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Probit Analysis for Lethality, Injury and Sterility in M_1 Generation of MDU 1 Barnyard Millet (*Echinochloa frumentacea*)

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ABSTRACT: MDU 1 is the recently released barnyard millet variety from Agricultural College and Research Institute, TNAU, Madurai. The duration of this variety is about 90–95 days. As the crop is mostly raised under rainfed conditions, early duration (75–80 days) barnyard millet varieties are preferred. Therefore, the MDU 1 barnyard millet was treated with the chemical mutagens to obtain desirable mutants. Since chemical mutagenesis produces more point mutations, Ethyl Methane Sulphonate (EMS) and Sodium Azide (SA) were selected for this study. The variety MDU 1 Barnyard millet was subjected to treatments of 5 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM and 60 mM of both the mutagens EMS and SA. Probit analysis was made and LD_{50} dose for EMS was 16.19 mM and 14.14 mM for Sodium Azide. Other characters such as survival percentage, shoot length, root length, plant height at maturity, pollen fertility and seed fertility were assessed in addition to germination percentage. All the characters were found to decrease with an increase in the dose of mutagens. This inferred that MDU 1 Barnyard millet responds well to chemical mutagens and there is scope for improvement. The established LD_{50} doses from this study could be utilized in a large-scale mutagenesis breeding programme for generating a wide range of mutants in barnyard millet.

Keywords: Barnyard millet, Ethyl Methane Sulphonate, Sodium Azide, LD_{50} value.

INTRODUCTION

Barnyard millet (*Echinochloa* species) has emerged as one of Asia's most important minor millet crops, with a significant increase in global production (Renganathan *et al.,* 2020). Unlike major crops, the area, production, and productivity of small millets are inadequately documented and are frequently reported as 'millets'. In India, barnyard millet is grown on 0.146 m ha with a production of 0.151 m t. Barnyard millet is a notable crop in India, Korea, Japan, and China, where it is utilized for both human food and fodder (Vetriventhan *et al.,* 2020). They are less vulnerable to biotic and abiotic stresses. In Tamil Nadu, it is cultivated in drylands and hilly areas by tribal farmers in Ramanathapuram, Madurai, Theni, Virudhunagar, Salem, Namakkal, Dindigul, Coimbatore, Thiruvannamalai, Villupuram and Erode districts (Nirmalakumari *et al.,* 2009). Despite the fact that Southern zones of Tamil Nadu have dominated barnyard millet farming in recent years, there is growing interest in millets in rice-growing areas of northern Tamil Nadu. Madurai 1 Barnyard millet was released in 2017. It is a pure line selection from the native landrace in Aruppukottai. It has a duration of 90 – 95 days and a yield potential of 1500 – 2000 kg/ha under rainfed conditions and 2000 – 2500 kg/ha under irrigation. A barnyard millet variety with a short

growing season of 75 to 80 days is required to fit into the present cropping system. The majority of farmers are looking for a barnyard millet with a duration of 75 to 80 days. Unlike hybridization and selection, mutation breeding has the advantage of improving a defect in an otherwise elite cultivar, without losing its agronomic and quality characteristics. Mutation breeding is the best alternative option for crop improvement in plants having small size florets which is very difficult for emasculation and hybridization. Induced mutagenesis approaches have the potential to produce novel variations across a variety of traits, which can be used directly as improved cultivars, as donor gene sources in hybridization programmes, or in the development of Near isogenic lines for testing agronomical and physiological hypotheses (Rutger *et al.,* 1976; Gowthami *et al.,* 2017). Chemical mutagenesis is a powerful tool used in mutation breeding programmes to improve a range of important characteristics. Chemical mutagens namely Ethyl Methane Sulphonate and Sodium Azide have been employed extensively to induce a wide range of functional variations. Chemicals mainly induce point mutations, rendering them ideal for developing missense and nonsense mutations, which would result in a sequence of change-of-function mutations (Anbuselvam *et al.,* 2010). EMS is the most extensively used chemical mutagen in plants due to its potency and flexibility of application. EMS alkylates guanine bases, leading to mispairing alkylated G pairs with T rather than C, resulting predominantly in G/C-to-A/T conversions (Bhat *et al.,* 2007). Because EMS generates a significant number of non-lethal point mutations (genome-wide), a relatively small mutant population (about 10,000) is sufficient to saturate the genome with mutations. The most important aspects of a mutant on breeding have been the induction of polygenic mutations that can improve yield and other important agronomic characters (Abdelnour-Esquivel *et al.,* 2020; Mamun *et al.,* 2020). The mutagenicity of Sodium Azide (SA) varies between species. Significant results have been reported in microorganisms, crops such as rice and barley, and SA is partially mutagenic in mammalian systems. Sodium Azide does not affect Neurospora, Drosophila, or *Arabidopsis thaliana*. SA, an ionic compound, induces mutation through an organic metabolite of the Azide chemical, L-azidoalanine, which is produced by the acetyl serine sulfhydrylase enzyme (Gruszka *et al.,* 2012). When the pH of the solution is maintained at 3, SA acts as a potent mutagen, and the predominant compound in this case is HN3. This uncharged HN3 molecule in the acid form of SA provides a greater degree of penetration across the cell membrane, which improves mutagenic efficiency (Sadiq and Owais, 2000). The LD_{50} of crops varies based on the species, variety, and portion of the mutation, as well as the material's water content. As a result, the current study was done to determine LD_{50} and to investigate the impact of EMS and Sodium Azide on biological materials in M_1 generation.

MATERIALS AND METHODS

The genotype used for mutagenic treatment is MDU 1 barnyard millet, which is a promising and leading barnyard millet variety cultivated in Tamil Nadu and the seeds were obtained from, Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Madurai. For mutagenesis, pure, well-filled, mature seeds (400 seeds each for each treatment) dried to 12% moisture content were selected. Pre-soaking the seeds (12 hours at room temperature) before mutagenic treatment improves physiological activation and it also enhances the imbibing capacity of seeds to absorb the EMS and SA. Water was decanted and dried for 6 hours in the shade. After pre-soaking was getting over, the seeds were treated with a fresh solution of Ethyl Methane Sulphonate and Sodium Azide prepared in phosphate buffer at pH 7.0 and pH 3.0 in different concentrations (05, 10, 20, 30, 40, 50 and 60 mM) for four hours under controlled conditions with intermittent shaking to ensure uniform treatment of seeds.

The EMS and Sodium Azide treated seeds of 25 each in each treatment were placed in roll paper towels for germination test under *in vitro* condition in three replications. In another set of treatments, 100 seeds each in three replications were sown in the field (*in vivo*) along with the control. Germination % (7 DAS), shoot length and root length (14 DAS) were observed for both in vitro and in vivo conditions and compared with the control. The number of plants that survived till maturity were scored from each treatment and recorded as % survival.

For pollen fertility testing, unopened flowers from ten randomly selected plants in each treatment were collected along with a control plant. Pollen grains were collected by gently tapping the stamen on a glass slide, then stained with 2 per cent Iodine-potassium iodide and examined under a stereomicroscope. Fully stained pollen grains were counted as fertile ones, whereas partially stained, malformed and void pollens were considered sterile. The plant exhibiting both types of pollen was classified as partially sterile. This classification is based on pollen assessment devised by Raj and Virmani (1988). The pollen fertility was calculated by using the formula,

$$\text{Pollen fertility (\%)} = \frac{\text{No. of round well stained pollens}}{\text{Total no. of pollens observed}} \times 100$$

Probit analysis. The LD_{50} values of Ethyl Methane Sulphonate and Sodium Azide for MDU 1 were determined based on the probit analysis (Finney, 1971, 1978). The probit function is the inverse cumulative distribution function (CDF) or quartile function associated with the standard normal distribution. The

procedure for the determination of LD_{50} using probit analysis is as follows.

1. Initially, mutagen doses were converted to log10 values.
2. Mortality percentage of seeds due to treatment doses were worked out and rounded to the nearest whole number.
3. By using Abbott's formula the corrected mortality percentage was calculated and rounded to the nearest whole number.

$$\text{Corrected mortality (\%)} = \frac{M_{observed} - M_{control}}{100 - M_{control}} \times 100$$

4. The corrected values were then transformed using the probit transformation.
5. Probit values (Y-axis) were plotted against Log10 concentration (X-axis), and a straight line passed through most of the plotted points to estimate the Log10 concentration associated with a probit of 5
6. Antilog to the Log_{10} value corresponding to the probit 5 was calculated to find out the LD_{50} for the particular mutagen under study.

RESULTS AND DISCUSSION

The variety MDU 1 of barnyard millet was selected to investigate the effects of various dosages of Ethyl Methane Sulphonate and Sodium Azide to determine LD_{50} values. LD_{50} values were determined with the help of probit analysis for the chemical mutagen used based on their germination under *in vitro* and *in vivo* conditions.

Determination of Lethal dose. The optimum dose is the dose that causes a maximum of mutation with a minimum of damage to the plant (Ramchander *et al.*, 2015; Veni *et al.*, 2017). In the present investigation, the LD_{50} value for EMS was 16.19 mM (Table 1 and Fig. 1) and for Sodium Azide it was 14.14 mM (Table 2 and Fig. 2) under *in vitro* conditions. This revealed that the LD_{50} is fixed more or less the same for both chemical mutagens (Talebi *et al.*, 2012). Therefore, the LD_{50} dose observed in this study is the optimum dosage for mutagenizing the barnyard millet seeds to induce mutations to produce viable mutants and maintenance of population for mutation breeding.

Table 1: Determination of lethal doses of Ethyl Methane Sulphonate in MDU 1 by Probit analysis.

Group	Dosage mM	Log dose	% Germination	% Corrected	Probits	LD_{50}
1	5	0.70	73.78	26.2	**4.36**	
2	10	1.00	53.78	46.2	**4.90**	
3	20	1.30	45.86	54.1	**5.10**	
4	30	1.48	43.12	56.9	**5.17**	16.19
5	40	1.60	38.22	61.8	**5.30**	
6	50	1.70	27.56	72.4	**5.60**	
7	60	1.78	20.45	79.5	**5.83**	

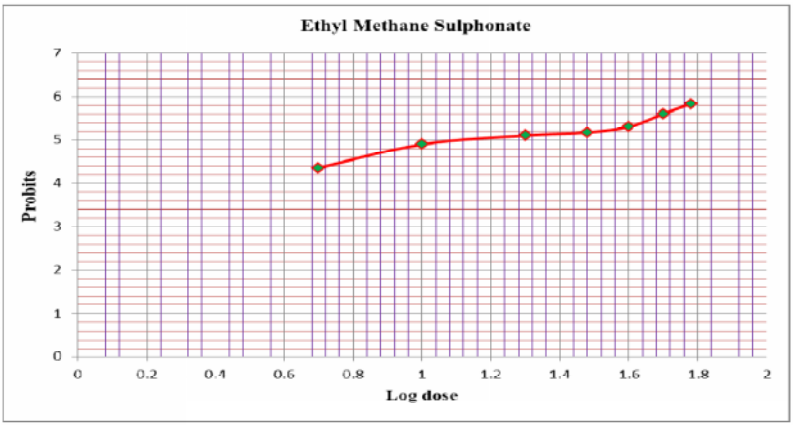

Fig. 1. Plots of Log doses versus probits from Table 1 for calculation of LD_{50} of Ethyl Methane Sulphonate.

Table 2: Determination of lethal doses of Sodium Azide in MDU 1 by Probit analysis.

Group	Dosage mM	Log dose	% Germination	% Corrected	Probits	LD_{50}
1	5	0.70	69.78	30.2	**4.48**	
2	10	1.00	52.45	47.5	**4.94**	
3	20	1.30	46.22	53.8	**5.09**	
4	30	1.48	40.89	59.1	**5.23**	14.14
5	40	1.60	33.78	66.2	**5.42**	
6	50	1.70	26.22	73.8	**5.64**	
7	60	1.78	16.45	83.5	**5.98**	

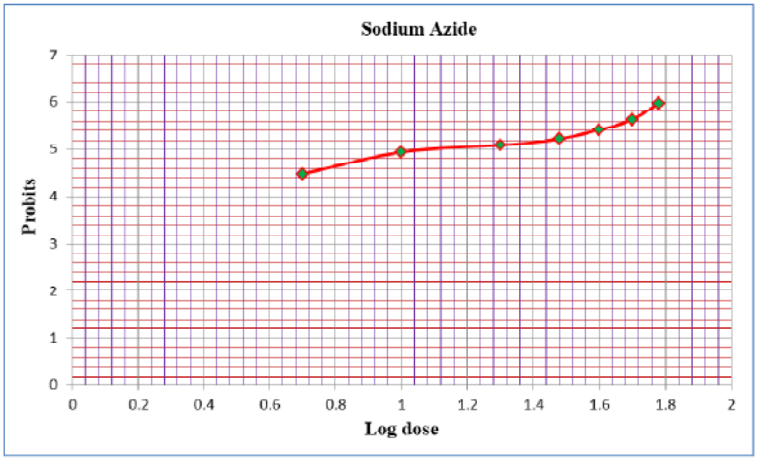

Fig. 2. Plots of Log doses versus probits from Table 2 for calculation of LD50 of Sodium Azide.

Impact of EMS and SA on germination and survival percentage. The responsiveness of MDU 1 barnyard millet to EMS and Sodium Azide treatments with respect to germination was observed (Table 3). The germination percentage tended to decline with the increased concentration and it exhibited a dose-dependent negative linear relationship. The germination percentage of MDU 1 under different dose concentrations was calculated based on the germinated seeds after treatment and compared with control (non-treated). In this study, the highest germination and survival percentage was recorded by the treatment 10 mM for both the chemical mutagen. The germination percentage values ranged from 57.06 (10 mM) to 29.24 per cent (50 mM) for EMS and 56.46 (10 mM) to 28.22 per cent (50 mM) for Sodium Azide. Survival of plants at maturity ranged from 52.42 (10 mM) to 26.68 per cent (50 mM) for EMS and 51.48 (10 mM) to 24.51 per cent (50 mM) for Sodium Azide. Mutagens had a significant effect on all of the treatments. This might be owing to the influence of mutagens on seed merismatic tissues (Ramya *et al.*, 2014; Ramesh *et al.*, 2019). This depicts the influence of EMS and Sodium Azide's mutagenic effect on germination. The increased activity of free radicals prevents seed germination, resulting in seed death. Furthermore, SA is known to block the enzyme-amylase, resulting in hormonal imbalance and germination failure (Dewi *et al.*, 2016). The survival of seeds was gradually reduced with an increase in the dose concentration of EMS and SA. The similar results were observed in rice (Vasline, 2013; Harding *et al.*, 2012).

Impact of EMS and SA on shoot length, root length and plant height on 30 DAS. The biological effects of mutagens are measured using seedling growth as an index. This may disrupt the energy flow in plants, resulting in mitotic inhibition, which contributes to seedling growth depression (Kleinhofs *et al.*, 1978). When compared to EMS, Sodium Azide treatments exhibited a greater reduction in shoot and root length (Table 3 and Fig. 3). All treatments of both mutagens exhibited significant differences in shoot and root length decreases over control. Shoot length showed a reduction in both EMS and SA as 92.70 per cent to 53.10 per cent and 87.40 per cent to 28.96 per cent, respectively. Likewise, reduction in root length ranged from 89.55 per cent to 53.06 per cent for EMS and 94.33 per cent to 31.66 per cent for Sodium Azide. Plant height reduction due to EMS treatment ranged from 90.52 per cent (10 mM) to 68.12 per cent (50 mM) and for Sodium Azide treatment ranged from 84.52 per cent (10 mM) to 64.80 per cent (50 mM), respectively. This revealed that all EMS and Sodium Azide treatments results in a progressive decline in plant height when compared to the control, and that this occurred in a linear and dose-dependent way (Tabasum *et al.*, 2011).

Impact of EMS and SA on pollen fertility and seed fertility. Several environmental factors affect panicle fertility, however, mutagens have been found as major contributors to increasing the amount of sterile florets (Awan *et al.*, 1980). The mutagen impact persisted during their reproductive period as well. Pollen and spikelet sterility were increasing in higher concentrations, as expected (Akilan *et al.*, 2019). The pollen fertility values were 89.23 per cent (10 mM) at the lower concentration and 45.03 per cent at higher concentration (50 mM) for EMS (Table 3 and Fig. 3). Similarly, the value for Sodium Azide was 91.09 per cent (10 mM) for lower and 48.80 per cent (50 mM) (Table 3 and Fig. 4) higher concentration respectively. Mutagens were shown to be effective in either reducing the mitotic index or increasing the frequency of micronuclei and pollen abnormalities. The results obtained are consistent with previous findings that SA treatments cause a significant degree of sterility in Barley in the M_1 generation (Mustafa, 1976). SA does not cause chromosomal aberrations in barley, but it does contribute to sterility by causing gene changes that result in gametic or zygotic lethality. Similar findings were also reported in blackgram (Surender and Vanniarajan, 2014). In most situations, meiotic anomalies are responsible for decreased pollen fertility in chickpea.

Table 3: Impact of Ethyl Methane Sulphonate and Sodium Azide on biological parameters of MDU 1 in M_1 generation.

Treatment	Germination (%) over control	Shoot length (%) over control	Root length (%) over control	Plant height on 30th DAS (%) over control	Survival of plants at maturity (%) over control	Pollen fertility over control (%)	Seed fertility over control (%)
Ethyl Methane Sulphonate							
10 mM	57.06	92.70	89.55	90.52	52.42	89.23	96.68
20 mM	48.65	84.18	85.07	83.70	43.70	81.05	90.04
30 mM	45.75	68.91	74.62	78.02	41.76	68.77	86.06
40 mM	40.55	59.86	61.35	72.56	37.53	60.71	83.18
50 mM	29.24	53.10	53.06	68.12	26.68	45.03	76.54
Sodium Azide							
10 mM	56.46	87.40	94.33	84.52	51.48	91.09	93.65
20 mM	49.75	75.71	83.33	80.36	46.22	83.24	89.12
30 mM	44.01	65.32	68.33	72.89	36.59	72.96	81.78
40 mM	36.36	48.05	37.6	69.74	33.66	55.65	75.74
50 mM	28.22	28.96	31.66	64.80	24.51	48.80	72.93

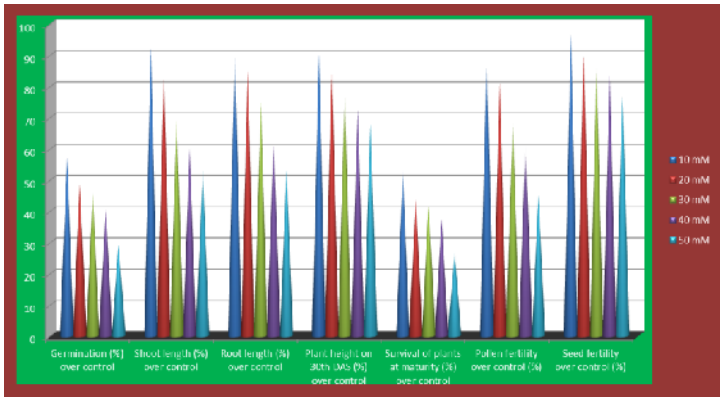

Fig. 3. Effect of different doses of EMS on germination, survival percentage at maturity, shoot length, root length, plant height at 30th DAS, pollen fertility and seed fertility of MDU 1.

Effective mutagens and treatments are required for the cost-effective application of the mutagen as a tool for mutation induction and direct/indirect use in a successful breeding programme. The LD_{50} dose is the ideal dosage for mutagenizing mutagens with the least amount of injury and the greatest number of viable mutants. This study revealed the actual injury to biological material caused by chemical mutagen (EMS and SA). Comparing EMS and Sodium Azide, the overall injury was less in EMS than in Sodium Azide. Optimum doses of mutagens are essential to induce desirable variable mutations. It is inferred from the present study that MDU 1 barnyard millet is responding well to chemical mutagens especially for EMS.

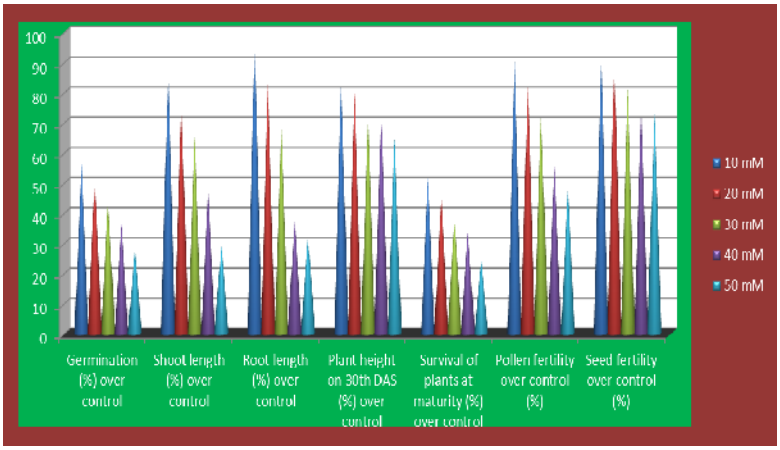

Fig. 4. Effect of different doses of Sodium Azide on Germination, survival percentage at maturity, shoot length, root length, plant height at 30th DAS, pollen fertility and seed fertility of MDU 1.

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