



GENERATION OF ETHYL METHANESULPHONATE (EMS) INDUCED MUTANT POPULATION OF *SOLANUM LYCOPERSICUM* CV. ARKA VIKAS

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ABSTRACT: Tomato is considered as a model system for Solanaceous plants because of its small genome size and availability of genomic resources. Understanding the pathways controlling different physiological processes and identification of the genes involved in regulating these pathways in tomato (*Solanum lycopersicum*) is a challenging task requiring several genetic resources and functional genomic analyses using mutants. Chemical mutagens like ethyl methanesulphonate (EMS) which brings about single nucleotide changes, are widely used for developing mutant populations. Here we have generated a comprehensive mutant population of tomato in Indian cultivar Arka Vikas. The EMS mutant population consisting of about 3,800 M₂ plants was visually phenotyped by using a Personal Digital Assistant (PDA). Digital recording of all the phenotypic variations were classified into 15 major categories and 48 sub categories based on SOL database, "The Genes That Make Tomatoes". The above mutant population is an excellent resource for both forward as well as reverse genetic studies. Generation of a tomato mutant population with allelic series would aid to functional genomics of plant growth and development.

Key Words: *Solanum lycopersicum* cv. Arka Vikas, Ethyl methanesulphonate (EMS), Mutants, Personal Digital Assistant (PDA).

INTRODUCTION

The cultivated tomato (*Solanum lycopersicum* L.), is one of the globally important crop for fresh market and food processing industry. It is also the second most consumed vegetable after potato (<http://faostatfao.org>). Tomato is rich source of several essential and beneficial nutrients in the human diet, such as antioxidants, vitamins and minerals [1]. Tomato is a representative model of Solanaceae, by virtue of its moderate size of diploid genome (950 Mb, n = 12), the same haploid chromosome number and high level of gene synteny with other Solanaceous plants [2- 4]. Tomato contains numerous genomic resources such as high density molecular maps, abundant collections of germplasm and mutants, highly efficient transformation protocols [5-8]. The fleshy berry-type fruits of tomato also makes it an excellent model for investigating fruit development, fruit ripening as well as metabolite analysis of sugar metabolism and carotenoid biosynthesis [9-15]. For these reasons tomato has been selected as a model plant for genomic studies in the Solanaceae family, and its genome sequencing has been completed (Tomato Genome Consortium, 2012). The number of tomato genes has been estimated to range from 30,000 to 40,000, and for several of these no function has been assigned [16]. Currently efforts are being made to elucidate the role of these uncharacterized genes in regulation of signaling pathways controlling the plant development particularly fruit ripening. The function of any uncharacterized gene can be best analyzed using mutants. A collection of nearly 1000 monogenic mutants of tomato in different backgrounds including spontaneous as well as induced mutants has been extensively used by researchers for functional genomics (<http://tgrc.ucdavis.edu>). In addition, more than 3000 mutants have been catalogued in the Solanaceae Genome Network in the name of "The Genes That Make Tomatoes" (<http://www.sgn.cornell.edu>). Recently, new tomato mutant collections were generated in different genetic backgrounds of tomato such as Micro-Tom, and cv Red Setter, [17-19].

Traditionally mutants have been used directly or indirectly to develop new crop varieties. Mutants are more acceptable for consumers than genetically modified organism (GMO). Mutants can also be used for gene discovery, controlling important traits and understanding the functions and mechanisms of the genes. Mutant population with a high density of mutations is an excellent resource for developing new varieties. The development of induced mutagenized resources are attempted by chemical (e.g., EMS), physical (e.g., X-ray or fast-neutron irradiation), or insertional mutagenesis (e.g., transposable elements or T-DNA) [18]. Generally, physical mutagens such as X-rays are less preferred for induced mutagenesis because they induce chromosomal breakages leading to larger deletions generating truncated genes and lethal alleles [21]. Among these ethyl methanesulfonate (EMS) is an effective and widely used chemical mutagen to induce point mutations [22]. It can generate sublethal and substerile alleles, thus an EMS generated mutant population is best suited for identifying more useful alleles of a specific gene of interest. Considering the need of mutants for basic and applied research, a public resource is required. In this study the large scale generation of tomato mutants was carried out to address this issue. In near future the demand for such mutants will increase to assign functions to the uncharacterized gene sequences that tomato genome sequencing project have generated.

Keeping this in view, we developed the EMS induced mutant resource and a systematic effort was made to evaluate the morphological effects of EMS on tomato (*cv. Arka Vikas*), to broaden its genetic base for selection of desirable genotypes for cultivation.

MATERIALS AND METHODS

Ethyl methanesulfonate (EMS) mutagenesis:

An Indian cultivar *Solanum lycopersicum cv. Arka Vikas* seeds, provided by the Indian Institute of Horticulture Research (IIHR), were subjected to ethyl methanesulfonate (EMS) mutagenesis. Batches of ~1000 seeds (M_0 seeds) were soaked in distilled water for 24 h at room temperature. After removing excess water, seeds were submerged in freshly prepared 500 ml solution of EMS (Sigma-Aldrich, St. Louis) at a concentration of 60 mM (0.75% w/v) for 24 h in dark with gentle shaking at $25\pm 2^\circ\text{C}$ [23]. The mutagenized seeds (M_1) were placed in muslin cloth bag and extensively washed under running tap water for 8 h. The M_1 seeds were sown in nursery bed containing red loam sandy soil prepared in the open field conditions. A batch of 100 seeds were used as a control and processed through the same procedures as mentioned above without EMS treatment. Two weeks after sowing, the percentage of seed germination and chimeric plants was observed to determine the effectiveness of mutagen affecting those events. Three weeks old M_1 seedlings were transplanted into open field and allowed to self-pollinate to produce M_2 seeds along with control plants. At the end of the fruit ripening stage, fruits were harvested and M_2 seeds were collected from individual M_1 plants to generate the M_2 families. The M_2 seeds were catalogued and stored at -20°C .

Phenotypic characterization of M_2 population:

About 10 seeds from each M_2 line were sown in germination trays filled with soilrite mix. Three weeks after germination, four individual plants (named as A, B, C and C) from each M_2 line were transplanted to the open field. Each M_2 plant was tagged with unique plant-ID barcode label. The M_2 plants were visually phenotyped according to 15 major categories and 48 sub categories defined previously by Menda et al., [6] by using a hand held Personal Digital Assistant (PDA). It has an inbuilt barcode laser scanner to identify individual plants using barcode labels with customized software, PHENOME, developed for large scale phenotypic data collection [24]. The observed phenotypic variations were captured using a digital camera (OLYMPUS CAMEDIA C-7070 wide zoom). All the recorded data from PDA and images were transferred to a Master-PC and analyzed to check the efficiency of EMS mutagenesis on different developmental stages of tomato.

RESULTS

Frequency of germination, plant survival and fertility rate in M_1 population:

Tomato *cv. Arka Vikas* is a fresh market variety and completes its life cycle within 140 days. It is also a highly productive variety with 35-40 tons/hectare yield. Based on the earlier reports of the Lethal Dose (LD) values and saturation of mutation in tomato, we used 60 mM (0.75% w/v) EMS concentration to mutagenize tomato. Since in seed propagated crops, mutagenic treatment of seeds is considered as the standard method, the seeds of *cv. Arka Vikas* were used as the starting material (M_0) for mutagenesis. Before generating the mutant population, we estimated the frequency of seed germination (Lethal Dose), plants survival and fertility rate in M_1 population. In this study, ~2,500 seeds were treated with 60 mM EMS and from these only 1,500 seeds germinated, showing 40% reduction in germination (LD_{40}) with respect to untreated control seeds (95%). Out of the germinated seedlings, 1,245 (83%) plants survived and produced M_2 seeds (Table 1). Several chimeric plants with yellow green or white patches on leaves were observed at seedling stage and at later stages of the M_1 generation which, directly indicates the effectiveness of mutagen. (Figure 1A-B).

Phenotypic evaluation of M₂ population:

For phenotype cataloguing and M₃ seed production, 1,245 M₂ families were grown with four plants from each line. Two weeks after germination appearance of albino and chlorotic seedlings indicated the efficiency of mutagen. Each M₂ plant was examined for visible phenotypic alterations at all developmental stages from seed germination to fruit ripening by using an electronic hand-held device PDA. The phenotypes of mutants were classified into 15 major categories and 48 sub categories. The frequency of mutant type was estimated as number of mutants observed out of total plants scored. The identified mutations were grouped on the basis of the trait affected. The percentage distributions of phenotypic variations are shown in pie diagram (Figure 2). A total of 158 (4.15%) plants showing morphological alterations, the most commonly observed phenotypes are related to the plant habit (0.65%), plant size (0.47%), leaf morphology (0.47%), fruit morphology (0.44%), leaf colour (0.39%) and the fruit colour (0.26%). Few plants showed some striking phenotypes like a wiry leaf, needle like leaf tip, leafy inflorescence, malformed fruits, beaked fruits, striped fruits and abnormal flowers (Figure 3A-G). One plant with huge number of fruits was also observed which may prove economically useful to the farmers (Figure 3H); further analysis of this plant is under progress. Another mutant plant showed drastically increased height and profuse branching (Figure 3I). Finally 3,800 M₂ plants survived and four different series of (named as A, B, C and D) M₃ seeds were obtained from individual M₂ plants.

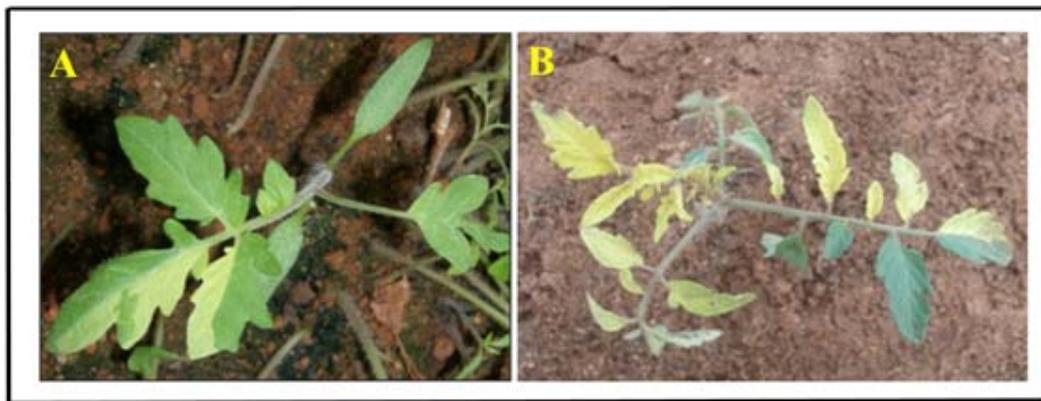


Figure 1: Examples of different chimeric plants observed in M₁ generation A: 3 weeks old seedling with yellow patches on leaves, B: Chimeric plant at later stages.

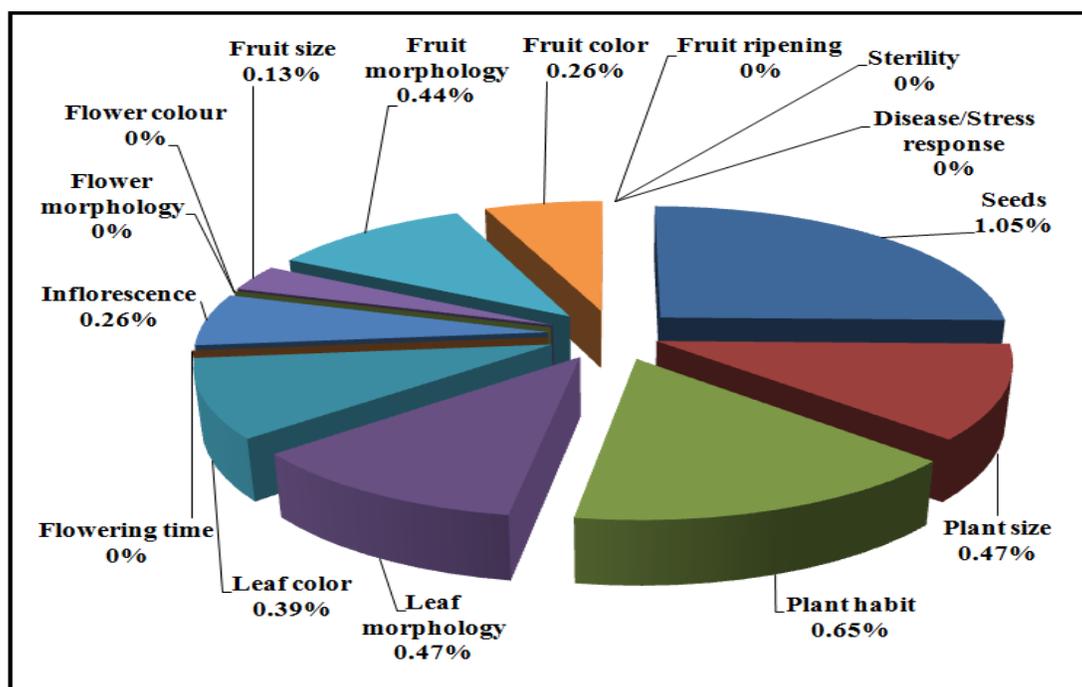


Figure 2: Classification of visible phenotypic variations observed in M₂ population. The variations were classified as 15 major categories.

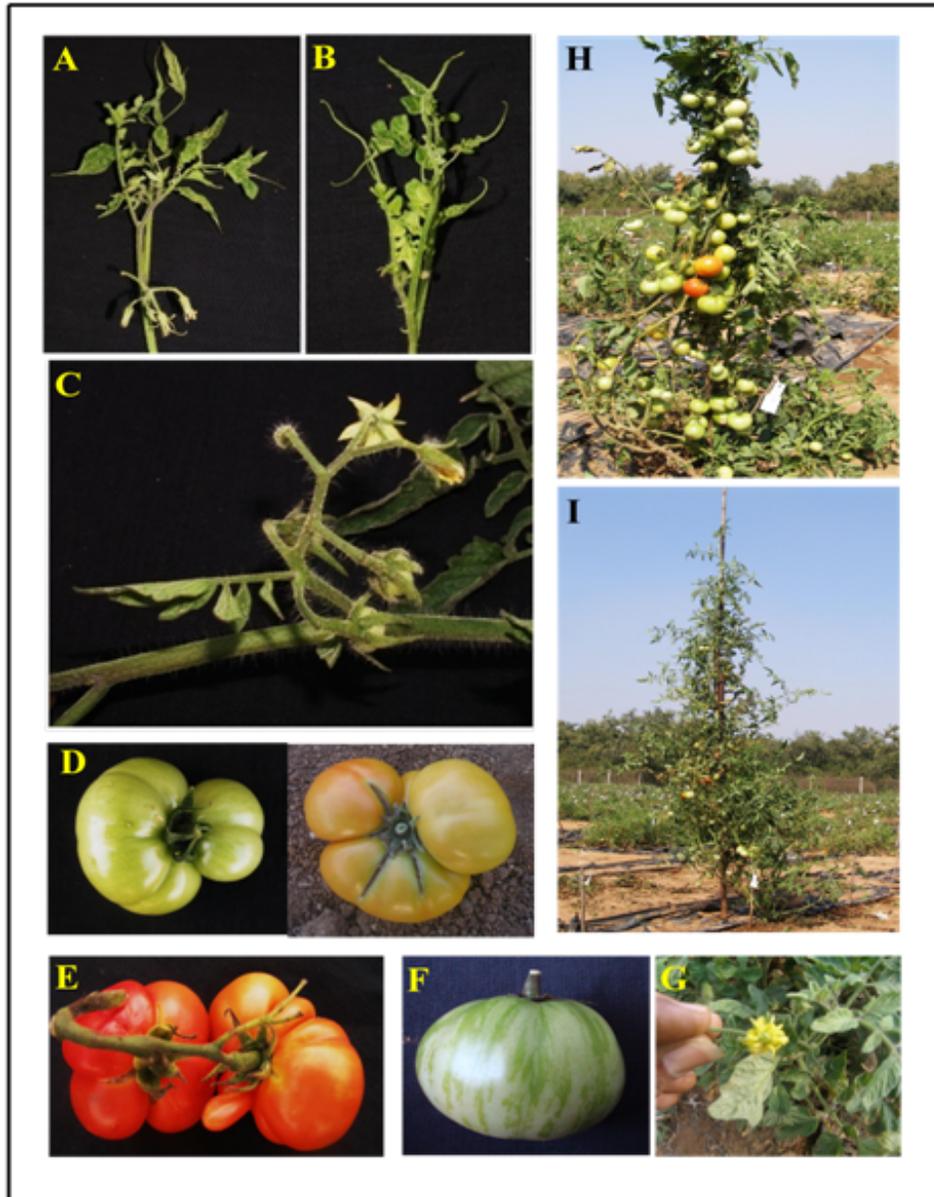


Figure 3: Examples of tomato mutant phenotypes observed in M_2 population A: Wierly leaf; B: Needle like leaf tip; C: Leafy inflorescence; D: Malformed fruit shape; E: Beaked fruit; F: Striped fruit; G: Abnormal flower; H: High yielding plant and I: Tall plant.

Table1: Effect of EMS treatment on seed germination, plant survival and fertility of plants in M_1 generation.

Month and Year	Cultivation condition	EMS Concentration/ Duration of treatment (hours)	Number of seeds treated	Number of seeds germinated (M1 seedling) (%)	Lethal Dose (LD)	Fertility of M1 plants (%)	Number of M2 lines (fertile)
Aug 2010	Open field	Distilled water, 24 hours	100	95.0	Control	90	---
Aug 2010	Open field	60 mM, 24 hours	~2,500	1500	40%	1,245 (83%)	3,800

DISCUSSION

EMS has been successfully used to introduce random single base changes in the genome of various organisms [25]. In the present study, we used 60 mM concentration of EMS to develop mutant population to mutagenize cultivar Arka Vikas tomato seeds based on the previous reports of the Lethal Dose (LD) values and saturation of mutation in tomato [6]. The inhibitory effect of chemical mutagen (EMS) on seed germination was noted for further studies. It was noted that 60 mM EMS lead to 40% (LD₄₀) reduction in seed germination compared to untreated control seeds (95%). The fertility of M₁ plants was found to be 83% with respect to control plants (90%). A strict correlation was observed between the EMS dose and the toxicity, the mutation density obtained and frequency of phenotypic alterations. Several groups have worked on such dose dependent inhibition of seed germination and survival of seedlings of tomato. Menda et al., [6] reported that the LDs in the *S. lycopersicum* cv. M82 were 15 and 90 when 0.5 and 1% EMS concentrations were used for mutagenesis, respectively. Watanabe et al., [17] observed LDs of 10, 20, and 57% when Micro-Tom seeds were treated with 0.3, 0.5, or 1.0% of EMS and finally 0.5% of EMS was chosen to develop the mutant population. They reported that the frequency of M₁ seedlings decreased with increasing EMS concentration. Minoia et al., [19] reported that Red Setter tomato 1.0% EMS treatment with LD₄₉ was more efficient to develop mutant population than 0.7% EMS treatment with LD₂₀. Saito et al., [20] used different EMS concentrations to develop the Micro-Tom mutant population and 1.0% of EMS with (LD₆₃) treatment represented the most efficient. While, the LD of Micro-Tom seedlings at 1.0% EMS was lower than that of cv. M82 [6], but higher than cv. Red Setter [19]. The LD observed in our population was higher than 0.7% and lower than that of 1% EMS treated cv. Red Setter and 1% EMS treated Micro-Tom [19,17].

In the present study, chimeric plants with yellow or white patches were observed in the M₁ generation which indicates the mutagen affected multiple cells in the embryo. The occurrence of mutation in a homozygous condition in some of these cells lead to yellow/white patches. In M₂ generation two different types of chlorophyll mutations were found viz., chlorina and xantha which indicate the preferential induction of certain type of mutations. Observation of chlorophyll mutations is considered one of the indicators for the assessment of the effectiveness of mutagens [26, 27]. The observation of chlorophyll mutations of physical and chemical mutagens has been reported in tomato [17, 18], sunflower [28], Phaseolus [29], blackgram [30] and chilli [27].

In brief 3,800 M₂ plants were obtained from EMS mutagenesis and phenotypic variations were digitally recorded from M₂ plants and classified into 15 major categories and 48 sub categories based on SOL database, "The Genes That Make Tomatoes". A total of 158 (4.15%) M₂ plants showing morphological variations were chosen as a mutant candidate based on primary screening. The frequency of phenotypic variations observed in our population varies compared to the 0.5% EMS treated population of cv. M-82 [15], 0.5% and 1% EMS treated population of Micro-Tom [28, 21] and 1% EMS treated population of cv. Red Setter [16], where they observed 42.5%, 10%, 19% and 29.8% respectively. This differences may be due to the effect of varietal difference on mutation induction. Based on the detailed spectrum and analysis of frequency of chlorophyll and viable mutants observed in M₂ generation, it is now well known that mutagenic effectiveness and efficiency depends not only on the type of mutagen and its dose, but also on the genetic architecture of an organism.

CONCLUSIONS

Natural genetic diversity often offers only fewer opportunities for exploitation and development of new varieties in any crop plant. Generation and characterization of mutation is the direct way to understand the complex physiological processes and genes involved in pathways. Development of an indexed collection of mutants for tomato is highly useful in elucidating the function of more genes. In present study, we have developed genetic resource of an Indian cultivar Arka Vikas by using EMS mutagenesis and this mutant resource will be highly useful in dissecting the mechanism underlying mutant phenotype. In addition above mutagenized population would also serve as a resource for high throughput reverse genetic studies to screen for point mutations in specific regions of targeted genes.

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