 2 showed an increase in growth index for Sakha 101 and Giza 177 recording 0.84 and 1.20 respectively in 5% PEG treatment and 0.684 and 0.718 respectively in 7% PEG treatment. In protocol 3, Sakha 101 and Giza 177 recorded 0.30 and 0.16 respectively in 5 % PEG treatment and 0.23 and 0.12 respectively in 7% PEG treatment.

TABLE 4. Calli parameters; callus induction % (CI %), fresh weight (FW), and growth index (GI) of Sakha 101 and Giza 177 at different EMS protocols and PEG treatments

Calli parameters	EMS protocols	PEG conc. (%)	Sakha 101	Giza 177
CI %	Control/ protocol 1	0	88.73 ± 9.08	86.06 ± 9.571
	Protocol 2	0	82.79 ± 12.39	63.76 ± 12.66
	Protocol 3	5%	78.88 ± 14.52	61.83 ± 16.67
		7%	44.38 ± 14.97	56.07 ± 15.05
F value	EMS		35.8^{***}	26.62^{***}
	PEG		70.99^{***}	1.76^{NS}
FW (g)	Control/ protocol 1	0	1.77 ± 0.40	1.068±0.295
	Protocol 2	0	0.72 ±0.14	0.608±0.158
	Protocol 3	5%	0.55 ± 0.16	0.462±0.106
		7%	0.22 ± 0.05	0.376±0.121
F value	EMS		245.95^{***}	81.5^{***}
	PEG		20.43^{***}	20.43^{NS}
GI	Control	0	1.24 ± 0.47	1.81 ± 0.95
	Protocol 1	5%	0.38 ± 0.15	0.82 ± 0.57
		7%	0.19 ±0.08	0.48 ± 0.51
	Protocol 2	5%	0.84 ± 0.47	1.2 ± 0.91
		7%	0.68 ± 0.44	0.72 ± 0.49
	Protocol 3	5%	0.3 ± 0.23	0.16 ± 0.09
		7%	0.23 ± 0.18	0.12 ± 0.04
F value	EMS		52.88^{***}	36.33^{***}
	PEG		5.28^{*}	6.8^{**}

*: P ≤ 0.05, **: P ≤ 0.01, ***: P ≤ 0.001, ^{NS}: non-significant.

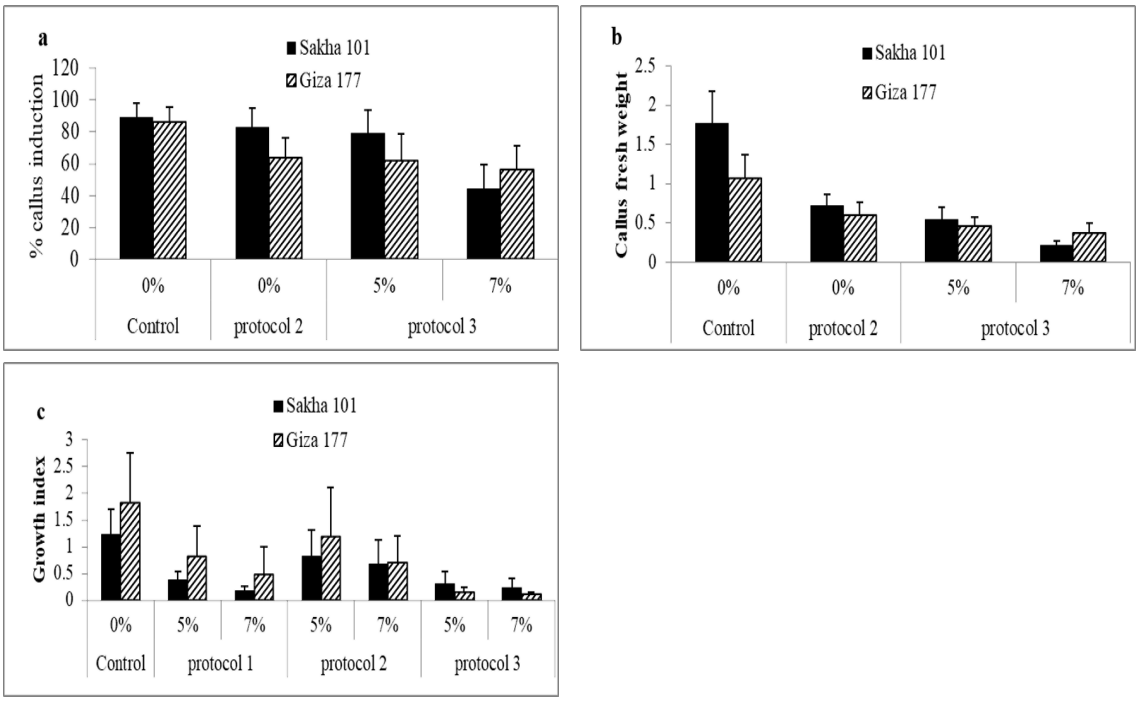

Fig. 6. callus induction % (a), fresh weight (b), and growth index (c) of Sakha 101 and Giza 177 at different PEG and EMS treatments

The expression of osmotic stress tolerance related genes in rice calli

Presently the expression of four genes as markers of processes related to water stress was studied. These comprised dehydrin, phytoglobin 1, 2, and 5 in the mutagenic calli treated with PEG. Expression of these genes was measured using quantitative real time PCR after two weeks of PEG-6000 treatment (7%) compared with the control (0% PEG). For Sakha 101 and Giza 177 different protocols, the relative gene expression (fold changes) of dehydrin, phytoglobin 1, 2, and 5 genes was significantly increased in the PEG treated calli compared with the control. Giza 177 showed higher expression levels of the four genes than Sakha 101 in protocol 1 and 2 (Fig. 7).

Protocol 2 showed that dehydrin, phytoglobin 2 and 5 were upregulated ($2^{-\Delta\Delta Ct}$ >1) in both Sakha 101 and Giza 177 but phytoglobin 1 was upregulated only in Giza 177 (Fig. 7). The results of qRT-PCR for expression levels of dehydrin, hemoglobin 1, 2 and 5 genes showed an increase by 1.7-fold, 0.97-fold, 1.13-fold and 1.1-fold respectively for Sakha 101, and by 2.13-fold, 1.33-fold, 1.27-fold, and 1.53-fold respectively for Giza 177 (Table 5). However, protocol 1 showed downregulation ($2^{-\Delta\Delta Ct}$ <1) for all examined genes in both Sakha 101 and Giza 177 except for dehydrin which was upregulated in Giza 177 only (Fig. 7). The transcripts levels of dehydrin, phytoglobin 1, 2 and 5 were increased by 0.67-fold, 0.73-fold, 0.53-fold and 0.63-fold respectively for Sakha 101 and increased by 1.53-fold, 0.73-fold, 0.7-fold and 0.93-fold respectively for Giza 177 (Table 5).

Regeneration

Calli grow normally when cultured in regeneration medim. Only calli of control and protocol 2 showed positive responses on regeneration medium, Sakha 101 showed regenerants from control and protocol 2 but Giza 177 appeared regenerants from control only. Plantlets from both mutagenized and control calli started regenerating just two months after the growing callus masses were transferred to regeneration media (Fig. 8). Few plantlets were obtained from both mutagenised and control calli. About 3 plantlets from control and nine plantlets from mutagenized calli were observed. Mutant plantlets did not show any apparent phenotypic differences with respect to control plantlets except one jar showed single albino plantlet (Fig. 8c). But the regeneration rate was higher in the mutagenized ones.

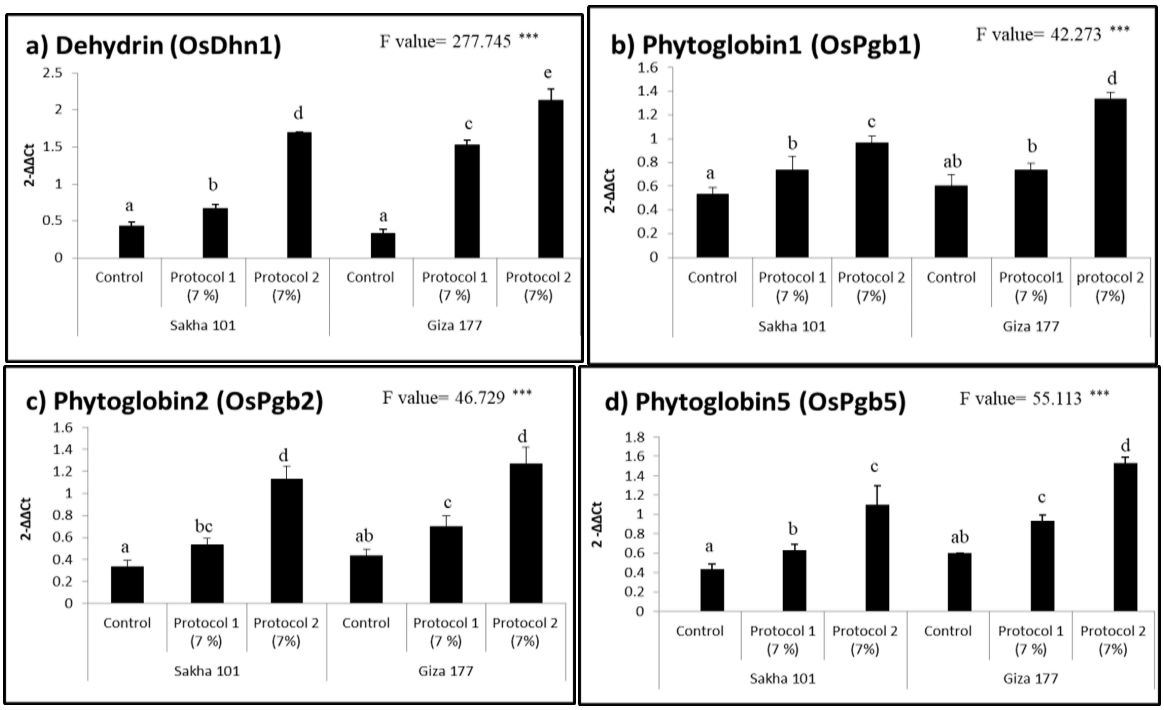

Fig. 7. Quantitative RT-PCR analysis of mRNA levels of dehydrin (a), OsPgb1 (b), OsPgb2 (c) and OsPgb5 (d) in Sakha 101 and Giza 177 during callus induction

TABLE 5. Relative expression of rice Dehydrin (OsDhn1), phytoglobin (OsPgb) 1, 2 and 5 genes in rice Calli of different EMS protocols after PEG treatment for Two weeks

Genotypes	Treatments	OsDhn1	OsPgb1	OsPgb2	OsPgb5
	Control	0.43 ± 0.05^{a}	0.533 ± 0.05^{a}	0.33 ± 0.06^{a}	0.43 ± 0.06^{a}
Sakha 101	Protocol 1 (7 %)	0.67 ± 0.06^{b}	0.733 ± 0.12^{b}	0.53 ± 0.05^{bc}	0.63 ± 0.06^{b}
	Protocol 2 (7%)	1.7 ± 0^{d}	0.967 ± 0.06^{c}	1.13 ± 0.12^{d}	1.10 ± 0.20^{c}
	Control	0.33 ± 0.06^{a}	0.60 ± 0.10^{ab}	0.43 ± 0.05^{ab}	0.60 ± 0^{ab}
Giza 177	Protocol 1 (7 %)	1.53 ± 0.05^{c}	0.73 ± 0.06^{b}	0.70 ± 0.10^{c}	0.93 ± 0.05^{c}
	Protocol 2 (7%)	2.13 ± 0.15^{e}	1.33 ± 0.05^{d}	1.27 ± 0.15^{d}	1.53 ± 0.06^{d}
F value		28.75^{***}	42.27^{***}	46.73^{***}	55.11^{***}

*: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ^{NS}: non-significant.

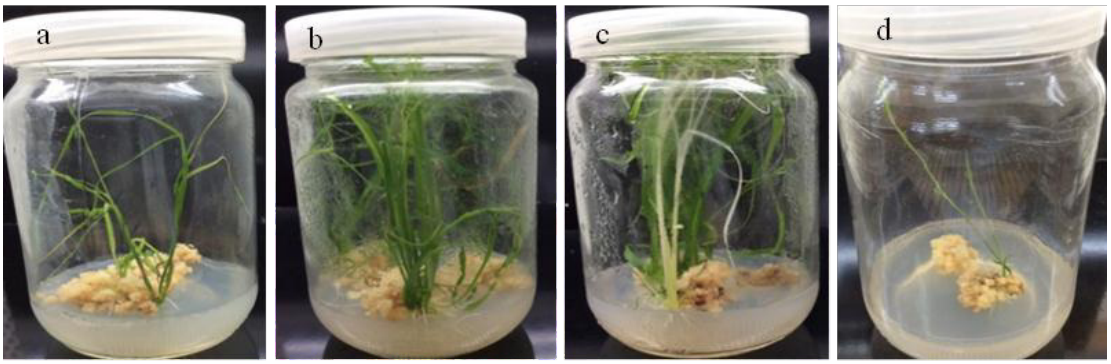

Fig. 8. Regeneration of rice calli of Sakha 101 (a) control, (b and c) protocol 2 and Giza 177 (d) control

Assessment of Genetic variability in rice cell lines using ISSR

The ISSR markers were used to assess the genetic variability of three putative mutant regenerants arising from *in vitro* regenerated plants of EMS and non-EMS treated calli compared to their genotypes (Sakha 101 and Giza 177) and a drought tolerant genotype (IET 1444). The thirteen primers (Fig. 9) identified 161 total bands among six genotypes and cell lines, including 96 polymorphic, 65 monomorphic and 32 unique bands. The 96 polymorphic bands accounted for 59.63 % of the polymorphism (Table 6). The highest polymorphism (70%) was detected by primer UBC 843 with bands of range size 105 to 890 bp, and primer HB 12 (69.23%) with bands of range size 107 to 976 bp, but the lowest (41.67 %) was detected with the primer HB14 with bands of range size 124 to 935bp. The number of amplified bands varied from eight (UBC 852) to sixteen (UBC 848 and HB 9). The size of the obtained fragments using all primers varied between 90 to 999bp. The largest fragment size (999bp) was obtained by using primer HB 9. While the smallest fragment size (90bp) was obtained when primers UBC 818 and UBC 836 were used. Except UBC 842, all ISSR primers showed that new markers were induced in regenerated new lines but were absent in their mother plants (Sakha 101and Giza 177). On the other hand, some bands appeared in mother plants (Sakha 101 and Giza 177) and disappeared in their regenerated new lines using all ISSR primers used except HB 14. Also, some primers showed common bands between new lines and IET 1444 and absent in their mother plants, these primers are UBC 812, UBC 814, UBC 836, UBC 840, UBC 848, UBC 852, HB 12, and HB 14.

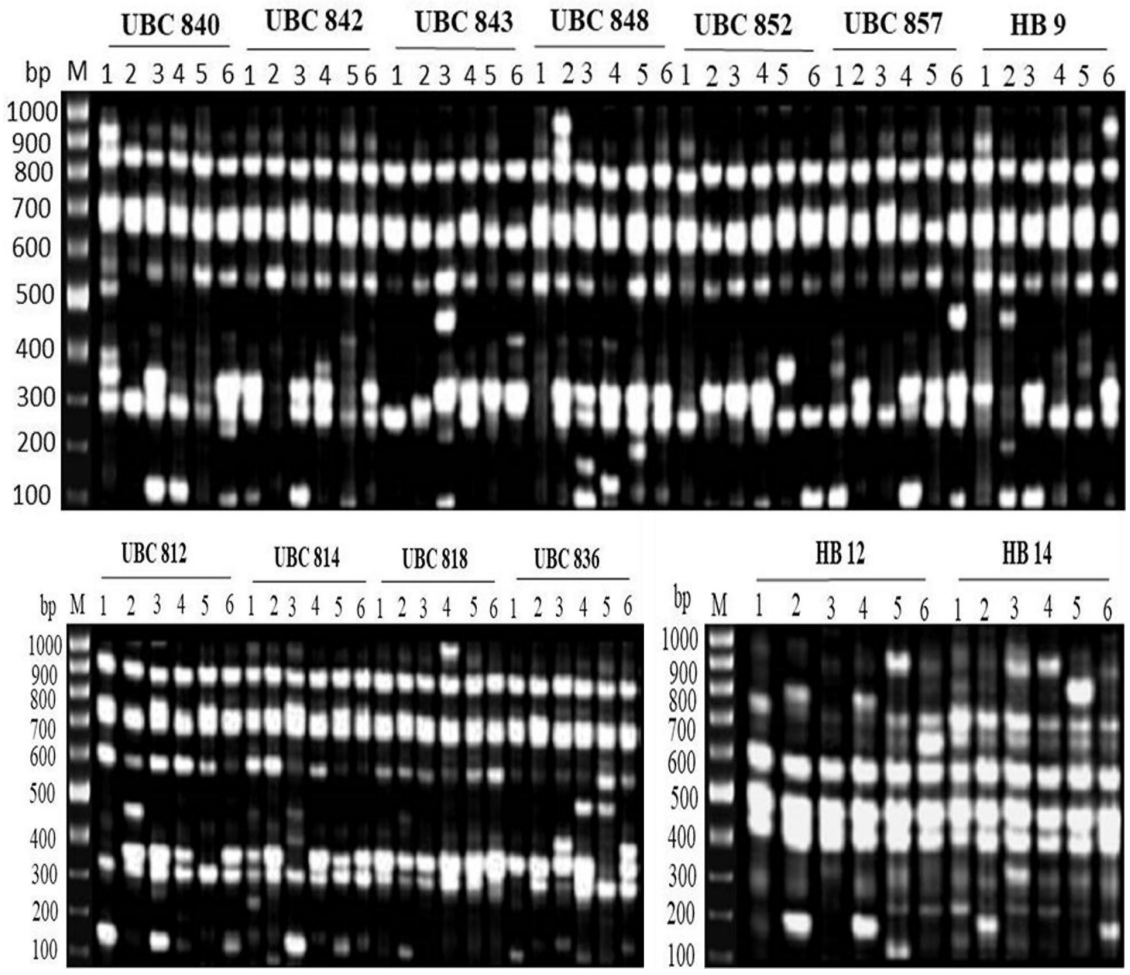

Fig. 9. ISSR profile of rice cell lines and their mother plants, (1) Sakha 101 (mother plant), (2) regenerated Sakha 101 (control), (3) regenerated Sakha 101 (protocol 2, 7 % PEG), (4) Giza 177 (mother plant), (5) regenerated Giza 177 (control), and (6) IET 1444 (wild type)

TABLE 6. Summary of genetic analysis of rice lines and their mother plants using ISSR

No.	ISSR primers	Total bands	Range of size (bp)	Monomor-phic bands	Polymor-phic bands	Unique bands	Polymor-phism %
1	UBC 812	10	124-993	5	5	2	50
2	UBC 814	13	99-969	5	8	3	61.54
3	UBC 818	12	90-964	6	6	3	50
4	UBC 836	14	90-915	6	8	2	57.14
5	UBC 840	13	110-934	6	7	2	53.85
6	UBC 842	12	108-912	5	7	2	58.33
7	UBC 843	10	105-890	3	7	5	70
8	UBC 848	16	110-948	6	10	5	62.5
9	UBC 852	8	108-878	3	5	2	62.5
10	UBC 857	12	110-908	4	8	1	66.67
11	HB 9	16	115-999	5	11	3	68.75
12	HB 12	13	107 -976	4	9	2	69.23
13	HB 14	12	124-935	7	5	0	41.67
Total		161		65	96	32	59.63

Cluster analysis

A dendrogram constructed from UPGMA cluster analysis of 13 ISSR primers is shown in Fig. 10. It separated the regenerants, their mother plants and the tolerant genotype into two main clusters at a dissimilarity distance of 1.11. The first cluster was subdivided at dissimilarity distance of 1.05 into a branch comprised Sakha 101 (mother plant), and a subcluster included Giza 177 (mother plant), and regenerated Giza 177 (control). In the second cluster, regenerated Sakha 101 (protocol 2, 7% PEG), and IET 1444 (wild type) were delimited together at dissimilarity distance of 1.00 but were separated from regenerated Sakha 101 (control) by a distance of approximately 1.056.

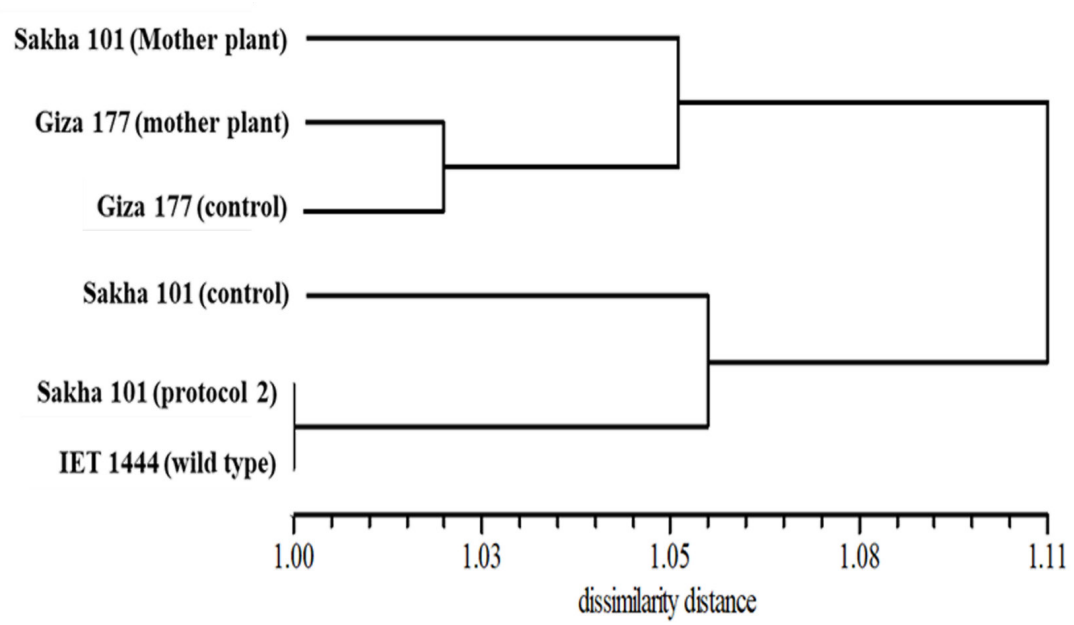

Fig. 10. Dendrogram analysis representing genetic distance between studied rice cell lines and their mother plants treated with EMS and PEG and without treatment as revealed by UPGMA cluster analysis based on ISSR fingerprinting

Discussion

Determining the mutagenic sensitivity of germinating seeds is an important aspect of mutation breeding because healthy crop growth and yield are dependent on seedling establishment (Nair & Mehta, 2014). The use of EMS mutagenesis, followed by in vitro, in vivo, and molecular screening of drought-resistant mutant clones, increased the efficiency of selection. Exposure to a mutagen should ideally result in a useful number of mutations without killing plants or impairing in vitro embryogenicity. Based on the median lethal dose (LD50), 1 % EMS for 6 hours was effective to generate whole rice mutants with a sufficiently high mutation density in Sakha 101 and Giza 177 in the present study. The LD50 is genotype dependent, as well as treatment time. The variable findings achieved in our study may be attributed to the treatment period and genotype (Opoku Gyamfi et al., 2022). The type of explant is also an important aspect in determining the best mutagen settings. EMS has been proven in studies to be capable of mutating both seeds and callus cells (Koch et al., 2012; Kumar et al., 2015).

The observed results showed that when EMS was used on rice grains to induce mutations, callus induction percentage and callus fresh weight also reduced with the increase of EMS concentration as compared to the control. This result is in agreement with that previous study of Mohammed & Ibrahim (2016) which revealed that a significant decrease in callus induction percentage and mean of callus fresh weight with increasing EMS concentration in two rice genotypes. The highest values of callus induction after control were at 0.5% EMS reached 56.86 and 60.25 in Sakha 101 and Giza 177 respectively. This finding is consistent with the findings of Lee & Lee (2002), who found that the incidence of callus induction was highest in rice anthers treated with 0.5% EMS for 10 days following culture.

Presently this study exhibited that germination percentage decreased as concentration of ethyl methane sulfonate increased for the determination of LD50. The decrease in percentage germination in the treatments could be attributable to the influence of EMS (Opoku Gyamfi et al., 2022). The 86% and 77.25% germination in Sakha 101 in Giza 177 respectively obtained in the control treatment may be caused by a decrease in viability of rice grains. In contrast to the control treatment, the germination percentage was low in all treatments in both Sakha 101 and Giza 177. Similar results were obtained by Talebi et al. (2012) who reported that reduction in germination percentage of rice grains occurred with corresponding increase in EMS concentration. Also results of Wattoo et al. (2012) indicated that the germination % decreases with the increasing dose of EMS in basmati rice. Opoku Gyamfi et al., (2022) also observed significantly reduced germination percentage in cowpea (*Vigna unguiculata* L.) as EMS concentration increased.

This study, aimed to establish drought-tolerant lines using in vitro selection incorporating EMS-induced rice variants (three different methods) via screening of calli on media stressed with varying levels of PEG (50 and 70g L^{-1}). The data revealed statistically significant differences in callus induction percentage, callus fresh weight, and growth index of rice cv. Sakha 101 and Giza 177 in vitro selection at 50 and 70g L^{-1} PEG. In all protocols, the control set (unselected treatment) showed higher callus induction percentage, fresh weight, and growth index than the water stressed medium (selected treatments). These findings agreed with those of Verma et al. (2013), who discovered a substantial difference in callus induction, callus development, and callus proliferation across PEG-induced rice treatments (30, 50 and 70g L^{-1}). The present findings were also consistent with those of Biswas et al. (2002), who discovered significant variations among five genotypes in the case of rice calli induced on stressed medium with varying PEG concentrations (5, 10, and 15g L^{-1}). For all five genotypes, callus induced in control sets was found to be healthier than callus induced in stressed medium. Callus induction was higher in the control set than in the stressed media in four genotypes. Three genotypes showed a decrease in callus induction percentage with increasing PEG concentrations supplemented in the callus induction medium.

Additionally Drought tolerance in rice was evaluated using qRT-PCR by determining PEG effect on mRNA expression level of four key drought-tolerance related genes (Dehydrin, phytoglobin 1, 2 and 5). Also the effect of EMS combined with somaclonal variation to generate tolerant lines of rice reflected in changes in transcriptional profiles of these genes under drought. However, most DHN genes exhibit considerable expression levels in seeds and

immature seedling stages in wheat and rice (Wang et al., 2014; Verma et al., 2017), the present study detected DHN expression in callus. The observed data revealed that dehydrin (OsDHN1) expression was increased in calli of both Sakha 101 and Giza 177 grown under drought stress (7 % PEG) and EMS treatments (protocol 1 and 2) compared to control (neither PEG nor EMS). This is consistent with the findings of Verma et al. (2017) who investigated the expression of DHN genes in rice shoots and roots following 1, 3, and 7 days of drought stress under control, moderate (10% PEG), and severe (20% PEG) conditions. According to their findings, all DHN genes were overexpressed in PEG treatments when compared to controls. Lee et al. (2004) also reported that various stresses significantly increased the expression of OsDhn1. While OsDhn1 was significantly and persistently expressed in response to drought stress, it was only transiently expressed in response to cold or salt stress.

Regarding the dehydrin gene was overexpressed in PEG treatment than control, suggesting their ubiquitous contribution to stress tolerance by scavenging reactive oxygen species (ROS). Dehydrins maintain the osmotic balance of cells and their chlorophyll contents, bind metals to scavenge ROS, and bind to DNA and phospholipids (Liu et al., 2017; Yu et al., 2018). Dehydrin have proven to be valuable in stress tolerance in various plant species (Kumar et al., 2014; Chiappetta et al., 2015; Bao et al., 2017; Hao et al., 2022),

The expression of phytoglobin (Pgb) genes and the localization of Pgb polypeptides in rice grown under normal and stress conditions have been studied. However, analysis by RT-PCR demonstrated that the hb1, hb2, and hb5 genes were expressed in embryonic and vegetative organs collected from rice plants grown in normal conditions (Arredondo-Peter et al., 1997; Garrocho-Villegas et al., 2008; Lira-Ruan et al., 2011). The detected results showed that rice phytoglobins 1, 2, and 5 were expressed in calli under both normal and drought-like conditions, although they were induced more with PEG treatment than under control. These findings was in accordance with Arredondo-Peter et al. (1997) and Garrocho-Villegas et al. (2008) who stated that rice nshbs expression was low under normal conditions. Hebelstrup et al. (2006) confirmed findings as they revealed that Pgbs are commonly expressed in meristematic regions of the plant and are frequently detected in areas where changes in the cellular state of tissue are occurring. They are thought to play an important role in the regular growth and development of plant organs.

The results revealed that the three rice hemoglobin genes 1, 2, and 5 were highly expressed under drought stress (7% PEG) in Sakha 101 and Giza 177 calli of EMS different protocols. Parallel to these results, Shankar et al. (2018) revealed that the expression profiles OsPgb1.1, OsPgb1.2, OsPgb1.3, and OsPgb1.4 were all induced under salt, drought, cold stresses, and ABA treatment. The Pgbs' substantial overexpression emphasises their relevance in the plant stress response pathway. Phytoglobins have been extensively investigated for their role in plant response to water stress in varied plant species (Hammond et al., 2020; Youssef et al., 2021; Chammakhi et al., 2022). The findings, together with the previous reports, show that OsPgbs overexpression improves tolerance to drought stress. Tolerance may be established by quenching ROS and reactive nitrogen species radicals and NO scavenging using Pgbs, which has been intensively investigated in recent years (Dordas, 2009; Hill, 2012; Baudouin & Hancock, 2013). NO and H_2O_2, which are more commonly associated with hypersensitive responses at higher concentrations, are also crucial in signalling pathways at lower concentrations (Romero-Puertas & Sandalio, 2016). As a result, NO/ROS homeostasis is critical for the normal functioning of cellular processes and the maintenance of active metabolism in plant cells (Hill, 2012). Moreover, other than drought, Pgbs have been studied for their sensitivity to several abiotic stressors as oxygen-limiting environment (Mira et al., 2016), flooding stress (Youssef et al., 2016; Fukudome et al., 2019), elevated levels of atmospheric nitric oxide (Zhang et al., 2019), cadmium (Bahmani et al., 2019), excessive moisture (Mira et al., 2021), and salinity (Youssef et al., 2022).

The genetic variability created through in vitro mutagenesis in rice (putative mutants and their corresponding mother plants) was efficiently assessed by ISSR markers. Presently this study reported 59.627% polymorphism using thirteen ISSR primers. All primers revealed polymorphism in all somaclons. The research propose that polymorphic fragments indicated by primers among regenerants resulting from in

vitro mutagenesis were most likely caused by EMS treatment. ISSR polymorphisms revealed the presence of genetic variations between plants regenerated from EMS-treated rice explants compared to mother plants. The observed polymorphisms were most likely caused by mutations in primer binding sites, which resulted in an increase or decrease in the overall number of primer binding sites, and hence the number of amplified fragments, however alterations within the amplified fragments would not be found (Reddy et al., 2002; Hofmann et al., 2004). Such frequency of somaclonal alterations reported in plantlets regenerated from in vitro mutagenesis can be used to improve rice biotechnologically.

Following the same trend, Perera et al. (2015) reported 42.85% polymorphism detected by ISSR among regenerated plants as a result of mutagenesis of Miscanthus x giganteus cultivar Freedom in vitro cultures. Such polymorphisms of ISSR showed presence of genetic variation among the regenerants originating from EMS mutagenic treatments. Using inter simple sequence repeat (ISSR) and sequence-specific amplification polymorphism (SSAP) molecular markers, Kour et al. (2014) also evaluated the genetic stability of the in vitro grown plants of *Artemisia absinthium*. They claimed that no variation was seen in the plants regenerated from the nodal explants and the both markers were able to detect the somaclonal differences in the callus regenerated plants.

Similarity coefficients reflected the genetic relationship between the rice regenerants and their mother plants. Based on dissimilarity coefficients ranging from 1 to 1.11, the UPGMA cluster results indicate that these putative mutants have a reasonably small genetic base. The cluster grouped the EMS mutant line of Sakha 101 with tolerant genotype IET1444. On the other hand it grouped Giza 177 regenerated line with its mother plant. This result revealed that the second protocol was more efficient in generating rice tolerant lines to drought in Sakha 101.

Conclusion

The present study employed tissue culture techniques and EMS's mutagenic ability to produce prospective drought-tolerant rice lines based on mutagenesis and somaclonal variation. Rice embryos were mutated and then selected for water stress tolerance. The findings suggest that EMS mutagenesis and in vitro osmotic pressure selection using PEG can be successfully used to develop rice with enhanced morphological and physiological responses to water stress. The results revealed genetic heterogeneity among the mutants, implying that the mutagen EMS at 1% is efficient for creating mutants for all attributes tested. The *in vitro* screening of EMS mutants with the creation of chemical drought using PEG-6000 to assess water stress tolerance could be a good track to developing drought-tolerant rice lines. The process has the potential to become a significant source in rice stress tolerance breeding attempts; however more research is needed at the molecular level to determine whether genetic alterations can result from EMS-caused mutation.

Competing interests The authors report no conflicts of interest regarding this work.

Authors' contributions: Haidy Nasser: conceptualization, experimental investigations, data interpretation, writing of the original draft. Mohamed S. Youssef: conceptualization, clarification of experimental methods, data interpretation. Aziza S. Elkholy: conceptualization, clarification of experimental methods, data interpretation, and reviewing. Soliman A. Haroun: supervision, conceptualization, data interpretation, reviewing, and editing the manuscript. All authors read and approved the final version.

Ethics approval: Not applicable

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إمكانية الطفرات الجينية لـ EMS والاختيار في المختبر لتحمل الجفاف باستخدام PEG والتوصيف الجزيئي للطفرات في الأرز (Oryza sativa L) باستخدام علامات qRT-PCR و ISSR

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يعتبر الجفاف مشكلة زراعية كبيرة تحتاج الى كثير من الجهد لمقاومة أثارها على انتاجية المحاصيل ، في هذه الدراسة، تم استخدام تقنية زراعة الأنسجة وإمكانية استخدام EMS كمادة مستحثة لاحداث طفرات، لتطوير خطوط خلايا الأرز المتحملة للجفاف. وتم استخدام أجنة ناضجة من نوعين من الأرز (سخا101 وجيزة 177) لتكوين الكالوس. وقد تمت دراسة ثلاثة استراتيجيات مختلفة باستخدام EMS كمادة مطفرة لتقييم وتحديد أفضل الطرق لانتخاب خطوط الخلايا المتحملة للجفاف. ولفحص استجابة الخلايا لـ PEG، تم استخدام الخلايا المعالجة بواسطة EMS بعد شهرين من الاستنبات الجيد ثم زرعت لمدة أسبوعين على وسط غذائي يحتوي علي تركيز 5٪ و7٪ من البولي ايثيلين جليكول PEG-6000. تم تقييم الاستجابة الجزيئية لـ PEG عن طريق الكشف عن التعبير الجيني للديهيدرين وفايتوجلوبين 1 و 2 و 5 بواسطة qRT-PCR. وكان التعبير الجيني النسبي للأربعة جينات مرتفعًا بشكل كبير في الخلايا المعالجة بـ PEG مقارنةً بالمجموعة القياسية لكلا النوعين. وأظهرت جيزة 177 مستويات تعبير أعلى للجينات الأربعة مقارنةً بسخا 101. وقد تم دراسة التغيرات الوراثية الناتجة عن استخدام المادة المطفرة EMS وزراعة الانسجة، تم استخلاص الحمض النووي وتنقيته معمليا ثم تمت دراسته باستخدام تقنية التكرارات البينية البسيطة المتكررة ISSR لثلاثة خطوط ناتجة من زراعة الانسجة ومقارنتها بنباتاتها الام (سخا 101 وجيزة) 177والصنف المقاوم للجفاف (IET 1444). وعكست معاملات التشابه العلاقة الجينية بين خطوط الأرز الناتجة من زراعة الانسجة ونباتاتها الأم. كما جمعت الشجرة المستمدة من بيانات الدنا ISSR بتجميع خط الارز المتحور من سخا 101 مع الصنف المقاوم IET 1444 .