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Characterisation and lethal dose determination of EMS induced mutation in *Gallong* jhum rice (*Oryza sativa* L.)

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Abstract

Chemical mutagenesis has been used to increase genetic diversity in crop plants. Among the chemical mutagens, Ethyl Methane Sulphonate (EMS) is stated to be the most effective and strong mutagen causing a high frequency of nucleotide substitution, as found in different genomes. Determination of mutagenic sensitivity is an important step in the mutation breeding program as it differs with species and varieties. Therefore, an attempt was made in the study to determine the lethal dose (LD_{50}) of EMS for *Gallong* jhum rice. A detailed phenotyping was performed after treating the rice with six different EMS concentrations (0%, 0.4%, 0.5%, 0.6%, 0.8%, 1.0% and 1.5%). Phenotypic and biochemical parameters on the M_1 generation were measured to determine EMS sensitivity for the *Gallong* rice. The results show that as the concentration of EMS increased, there was a decrease in germination, seedling height, root length, and emergence under field conditions compared to the control in the M_1 generation. The result showed that 0.8% concentration of EMS induced mutation at saturated level in the rice seeds, while 1 to 1.5% concentrations of EMS were detrimental for the growth and development. The results concluded that 0.8% EMS concentration as LD_{50} could be used in rice mutational programs.

Keywords: Lethal Dose; Mutagenesis; Ethyl Methane Sulfonate; *Oryza sativa*

1. Introduction

The practice of cultivating jhum in the hilly areas of Bangladesh, especially in the Chittagong Hill Tracts (CHTs) comprises about 10% of the country's total area [1,2]. A study estimated a total of 40,000 households' practice jhum cultivation in CHTs on which about 143000 people depend for their subsistence. Along with 5 to 8 other crops grown in a Jhum field, rice is the primary crop. In CHTs more than 300 jhum rice varieties are found and those are conserved in BRRI [3]. Rice (*Oryza sativa*) is a widely consumed cereal crop [4] and low production in jhum results in acute poverty and, malnutrition in the upland community of CHTs. Good quality seed of jhum crops is needed to be produced to maximize the production of it. Genetic improvement of the crops is essential in addressing global nutrition and food security [5]. Particularly, the genetic improvement of jhum rice to increase its yield in the non-irrigated hilly lands is needed on a priority basis in the light of the emerging challenges of growing population, decreasing lands, and escalating threats posed by drought in CHTs.

Genetic variation and subsequently its improvement can be created by germplasm collection, domestication, plant introduction, hybridization, (intra, inter varietal, distant, somatic etc.) mutation, polyploidy, soma clonal variation and genetic engineering for superior variety selection [6]. Artificial treatment of plant materials significantly produces mutations in higher frequencies in cultivated crops especially in diploids [7]. Rice mutants have been created using a variety of approaches, including physical and chemical mutagenesis [8,9], DNA tagging with T-DNA [10,11], retrotransposons and transposons [12-15], and enhancer trapping [16,17]. The Rice Functional Genomic Express Database integrated eleven rice mutant libraries created by transposon tagging and T-DNA carried out in different institutions. EMS is said to be the most effective and powerful mutagen among chemical mutagens [18,19] which causes point mutations in plants [20]. EMS could be used to create new varieties with higher yields and

improved agronomic traits [21]. EMS alkylates guanine bases, causing alkylated G pairs to mis-pair with T instead of C, culminating in G/C to A/T transitions [8]. Point mutation density can be as high as four mutations per Mb [22,23]. A few researches have been carried out to produce EMS-induced rice mutant populations over the last few years in different countries [8]. The term "mutagenic effectiveness and efficiency" refers to how well a mutagen's mutagenic effect is measured [24]. Mutagenic effectiveness measured through the frequency of mutations induced by a unit dose of mutagen [25]. The primary objective of any mutational breeding program is to determination of a suitable dose for a particular cultivar [26]. Higher doses produce a very drastic effect that may lead to the death of an organism whereas a relatively lower dose often results in altered growth characteristics [27,28]. Mutagenic efficiency refers to the proportion of mutations to other associated undesirable biological effects such as lethality and sterility, gross chromosomal aberrations, induced by the mutagen. For avoiding the loss of experimental materials needs to determine LD_{50} before massive exposure of similar materials [18]. The mutagenic dose which inhibits survival to 50% (LD_{50}) or growth to 50% (GR_{50}) is considered as optimum [25]. LD_{50} is firstly calculated which can be used as the best dose for inducing mutations [29] and in which the highest frequency of mutation occurs [18,26]. The lethal dose is different between species to species and within species. For example, LD_{50} in EMS concentration 0.50% for MR219 rice [30], 0.80% for ADT 43 & ADT 45 rice [31], 42.65mM and 44.6mM for CO.1 Pitchi and CO.1 Mullai respectively [18], 0.8% was chosen for upland rice variety Nagina22 [32], 0.25% and 0.50% for Basmati Rice [33] and values ranged from 0.60% to 0.80% for ADT 43 and IR 64 [34]. Thus, the present study was undertaken to induce the mutation in *Gallong* jhum rice for the determination of lethal dose (LD_{50}) and for using the mutant seeds in future experiments.

2. Methodology

2.1 Materials

Gallong Jhum rice was collected from Rangamati, Bangladesh which was cultivated in jhum land. The experiments were conducted from November 2018 to April 2019 at Shahjalal University of Science and Technology (SUST), Sylhet, Bangladesh. The seeds were sown at the field research station located beside the IICT building of SUST, and an *in vitro* study was performed at the Laboratory of Tissue Culture of the Department of Genetic Engineering and Biotechnology, SUST, Sylhet, Bangladesh.

2.2 EMS treatment

The seeds were treated with different concentrations of EMS and only phosphate buffer was used as control. The different concentrations were 0.4%, 0.5%, 0.6%, 0.8%, 1.0% and 1.5%, prepared with phosphate buffer having pH 7. Approximately 210 rice grain was added in a falcon tube with 40ml of buffer solution followed by the addition of EMS in the falcon tube at the ratio of 0.4%, 0.5%, 0.6%, 0.8% 1.0%, 1.5% and 0% with micropipette gradually. 1.5% EMS was considered as negative control and buffer solution without EMS (0% EMS) was considered as a positive control. All the treated seeds in the falcon tube were put in the shaker for 12 hr at 120 rpm overnight. After 12 hr the tubes were removed from the shaker and washed several times with distilled water. In every washing step, the liquid was poured from the falcon tube into a container with 7.5×vol. of 0.5M NaOH. The fields were prepared 2-3 days before the EMS treatment and the seeds were sown immediately after EMS treatment.

Table-1. Preparation of EMS concentrated solution by adding different concentrations of EMS with buffer solution for *Gallong* rice seed mutagenesis.

EMS concentrations	Preparation of EMS solution	Seed
Control	40ml buffer solution only	Approximately 210 seeds were soaked in each EMS concentration
0.4%	40ml buffer + 160µl EMS	
0.5%	40ml buffer + 200µl EMS	
0.6%	40ml buffer + 240µl EMS	
0.8%	40ml buffer +320µl EMS	
1.0%	40ml buffer + 400µl EMS	
1.5%	40ml buffer + 600µl EMS	

2.3 Field experiment

Total 210 mutated seeds (M_0) having three replications of each treatment along with the control were sown to raise M_1 generation in the field. Ten days after sowing, the number of seeds that germinated under these conditions was recorded. Chlorophyll and anthocyanin content of 30-day-old seedlings were determined spectrophotometrically. Any deviation in the characters of treated plants from the control plants were screened and marked. The morphological variations were observed and recorded. Later, all the 30-day-old seedlings were transplanted in a well-prepared randomized block designed field. Before transplanting, the data on the shoot length, root length, branch number, root number and leaf number were recorded for ten randomly selected plants from each treatment.

2.4 Parameters studied

2.4.1 Germination percent (%)

210 seeds of *Gallong* rice from each treatment of EMS were grown in the field. Untreated controls were also sown for comparison. Observations on germination were recorded at 15th day after sowing in each treatment. According to Vasline [35], germination percent (%) was computed using the following formula:

$$\text{Germination Percent (\%)} = \frac{\text{Number of seed germinated}}{\text{Total number of seed sown}} \times 100$$

2.4.2 Lethal dose

The lethal dose (LD) was calculated [36] as follows:

$$\text{LD\%} = \left(1 - \frac{\text{Germination rates of EMS treated seeds}}{\text{Germination rates of control}}\right) \times 100$$

2.4.3 Injury percent (%)

In comparison to the control, the percent reduction in plant height was used to determine the seedling injury. Injury percent calculated as follow [37]:

$$\text{Injury Percent (\%)} = \frac{\text{Control} - \text{Treated}}{\text{Control}} \times 100$$

2.4.4 Survival ratio

The number of surviving plants in each treatment was counted at 42 days (after sowing) and the survival percentage was computed using the formula [37]:

$$\text{Survival Ratio (\%)} = \frac{\text{No. of seedlings survived}}{\text{No. of seeds germinated}} \times 100$$

2.4.5 Total chlorophyll content

Total Chlorophyll Content (TC) of 30-day-old seedlings was determined spectrophotometrically and calculated using the following equation:

$TC\ (mg/g) = [20.2(A_{645}) + 8.02(A_{663})] \times V/1000W$

2.4.6 Total anthocyanin content

Total Anthocyanin content of 30-day-old seedlings was determined spectrophotometrically and calculated using the following equation:

Total Anthocyanin Content $= [OD_{530} - 0.25 \times OD_{657}] \times TV/[dwt \times 1000]$

2.4.7 Determining the leaf colour mutation

Albina: Leaves are white, lack chlorophyll.
Xantha: Leaves that have little or no chlorophyll but are yellow due to carotenoid pigmentation.
Viridis: Seedling with whitish tips of leaves and are lethal.
Striata: They have green longitudinal bands that alternating with transverse yellow or white bands.
Other observations recorded were branch number, root length, root number and leaf number at 30th day after germination.

2.5 Data analyses

With the help of the computer application SPSS, the acquired data were statistically evaluated using the "Analysis of Variance Technique.", post- ANOVA comparisons were carried out by Tukey and the significance of mean difference was conducted by using SPSS. For all of the tested parameters, the least significant difference test ($P < 0.05$) was used to look for significant changes between the treatments.

3. Results and discussion

3.1 Germination percent (%)

The number of germinated seeds (%) under different EMS concentrations is shown in Figure 1. In the M_1 generation of treated seeds, an increase in EMS concentration resulted in a significant reduction in seed germination. Control had the highest germination rate (60%) while 1.5% concentration resulted the lowest germination rate (0%). It was observed that as the EMS concentration was raised, seed germination decreased significantly ($P < 0.001$). The seeds might have absorbed the mutagen, which then entered into the meristematic regions and damaged the germ cell, resulting in a reduction in germination [38]. Also, a reduction in germination could be due to cell constituent damage [39], delay or inhibition of physiological and biological processes or alteration of enzyme activities [30].

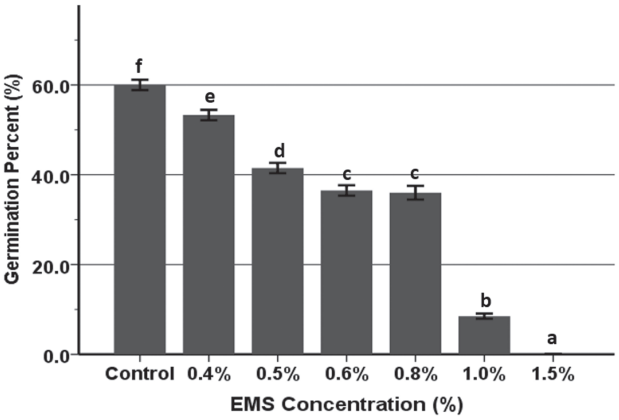

Figure 1: Effect of different concentrations of EMS mutagenesis on seed germination. Different letters indicate significant differences between the treatments ($P < 0.05$).

3.2 Median lethal dose

When the seeds were treated with 0.8% EMS, the lethal dose value was 40.47% (Figure 2), which was near to LD_{50}. On the other hand, in 0.4%, 0.5%, 0.6%, 1.0% and 1.5% EMS concentrations, the corresponding LD_{50} were 11.72%, 30.84%, 39.17%, 85.84% and 100% respectively. To develop a high frequency of desirable mutations in mutation breeding programs, selecting an effective and efficient mutagen concentration is essential [40,41]. For chemically induced mutagenesis, the median lethal dose (LD_{50}) is a critical parameter. For high frequency of mutation induction, median lethal dose determination is important. Based on the growth reduction of seedlings in response to various concentrations of EMS, the present experiment determined the LD_{50} as 0,8% for the Gallong rice.

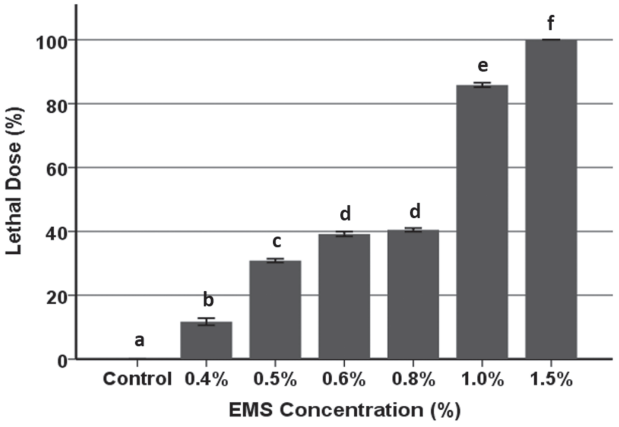

Figure 2: Effect of EMS concentrations on lethal dose determination. Different letters indicate significant differences between the treatments ($P < 0.05$).

3.3 Effect on plant growth

The biological effects of EMS can be determined using plant height as an important indicator. Shoot and root length at seedling stage (Figure 3 & Table 2) of jhum rice showed different responses while exposed to EMS treatment. Both shoot and root length showed a decreasing trend with increasing EMS concentration. However, the response varied; the length of root under 0.4% treatment was as same as control while the length of shoot under the treatments 0.4%, 0.5% and 0.8% had similar responses.

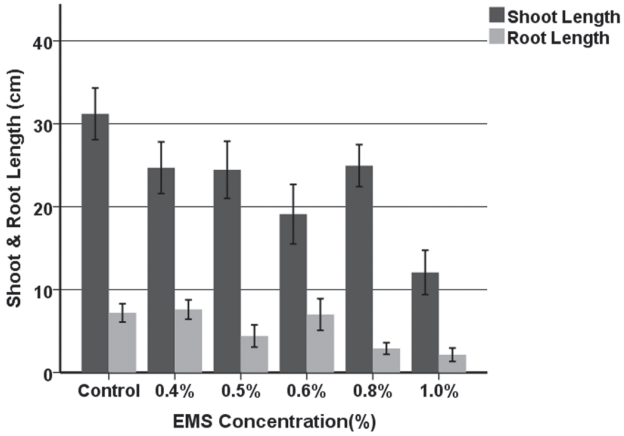

Figure 3: Effect of different EMS concentrations on shoots and root length on seedling stage.

Leaf and root number were significantly high ($P < 0.05$) in 0.4% EMS treated seedlings, while the lowest branch and leaf number were recorded in 0.6% EMS treated seedling. Again, the lowest root number was found in 1.0% EMS treated seedlings (Figure 4 & Table 2).

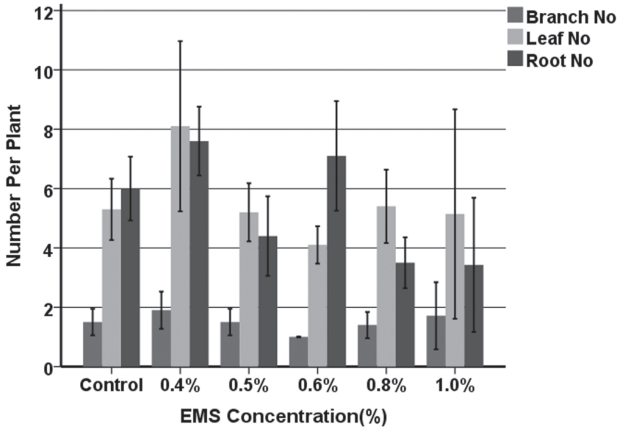

Figure 4: Effect of different EMS concentrations on branch, leaf and root number.

Table-2. Quantitative variation of different EMS treatments with respect to shoot length, root length, branch number, leaf number and root number.

EMS concentrations	Shoot length (cm)	Root length (cm)	Branch number	Leaf number	Root number
Control	31.20 d	7.20 c	1.50 ab	5.30 ab	6 bc
0.4%	24.70 c	7.60 c	1.90 b	8.10 b	7.60 c
0.5%	24.45 c	4.40 b	1.50 ab	5.20 ab	4.40 ab
0.6%	19.10 b	7 c	1 a	4.10 a	7.10 c
0.8%	24.95 c	2.90 ab	1.40 ab	5.40 ab	3.50 a
1.0%	12.07 a	2.14 a	1.71 ab	5.14 ab	3.43 a
Mean	22.75(±6.49) ***	5.21(±2.38) ***	1.50(±.30)	5.54(±1.34) *	5.34(±1.82) ***

Values followed by the same letters are not significantly different according to Duncan Multiple Range Test (DMRT) at P values ($P < 0.05$). Asterisks indicated significant (* $P<0.05$, ** $P<0.01$, *** $P<0.001$) difference of variables between the EMS concentrations according to ANOVA. The value inside the first bracket indicated standard deviation.

In the present investigation of the growth behaviour (shoot and root length, branch, leaf and root number, etc.) in comparison to the control population, the mutant population showed a wide range of variations in the traits studied here. This could be due to the EMS-induced biological damage to the embryo, which then reflected on the plant's growth behavior until maturity. In terms of genetics, the probability of desirable phenotypic modification in the M_1 generation is extremely low, and only dominant mutations can be identified [42]. The phenotypic mutation was observed in the current investigation among 210 mutagenised seeds during the M_1 generation.

3.4 Injury percent (%)

The frequency of mutations is associated with the degree of injury caused by EMS. Among the six EMS treatments, injury percent (IP) was significantly ($P < 0.001$) increased with increasing EMS concentrations (Figure 5). The highest seedling injury (100%) was observed at 1.5% EMS concentrations. The frequency of mutations is connected with the severity of injury induced by EMS. Arisha et al. [41] Aslam et al. [43] observed that the percent of injury increased with increasing EMS concentration.

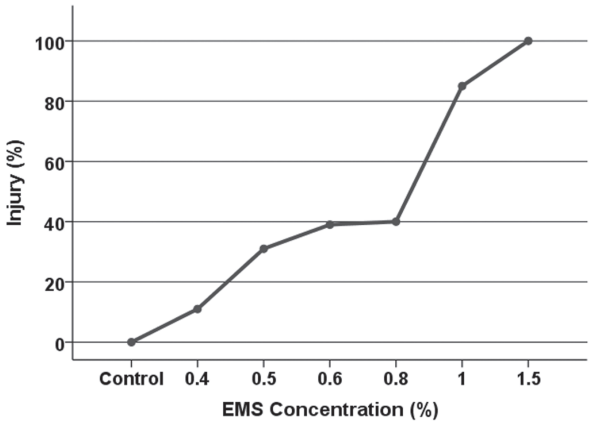

Figure 5: Effect of different EMS concentrations on plant injury.

3.5 Survival ratio

The survival ratio showed a significant ($P<0.001$) difference among the treatments. Maximum survival was observed in the control group and subsequently reduced with increasing EMS concentrations (Figure 6). The present study showed the lowest survival rate at 1.5% EMS concentration among all treatments while the control had a 96% survival ratio. Talebi et al. [30] and Vasline [34] reported that the survival percent of M_1 plants decreased considerably with an increase in EMS concentrations. Increasing EMS concentrations might damage the embryo or alter the physiological and

biological processes that could drive seedling growth, resulting in embryos becoming weak or dying, lowering the survival ratio, depending on the severity of the embryonic damage [40].

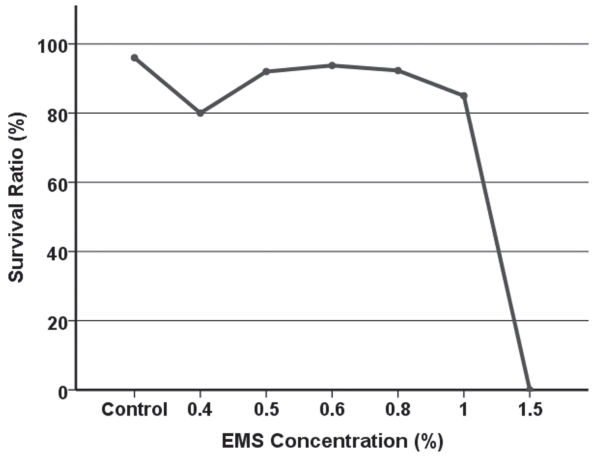

Figure 6: Effect of different EMS concentrations on plant survival ratio.

3.6 Chlorophyll mutation

Usually, chlorophyll mutations were scored when seedlings were 7-15 days old [44] and the primary leaves showed deficiency in chlorophyll. The frequency of chlorophyll mutations varied with different doses of EMS. The highest mutagenic frequency found in 0.8% EMS treated seedling while control, 0.4%, 0.5%, 0.6% and 1.0% mutagenic frequency were correspondingly 0%, 2.22%, 9.64%, 8% and 11.76% (Table 3 & Figure 7). The abnormality of chlorophyll is easily visible and quantifiable and one of the strategies to confirm the induction of that mutation in the treatments. In our study, mutation frequency was calculated from different chlorophyll mutations (Striata, Albina, Xentha and Viridis) observed in seeding from EMS treated seeds. It was found that 0.8% has a high mutation frequency (12.5%). On the other hand, control has 0% mutation frequency. Mishra et al. [45], Sharma et al. [25], Vasline et al. [31] reported that chlorophyll mutations increased with the increase in doses of EMS.

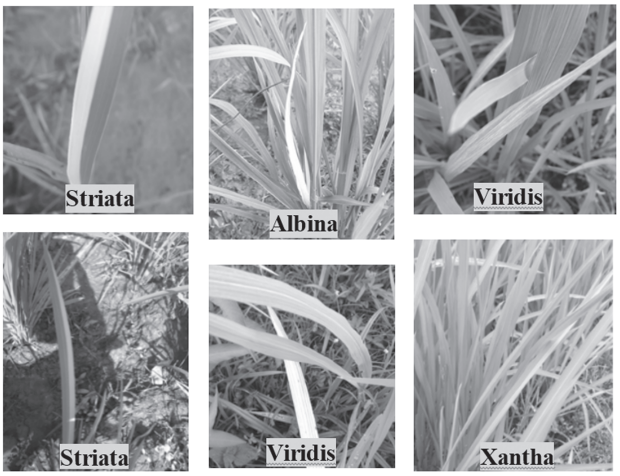

Figure 7: Different types of colour mutation after EMS treatment on *Gallong* rice seedling.

The effect of EMS on the chlorophyll content of plant leaf was also confirmed by measuring spectrophotometrically. Chlorophyll content of EMS treated seedling decreased with increasing rate of EMS except for 0.8% (Figure 8). On the contrary to the observed trend, 0.8% EMS concentrated seedlings showed the highest chlorophyll content (0.551mg/g).

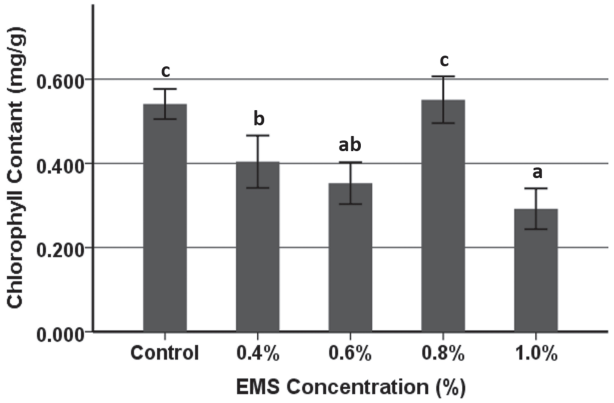

Figure 8: Effect of different EMS concentrations on chlorophyll content. Different letters indicate significant differences between the treatments ($P < 0.05$).

Table-3. Mutagenic frequency of chlorophyll mutation from EMS treated seedling.

EMS concentration	Albina	Striata	Xantha	Viridis	Number of chlorophyll mutants	Number of plants observed	Mutagenic frequency
Control	0	0	0	0	0	120	0
0.4%	1	1	0	0	2	90	2.22
0.5%	4	1	2	3	8	83	9.64
0.6%	1	1	2	2	6	75	8
0.8%	4	2	2	2	9	72	12.5
1.0%	1	1	0	0	2	17	11.76

3.7 Anthocyanin content

The amount of anthocyanin in leaves can be explored as a key indicator for EMS induced mutation. When plants are under high stress, they accumulate anthocyanin. With increasing EMS concentration, anthocyanin content was also increased (Figure 9) except 0.4% EMS treated seedlings. The highest anthocyanin content was (0.0093mg/g) found in 1.0% EMS treated seedlings. Increasing EMS concentration, anthocyanin content was also found to accumulate in seedling leaves. This signifies that the stressful condition of the seedling is related to the increased EMS concentration. Anthocyanins often correlate with enhanced stress tolerance [46] and may have a protective role in plants against extreme stress [47].

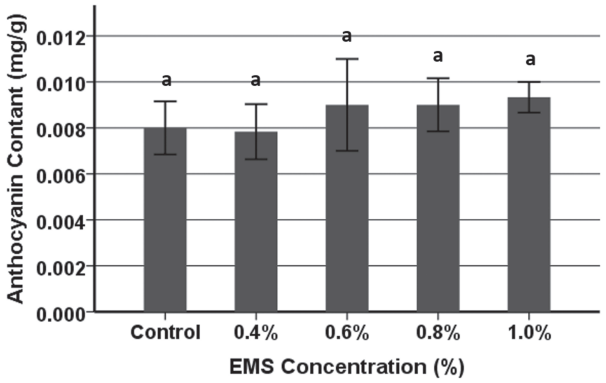

Figure 9: Effect of different EMS concentrations on anthocyanin content. Different letters indicate significant differences between the treatments ($P < 0.05$).

4. Conclusions

Chemical mutagens can be used in plant breeding to increase the genetic variability of plants. The effective use of mutagens depends on factors such as type of mutagens, plant species or varieties, plant materials, the dose and dose rate, environmental conditions during the treatments etc. Determination of median lethal dose is important for resulting in high mutational frequency. In this experiment, we found that 0.8% EMS treated seedling of *Gallong* rice was almost near to LD_{50}. The results suggested that the optimum dose of EMS concentration for *Gallong* rice is 0.8% for seed treatment. This optimum mutagen dose determined could be useful for mutation breeding studies in *Gallong* jhum rice improvement. The mutagenised seeds generated in this study could serve as a base population to study rice yield and grain quality in near future.

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