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Determination of Lethal Dose (LD_{50}) and Mutagenic Effects of Ethyl Methane Sulfonate (EMS) on Germination and Survival of Seedlings in *Stevia rebaudiana* Bertoni

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Abstract

Stevia rebaudiana Bertoni belongs to Asteraceae family and is known for production of sweet glycosides, an alternative to sugar. The impact of Ethyl Methane Sulfonate (EMS) in developing new stevia mutants was carried out at Department of Horticulture University of Agricultural Sciences, GKVK, Bengaluru. Seeds of stevia cv. CIM-Madhu were collected and exposed at different durations of 1, 3 & 6 hrat 0.1 per cent, 0.2 per cent, 0.4 per cent, 0.6 per cent, 0.8 per cent and 1.0 per cent EMS concentrations to determine the LD_{50} value. Germination percentage of non-mutagenized seeds was maximum (70%) which was followed by 0.1 per cent concentration of EMS with 68 per cent germination and 2.86 per cent reduction over the control. Survival percentage was 93 per cent in control which was followed by 85 per cent at 0.1 per cent of EMS. Exposure of non-mutagenized seeds took minimum days for germination. However, as the EMS concentration increases, days taken for germination tend to get longer. Treatment with a 1.0 per cent EMS concentration exhibits the most pronounced delay in germination. LD_{50} a dose that causes 50 per cent mortality to the seeds was found at 0.8 per cent EMS concentration. After ascertaining LD_{50} value in both the treatments, mutation was induced to create variability for morphological and other desirable traits.

Keywords : Ethyl methane sulfonate, Lethal dose (LD_{50}), Stevia, Germination & Survival

Stevia (*Stevia rebaudiana* Bertoni) belongs to the family Asteraceae and is native to Paraguay and South-West Brazil. It is also called as sweet leaf, sugar leaf, sweet honey leaf, rebiana, sweet herb and methitulsi. The plant got global attention due to the extreme sweet taste of the leaves and aqueous extracts. This unique sweetness is due to diterpene glycosides namely Stevioside, Rebaudioside A, B, C, D, M and six other compounds which are having insulin balancing properties (Kumar *et al*., 2014).

China stands as a world leader in stevia production and supply. The stevia being cultivated in Malysia, Paraguay, Kenya, United States, Vietnam, Brazil, India, Argentina and Columbia. In India, Madhya Pradesh, Punjab, Andhra Pradesh, Karnataka, Chhattisgarh and Maharashtra are the major stevia growing states (Annonymous, 2023). The cultivation of stevia in India is hampered because it produces flowers at an early stage under Indian photoperiod condition, thus leading to poor leaf yield. Additionally, meager breeding work has been done in developing suitable cultivars having photo insensitive characters.

Assessment of genetic variability is the first step in any crop improvement programme and it can either be created through hybridization or induced mutation. Induced mutagenesis is a formidable tool for generating a lot of inherent genetic variation to develop new high yielding varieties. Mutation

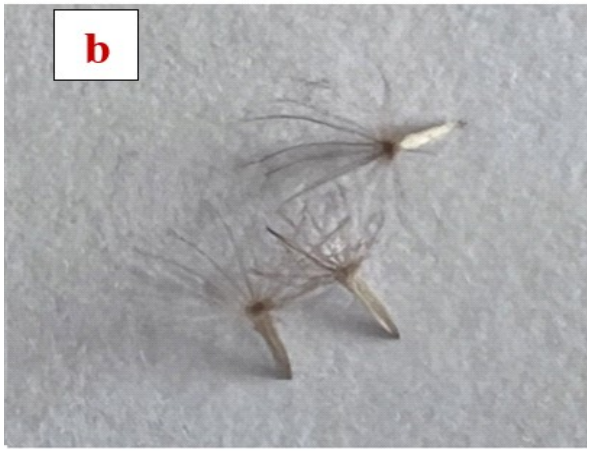
breeding is one of the approaches to create variability through novel recombination's using both chemical and physical mutagens (Smitha *et al*., 2022). Mutation can occur spontaneously or resulting from exposure to radiation or chemicals. Mutation studies in many of the crops showed that it is quite useful for induction of variability and development of cultivars with improved traits. (Alka *et al.,* 2013 and Mogali *et al*., 2016).

Mutagenic agent like EMS has been widely used for the development of assorted traits of crops but the success of mutation depends on its dose applied. Usually, mutagen treatments scale back seed germination, rate of growth, vigour and fertility. There's substantial killing of plants throughout completely different stages of development, so significantly reduces the survival of ensuing plants. The dose needed for prime agent potency depends on properties of the mutagenic agents and material treated (Jayashree *et al*., 2022). Hence, an overdose may kill too many treated individuals and lesser dose can turn out fewer mutations. The optimum dose can turn out the high frequency of mutations and cause minimum killing that varies with crop species and agent used (Badere *et al*., 2007). Therefore, determination of the LD_{50} (Lethal Dose), a dose that causes 50 per cent mortality to the seeds is critical. The LD_{50} is completely different between species and varieties in a species (Aney, 2013). Therefore, this study was carried out to determine the LD_{50} of stevia cultivar CIM-Madhu with EMS and its effect on seed germination and survival of seedlings.

Material and Methods

The study was carried out at Department of Horticulture, University of Agricultural Sciences, GKVK, Bengaluru during 2021-2022. Seeds of stevia cv. CIM-Madhu were treated with different concentrations of EMS with varied duration of exposure. Based on the color of the seeds, viable (dark colored**)** and non-viable (pale or clear colored) seeds (Plate 1) were separated manually and 85 viable seeds were used for each treatment with 3 replications. To treat with EMS, the stock solution of 5 per cent EMS was prepared along with 2 per cent Dimethyl sulfoxide (DMSO) by dissolving them in the required quantity of sterile 0.1M phosphate buffer. All the seeds were cleaned using running tap water before the treatment. The seeds were thoroughly immersed in the EMS solutions of different concentrations at 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 percentage in a dimly lit room and were left in the solution at different duration of exposure for 1, 3 & 6 hours (Table 1). After the treatment, seeds were kept in running tap water for about 15 minutes to remove excess EMS. These treated seeds were then placed on a sterile brown paper to absorb the excess water. The treated and untreated (control-water soaked) seeds were sown in media containing sand, soil and Farm yard manure (FYM) in the ratio 1:2:1 with coir pith on top.

(a) Viable seeds are dark colour



(b) Non- viable seeds are pale colour

Plate 1: Stevia seeds

TABLE 1

Treatment details on induction of Ethyl methane sulfonate (EMS) to stevia seeds

Sl. No.	Concentration of EMS	Duration of exposure
T_1	Control- Water treated Non-mutagenized - CIM-Madhu	1 hr, 3 hr and 6 hr
T_2	EMS @ 0.1%	1 hr, 3 hr and 6 hr
T_3	EMS @ 0.2%	1 hr, 3 hr and 6 hr
T_4	EMS @ 0.4%	1 hr, 3 hr and 6 hr
T_5	EMS @ 0.6%	1 hr, 3 hr and 6 hr
T_6	EMS @ 0.8%	1 hr, 3 hr and 6 hr
T_7	EMS @ 1.0 %	1 hr, 3 hr and 6 hr

Observation on germination percentage, days taken for germination and survival percentage were recorded and the LD_{50} was determined by plotting a simple regression graph of seedling survival percentage against EMS concentrations.

Statistical Analysis

The mean values of germination percentage, days taken for germination and survival percentage, of seeds in each replication were used for Fisher's method of analysis of variance (ANOVA). The analysis of variance for individual character was carried out using the percentage values of replications following the method given by Panse and Sukhatme (1967). The significance of the differences among all the treated lines was tested by F-test using the error variance. The complete data was analysed using completely randomized design (CRD).

RESULTS AND DISCUSSION

Germination Percentage

Germination of stevia seeds was non-significant with 1 hour and 3 hours of dip of EMS (Table 2 & 3). At 6 hour of dip with different concentrations of EMS, significant variations in germination percentage was observed (Table 4). Out of 85 seeds sown, 60 seeds were germinated with 70 per cent of germination in non-mutagenizedseeds (control), which is followed by 0.1 per cent concentration of

TABLE 2

Effect of EMS treatment for 1 hour on germination percentage of stevia

Treatment	No. of seeds germinated out of 85 seeds sown	Germination %	Per cent reduction over control
T_1 - Control	58	68	0.00
T_2 - 0.1 % EMS	57	67	1.47
T_3 - 0.2 % EMS	55	65	4.41
T_4 - 0.4 % EMS	53	62	8.82
T_5 - 0.6 % EMS	57	67	1.47
T_6 - 0.8 % EMS	58	68	0.00
T_7 - 1.0 % EMS	53	62	13.24
S. Em. ±	-	-	-
C.D.	NS	NS	-

TABLE 3

Influence of EMS treatment for 3 hour on germination percentage in stevia

Treatment	No. of seeds germinated out of 85 seeds sown	Germination %	Per cent reduction over control
T_1 - Control	55	65	0.00
T_2 - 0.1 % EMS	54	64	1.54
T_3 - 0.2 % EMS	51	60	7.69
T_4 - 0.4 % EMS	48	56	13.85
T_5 - 0.6 % EMS	49	58	10.77
T_6 - 0.8 % EMS	53	63	24.62
T_7 - 1.0 % EMS	48	51	21.54
S. Em. ±	-	-	-
C.D.	NS	NS	-

TABLE 4

Effect of EMS treatment for 6 hour on germination percentage in stevia

Treatment	No. of seeds germinated out of 85 seeds sown	Germination %	Per cent reduction over control
T_1 - Control	60	70	0.00
T_2 - 0.1 % EMS	58	68	2.86
T_3 - 0.2 % EMS	53	62	11.43
T_4 - 0.4 % EMS	46	54	22.86
T_5 - 0.6 % EMS	42	49	30.00
T_6 - 0.8 % EMS	38	45	35.71
T_7 - 1.0 % EMS	33	39	44.29
S. Em. ±	3.46	4.06	-
C.D.	10.62	12.44	-

EMS with 68 per cent germination and 2.86 per cent reduction over the control in which 58 seeds germinated out of 85 sown. The most significant reduction in germination was observed with EMS at 1.0 per cent concentration, resulting in a germination per cent of 39 and representing a 44.29 per cent reduction over the control. This reduction in germination percentage was consistent with increasing EMS concentrations.

It may be due to EMS tends to decrease germination rates in seeds as it causes mutations in the genetic material of the seeds, particularly in the DNA. These mutations can disrupt the normal genetic processes involved in seed germination. This disruption may affect various processes, including cell division, hormone regulation and metabolic pathways that are essential for germination. When concentration of EMS increases, the likelihood of inducing multiple mutations in seeds also rises. These multiple mutations can result in a higher percentage of seeds with severe abnormalities, leading to reduction in germination. Sometimes mutations induced by EMS can be lethal or severely detrimental to the development of the seed. Seeds with such mutations may not be able to complete the germination process, resulting in reduced germination. All these may be possible reasons for reduction in germination percentage with increasing EMS concentrations. Similar results were observed by Zuraine *et al.,* 2019 in stevia and Tawfik *et al.,* 2021 in *Cassia occidentalis*.

Days taken for Germination

The effects of EMS on the days taken for germination is presented in Table 5. At 1 hr and 3 hr of dip effect of EMS on days taken for germination was found to be non-significant. Water soaked seeds for 6 hours took minimum days (10.80) for germination. However, as the EMS concentration increases, days taken for germination tend to get longer. Treatment with a 1.0 per cent EMS concentration exhibits the most pronounced delay in germination, with times of 22.10 days, for 6 hours of exposure. These results indicate that higher concentrations of EMS and longer exposure durations are associated with a considerable extension in the time required for seed germination. Similar results were obtained by Barman *et al*., 2015 in Jamun. This delay in germination is due to EMS induced mutations, potentially alters the crucial genes governing germination. The genetic changes introduced by EMS can impede the efficiency of cellular processes vital for germination, resulting in a slower progression through germination stages thus leading to extended germination period.

TABLE 5

Influence of EMS on days taken for germination of stevia seeds

Treatment	Days taken for germination		
	1 hr	3 hr	6 hr
T_1 - Control	13.68	11.52	10.8
T_2 - 0.1 % EMS	14.96	16.44	17.6
T_3 - 0.2 % EMS	13.91	15.4	16.5
T_4 - 0.4 % EMS	14.98	17.2	19.98
T_5 - 0.6 % EMS	13.1	18.4	20.5
T_6 - 0.8 % EMS	14.18	17.89	21.98
T_7 - 1.0 % EMS	15.98	17.28	22.21
S.Em. ±	-	-	1.361
C.D. @ 5%	NS	NS	4.169

Seedling Survival Percentage

The impact of EMS on the seedling survival percentage of stevia following a 6-hour exposure at different concentrations was found significant (Table 6). The Non-mutagenized seeds (control) exhibited maximum survival percentage of 93.00 which is followed by 0.1 per cent concentrations of EMS with 85 per cent survival and 8.60 per cent reduction over the control. While, treatments with increasing EMS concentrations ranging from 0.1 per cent to 1.0 per cent showed progressively greater reductions in seedling survival percentage with the highest concentration of 1.0 per cent of EMS leading to a 54.84 per cent reduction over the control and survival of 42 per cent. These findings suggest that as the EMS concentration increases,

TABLE 6
Effect of EMS on seedling survival percentage in stevia

Treatment	Survival %					
	1 hr	Per cent reduction over control	3 hr	Per cent reduction over control	6 hr	Per cent reduction over control
T_1 - Control	92	0.00	90	0.00	93	0.00
T_2 - 0.1% EMS	88	4.35	89	1.11	85	8.60
T_3 - 0.2% EMS	85	7.61	85	5.56	79	15.05
T_4 - 0.4% EMS	87	5.43	88	2.22	68	26.88
T_5 - 0.6% EMS	90	2.17	84	6.67	55	40.86
T_6 - 0.8% EMS	85	7.61	86	4.44	48	48.39
T_7 - 1.0% EMS	87	5.43	83	7.78	42	54.84
S.Em. ±	-	-	-	-	5.01	-
C.D. @ 5%	NS	-	NS	-	15.35	-

the adverse impact on stevia seedling survival becomes more pronounced.

The decreased survival percentage of seedlings treated with EMS is likely due to the fact that EMS induce mutations that are harmful or detrimental to the normal functioning of the plant and these mutations can disrupt essential biological processes, such as growth, development and metabolism. Some mutations may affect critical genes involved in the survival of the seedlings, leading to reduced survivability. Similar results were found by Purente *et al*., 2020 in Chrysanthemum.

Stevia seeds were exposed with different concentration of EMS at 0.1 per cent, 0.2 per cent, 0.4 per cent, 0.6 per cent, 0.8 per cent and 1.0 per cent at different durations *viz*., 1, 3 & 6 hr. Among the 3 durations of exposure, significant results were obtained at 6 hrs of exposure with different concentration of EMS for germination percentage, days taken for germination and survival percentage (Fig. 1). Further to determine LD_{50} value 6 hours of exposure with different concentration of EMS was used.

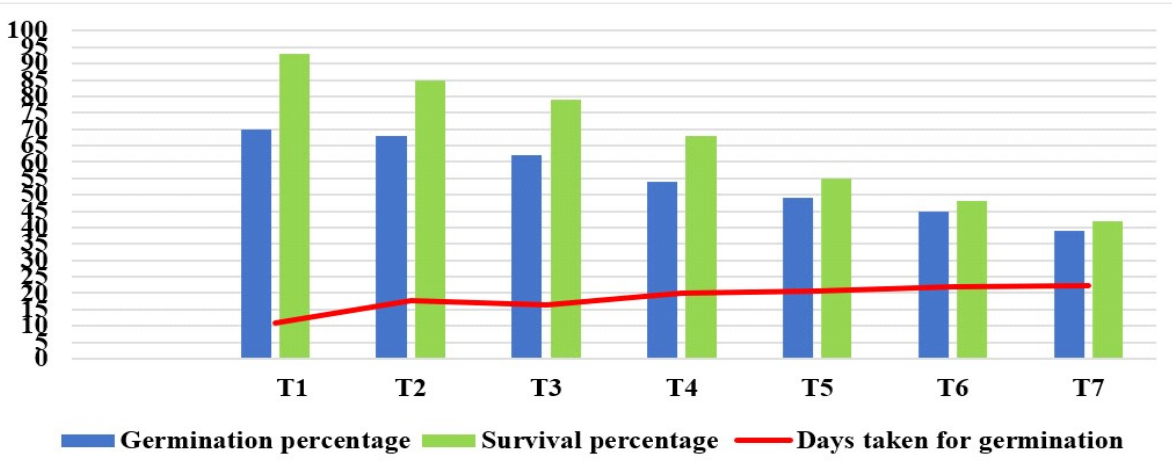


Fig. 1 : Effect of EMS on germination, days taken for germination and survival percentage in *Stevia rebaudiana* Bertoni at 6 hours of dip

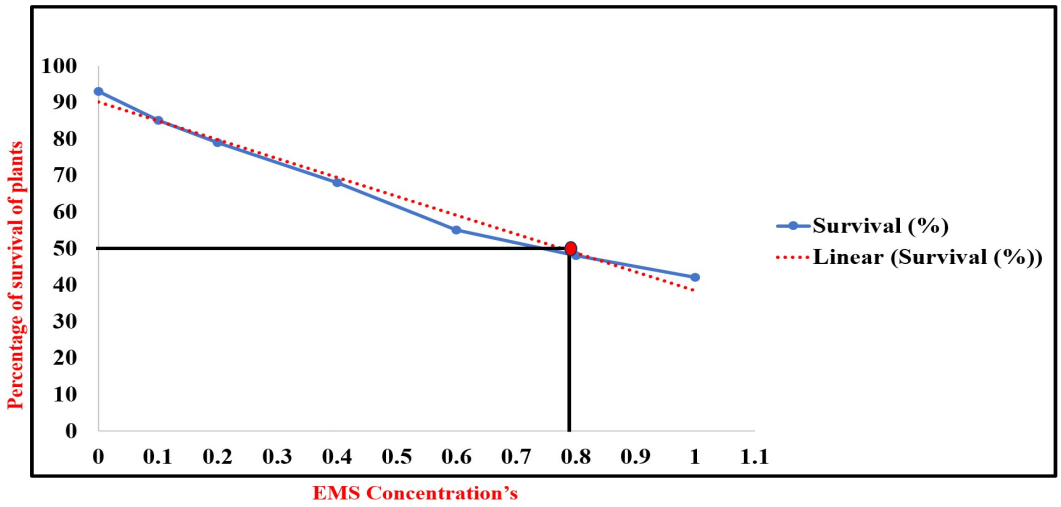


Fig. 2 : Plot of log doses of EMS vs survival percentage of plants to determine LD_{50}

Determination of LD_{50}

The determination of LD_{50} value for any mutagen is essential to produce maximum viable mutants with minimum damage to the plant. The LD_{50}value was determined by plotting a simple regression graph of seedling survival percentage against EMS concentrations (Fig. 2). As the EMS concentration increases, significant reduction in seedling survival was noticed. LD_{50} a dose that causes 50 per cent mortality to the seeds was found to be at 0.8 per cent of EMS concentrationfor stevia cv. CIM-Madhu. Similar results were obtained by Yadav and Krishna (2013) in cumin.

To develop M_1 populations of stevia cv. CIM-Madhu, determination of LD_{50}, germination and survival percentages are crucial parameters. There are limited studies on determination of LD_{50} for EMS in stevia using seeds, so the findings of this study on LD_{50} could be used as reference for initiating mutation breeding in other cultivars of stevia and also for improvement of specific traits by mutation breeding. It can be concluded from findings of present study that the increased concentration of EMS has resulted in minimum germination and survival percentage as compared to the control plants.

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