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Impact of the Chemical Mutagen Ethylmethane Sulfonate (EMS) on the Reproductive Characteristics of Clusterbean Plants (*Cyamopsis tetragonoloba* Linn.)

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ABSTRACT: The plants of clusterbean were planted using either the seed soaked on ethyl methane sulfonate (EMS) or water over 6 h and cultivated under varying salinity levels (0, 4, 8 and 12 dS m^{-1}) with the non-saline controls. It was observed in terms of percent germination of the seed, time to reach the final germination, morphological changes in the arrangement and shape of the leaves, number of clusters of flowers on the plant, number of flowers on the plant, pollen germination and viability, number of pods on the plant, number of seeds on the plant, and weight of the 100 seeds. The results revealed that EMS-treated seeds experienced reduced seed germination and plant height as well as slowed seed germination, pollen germination (in vitro) and pollen tube length under non-saline conditions. EMS treatment generated mutants that had bountiful growth in vegetation at salinity of 8 and 12 dS m^{-1}. It was also able to cause morphological changes of stigma, which shifted between capitative stigma and subapical stigma, and crescent stigma, both in saline and non-saline conditions. Trichomes also covered these plants in certain instances. Plants germinated under non-saline conditions on EMS-treated seeds produced fewer pods and their weight of 100 seeds reduced. Nevertheless, mutants which grow under salinity level of 12 dS m^{-1} recorded more flowers, pods, seed yield per plant and test weight of 100 seeds than did their counterparts which grow on untreated seeds. Such results indicate that mutagenesis caused by EMS could lead to the emergence of clusterbean genotype that has a better tolerance to saline conditions.

Keywords: EMS, clusterbean, mutants, salinity, pollen.

INTRODUCTION

Soil salinity is one of the key abiotic stressors that influence crop production as it is among the most critical limiting factors that reduce the productivity and distribution of crop plants (Li *et al.*, 2022) Seldom is any climatic zone unconditionally devoid of salinization; the problem is however mostly related to arid and semi arid lands. Salinity of soil is a big agricultural challenge in the state of Haryana and has been impacting over 320, 000 hectares of land which is roughly 10 percent of the agricultural land in the state. This issue has grown serious over the last several years and a 35-80 percent growth in saline impacted lands in the last 20 years. The FAO Global Map of Salt-Affected Soils (GSAmap) expands 118 countries and gives salinity estimates of about 424 million hectares of topsoil (0-30 cm) and 833 million hectares of subsoil (30-100 cm). Salinization of soil has thus become a significant ecological and agricultural issue across the world and has been rated as one of the most common causes of land degradation and desertification (Ding *et al.*, 2011). Most recent estimates have shown that over 6 percent of the world land is affected by soil salinity, with approximately 19.5 percent of the irrigated agricultural land and 2.1 percent rain-fed agricultural land being affected. The salinity action of loss of cultivable land is in direct opposition to the rising food demand of the world population which is estimated to be around 1.5 billion people by the year 2030. A number of mechanical and biochemical methods have been devised to salvage saline soils but most of these are expensive and can hardly be applied to large scale basis. Saline soils have a low soil fertility, poor plant growth and poor crop yield due to the unusual biological and physicochemical characteristics of the soils. The effects are less fertility in the soil, lower yield of crops, waste of farming resources, ecological instability, and the presence of secondary environmental risks (Wang *et al.,* 2021; Wu *et al.*, 2018; Li *et al.,* 2014). Restraints of Traditional Breeding and Somaclonal Variation to Salt Tolerance. The other more sustainable solution to the issue of soil salinity is the breeding of crops whose salinity level is more resistant. The tolerance to salinity on the other hand is a multifaceted polygenic character which takes into consideration numerous physiological and biochemical pathways, hence making its enhancement in the traditional breeding method complicated and time-intensive (Epstein *et al.,* 1980). In addition to that, most of the crops that are of the Fabaceae family are characterized by low genetic variation within the genotypes that are available to them thus limiting the success of breeding programs that are traditional based. In vitro culture of tissues has been as an alternative measure to enhance genetic variation. Such methods can also produce somaclonal changes, which might cause enhanced abiotic stress tolerance like salinity (Kirti *et al.,* 1991; Lutts *et al.,* 1999). Nevertheless, somaclonal variation is not always effectively utilized, because it is not easy to regenerate complete plants with the use of a selected cell line, especially with leguminous crops. Consequently, there is a need to apply alternative methods that have the capacity to foster broader genetic diversity to enhance salinity tolerance in these crops. Reasoning of EMS Mutagenesis in Clusterbean and Objectives of the Study. Mutation breeding using chemical mutagens is one potentially useful approach to the creation of new genetic variability (Unan *et al.,* 2021). The concept of mutation breeding has emerged as a powerful method of improving crops especially the crops with a small genetic base (Acharya *et al.,* 2007). The ethyl methane sulfonate (EMS) is one of the commonest chemical mutagens that is extensively applied as an alkylating agent since it creates point mutations by inserting alkyl groups on nucleotides, leading to base substitutions and genetic diversity (Sabetta *et al.,* 2011; Arisha *et al*., 2014; Gillmor and Lukowitz 2020). EMS is a very powerful mutagen since it causes a high percentage of point mutations and relatively low percentages of chromosomal aberration (Hajra, 1979; Van Harten, 1998). EMS mutagenesis has been able to generate useful mutants in various crops resulting in increase in yield, quality, and tolerance to stress. An example can be given of mutants that were produced with higher yield and content of ascorbic acid in Capsicum annuum as a result of mutagen treatment (Pillai and Abraham 1996). On the same note, Basu *et al.* (2008) got fenugreek mutants with early maturation, high seed yield, seed quality improvement, and determinate growth habit after treatment with EMS. EMS mutagenesis has also been applied in improving seed quality characteristics like protein and oil content in peanut (Wang *et al.,* 2007). Moreover, EMS-mutated calli have been used to derive NaCl-tolerant sweet potato lines (Luan *et al.,* 2007), and EMS-mutated mutants of sugar cane have also been made by subjecting the calli to NaCl stress in vitro after EMS treatment and in vitro selection (Mallikarjun *et al.,* 2008). Clusterbean (*Cyamopsis tetragonoloba* L.) is a useful multipurpose legume crop that is drought-tolerant and has wide applications in production of fodder, vegetables, green manuring, and grain production. Its productivity is also negatively influenced by the salinity of the soil, even when it is adapted to dry conditions. Based on these positive accomplishments and the necessity of attaining salinity-tolerant genotypes, the current research has been carried out with an aim of studying the possibility of enhancing salinity tolerance of clusterbean using EMS-induced mutagenesis and to determine the growth and yield potential of EMS-treated clusterbean plants at varying salinity conditions.

MATERIALS AND METHODS

Seeds of clusterbean (*Cyamopsis tetragonoloba* L.) cultivar HG 2-20 were obtained from the Forage Section, Department of Genetics and Plant Breeding, CCS Haryana Agricultural University (CCSHAU), Hisar, India. The cultivar HG 2-20 was originally developed by CCSHAU, Hisar and was used in the present study as the experimental material.

A total of approximately 200 seeds were used for the experiment, with equal numbers allocated to control and EMS treatments. Preliminary studies indicated that soaking seeds in 0.5% ethyl methane sulfonate (EMS) solution for 6 h delayed germination but allowed optimal germination under non-saline conditions, whereas longer exposure adversely affected root growth.

Therefore, seeds were soaked in 0.5% EMS solution for 6 h at room temperature. After treatment, the EMS solution was drained and the seeds were thoroughly washed with sterile distilled water for at least 30 min to remove residual mutagen. Seeds soaked in distilled water for the same duration served as the control treatment.

The experiment was conducted in pots arranged in a Completely Randomized Design (CRD). Both control and EMS-treated seeds were grown under four salinity levels: 0 (non-saline control), 4, 8 and 12 dS m^{-1}.

Each treatment consisted of three replicates, with five pots per treatment and three plants maintained per pot after thinning.

Salinity treatments were imposed using sodium chloride (NaCl) solutions prepared to achieve the desired electrical conductivity (EC) levels. The salinity levels were monitored using a conductivity meter and maintained throughout the experiment by periodic irrigation with the respective NaCl solutions.

The number of days required for completion of germination was recorded for each treatment.

Plant morphological characteristics were recorded prior to the onset of flowering. During the flowering stage, the following observations were recorded:
— Number of floral clusters per plant
— Number of flowers per plant
— Pollen diameter
— Pollen viability, determined using the method of Hauser and Morrison (1964)
— Pollen tube growth
— In vitro pollen germination
— Stigma shape
— Number of ovules per pistil

For in vitro pollen germination, a semi-solid medium containing 35% sucrose, 300 ppm calcium nitrate, 100 ppm boric acid, and 0.8% agar-agar was prepared.

Fresh pollen grains were inoculated onto the medium in Petri plates and incubated at 30°C for 4 h in the dark. After incubation, pollen germination percentage and pollen tube growth were recorded under a microscope.

Yield and Yield-Related Parameters

At maturity, the following yield-related parameters were recorded:
— Number of pods per plant
— Number of seeds per plant
— Pod length
— 100-seed weight (test weight)
— Seed yield per plant

All experimental data were subjected to statistical analysis using analysis of variance, a significance level of $P \leq 0.05$. Statistical analysis was performed to determine the significance of differences among treatments for all measured parameters.

RESULTS AND DISCUSSION

Soaking of the guar seeds in 0.5% EMS for 6 h reduced germination compared with that of the control. Seed germination was also delayed (Table 1) under both saline and nonsaline conditions. Although salinity decreased the germination of EMS-treated seeds, germination was greater at 4 dS m^{-1} salinity than at other salinity levels under non-saline conditions. A similar reduction and delay in EMS-treated seeds has been reported (Gohal *et al.,* 1970). Sharma (1965) also reported decreased germination in sorghum seeds soaked in 0.5% EMS for 6 h. Reduction in the seed germination may be due to the effect of mutagenesis on meristematic tissues of the radicle or plumule. An increase in the percentage of germination was observed in both treatment *i.e.* 0.05% and 0.10% EMS Concentration (Mahamune, 2021). This increase of germination after treating with mutagen may be attributed to various physiological changes. Interestingly, EMS treatment significantly reduced plant height under non-saline conditions and 4dS m^{-1} salinity, but some plants (mutants) raised under higher salinities of 8 and 12 dS m^{-1} salinity had plant heights and leaf areas comparable to those of the control, and all these plants were fertile (Fig. 1). The seedling height decreased with respect to increase in the mutagenic concentration. And the maximum decrease in height was observed in the 0.15% treatment (Mahamune, 2021). (Guar plants normally produce two cotyledonary leaves, 3 simple leaves and three trifoliate leaves. EMS treatment disrupted this sequence. Some of the mutants produced two simple leaves followed by a trifoliate leaf, which was again followed by a simple leaf and finally, trifoliate leaves. Under saline conditions, a similar sequence of leaf production was evident. In one of the mutants raised under 12dS m^{-1} salinity, four simple leaves were produced before the trifoliate leaves were produced. Another mutant even produced a tetrafoliate leaf. In addition, thickening, broadening, shortening and crinkling of leaves were also evident. These morphological variations in leaves are indicators of effective mutagen treatment. EMS-mediated changes in the leaf arrangement and shape have been reported in Capsicum annuum (Jabeen and Mirza 2004).

Ethylmethane sulphonate or salinity had no significant effect on the initiation of flowering, but flowering was completed early in EMS-treated plants raised under nonsaline conditions and at relatively low salinity (4dSm^{-1}). Guar plants generally produce 3-5 clusters of flowers, while treating seeds with EMS reduced the number of clusters to 2-3 under nonsaline and saline conditions. The number of floral buds and flowers per plant decreased drastically in the plants raised from EMS treated plants. It decreased with increasing level of salinity in plants raised from treated and untreated seeds; the reduction was greater in the later cases. The percentage of floral buds transformed into flowers was greater in plants raised from untreated seeds under saline conditions than in those raised from EMS-treated seeds under both saline and nonsaline conditions. The pollen grains were 37.44 µm in diameter and 95.76% viable. Neither of these parameters was significantly affected by salinity or EMS treatment. However, Sanders *et al.* (1999) observed non dehiscing, late dehiscing and pollenless mutants of *Arabidopsis thaliana* following EMS treatment. Tube growth and in vitro pollen germination decreased in EMS-treated plants raised under saline or nonsaline conditions; the reduction was more pronounced in the pollen grains of plants from EMS-treated seeds than in those from untreated seeds (Table 2). Interestingly, EMS treatment induced changes in the shape of sigma phase. It changed. The shape of the plants ranged from capitate to subapical, crescent shaped and well-developed (Fig. 2). These strains were functionally defective because they did not support pollen germination; hence, a reduction in yield in both saline and nonsaline conditions was evident. Komaki *et al*. (1988) also observed aberrant structures of stigmas in Fl82 and Fl89 mutants of *Arabidopsis thaliana*. The stigma had two clumps of papillae and horn-shaped projections.

The number of seeds and number of pods per plant and the test weight of 100- seed decreased in the plants raised from the EMS-treated plants compared with those raised from the untreated plants under nonsaline conditions. Salinity adversely affected these parameters; the difference was more pronounced in the untreated plants than in the treated plants. Seed yield/plant decreased with salinity in untreated seeds. Seed treatment with EMS led to a significant reduction in seed yield under nonsaline conditions, while an increase in yield was evident in plants treated with 12dSm^{-1} compared with untreated plants (Table 3). The percent pod set remained unchanged up to 8 dSm^{-1} salinity in plants raised from untreated seeds and then decreased significantly at 12dSm^{-1}. The percent pod set was relatively greater in the plants raised from EMS-treated seeds than in those raised from untreated seeds, and the value was greatest at 12dSm^{-1}. Percentage of pollen sterility demonstrated a steady increase within each treatment with increasing concentrations of the mutagen. At highest concentration of EMS, maximum sterility was observed. (Mahamune, 2021). Pod length decreased in plants raised from EMS-treated seeds under both saline and nonsaline conditions, and the difference was greater in the former than in the latter. Seed set and seed and pod characteristics were not significantly affected by EMS treatment, which was also shown by Gohal *et al.* (1970). However, in Luhua 11 cv. of peanut, EMS produced both longer and shorter pod mutants (Wang *et al.,* 2007). It also increased the protein and oil content of the seeds. Francois *et al.* (1990) reported that the seed yield of two cvs. of guar was not affected by salinity levels up to 8.8 dSm$^{-1,}$ and each unit increase in salinity above this reduced yield was 17%. These authors further showed that a reduction in the number of pods/plant and seed weight are the primary factors contributing to reduced yield. However, such yield reductions in cowpea resulted from a decrease in the number of pods (West and Francois 1982).

It is thus evident that EMS treatment inhibited plant growth and yield parameters under nonsaline conditions. However, this treatment improved the plant height and leaf area of plants raised under 8 and 12 days of Sm^{-1} salinity. The number of flowers and pods and the seed yield per plant and the test weight of 100 seeds were greater for the mutants growing under 12dSm^{-1} salinity than for their corresponding counterparts from untreated seeds. These increases in yield parameters under saline conditions were definitely lower than those under the nonsaline untreated control, but EMS treatment offers a new avenue for harvesting relatively better yields under relatively high salinity conditions.

Table 1: Effect of seed treatment with EMS (0.5%) on seed germination (%) and days required for completion of germination in guars raised under saline conditions.

Parameters	Untreated seeds				Treated seeds			
	C	4	8	12	C	4	8	12 dSm^{-1}
Seed Germination (%)	100	87.4	87.6	79.3	84.47	89.5	50.67	49.33
Days Required to complete Germination	5	5	5.2	5.7	9	9	11	11

Table 2: Effect of seed treatment with EMS (0.5%) on the pollen germination (%) and tube length (µm) of guar under saline conditions.

Parameters	Untreated seeds				Treated seeds				CD at 5% LS
	C	4	8	12	C	4	8	12	
Pollen germination	89.92	88.45	85.19	74.4	82.15	80.48	66.79	35.75	7.32
Tube length	503.9	469.33	393.65	309.76	388.8	486.3	347.9	232	31.7

Table 3: Effect of seed treatment with EMS (0.5%) on different reproductive characteristics of guar plants raised under saline conditions.

Parameters		Untreated seeds				Treated seeds			
	C	4	8	12	C	4	8	12 dSm^{-1}	CD at 5%
No. of floral buds/plant	47.4	41.3	29.8	18.3	27.2	36	35	36.2	5.1
No. of flowers/plant	38.1	35.8	24.7	16.2	18.3	23.6	24.1	22.2	3.9
Buds transformed into flower (%)	80.4	86.7	89.6	88.5	67.3	58.3	68.8	58.6	
No. of ovules/pistils	8.6	9.2	7.6	7.2	7.5	7.6	7.2	7.2	0.8
No. of pods/plant	19.2	18.4	13.6	6.2	10.8	13.4	14	14.2	3.4
Pod set (%)	50.4	51.4	50.9	38.3	59	56.8	55.6	63.9	
Length of pods (cm)	5.82	5.84	5.12	4.87	3.77	4.79	4.6	5.4	0.7
No. of seeds/pods	7.3	7.22	6.89	5.36	7.08	7.39	6.87	6.97	0.3
Seed yield/plant(g)	4.12	3.74	2.8	1.51	1.91	2.39	2.67	2.15	0.2
Test weight of seeds (g)	2.94	2.81	2.66	2.45	2.5	2.89	2.98	2.85	0.2

Fig. 1. Effect of soaking in 0.5% EMS for 6 h on the growth of plants raised under $4dSm^{-1}$ (B), $8dSm^{-1}$ (E) and $12dSm^{-1}$ (F) salinity conditions. A represents the nonsaline control.

Fig. 2. Effect of seed soaking in 0.5% EMS for 6 h on the shape of the stigma in guars.

CONCLUSION

Treatment of guar (*Cyamopsis tetragonoloba*) seeds with the mutagen Ethyl Methane Sulfonate reduced and delayed germination and decreased seedling height, indicating inhibitory effects on early growth. EMS treatment also induced several morphological variations such as altered leaf sequence, tetrafoliate leaves, and changes in leaf shape, confirming effective mutagenesis.

Reproductive traits such as number of flower clusters, buds, and pods were reduced in EMS-treated plants, and pollen germination was also affected. Consequently, yield parameters like pods per plant, seeds per plant, and 100-seed weight declined under non-saline conditions.

However, some EMS-induced mutants showed relatively better growth and yield performance under higher salinity levels (8 and 12 dS m^{-1}). Thus, although EMS generally inhibited growth under normal conditions, it generated useful genetic variability and may help in developing guar lines with improved tolerance to saline environments.

FUTURE SCOPE OF STUDY

Evidence in the current paper showed that ethyl methane sulfonate (EMS) treatment resulted in morphological and reproductive differences in clusterbean and generated mutants with relatively improved growth and yielding performance at elevated salinity levels (8 and 12 dS m^{-1}). Even though EMS treatment decreased the germination, plant height, and yield parameters under non-saline conditions, some of the mutants exhibited better vegetative growth, leaf formation, and comparatively high yield parameters under saline conditions, thus showing that EMS mutagenesis has potential in the development of salinity tolerant genotypes. Further investigations should be based on the identification and stabilization of promising EMS-induced mutants that will work better in saline environments. These mutants can be tested over several generations (M2, M3 and further on) to determine the stability and inheritability of the desirable traits. It also needs more researchers to understand the physiologic and biochemical processes of the salinity tolerance of the selected mutants, such as the accumulation of ions, osmotic adjustment and antioxidant activity. Molecular and genomic research can also be done in order to determine the genes related with the salinity tolerance caused by EMS mutagenesis. Moreover, field trials in multi-location with different salinity conditions are to be conducted to test the agronomic performance of potential mutants and yield stability. These studies will be used to establish their appropriateness in breeding programs that will produce salinity-tolerant clusterbean varieties. In general, the results of the given study show that the mutagenesis induced by EMS may prove to be a useful instrument in increasing the genetic variability and in creating clusterbean varieties that would yield decent crops in saline soil environment.

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