Effects of Ethyl Methane Sulphonate on Seed Germination and the Vigour Index of Wheat (*Triticum aestivum* L.)

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ABSTRACT

We conducted laboratory experiments using ethyl methane sulphonate (EMS) to induce mutation in wheat seeds. We subsequently performed inter-simple sequence repeats (ISSR) analysis to determine the mutational effects of EMS on seed germination and on the vigour index of wheat seeds. ISSR was assayed to determine genetic diversity using eight different concentrations of EMS) and 18 markers that enabled the treatment results to be compared. Among the 18 ISSR markers, marker 5 was associated with a high level of polymorphism (96.87%), whereas a lower level of polymorphism (32.81%) was associated with marker 16. All of the primers applied in the study enabled differentiation of the treatments. The results obtained using the primers numbered 3, 5, 7, 11, 12, 13, 15, 16 and 17 were satisfactory, whereas those obtained using the remaining nine primers were slightly better. There was a significant increase in the seed germination percentage (91%) at an EMS concentration of 1.50%, used in Treatment 7, with notable increases in the lengths of shoots (13 cm) and roots (9 cm). Moreover, at an EMS concentration of 1.50%, the fresh and dry weights of the seedlings increased significantly by 0.2353 g and 0.016 g, respectively. We conclude that the use of ISSR and ISSR markers to determine EMS mutagenesis is effective for analysing mutated wheat differentiation and for determining the effects of EMS on seed germination and the vigour index of wheat.

Keywords: *ethyl methane sulphonate, germination, inter-simple sequence repeats, mutation, seed, wheat*

Introduction

Wheat (Triticum aestivum L.) is one of the world's most important cereal crops and a staple crop for about 35% of the global population[1]. The main wheat-cultivating areas in China encompass five climatic ecological regions and 20 geographic regions[2]. Chemical or physical mutagenesis is often used to generate mutant plant repositories[3]. In mutagenesis induced by ethyl methane sulphonate (EMS), guanines are alkylated, and thymines are often mispaired with 0-6-ethyl G (instead of cytosine) in the complementary strand[4].

Inter-simple sequence repeats (ISSR) is a methodology that has gained currency in the area of plant improvement. The reason for its utility in plant breeding lies in the close linkages that exist between certain DNA markers and important agronomic traits[5]. The use of ISSR primers that can be anchored to one or two nucleotides ending either with 5′ or 3′ can reveal variations in the numerous microsatellite regions. This methodology is widely applied because it is inexpensive, easy, and quick to both develop and use; does not require prior knowledge of DNA sequences; and the quantity of the DNA template that is initially required is minimal[6]. ISSR markers have been applied widely, mainly in the field of plant genetics[7]. Because of the high level of polymorphism and reproducibility associated with this fingerprinting method, it has been widely used, especially about plants[8]. For example, ISSR markers have been used to determine the genetic diversity of Eragrostis tef[9], Cicer[10], and wild rice[11]. Moreover, this method has been applied in the study of genetic relationships and phylogenetic analysis of various crops[12]. The application of ISSR entails several advantages. Apart from the low quantities of DNA templates that are required, sequence data are not needed for primer

construction. Other advantages include the random distribution of the markers throughout the genome, with numerous informative bands generated per reaction. Further, this method is reliable and reproducible[13].

Although many independent studies of either genetic or chemical profiles of a wide variety of plants have been conducted in recent years, there have been few attempts to integrate analyses of the chemical and genetic diversity of plants of different resources. Importantly, the use of a combination of methods could shed light on secondary metabolites and genetic differentiation between plant species[14-15]. The source of polymorphism for both markers is identified based on the presence or absence of an amplified fragment of a particular length between different individuals, which is mainly caused by sequence variation in the primer annealing sites and/or the presence or loss of a restriction site[16]. This methodology has rapidly gained acceptance and is widely applied by researchers engaged in various fields of plant improvement[17]. In particular, it has been widely applied in studies of genetic diversity and the genetic structure of populations[18].

Specifically, the ISSR methodology, entailing the use of 3′ or 5′ anchored primers for increasing levels of precision and reproducibility, is widely applied in the area of plant improvement. The ISSR technique has been used as a technique for enabling identification in studies conducted for many crops[5]. Seed germination and the emergence of seedlings are likely to be the most important phenological events influencing the successful establishment of field crops[19].

Therefore, we applied the ISSR-PCR methodology in a study aimed at determining the extent of EMS mutagenesis and assessing the effect of EMS on seed germination and the vigour index of wheat.

Materials and methods

Seed material from the Chinese spring cultivar of *Triticum aestivum L.* was collected for this experiment, which was conducted in the Guangdong Provincial Key Laboratory of Plant Molecular Breeding at South China Agricultural University.

EMS Mutagenesis

Common wheat seeds were placed in a 500 ml flask and ultrapure water was added to the 5 cm level above the seeds (approximately 100 ml). The seeds were soaked overnight at room temperature for a period of 20 hours. Subsequently, the water was decanted and 50 ml of water with EMS at concentrations of 0.25%, 0.50%, 0.75%, 1%, 1.25%, 1.5% and 2% (v/v), respectively, were added. The seeds were then incubated for 12 hours at room temperature, followed by decantation of the EMS solution and rinsing with 100 ml of ultrapure water (five times for 4 minutes each time) and 200 ml of ultrapure water (four times for 15 minutes each time). They were then rinsed under running tap water for 4 hours before being planted in Petri dishes where they were germinated on filter paper soaked in distilled water under white light at 20 °C in a growth chamber. All of the treatments were subsequently assessed 7 days after seeding.

Extraction of DNA

At the end of the first week, seedlings from all of the treatments were collected for extraction of DNA using the TPS method. The wheat sequences were obtained from the NCBI database (Table 1, Fig. 1).

Table-1: Wheat sequences obtained from the NCBI database and the "gi" for the wheat and their individual sequence lengths.

Plant	Accession	Sequence length (nucleotide)	Sequence length (amino acid)
Wheat	CBUC010000012.1	1145465bp	381821

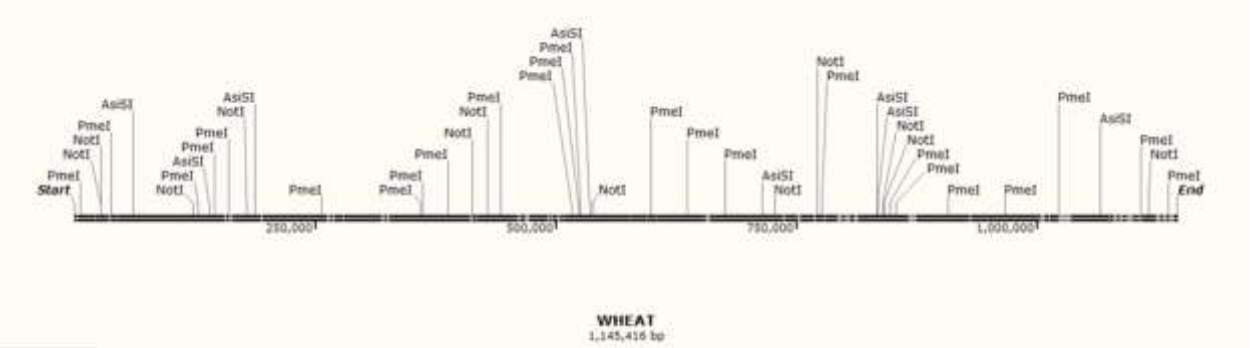

Fig-1. Wheat map sequences.

ISSR amplification and percentages of polymorphic bands

PCR amplification was performed with primers (sourced from Guangzhou Ruibiotech Company). The experiment was carried out using 18 ISSR primers (Table 2). Amplification reactions were conducted using a 25 µl solution containing 12.5 µl of the PCR mixture Initial denaturing was conducted for 7 minutes at 94 °C, followed by 45 cycles of denaturing at 94 °C, each lasting 30 seconds, then 45 seconds of annealing at 52 °C, a further 2 minutes at 72 °C and a final 7 minutes at 72 °C. PCR products were analysed by performing gel electrophoresis in 1.5% agarose. The gel was stained with ethidium bromide and digitally photographed under ultraviolet (UV) light.

Table-2: ISSR primers used in this study.

Primer number	Primer code	Primer Sequences	Temperature (°C)
1	ISSR1	AGX8	52
2	ISSR2	ACX8	52
3	ISSR3	AGX8T	52
4	ISSR4	AGX8A	52
5	ISSR5	AGX8C	52
6	ISSR6	AGX8G	52
7	ISSR7	ACX8T	52
8	ISSR8	ACX8A	52
9	ISSR9	ACX8C	52
10	ISSR10	ACX8G	52
11	ISSR11	AGX8RT	54
12	ISSR12	AGX8RA	54
13	ISSR13	AGX8RC	54
14	ISSR14	AGX8RG	54
15	ISSR15	ACX8RT	54
16	ISSR16	ACX8RA	54
17	ISSR17	ACX8RC	54
18	ISSR18	ACX8RG	54

% of polymorphic bands = (No. of polymorphic bands/ Total no. of bands) X 100%

Percentage of seed that germinated, length of shoots and roots, fresh weight (g) of seedlings and vigour index

At the end of the first week following seeding, the percentage of seeds that had germinated and the vigour index were calculated. Further measurements were taken of the shoot and root lengths (in cm) and the fresh and dry weights (g) of the seedlings. A total of 100 seeds from each of the EMS concentrations had germinated in the growth chamber. The percentage of germination treatment was calculated 7 days after seeding. The values for seed germination (%) were calculated using the following formula (ISTA, 1999).

GP = (germinated seeds/total tested seeds) x 100 %

where GP = germination percentage

Seed vigour index was calculated using the following formula:

VI = PG × SDW

where VI = Vigour index; PG =percentage of germinated seeds and SDW = seedlings' dry weight (average)

To measure root and shoot lengths, 10 plants were randomly selected from the seedlings on the eighth day following seeding. The roots and shoots were then removed from the seedlings and their lengths were measured.

After measuring the lengths of the roots and shoots, the fresh and dry weights of the seedlings were recorded.

ANOVA test

The ANOVA test was performed on the collected data using the Statistical Program for Social Sciences (SPSS) software. The mean differences in the treatment means were obtained by performing the Least Significant Difference (LSD) test[20].

Results

Seven days after seeding, DNA was extracted from the seedlings using the TPS method. Assaying was performed using 1% agarose gel containing 0.1 ugl-1 ethidium bromide and was visualized with UV light. The 18 primers were formulated with reference to SnapGene software (Table 2).

ISSR band patterns in wheat

Out of the 18 ISSR primers used in the experiment, nine produced DNA fragments that were scrabbled. The sizes of the amplified products ranged from 250 bp to 2 kb. Mutations occurring with primer 3 presented characteristic band patterns that were revealed by 1.5% agarose gel stained with ethidium bromide and digitally photographed under UV light (Figer 3).

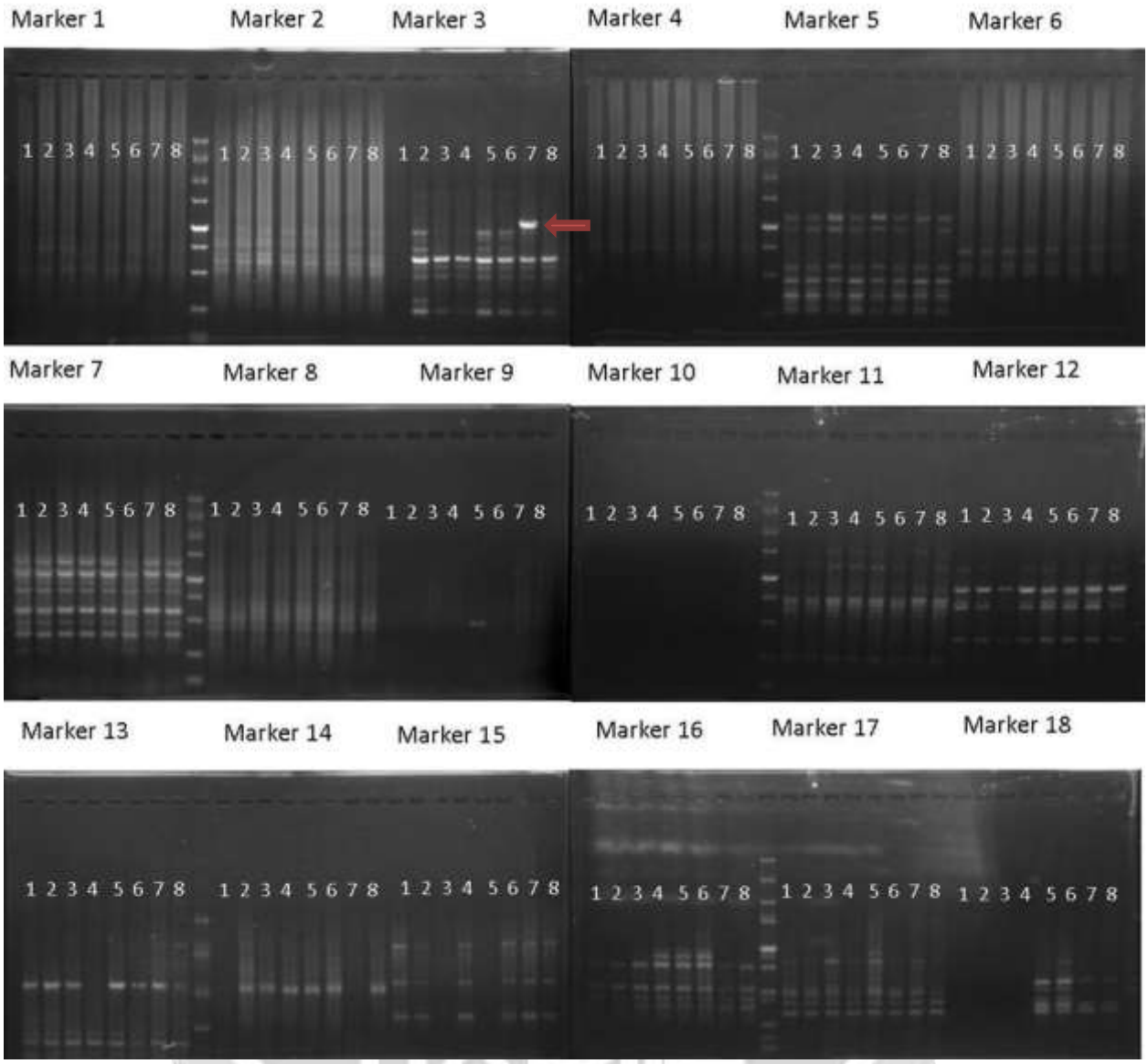

Fig-3: A comparison of the electrophoresis profiles obtained for common wheat, amplified using nine primers and a 5000 bp DNA Ladder. "M" indicates the molecular-size marker. Polymorphic bands associated with the eight EMS concentrations can be compared in the profiles. Mutation is indicated by a red arrow

All nine of the scrabbled ISSR primers were polymorphic and reproducible. They produced differing numbers of DNA fragments, depending on their simple sequence repeat motifs. PCR amplification, with ISSR sequences used as primers, produced 456 fragments, 344 (75.43%) of which were polymorphic. The polymorphism ranged between 96.87%, obtained for primer 5 to 32.81% obtained for primer 16 (see Table 3).

Table-3: The total number of loci, polymorphic loci and the percentage of polymorphism.

Primer code	Total loci	Polymorphism loci	Polymorphism (%)
3	72	42	58.33
5	64	62	96.87

7	56	50	89.28
11	56	48	85.71
12	40	36	90.00
13	40	21	52.50
15	24	18	75.00
16	64	39	32.81
17	40	28	70.00

Effects of EMS on the seed germination percentage and vigour index

As shown in Table 4 and Fig. 4, there were significant increases in seed germination (91%) and in the vigour index (1.456) at an EMS concentration of 1.50%.

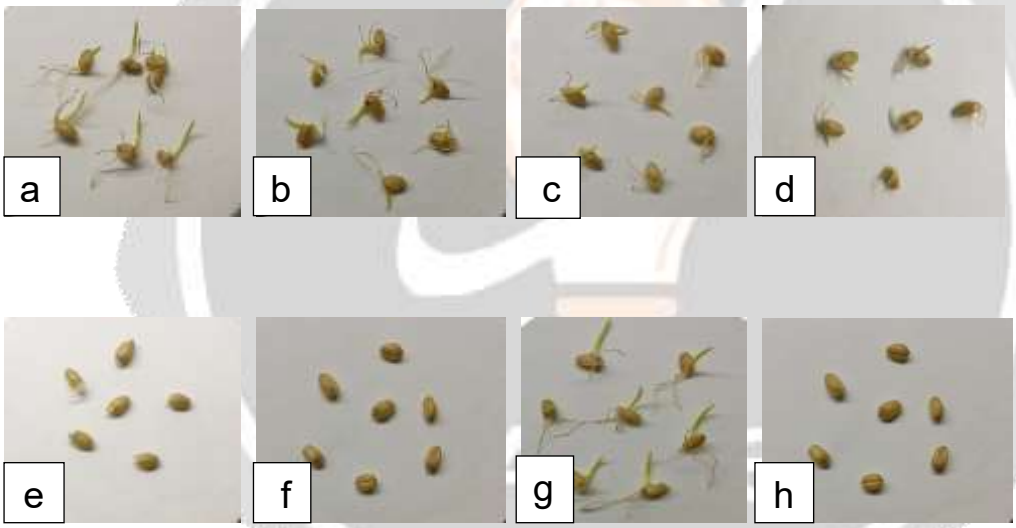

Fig-4: Effects of EMS concentrations on wheat seed germination and the vigour index 4 days after planting at EMS concentrations of 0% (a), 0.25% (b), 0.50% (c), 0.75% (d), 1.00% (e), 1.25% (f), 1.50% (g) and 2.00% (h).

Table-4: Effect of EMS concentrations on wheat seed germination and the vigour index 7 days after seeding.

Treatment number	EMS concentration (%)	Germination of seed (%)	Vigour Index
1	0.00	85.0 ab ± 5.0	1.275
2	0.25	80.5 ab ± 5.6	1.207
3	0.50	77.0 bc ± 3.0	1.078
4	0.75	71.0 cd ± 1.5	1.065
5	1.00	63.0 de ± 3.0	0.819
6	1.25	56.0 e ± 5.6	0.728
7	1.50	91.0 a ± 1.0	1.456
8	2.00	50.0 f ± 2.0	0.600

Note: Duncan's Test showed that the difference was at a significance level of 0.1.

Effects of EMS concentrations on the lengths of shoots and roots and on the root/shoot ratio

As shown in Fig. 5 and Table 5, there were significant increases in the lengths of shoots (13 cm) and roots (9 cm) at an EMS concentration of 1.50%.

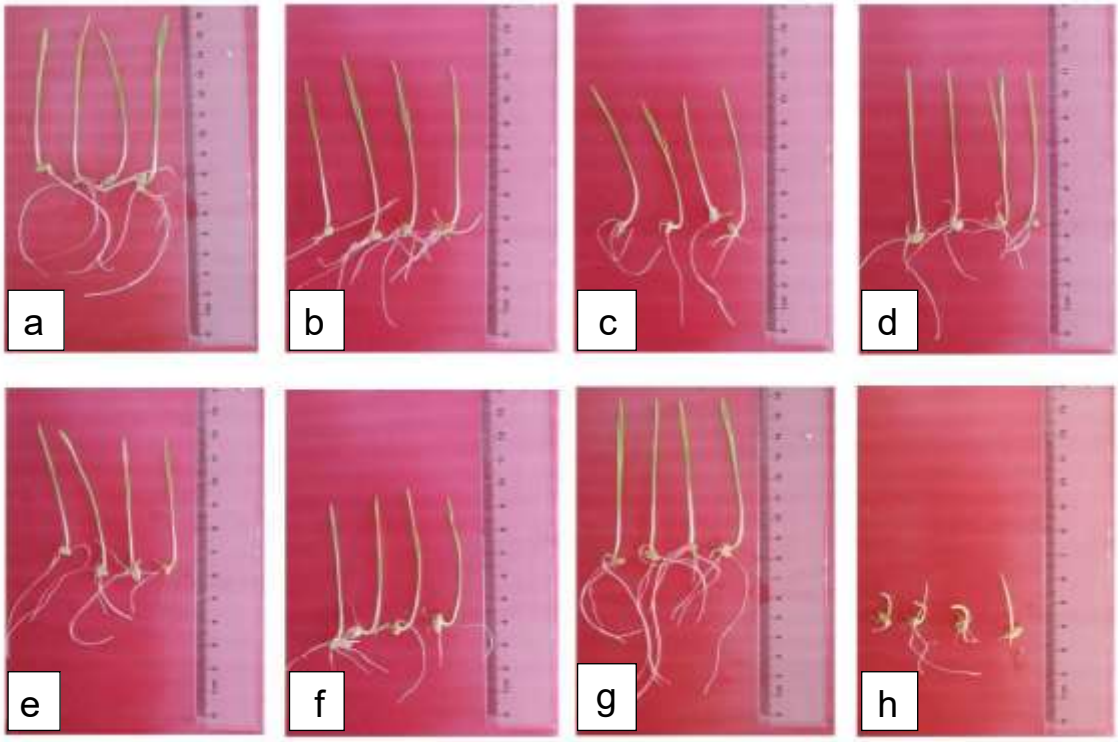

Fig-5: Effects of EMS concentrations on the lengths of shoots and roots of wheat seeds (in cm) after 1 week at EMS concentrations of 0% (a), 0.25% (b), 0.50% (c), 0.75% (d), 1.00% (e), 1.25% (f), 1.50% (g) and 2.00% (h).

Table-5: Effects of EMS concentrations on the lengths of shoots and roots and shoot/root ratios of wheat seeds 7 days after seeding.

Shoot length (cm)	Root length (cm)	Root/shoot ratio
12.5 ab ± 0.5	8.5 a ± 0.5	0.68
11.0 bc ± 1.0	8.0 ab ± 1.0	0.72
10.5 bc ± 0.5	7.6 abc ± 0.6	0.72
10.5 bc ± 0.5	7.5 abc ± 0.5	0.72
10.0 c ± 1.0	7.5 abc ± 0.5	0.75
9.5 c ± 0.5	6.5 bc ± 0.5	0.68
13.0 a ± 1.0	9.0 a ±1.0	0.69
9.0 c ± 1.0	6.0 c ±1.0	0.66

Note: Duncan's Test, showed that the difference was at a significance level of 0.1.

Effects of EMS concentrations on fresh and dry weights of seedlings

Table 6 shows that both the fresh and dry weights of the seedlings increased significantly with Treatment 7 (an EMS concentration of 1.50%) by 0.2353 g and 0.016 g, respectively

Table-6: Effects of different EMS concentrations on fresh and dry weights of wheat seedlings after 7 days

Fresh weight (g)	Dry weight (g)
0.2257 a ± 0.01	0.015
0.2221 a ± 0.01	0.015
0.2169 a ± 0.01	0.014
0.1813 b ± 0.01	0.015
0.1598 bc ± 0.01	0.013
0.1534 cd ± 0.01	0.013
0.2353 a ± 0.01	0.016
0.1298 E ± 0.01	0.012

Note: Duncan's Test showed that the difference was at a significance level of 0.1.

Discussion

The ISSR technique is a PCR-based method, entailing amplification of a DNA segment that occurs at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite directions. Microsatellites, usually 16–25 bp long, are applied as primers within a single primer PCR reaction targeting multiple genomic loci to amplify mainly inter-ISSR sequences of different sizes. Fig. 3 shows the electrophoretic results. Low molecular-weight bands were included in the analysis, but very faint bands were omitted. The electrophoretic patterns for several amplifications were identical when the same primer and the same PCR programme were applied at different EMS concentrations. Therefore, the use of ISSR markers was found to be valuable for differentiating mutations in wheat. All of the treatments demonstrated effective differentiation by each of the primers. Whereas the primers numbered 3, 5, 7, 11, 12, 13, 15, 16 and 17 produced satisfactory results, the results obtained with the remaining nine primers were slightly better. Ng and Tan, (2015) have discussed in detail the effects of using these different primers for generating ISSR bands[13]. Our results endorse those of Rajeswari et al., (2014)[21], who detected 87.9% of polymorphism in 19 cashew accessions with ISSRs. Refika and Akçali (2020) suggested that the ability to resolve genetic variation may be more directly related to the degree of polymorphism detected using the marker system[22]. Goulao et al. (2001) found that the ISSR method is more reproducible than other methods, producing more complex marker patterns. It is therefore useful for differentiating between and identifying closely related cultivars[23].

The germination rate and vigour index increased in the seventh treatment with an EMS concentration of 1.50% as a result of mutation (Figer 3), but the germination rate decreased with any further increase in EMS concentration (see Table 4). Similar results have been reported for cereals and pulses[24-25]. A decrease in the germination rate could be caused by the inhibition of DNA synthesis or other physiological damage after mutagenic (EMS) treatments.

The lengths of the shoots and roots and the fresh and dry weights of seedlings increased with the use of EMS at 1.50% concentration (Tables 5 and 6), but these measurements decreased when the concentration of EMS mutagen increased further. EMS has a stimulatory effect on shoot length because of its antagonistic effect on root length. Therefore, significant decreases in shoot and root lengths occurred at other EMS concentrations. This chemically induced growth mainly occurred as a result of cell death and the suppression of mitosis in the different treatments. Therefore, high doses of EMS mutagens at a particular concentration are suitable for obtaining positive biological effects. In another side the indicated that with increase in other concentrations of EMS damage in seedling increases similar reports were given in *Lycopersicon esculentum*[26] and in tomato[27]. From the above results, we can conclude that EMS is capable of causing damage to plants at a molecular level and may induce mutation at higher concentrations, with the likelihood of greater damage and the presence of more variables. Similarly it has been shown that EMS can induce mutations in chilli plants[28].

CONCLUSION

EMS successfully induced mutation as indicated by biological parameters and effects on the traits of wheat seedlings. ISSR primers can be easily designed or customized to fit the needs of an experiment following a thorough consideration of the findings of this study.

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