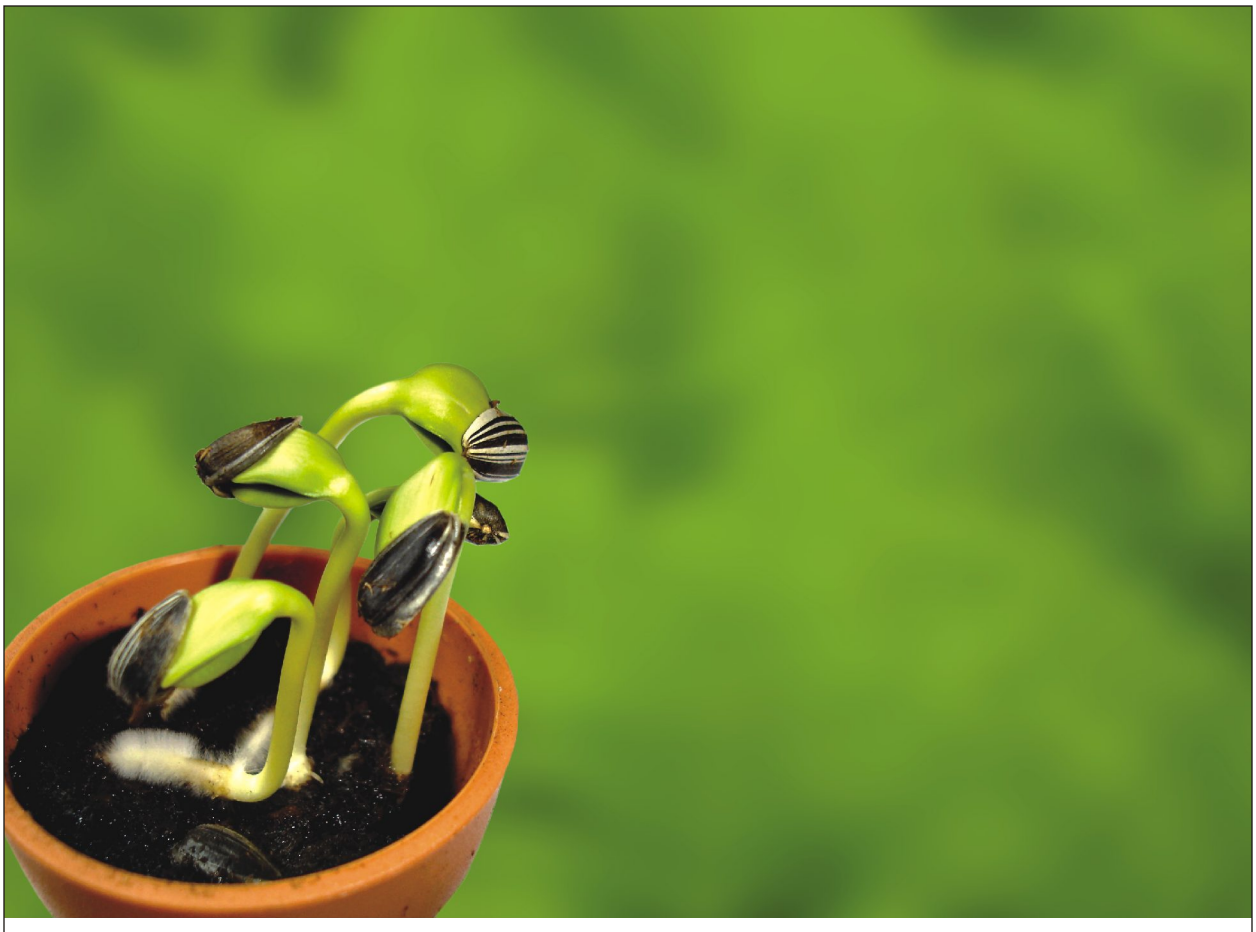

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Research Article
Mutagenic Effects of Ethyl Methanesulfonate on Morpho-Physiological Traits of Local Rice (*Oryza sativa* L.)

Muktar Hossain, Rotna Khatun, Nilufa Akhter Banu and A.K.M. Nazmul Huda

Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia, Bangladesh

Abstract

Background and Objective: Locally adapted varieties are the genetic resources used to develop bettered varieties. Chinekani, a local rice variety with a pleasant aroma and great quality is cultivated in the salty region of Bangladesh. However, no improved rice variety or mutant was included in a mutant database of FAO from this local rice variety. Therefore, the main objective of this study is to evaluate the effects of Ethyl Methane Sulfonate (EMS) on creating a mutant population of this local rice variety. **Materials and Methods:** To evaluate the effect of ethyl methanesulfonate on genes of local rice relating to the morpho-physiological and yield-related character, different concentrations of EMS (1, 2, 3, 4 and 5%) for a three-time duration (6, 12 and 24 hrs) was applied and its effect was observed till harvesting. **Results:** In this study, LD_{50} was observed at 3% concentration for 6 hrs based on germination rate indicated higher concentration of EMS requirement for successful mutation breeding. Moreover, total chlorophyll (a+b) content remained unchanged and carotenoid content was significantly decreased in all treatments compared with control pointed to the non-mutagenic effects of EMS on genes responsible for chlorophyll as well slowing down effect on carotenoid related gene(s). In M_2 generation, significantly increased leaf area, meaningful tiller number per panicle and a significant number of grains per panicle were found among the mutants. **Conclusion:** Findings of this investigation can be exploited for future rice breeding programs. Novel mutant alleles and their functional gene expression analysis employing reverse genetic tools might be discovered using these germplasms.

Key words: EMS, mutation, mutant, lethal dose, genetic, chinekani, hydrolysis

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Corresponding Author: A.K.M. Nazmul Huda, Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia, Bangladesh

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Rice is an important crop for more than one-third of the world's population and provides over 21% of the calorific needs of the world's population and up to 76% of the calorific intake of the population of South East Asia[1,2]. However, increased rice production is demanded due to the geometric growth rate of the global population, particularly in countries where rice is the major stable food resource. Currently, rice genes can be functionally annotated after completion of the rice genome sequence and the availability of large collections of phenotypic data[3-5]. However, locally adapted varieties that farmers have grown for generations are the genetic resources of rice used to develop bettered rice varieties.

There are numbers of nutritious and fast-growing local rice varieties that usually don't grow in every region of the world. Chinekani is such a type of local rice variety that is cultivated in the salty region of Satkhira district in Bangladesh. This variety has great market demand due to its pleasant aroma and great quality. This variety is an ideal plant material for the rice breeding program as it contains three major criteria of breeding such as pleasant aroma, nutritive value and salt tolerance mechanism. No rice variety was observed with lots of desirable characters like Chinekani. So, this variety needs to be used in generating a mutant population for future rice breeding program. The introduction of mutation in the Chinekani rice genome is one of the strategies that can be used to increase diversity. Over the last few years, several new projects have been done to produce EMS induced rice mutant population in research institutes[6]. Advantages of mutations are employed in plant mutation breeding to give rise to desirable variation for crop improvement[7]. Gene mutagenesis alters nucleotide sequences, giving up the production of novel alleles. Physical agents (such as UV, X-ray radiation and fast neutron), chemical mutagens [such as EMS, Nitrosomethylurea (NMU), N-ethyl-N-nitrosourea (ENU)], biological agents such as T-DNA and transposons and transgenic technologies such as CRISPR-Cas9, TALENs are the four common mutagenesis methods[8].

Mutagenesis is the most successful and efficient technique for improving agronomic and physiological characteristics of rice variety. According to Food and Agriculture Organization (FAO), an official mutant database since 1996, 2300 varieties have been improved through mutagenesis among them 501 rice varieties[9]. However, Chemical mutagens (EMS, diepoxybutane (DEB) etc.) and irradiation (Gamma rays, X rays etc.) are the two most widely used in mutagenesis which induces a large number of functional variations in rice. Moreover, chemical mutagens induce mainly point mutations and irradiations normally induce chromosomal rearrangements and deletions[6,10]. Ethyl Methane Sulfonate (EMS) is one the most common mutagen used today. EMS produces random mutations in DNA by nucleotide substitution, specifically by guanine alkylation[10]. It forms O^6-ethylguanine by modifying guanine through the alkylation of its ethyl group. This modified guanine bind with thymine instead of cytosine. So G: C base pair can become an A: T pair (a transition mutation) after subsequent rounds of replication.

Using EMS is advantageous because genetic screening of its effects is possible in various organisms including *Drosophila melanogaster* and *Arabidopsis thaliana*[6]. Ethyl methane sulfonate is a chemical mutagen that is frequently used for seed mutation because it is effective and induces high-frequency point mutations, some of which lead to a novel stop codon for different genes[10,11]. Aside from its effectiveness, EMS is also relatively easy to handle compared to other chemical mutagens such as nitrous compounds and can be detoxified via hydrolysis for disposal[7]. Point mutations produced by EMS can be detected using new technologies such as next-generation sequencing and TILLING (Targeting Induced Local Lesion IN Genome)[12]. However, the mutagenic effect of EMS on local rice varieties like Chinekani is still unknown to us. Moreover, no improved rice variety was included in a mutant database of FAO from this local rice variety Chinekani.

Therefore, the main objective of this study is to evaluate the effect of EMS on creating a mutant population of this local rice variety.

MATERIALS AND METHODS

Study area: The study was carried out at the experimental field and Plant Biotechnology Lab of Department of Biotechnology and Genetic Engineering, Islamic University, Bangladesh from April, 2018-October, 2019.

Methodology: Healthy and dried seeds were subjected to 6, 12 and 24 hrs treatment by five different concentrations (1, 2, 3, 4 and 5%) of EMS. For each treatment 300 seed was used. The treated seeds were thoroughly washed in running tap water for half an hour to remove the residual of mutagen sticking to the seed coat. Another 300 seed was kept untreated to act as a control for comparison. Seedlings including control were sown in pots to rise M_1 generation. In the M_2 generation, only seven populations (each population

Table 1: Agronomical and yield-related character measurement procedure

Agronomic characters measured	Method of measurement
Plant height	Height from the base of the plant (soil line) to the tip of panicles of the main tillers at first flowering
Stem shoot diameter	Diameter of the base of the plant at first flowering
Flag leaf length	Length of the leaf of the main tillers at first flowering
Flag leaf width	Width of the leaf of the main tillers at first flowering
Panicles length	Number of panicles length per individual at maturity
Total grain number	Total seeds produced from panicles
Filled grains per panicles	Number of seeds fill per panicles
Grain length	Length of each seed produced from plant
No. of tillers	Number of tillers determined at first flowering for each individual
1000-grains weight	Weight of 1000 seeds per plant

contains fifty plants) were raised from seven desirable mutant plants of the M_1 generation. Observations of yield, agronomic and physiological characters will be made at corresponding stages of the plant. Agronomical and yield-related characters induced by EMS were measured according to the procedure mentioned in Table 1.

Physiological characters (chlorophyll and carotenoid): Chlorophyll and carotenoid content were measured followed by Huda *et al.*[13]. Shortly, small parts of the leaf were cut from both controls and treated rice plant and measured. The leaves were ground with 80% (v/v) acetone using a mortar pestle after washing with distilled water. The ground samples were centrifuged and optical densities of supernatant were taken using a spectrophotometer. The concentration of chlorophyll and carotenoid were determined according to the following Eq.:

$$\text{Chl. A} = 11.75\times\text{absorbance } 662 - 2.35\times\text{absorbance } 645$$

$$\text{Chl. B} = 18.61\times\text{absorbance } 645 - 3.96\times\text{absorbance } 662$$

$$\text{Carotene} = 1000\times A470 - 2.270\times\text{Chl a} - 81.4\times\text{Chl b} \div 227$$

Statistical analysis: All experiments were performed in a completely randomized block design having independent replications for each treatment. Statistical significance was set at $p = 0.05$ by one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) using SPSS Statistics 20 Software. Further, a graphical presentation for LD_{50} was prepared using micro soft Excel.

RESULTS

Lethal dose: EMS treatment of different concentrations along with duration was evaluated on local rice variety Chinekani to determine the lethal dose 50 based on germination rate and 3% concentration of EMS treatment for 6 hrs was found to be the concentration for LD_{50} (Fig. 1). The result of this investigation also indicated that the germination was decreased for an increased dose of EMS.

Mutagenic effect of EMS on morpho-physiological characters: The plant height was significantly increased at lower concentration (1 and 2%) with a 6-12 hrs treatment duration compared to the control plant. But when this concentration was increased height was decreased compared to control as well as low concentration treated plant (Table 2). Moreover, significantly increased leaf length as well as leaf breath compare to non-treated control plant was observed at 2 and 3% concentration of EMS. Furthermore, stem shoot diameter that inhibits lodging was significantly enhanced at 2 and 3% EMS concentration for 6 hrs duration compared to control. Further, chlorophyll content was remaining steady at all treatments. However, carotenoid content was decreased in all of the treatment compared to a control plant.

Mutagenic effect of EMS on yield-related characters: Panicle length was significantly increased under the EMS treatments of 1% for 6 and 24 hrs, 2% for 6 hrs, 3% for 12 hrs and 5% for 6 hrs compared to the control plant (Table 3). Total 3% for 12 hrs and 5% for 6 hrs treatments showed the better result among the treatments. Similarly, 1% for 6 hrs, 2% for 6 hrs, 3% for 12 hrs, 5% for 6 hrs and 5% for 24 hrs treatment enhance tiller number significantly compared to control though the highest tiller number was observed under the treatment of 5% for 6 hrs and 5% for 24 hrs. In this study decreased tiller number compared to control was also observed under the treatment of 3% for 6 hrs, 3% for 24 hrs and 4% for 12 hrs. Furthermore, the number of grains per panicle was also increased compared to control under the treatments of 3% 12 hrs and 5% 6 hrs. But this number was decreased under the treatment of 1% 12 hrs, 1% 24 hrs, 3% 24 hrs, 4% 6 hrs and 4% 12 hrs. In addition, 1000-grains weight, in 1% 12 hrs and 3% 24 hrs treatment were significantly increased.

Variants in M_2 generation: Based on the results in M_1 generation, a total of seven mutants are found to be suitable for desirable variation and was used to bring forth M_2 generation (designated mutant 1-7) to achieve the variant

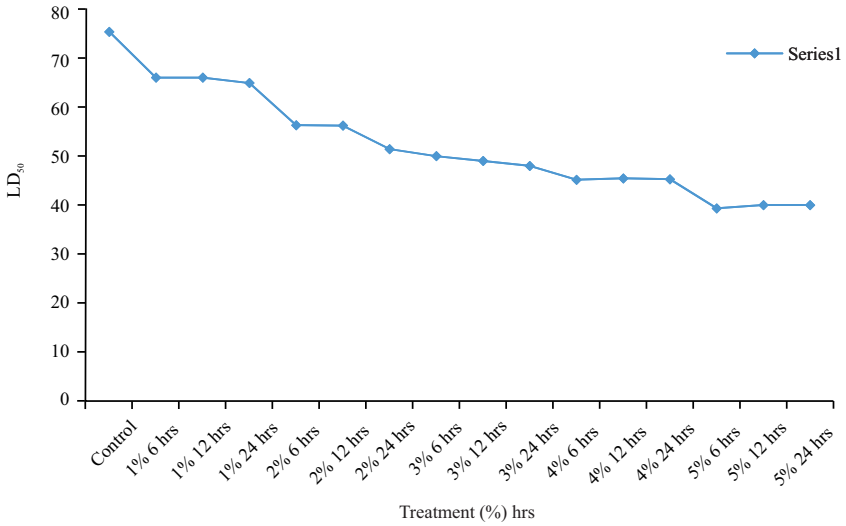

Fig. 1: Lethal dose (LD_{50}) fifty of seeds germination

Table 2: Mutagenic effect of EMS on morpho-physiological characters

Treatment (%) hrs	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Stem shoot diameter (cm)	Chlorophyll (µg mg^{-1})	Carotenoid (µg mg^{-1})
(1) 6	105.50±13.22^{bcd}	34.87±7.36^{abc}	1.50±0.17^{cd}	0.75±0.12^{cd}	882.33±38.72^{a}	59.43±1.24^{ab}
(1) 12	96.25±14.93^{abc}	34.88±7.37^{abc}	1.36±0.37^{bcd}	0.60±0.10^{abcd}	1046.52±82.52^{ab}	68.92±8.44^{bc}
(1) 24	92.50±2.21^{abc}	34.81±7.56^{abc}	1.25±0.19^{abc}	0.52±0.10^{ab}	964.36±49.68^{ab}	66.11±2.11^{abc}
(2) 6	109.38±16.67^{cd}	39.25±7.78^{bcde}	1.38±0.12^{bcd}	0.76±0.23^{d}	1019.81±41.01^{ab}	69.23±7.39^{bc}
(2) 12	105.38±16.67^{cd}	45.12±7.34^{e}	1.20±0.15^{ab}	0.62±0.15^{abcd}	896.88±70.69^{a}	58.35±2.25^{ab}
(2) 24	97.50±20.17^{cd}	39.25±9.99^{bcde}	1.02±0.22^{a}	0.49±0.13^{ab}	1061.83±128.16^{ab}	69.29±11.34^{bc}
(3) 6	101.75±10.70^{d}	43.87±7.59^{de}	1.30±0.26^{abc}	0.59±0.09^{abcd}	1187.29±193.73^{b}	75.56±15.22^{bc}
(3) 12	120.00±15.10^{d}	43.13±5.08^{cde}	1.58±0.46^{d}	0.65±0.18^{bcd}	895.51±222.66^{a}	50.01±13.07^{a}
(3) 24	101.00±13.93^{bc}	30.00±7.23^{a}	1.30±0.17^{abc}	0.61±0.27^{abcd}	1017.12±174.49^{ab}	68.19±11.14^{abc}
(4) 6	95.00±9.00^{abc}	33.00±11.74^{ab}	1.22±0.21^{abc}	0.53±0.14^{ab}	1067.98±201.39^{ab}	69.68±13.44^{bc}
(4) 12	97.33±11.14^{bc}	29.33±7.57^{a}	1.19±0.18^{ab}	0.63±0.13^{abcd}	934.68±120.44^{ab}	63.80±11.32^{abc}
(4) 24	101.29±17.55^{bc}	43.00±6.78^{cde}	1.28±0.17^{abc}	0.61±0.18^{abcd}	1010.69±234.78^{ab}	62.51±17.35^{ab}
(5) 6	119.75±10.93^{b}	36.00±8.63^{abcd}	1.25±0.14^{abc}	0.65±0.12^{bcd}	995.90±91.68^{ab}	58.01±3.61^{ab}
(5) 12	80.63±15.92^{a}	32.88±6.62^{ab}	1.22±0.13^{abc}	0.45±0.11^{a}	1182.40±188.64^{b}	75.09±15.95^{bc}
(5) 24	88.38±16.81^{a}	36.13±3.18^{abcd}	1.63±0.29^{d}	0.57±0.16^{abc}	1068.03±196.96^{ab}	62.39±15.79^{ab}
Control	88.36±17.25^{ab}	30.50±5.07^{ab}	1.36±0.27^{bcd}	0.60±0.09^{abcd}	1167.86±116.61^{b}	81.61±7.10^{c}

Different letters indicate significant differences between mean±SD of treatments (n = 50) at a $p \leq 0.05$ significant level

Table 3: Mutagenic effect of EMS on yield-related characters of local cinnekani rice variety

Treatment (%) hrs	Panicle length (cm)	No. of tiller per panicle	Grains per panicles	Grain weight (g) (1000 seed)
(1) 6	22.75±1.49^{def}	9.25±1.04^{efg}	115.38±20.90^{abcd}	13.93±4.41^{ab}
(1) 12	19.88±2.17^{abc}	7.63±1.0 6^{bcd}	85.75±19.71^{a}	15.93±4.20^{b}
(1) 24	21.75±2.19^{cde}	8.00±1.51^{cde}	93.13±28.42^{a}	11.43±1.87^{a}
(2) 6	23.50±2.30^{ef}	9.00±1.41^{def}	156.25±46.12^{cdef}	14.06±4.93^{ab}
(2) 12	19.63±2.44^{abc}	7.86±1.36^{cde}	124.50±49.75^{abcde}	10.68±.37^{a}
(2) 24	20.88±2.64^{bcd}	7.63±1.78^{bcd}	122.63±34.47^{abcde}	10.43±.41^{a}
(3) 6	20.25±2.25^{abc}	6.25±1.49^{ab}	118.50±54.72^{abcde}	13.81±6.02^{ab}
(3) 12	24.38±2.26^{f}	9.12±1.73^{defg}	168.13±63.31^{ef}	10.75±.88^{a}
(3) 24	18.13±2.42^{a}	5.50±1.07^{a}	79.25±33.24^{a}	15.87±6.96^{b}
(4) 6	20.58±3.20^{abcd}	7.58±1.68^{bcd}	97.25±3e1.33^{ab}	13.50±4.40 ^{ab}
(4) 12	18.83±2.29^{ab}	7.00±0.76^{bc}	110.38±41.05^{abc}	13.93±5.42^{ab}
(4) 24	19.58±2.82^{abc}	7.58±1.59^{bcd}	124.38±34.81^{abcde}	10.50±.46^{a}
(5) 6	24.63±1.68^{f}	10.13±1.13^{fg}	195.25±54.23^{f}	11.50±1.16^{a}
(5) 12	19.38±1.60^{abc}	8.88±.99^{def}	112.63±27.85^{abcd}	14.18±4.73^{ab}
(5) 24	19.38±1.78^{abc}	10.50±.92^{g}	144.63±50.53^{bcde}	10.18±.70^{a}
Control	18.13±1.25^{ab}	8.00±1.20^{cde}	162.13±66.82^{def}	10.68±1.63^{a}

Different letters indicate significant differences between mean±SD of treatments (n = 50 selected randomly) at a $p \leq 0.05$ significant level

Table 4: Value-added traits in M_2 generation

Population name	Height (cm)	Leaf length (cm)	Leaf width (cm)	Panicle length (cm)	No. of tiller per panicle	Grains per panicle	Grain weigh tin (g) (1000 seed)
Control	83.25±5.57^{cd}	18.32±0.703^{a}	1.36±0.28^{bc}	22.88±2.36^{b}	6.88±0.99^{a}	114.88±23.12^{c}	21.11±1.94^{a}
Mutant-1	90.75±2.61^{d}	18.00±1.07^{a}	1.48±0.18^{c}	22.50±2.98^{b}	8.00±1.06^{bc}	137.25±11.05^{d}	21.14±2.49^{a}
Mutant-2	74.25±9.21^{b}	18.62±3.33^{a}	1.10±0.076^{a}	23.00±2.62^{b}	7.88±0.99^{b}	97.88±7.36^{a}	20.98±1.29^{a}
Mutant-3	82.63±5.39^{c}	33.25±4.03^{c}	1.13±0.052^{a}	20.63±2.45^{b}	7.25±0.88^{ab}	108.63±14.36^{bc}	22.31±1.84^{a}
Mutant-4	82.25±11.75^{c}	33.13±5.67^{c}	1.08±0.089^{a}	20.50±3.34^{b}	7.63±1.18^{ab}	112.50±24.53^{bc}	20.12±3.14^{a}
Mutant-5	65.75±3.77^{a}	25.13±5.4^{b}	1.21±0.135^{ab}	17.00±1.85^{a}	9.63±0.74^{d}	83.50±10.39^{a}	22.66±3.4^{a}
Mutant-6	84.25±4.68^{cd}	24.63±4.937^{b}	1.52±0.23^{c}	15.88±1.55^{a}	9.38±0.52^{d}	86.75±6.21^{a}	20.24±2.29^{a}
Mutant-7	72.13±9.24^{ab}	22.75±2.66^{b}	1.16±0.052^{a}	17.88±1.81^{a}	8.88±0.64^{cd}	91.75±34.96^{a}	20.42±1.48^{a}

Different letters indicate significant differences between mean±SD of treatments (n = 50 selected randomly) at a $p \leq 0.05$ significant level

population with desirable yield-related characters (Table 4). Among them, three mutant plants generated from 3% EMS (6 hrs) treatment and four mutant plants generated from 3% EMS (12 hrs) treatment.

In mutant population-3-7, significantly increased leaf area and meaningful tiller number per panicle were found in all mutant populations compared with control plant. However, compared with the control plant a significant number of grains per panicle was found in mutant population-1. Furthermore, plant height significantly enhanced in mutant-1 as well as significantly reduced in mutant-5 population compared with non-mutated population. No significant difference among the mutant populations was mention able compared with the control plant in the case of panicle length and weight of 1000 seed of the mutants.

DISCUSSION

Chemical mutagens have been used for a long time in a breeding program and its mutagenesis is a proven way of creating a variety of characters in crop plant within a shorter time compared to another mutagenesis in conventional breeding program[14]. In this present investigation, Ethyl Methane Sulphonate (EMS) was used as a mutagen to create variation among the morpho-physiological and yield-related character as it was well-known chemical mutagen responsible for point mutation[10]. Its effect was also evaluated on different cultivated crop plants such as Basmati rice, *Stevia rebaudiana*, cowpea, peas to create variation[14-17]. Application of various concentrations of EMS and different exposure duration enhances the chance of variation caused by mutation[10].

The potency of mutagen is an important factor in mutation breeding to make a breeding program successful[18]. However, the efficacy of mutagen depends on the concentrations and the materials being treated. So, it is crucial to choose and optimize the concentration of the mutagen before treating the bulk materials to assure high mutation frequency and at the same time obtain enough viable seeds. Five different concentrations of EMS (1, 2, 3, 4 and 5%) for three-time duration (6, 12 and 24 hrs) were applied in this investigation. In this study, high concentrations of EMS give higher mutation frequency with detrimental effect to plants which was also supported by previous research[10,19]. Moreover, the survival rate will be higher with a low frequency of mutation at a lower concentration of mutagen which was found in our investigation[20]. In a comparative study, LD_{50} dose of EMS was found 0.3% in Malaysian rice and 26.40 mM (0.38%) in soybean[10,21]. But in this present investigation, LD_{50} was observed at 3% concentration for 6 hrs duration indicating a higher concentration of EMS is suitable for a successful mutation breeding program in this local rice variety.

Several variations in morpho-physiological and yield-related traits including plant height which is mostly utilized as an index of different physical and chemical mutagens to identify the biological influences was observed due to 3% EMS treatment for 6 hrs duration[6]. However, chlorophyll content ($\mu g\ mg^{-1}$) remains unchanged where carotenoids content ($\mu g\ mg^{-1}$) was found to be decreased significantly compared with control in all treatments. From this finding, it is assumed that genes that are responsible for chlorophyll are positioned at heterochromatin especially at facultative heterochromatin, which can be changed only in response to cellular signals and gene activity[22]. It is also assumed that EMS slowdown the activity of carotenoid related gene (s) by substituting the cytosine in place of guanine at the regulatory region. In M_2 generation seven mutant populations were generated with desirable leaf area, meaningful tiller number per panicle and a significant number of grains per panicle. In a breeding program, a larger leaf surface area is an important desirable character as it increases the photosynthesis rate by receiving maximized sunlight for photosynthesis[21]. Similar phenotypic changes also observed in Caladium and tomato[23,24]. In evaluating the yield potential of these mutants, it will be important to evaluate the heritability of mutants in a multi-location yield trial that incorporates appropriate experimental design to determine if these mutations will consistently perform across different environments[25].

CONCLUSION

In this study, EMS was used to evaluate its effect on DNA mutation in local rice variety. This investigation indicates that the local rice chinekani is more resistant against EMS as the LD_{50} value of this experiment is quite high compared to previous investigations. Moreover, findings propose that EMS has no effect on genes responsible for chlorophyll but has a slowing down effect on carotenoid related gene (s).

SIGNIFICANCE STATEMENTS

In this investigation, the effect of EMS on the physiological parameters of local rice variety was studied and addressed clear novel hypotheses of interest to a general plant breeder audience that were not studied earlier. Furthermore, mutants with value-added traits (yield-related) will be available for the scientific community access and serve as a public genetic resource for research and breeding programs.

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