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Determination of LD_{50} dose for ethyl methane sulphonate induced mutagenesis in Bhagwa Pomegranate

Mandeep Rawat, V. P. Singh, S. K. Verma, Ratna Rai, Ranjan Srivastava

Abstract

Mutation breeding utilizes both chemical and physical mutagens for inducing variability among existing genotypes. In mutation breeding, the estimation of LD_{50} is the initial step to finding the optimal dose to determine the best dose to produce a better revival of mutants with little population diminution. Pomegranate is a valued fruit and has immense nutraceutical and pharmaceutical properties. Its hardy nature, low maintenance, wider adaptability, high yield with better keeping quality, and better remuneration make it the choicest fruit to be grown in non-traditional areas in Uttarakhand. Pomegranate cv. Bhagwa is a high yielding variety, soft seeded, and have attractive aril colour. In the current experiment, mutagenesis was performed on hardwood cuttings of 'Bhagwa' pomegranate using alkylating agent Ethyl methane sulphonate (EMS). A total of 7 different treatments ranging from 25 mM to 150 mM were tested against control for their efficiency in mutant generation. The results revealed that there is a gradual decrease in the survival percentage of cuttings with increasing doses of mutagenic treatments. Based on the survival percentage of the treated cuttings, the probit curve analysis determined that the LD50 dosage of EMS for hardwood cuttings for Bhagwa pomegranate was 51.82 mM. Furthermore, reduction in chlorophyll content was observed with the application of higher doses of EMS.

Keywords chemical mutagenesis, ethyl methane sulphonate, LD_{50}, mutation breeding

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Authors:
M. Rawat, V. P. Singh ✉, R. Rai, R. Srivastava
Department of Horticulture, G. B Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

S. K. Verma
Department Genetics and Plant Breeding, G. B Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

✉ hortvp@gmail.com

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Introduction

Pomegranate (*Punica granatum* L.) was earlier categorized as a member of the family Punicaceae but recently reclassified under the family Lythraceae [1] has two species *P. protopunica* and *P. granatum* [2]. The crop improvement work is mainly focused to *P. granatum* due to the commercial preference as its fruits are more delicious to eat, good in taste, have high juice content and fruits are attractive and bigger. It is an important arid zone fruit crop and is well adapted to diverse climatic conditions in several countries. Pomegranate fruit is highly nutritive and every part of this plant contains various beneficial bioactive compounds. Bark, leaves, flowers, and fruits of pomegranate have antimicrobial properties, are helpful in reducing blood pressure, and are effective

against diabetes and cancer [3]. Fruits of the pomegranate can be eaten fresh or processed to make syrup, dessert, jelly, anardana etc. [4]. Not much success has been achieved in the genetic enhancement of pomegranate through conventional breeding methods due to several difficulties like long juvenile period, polygenic traits, and intricate genetic system [5]. Also, the lack of knowledge about the type and extent of genetic variability and the relationships between important traits would make it difficult to design an efficient breeding strategy for its genetic improvement [6]. Over the years, numerous crop plants have successfully undergone mutation breeding to create genetic diversity and breed new kinds. Mutation breeding has been considered as one of the reliable sources to create genetic variation, providing unique germplasm and the starting point for plant breeders [7]. However, the prevalence of occurring natural mutation is extremely less and sometimes difficult to detect. Artificially induced mutation can be carried out using physical (α-rays, β-rays, γ-rays, fast moving neutrons, UV-rays etc.) and chemical (EMS, methyl methane sulphonate, sulphur mustard etc.) mutagens. In contrast to ionizing radiation, which typically causes chromosomal rearrangements and deletions, chemicals primarily cause point mutations. Among these chemical mutagens, EMS is one of the most powerful and often used chemical mutagens to cause point mutations [8]. EMS alkylates guanine bases and causes mispairing of alkylated guanine (G) pairs with thymine (T) in place of cytosine (C), leading to transitions that are predominantly between G/C- and -A/T [9]. Furthermore, an EMS developed mutant population is ideally suited for identifying more advantageous alleles of a particular gene of interest since it can produce sub-lethal and sub-sterile variants.

The current investigation was taken up as the initial phase for the mutation breeding in 'Bhagwa' pomegranate which is cultivated in many parts of India for its superior quality fruits. Further, this crop is now being grown in non-traditional areas as in the plains of Uttarakhand due to its high value and fancy nature [10]. Bhagwa is a popular, high yielding with quality fruit and is being cultivated mainly in the state of Maharashtra, Gujarat, and Karnataka. In north India, cultivation of Bhagwa is more or less is relatively recent and is adversely affected by poor orchard management, and many biotic and abiotic stresses [11]. Therefore, a mutation breeding strategy was adopted to obtain some useful mutants which are having suitability for Uttarakhand plains. The present study was executed to determine the LD_{50} of EMS and to eventually obtain some desirable mutants of pomegranate cv. Bhagwa.

Methodology

In the present investigation one year old hardwood cuttings of pomegranate cv. Bhagwa was treated with six doses of EMS (25 mM, 50 mM, 75 mM, 100 mM, 125 mM, and 150 mM) tested against the control. A freshly prepared solution of EMS was used for treatment and post-treatment of sodium hypochloride as a quick dip was given in order to remove the traces of EMS. The cuttings were taken from the Bhagwa mother plants block maintained at Horticulture Research Centre, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand. Cuttings were pre-soaked for one hour and then dipped in EMS solution for 8 hours and 16 hours. After dipping, these hardwood cuttings were washed for one hour in running tap water in order to remove traces of EMS. After the treatment, cuttings were planted in root trainers containing growing media consisting of soil: vermicompost : sand in a ratio of 1:1:1. The percentage survival, leaf area and total chlorophyll content were estimated at eight weeks after sprouting of cuttings. The experiment was organized in a Randomized Block Design and replicated thrice. Using a probit analysis and comparing the survival of treated cuttings to control cuttings, the LD_{50} dosage was determined. Leaf area was measured by using the petiole plant leaf area meter app version 2.0.1. The leaf chlorophyll content was measured by the method suggested by Hiscox and Israelstam [12]. After taking the initial observations, the plants were looked after for distinct analysis.

Probit analysis

The LD_{50} value for EMS was calculated based on the probit analysis [13]. The probit function is the reciprocal of the cumulative distribution function (CDF) or quantile function linked with the standard normal distribution.

Analysis of variance

To determine the LD50, data were analyzed using the analysis of variance approach using R-studio 4.2.3 software. The LD50 was estimated through the simple linear regression model by fitting the straight line equation

$$y = a + bx$$

Where, y is the response variable (percent survival) and x is the independent variable (irradiation dose), while 'a' and 'b' represent the slope and constant, respectively.

Results and Discussion

Worldwide, mutation techniques have made a substantial contribution to plant improvement and significantly improved the productivity and economic worth of various crops [14]. However, the effectiveness of these techniques differs significantly according to the genetic makeup of the material, its physiological state, and that of its organs, as well as how the material was handled both, before and after the mutagenic treatment [15-16]. In view of the above, the results of the study carried out to determine the LD_{50} dose of Bhagwa pomegranate was presented here under.

Determination of LD50 dose for hardwood cuttings

The results obtained with EMS treatments showed a gradual decrease in the survivability of cuttings with the increase in doses of mutagenic agents as previously mentioned by many researchers [17-19]. The LD50 for EMS was calculated on the basis of the survival percentage of treated cuttings in contrast with that of untreated cuttings (Table 1). The highest percentage of survival was recorded in control i.e. 96.29 percent and the least was obtained with 150 mM treatment i.e. 27.78 percent (Table 1). There was a significant reduction in the sprouting percentage of hardwood cuttings as the mutagenic dose increased as shown in Table 2. In this case, the LD_{50} value for EMS was obtained at 51.82 mM (Figure 1). In general, lower and moderate doses of EMS gave hardwood cuttings a better chance of surviving than the higher dosages. The two different dipping timings were taken i.e. at 8 hours and at 16 hours just to determine the suitable timings. It was observed that none of the cutting survived at dipping time of 16 hours, suggesting that treatment should be given for 8 hours in the case of hardwood cuttings. However, the survivability of planting material differs with nature, type of propagating material, concentration of mutagen, and the prevailing abiotic conditions [20-22].

Table 1. Effect of EMS on survival percentage of hardwood cuttings of Bhagwa pomegranate

SN.	Treatments	Survival percentage	Per cent survival over control	Per cent reduction over control
1.	T_1 (Control)	96.296	100.00	-
2.	T_2 (25 mM)	60.18	62.50	37.50
3.	T_3 (50 mM)	52.38	54.39	45.60
4.	T_4 (75 mM)	42.86	44.50	55.50
5.	T_5 (100 mM)	32.22	33.46	66.54
6.	T_6 (125 mM)	30.00	31.15	68.85
7.	T_7 (150 mM)	27.78	28.84	71.16
SEd		**4.868**		
CD@ 5%		**15.167**		

Table 2. Probit analysis for determining LD_{50} dose in hardwood cuttings of Bhagwa pomegranate

SN.	Treatments	Log_{10} of doses	Survival percentage	Mortality over control	Empirical probit unit	LD_{50} Dose
1.	T_1 (Control)	-	100.00	-	-	51.82 mM
2.	T_2 (25 mM)	1.39	62.50	37.50	4.67	
3.	T_3 (50 mM)	1.69	54.39	45.60	4.87	
4.	T_4 (75 mM)	1.87	44.50	55.50	5.13	
5.	T_5 (100 mM)	2.00	33.46	66.54	5.41	
6.	T_6 (125 mM)	2.09	31.15	68.85	5.50	
7.	T_7 (150 mM)	2.17	28.84	71.16	5.55	

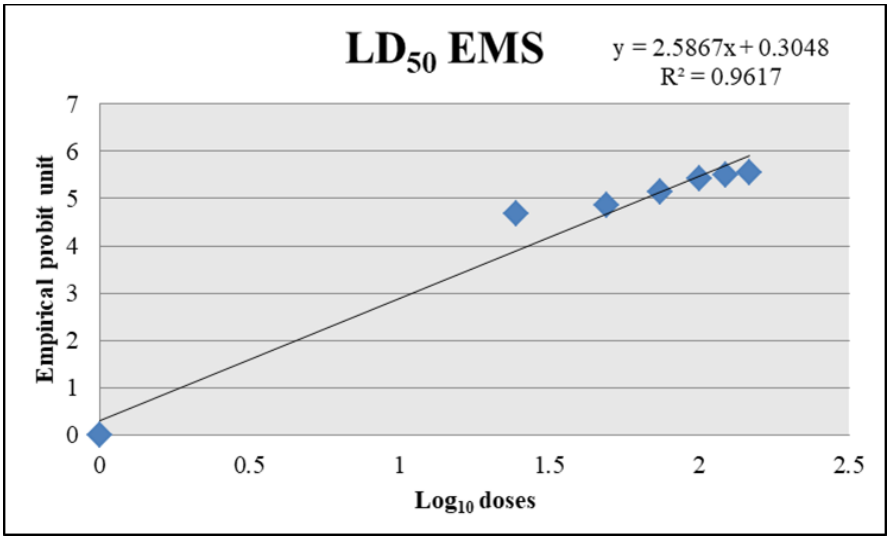

Figure 1. Probit analysis based on the corrected mortality rate of Bhagwa pomegranate

In hardwood cuttings of *Jasminum grandiflorum* for cultivars Arka Arpan, White Pitchi, and ACC Jn-1 for EMS obtained LD_{50} doses 40.7 mM, 37.15 mM, and 42.66 mM, respectively [23]. Further, the LD50 doses of 42.65mM and 44.6mM for two cultivars of jasmine Co1 Pitchi and Co1 Mulla, respectively have been reported by Ghosh and Ganga [24]. Mutagenic sensitivity depends on the source of irradiation, the dose rate, and the plant material [25]. The higher LD_{50} doses in 'Bhagwa' pomegranate as compared to various jasmine cultivars might be because hardwood cuttings of pomegranate are harder than those of jasmine. The reason for low sprouting in the present study on mutagenic treatments may be due to postponement in the initiation of mitosis and chromosomal abnormalities induced activation of enzymes [26].

Impact of EMS doses on leaf area and chlorophyll content of plants

Results obtained regarding leaf area of EMS-induced Bhagwa pomegranate mutants showed significant variation from that of control. The maximum leaf area was recorded with 50 mM mutagenic treatment i.e. 29.60 cm^2 which was significantly higher to that of the control i.e. 23.07 cm^2 (Table 3). The lowest leaf area was noted with the highest mutagenic dose of 150 mM (15.50 cm^2) which was significantly lower than other treatments except T_6 (125 mM). It is further conspicuous from the table that there was no significant difference exists between the control and 25 mM treatment. Further, through statistical analysis, it was found that 75 mM and 100 mM treatments were non-significant with each other. Similarly, decreased leaf area in Kinnow mandarin was earlier documented by Mallick et al., [27] as they noted the reduction of 17.64 percent leaf area over control with 0.5 percent of EMS treatment. In other studies, comparable results were also disclosed by Hearn

Table 3. The effect of different EMS doses on leaf area and chlorophyll content of Bhagwa pomegranate

SN.	Treatments	Leaf area (cm^2)	Chlorophyll a	Chlorophyll b	Total chlorophyll content (mg/g FW)
1.	T_1 (Control)	23.06±0.56^{abc}	4.36±0.01^{a}	0.34±0.05^{b}	4.70±0.05^{a}
2.	T_2 (25 mM)	22.33±0.61^{abc}	2.42±0.16^{b}	0.65±0.15a	3.07±0.03^{b}
3.	T_3 (50 mM)	29.60±3.45^{a}	2.19±0.13^{bc}	0.62±0.09^{a}	2.82±0.04^{c}
4.	T_4 (75 mM)	26.20±6.70^{ab}	2.31±0.06^{bc}	0.33±0.03^{b}	2.64±0.04^{d}
5.	T_5 (100 mM)	26.90±0.65^{ab}	2.27±0.02^{bc}	0.31±0.00^{b}	2.59±0.02^{d}
6.	T_6 (125 mM)	17.70±2.06^{bc}	2.17±0.01^{c}	0.20±0.00^{b}	2.38±0.01^{e}
7.	T_7 (150 mM)	15.50±1.27^{c}	1.88±0.01^{d}	0.24±0.01^{b}	2.13±0.02^{f}
	SEd	**2.785**	**0.070**	**0.060**	**0.029**
	CD@ 5%	**6.136**	**0.155**	**0.312**	**0.065**

[28] in orange and grapefruit, Murthy et al., [29] in mulberry, and Anitha et al., [30] in Bougainvillea cv. Lalbagh and Prabhukumar et al., [31] in *C. inodora*. The reduction in leaf area with higher doses of mutagen might be due to more cellular injury thus resulting to the deterioration of endogenous auxin [32].

In the case of leaf chlorophyll content, it was enviably observed that with increasing doses of EMS, leaf chlorophyll contents were decreased. Maximum total chlorophyll content (4.7±0.05) was observed with control which was followed by 25 mM (3.07±0.03) and 50 mM (2.82±0.04) treatments (Table 3). The lowest total chlorophyll content was observed with 150 mM treatment. While in the case of chlorophyll a and chlorophyll b maximum contents were observed with control (Table 3); however these values were lower than control values which were followed by 25 mM and 75 mM as well as 25 mM and 75 mM treatments, respectively. The presented results of reduction in chlorophyll in EMS-treated plants were found close to the outcomes of Bidabadi et al., [33] in bananas and Kumar et al., [34] in Kinnow mandarin. At higher mutagenic doses, the reduction in photosynthetic pigments may be caused by pigment oxidation and chloroplast damage, which would reduce catabolic and enzymatic activities in leaves [35].

Conclusion

In the present investigation the LD_{50} dose for pomegranate cv. Bhagwa was found to be 51.82 mM. The outcome of the present investigation supported that the influence of EMS altered the leaf physiology. The present findings disclosed that the lower concentration of EMS influenced the leaf attributes to a lesser extent and as the mutagenic doses increased, more drastic changes were observed on cutting survival, leaf area, and chlorophyll content. This suggested that at higher mutagenic dosages cell organelles degrade, which disrupted cellular mechanisms because of free radicals build up in the plant tissues. Furthermore, the scope for a thorough examination of the physiological traits of the desirable mutants obtained in the vegetative phase has increased due to the broad range of variations caused by EMS. Hence, it can be concluded that EMS can develop mutants in pomegranate and these mutants can be used as commercial cultivars after proper agronomic and quality related tests.

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