Determination of LD_{50} of Ethidium Bromide for Induction of Mutation in Marigolds

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Abstract

Mutation is an effective way to increase genetic variability in plant, which can be done in two ways: physical mutation and chemically induced mutation using a chemical mutagen. Ethidium bromide (EtBr) is the most efficient chemical mutagen, inducing mutation in the mitochondria, chloroplasts and nucleus DNA. To increase genetic variance in marigolds, the appropriate concentration level and soaking periods of EtBr must be identified, which was the purpose of this study. Marigold seeds were soaked with 0, 250, 500, 750 and 1000 mg/l of EtBr for 24, 48 and 72 hours. The results showed that the most appropriate concentration of EtBr for inducing mutation in marigolds (LD_{50}) was 623.81 mg/l for 48 hours. However, increasing the concentration level of EtBr decreased the growth of the marigolds, as measured by the height of the tree, and, further, EtBr markedly reduced the survival percentage, especially at high concentration levels.

Keywords: mutagen, genetic variance, lethal dose

Introduction

Ethidium bromide (EtBr) is an intercalation agent commonly used as a fluorescent tag for nucleic acid stains in molecular biology laboratories for techniques such as agarose gel electrophoresis (Ohta, Tokishita, & Yamagata, 2001). EtBr is also a mutagen as it inserts itself between the double strands of DNA and causes deformation (Waring, 1995). EtBr strongly binds to both DNA and RNA, with one molecule of EtBr bound for every 4 or 5 nucleotides (Hsu, 1968). In bacteria, EtBr has been shown to inhibit replication, thereby interfering with both DNA and RNA synthesis (Waring, 1965) and causing frameshift mutation (McCann & Ames, 1976). These effects are presumably due to the interaction of EtBr and genomic DNA, causing errors during DNA replication (Fukunaga, Cox, Sprecken, & Yielding, 1984). In yeast, EtBr causes damage to the mitochondrial DNA and induces petite mutants. Petite mutants often lack mitochondrial DNA or possess it in abnormal amounts or with abnormal density (Goldring, Grossman, Krupnick, Cryer, & Marmur, 1970). EtBr induces petite mutation interference with mitochondrial DNA synthesis and also causes or permits degradation of mitochondrial DNA in yeast (Whittaker, Hammond, & Luha, 1972). Levy and Ashri (1975) found that, in plants, the mutagenic efficiency of EtBr was much higher than that of ethyl methane-sulfonate (EMS), where there were differences in the mutation phenotype between EMS and EtBr. EtBr effected plant size mutation and chlorophyll mutations, while for EMS, the opposite occurred. There were also phenotype differences between the stages treated. Growth mutations, which may be cytoplasmic or nuclear, were induced by both mutagens; EtBr and EMS. Because EtBr has a different chemical mode of action to EMS, it is stable in solution and is a very efficient mutagen.

Mutation breeding has been widely used for improvement of traits of crop plants. The important strategy in mutation breeding is to develop well-adapted plant varieties by altering one or two major agronomic metrical traits. Mutation induction offers significant increases in crop production (Kharkwal & Shu, 2009) and the possibility of inducing desired traits that are not found in nature. The specific purpose of mutation breeding

programs is to increase the genetic variability and develop desired traits in various crop plants. This has been proven to be possible and viable by a number of scientists (Burton & Hanna, 1976; Levy & Ashri, 1975). The essential element of mutation breeding program is the selection of effective and efficient mutagens that produce a high frequency of desirable mutations. Several factors such as the properties of the mutagens, duration of the treatment and concentration levels of the mutagen, and other factors, influence the effect of mutagens. Mutation can be induced in both the seeds, and the vegetative propagated plants, by treatment with a chemical mutagen. The mechanism of mutation induction is that mutagen treatment breaks the DNA in the nuclear, mitochodria and chloroplast during the process of DNA repair mechanism. New mutations occur randomly and are heritable. Mutagen agents have been used to induce useful phenotypic variation in plant, and has successfully used in more than 2543 mutant cultivars from 175 plant species in 50 countries (Chopra, 2005).

Normally, mutagens can reduce seed germination, growth rate, vigour and fertility, so identifying the most effective dose required for high mutagenic efficiency depends on both the properties of the mutagenic agent and the type of plant. An overdose of mutagen might kill many treated plants and an underdose will produce fewer mutant plants. The optimum dose (lethal dose 50%; LD_{50}) will produce a high frequency of mutations and cause minimum mortality. LD_{50} is the optimun concentration, but it varies with crop species and mutagen used (Singh, 2000). This, therefore, is the contribution of our research to plant science: the identification of the suitable dose of LD_{50} of a particular mutagen for maximum effectiveness in inducing mutations in a specific crop.

No information currently exists on the optimum dose of EtBr mutagene for marigold plants, which is the reason that marigolds were selected for the study. The concentration level and soaking periods of EtBr that would result in LD_{50} in marigold plants, and the effect of EtBr on marigold plant growth, were the specific issues determined.

Methods and Materials

Royal Orange marigold seeds from AFM Flower Seeds Thailand Company were used as the study sample. The study was conducted at Rajamangala University of Technology Lanna, Phitsanulok. EtBr was used as the chemical mutagen for induction of mutation. The study was conducted under controlled conditions in the laboratory with three replications, with a control sample of marigold seeds that were treated with distilled water only, for 24 h. In each replication, a sample of twenty marigold seeds was first presoaked in water for 2 h and then treated with EtBr at concentration levels of 250, 500, 750 and 1,000 mg/l, for 24, 48 and 72 h at room temperature. The treated marigold seeds were then washed 3 times under running tap water to remove residual EtBr, and were then placed on planting material which consisted of peat moss and Vermiculite in a ratio of 3 : 1. Water was applied once a day to maintain moisture in this planting material. The number of surviving marigold plants were recorded on the 30^{th} day after planting (DAP). The surviving marigold plants at the 30^{th} DAP were counted for each replication, and the survival percentage of the marigold plant was calculated from the three replications at each level of concentration, and the data was analyzed to determine LD_{50} using Microsoft Excel for calculating the linear regression equation from the survival percentage of the samples, described by Deepak, Kadambari, and Abha (2011).

The effect of the treatment with EtBr on plant growth was also studied. The same set of concentration levels of the EtBr, and the soaking periods, that had been used, and described above, were used in this aspect of our

study. The surviving marigold plants, in each treatment, were grown in a greenhouse under natural light. The stem length of each plant was measured at the 30th DAP, and those measurements were analyzed by One Way Analysis of Variance (ANOVA), using an α of 0.05, and comparing the means of the stem lengths of the plants, in each treatment, was done according to Duncan's method. The data are presented as means and standard error.

Results

1. Effect of concertation level and soaking periods on marigold seed in EtBr on survival percent of marigold plant.

Effect of EtBr concertation level and soaking periods of marigold seed on survial percent of plant was studied. Marigold plant seeds were treated with 4 different concentrations of EtBr (250, 500, 750 and 1,000 mg/l) for 24, 48 and 72 h. Percents of marigold plant survival on the 30th DAP were shown in Table 1. Almost all of marigold seed soaking with EtBr sprouted slower than the control group. It was found that survial percent of marigold plant treated with EtBr concentration of 250, 500 and 1,000 mg/l for 48 h and 250 mg/l for 48 h were similar to the control group but survial percent of marigold plant treated with EtBr concentration of 750 mg/l for 24h, 500, 750 and 1,000 mg/l for 48 and 250, 500, 750 and 1,000 mg/l for 72 h was significantly ($P<0.05$) lower than the control group (Table 1).

Table 1 effect of concentration level and soaking periods in EtBr on survival percent of marigold plant

Concentration (EtBr) (mg/l)	Time (h)	Number and percent of surviving marigold plant						mean	S.D.
		1		2		3			
		number	percent	number	percent	Number	percent		
0	24	16	100.00	9	100.00	13	100.00	100.00^{ab}	0.00
250	24	15	93.75	10	111.11	15	115.38	106.75^{a}	11.46
500	24	10	62.50	7	77.78	9	69.23	69.84^{abc}	7.65
750	24	5	31.25	6	66.67	9	69.23	55.72^{cde}	2.08
1,000	24	6	37.50	8	88.89	9	69.23	65.21^{abc}	1.53
250	48	8	50.00	5	55.56	9	69.23	58.26^{bcd}	2.08
500	48	8	50.00	5	55.65	7	53.85	53.14^{cde}	1.53
750	48	4	25.00	5	55.56	6	46.15	42.24^{cde}	1.00
1,000	48	8	50.00	4	44.44	5	38.46	44.30^{cde}	2.08
250	72	6	37.50	4	44.44	1	7.69	29.88^{cde}	2.52
500	72	5	31.25	3	33.33	0	0.00	21.53^{de}	2.52
750	72	6	37.50	0	0.00	2	15.38	17.63^{de}	3.06
1,000	72	3	18.75	1	11.11	1	7.69	12.52^{c}	1.16

Identical letters mean that differences between values are not significant at P=0.05, using Duncan

2. Concentration level of EtBr that make the 50% lethal for marigold plant at soaking periods of 24, 48 and 72 hr

Analysis of the correlation between EtBr concentration and marigold plant survival by linear regression equation were analyzed for determination of LD_{50}. LD_{50} is the amount of concentration of EtBr which caused 50% of marigold plant death of when compare with their control group at 24, 48 and 72 h. At soaking period

of 24 h, a simple linear regression was calculated to predict EtBr concentration based on surviral percent of marigold plant. A significant regression equation was found ($F(1,13) = 13.076$, $p = 0.000$), with an R^2 of 0.501. Participant predicted EtBr concentration to equal to 1326.312 - 10.394 (percent of marigold plant survival)% when EtBr concentration was measured in mg/l. Participant's percent of marigold plant decreased by -10.394 for each mg/l of EtBr concentration at soaking periods of 24 h (Table 2). LD_{50} value was determined with the help of linear regression equation analysis based on survival percent of the marigold plants after treatment with different concentration of EtBr, compared with the untreated control. Optimum dose is the dose that causes maximum mutation with minimum damage to the plant. At soaking periods of 24 h, from the linear curve analysis, the LD_{50} value for EtBr concentration was 806.62 mg/l (Figure 1).

Table 2 analysis of variance concerning percent of marigold plant mortality and EtBr concentration at soaking periods of 24 h

model	b	SEb	β	t	p-value
constant	1326.312	238.768		5.555	0.000
Percent of marigold plants survival	-10.394	2.874	-0.708	-3.616	0.000
SEcut = ±268.515					
R = 0.708; R2 = 0.501; F = 13.076; p-value<.01					

b = un-standardized beta coefficient, SE_b = standard error, ß = standardised beta coefficient, t = t-test statistic, p-value = significance value, SE_{cut} = standard error of the estimate, R = correlation analysis, R^2 = R square (coeffiecient of determnation), F = F-test statistic

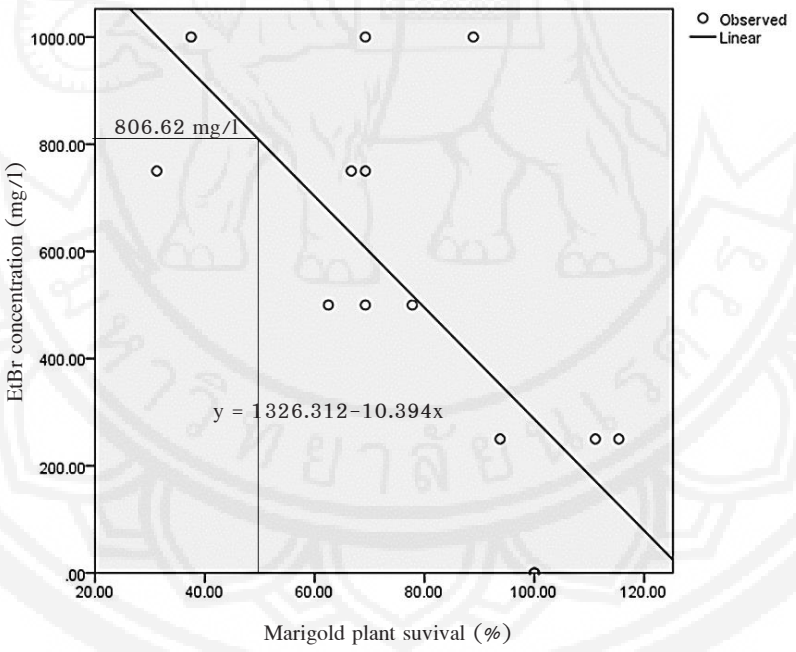

Figure 1 plot of EtBr concentration versus percent of marigold plant survival for calculation of LD_{50} at soaking periods of 24 h.

When soaking marigold seed of 48 h, a simple linear regression was calculated to predict EtBr concentration based on percent of marigold plant survival. A significant regression equation was found ($F(1,13) = 25.081$, $p = 0.000$), with an R^2 of 0.658. Participant predicted EtBr concentration to equal to 1269.301 - 12.910 (percent of marigold plant survival)% when EtBr concentration was measured in mg/l. Participant's percent of marigold plant decreased by -12.910 for each mg/l of EtBr concentration at soaking periods of 48 h (Table 3).

At soaking periods of 48 h, from the linear curve analysis, the LD_{50} value for EtBr concentration was 623.81 mg/l (Figure 2)

Table 3 analysis of variance concerning percent fo marigold plant mortality and EtBr concentration at soaking periods of 48 h

model	b	SEb	β	t	p-value
constant	1269.301	164.146		7.733	0.000
Percent of marigold plants survival	-12.910	2.581	-0.811	-5.002	0.000
SEcut = ±222.078					
R = 0.811; R2 = 0.658; F = 25.018; p-value<.01					

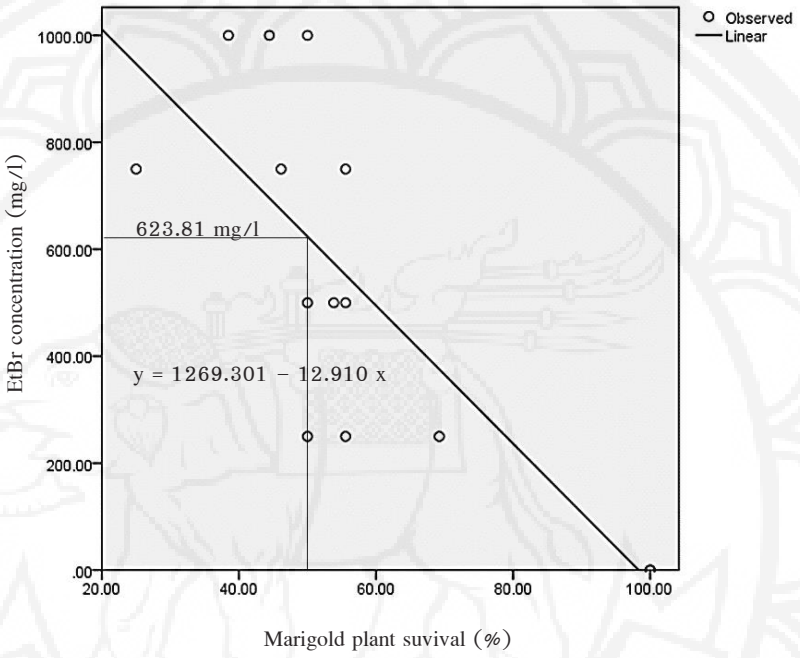

Figure 2 plot of EtBr concentration versus percent of marigold plant survival for calculation of LD_{50} at soaking periods of 48 h.

When soaking marigold seed for 72 h, a simple linear regression was calculated to predict EtBr concentration based on percent of marigold plant survival. A significant regression equation was found ($F(1,13) = 18.437$, $p = 0.000$), with an R^2 of 0.586. Participant predicted EtBr concentration to equal to 784.357-7.832 (percent of marigold plant survival)% when EtBr concentration was measured in mg/l. Participant's percent of marigold plant decreased by -7.832 for each mg/l of EtBr concentration at soaking periods of 72 h (Table 4). At soaking periods of 72 h, from the linear curve analysis, the LD_{50} value for EtBr concentration was 806.62 mg/l (Figure 3)

Table 4 analysis of variance concerning percent of marigold plant mortality and EtBr concentration at soaking periods of 72 h

model	b	SEb	β	t	p-value
constant	784.357	91.443		8.578	0.000
Percent of marigold plants survival	-7.832	1.824	-0.766	4.294	0.000
SEcut = ±244.218					
R = 0.766; R^2 = 0.586; F = 18.437; p-value<.01					

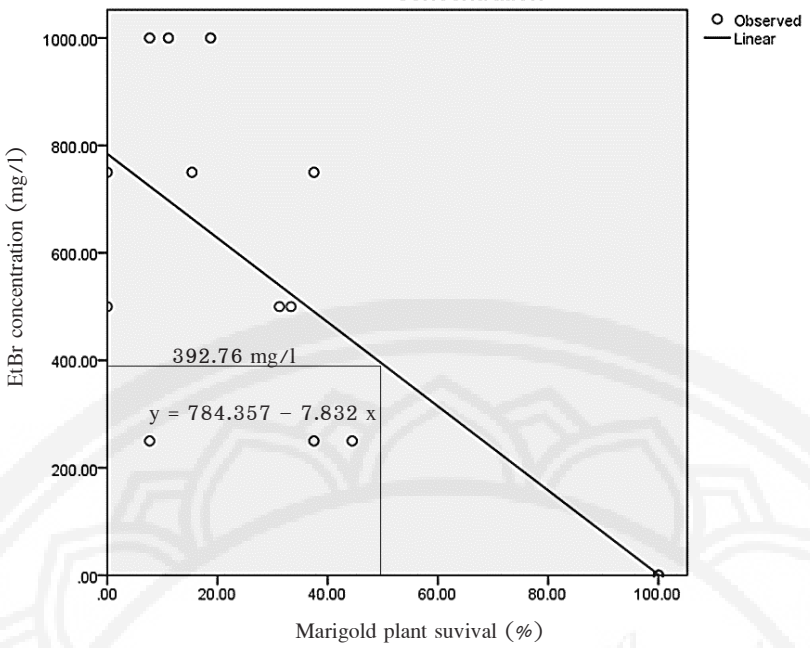

Figure 3 plot of EtBr concentration versus percent of marigold plant survival for calculation of LD_{50} at soaking periods of 72 h.

3. effect of concentration level and soaking periods in EtBr on marigold plants growth

the effect of EtBr on growth of marigold was shown in Fig. 4. It was found that when the concentration of EtBr increased, the average height of marigold plant also decreased, especially in the group of seed soaking with EtBr at 1000 gm/l for 48 hours. The lowest average height was 1.72 cm. Moreover, the marigold plant average height of the group of seed soaking with EtBr at 250 and 500 mg/l for 24 h were not significantly different from their control group. However, the marigold plant average height of the groups of seed soaking with EtBr at 750 and 1000 mg/l for 24 h and 250, 500, 750 and 1000 mg/l for 48 h was significantly lower than their control group (Fig. 4). The number of surviving marigold plant was very low in groups of seed soaking with EtBr at 250, 500, 750 and 1000 mg/l for 72 h. So, they did not display data.

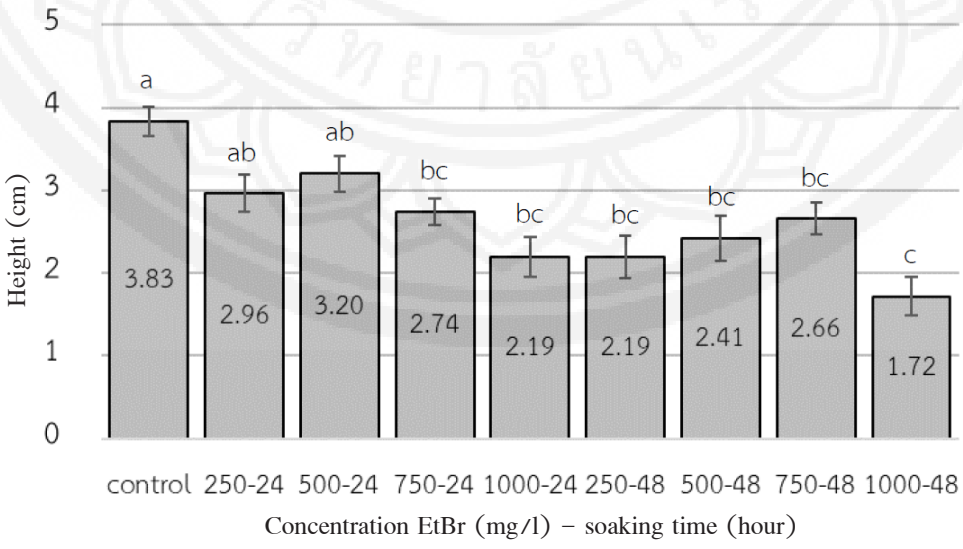

Figure 4 effect of EtBr concentration and soaking periods on height of marigold plant

Discussion

Mutation induction is a tool for generation of genetic variation to create new characteristics. The induction of mutations has higher potential to create new characteristics than the occurrence of natural mutations (Chopra, 2005). For both physical and chemical mutation induction, LD_{50} values are taken into consideration because this value will make the chances of mutation high (Anbarasn, Sivalingam, Rajendran, Anbazhagan, & Chidambaram, 2013). The success of mutation using chemical mutagen depends on concentration level and soaking periods. The low concentration of chemical mutagen may not cause mutation and, therefore, there are no changes in mutated seeds. On the other hand, high concentration can cause death of the mutated seed, sterility and other deleterious effect. Thus, we must first determine the LD_{50} mortality of the seeds or safe dose concentration where 50% of the seeds can survive (Roslim, Herman, & Fiatin, 2015). Therefore, this study determined concentration level of EtBr causing 50% mortality of marigold plant. The R^2 value of simple linear regression of soaking marigold seeds for 48 h was the highest. Thus, the optimal equation for regression is y = 1269.301 – 12910 (X). The equation shows that using EtBr in the induction of mutation should be based on the EtBr concentration of 623.81 mg/l by soaking the marigold seeds for 48 h. Considering the growth of marigolds, it was found that the EtBr concentration of 250 – 1000 mg/l and soaking seeds for 48 h can cause significant growth alteration of marigold plant from the control group. Therefore, the EtBr concentration of 623.81 mg/l for 48 h would have changed phenotype of the marigold. The success of using chemical induced mutation depends on concentration level and time of exposure.

The experiments showed that when EtBr concentration and soaking periods increased, the survival of marigolds decreased because marigold seeds can absorb more EtBr into seed. Based on study of EtBr uptake by two types of peanut seed and its relationship to varietal sensitivity and mutagenic efficiency, it was found that soaking periods affected on absorption of EtBr into part of peanut seed such as testas, cotyledons and plumules plus radicles. The longer seed was soaked in EtBr, the more EtBr was absorbed into seed parts. This would increase efficiency of the mutation when EtBr was absorbed into embryo (Levy, Ashri, & Rubin, 1978). It is interesting to note that soaking seed at EtBr concentration of 250 mg/l for 24 h had germination percentage of marigold seed over 100 % when compared to the control group because EtBr at this concentration to sterilize marigold seed surface. It can be seen from the absence of fungus on marigold seed soaked with EtBr 250 mg/l for 24 h. However, marigold seed soaked with distilled water for 24 h was found to have fungus while soaking the seed in distilled water. In the previous study, there was a report that EtBr can eliminate bacterial (Miki & Hard, 2013). When compare marigold plant average height of the groups of seed soaking with EtBr at 250 and 500 mg/l for 24 h with the control group, no significant difference was found. This suggests that soaking seed at EtBr concentration of 250 mg/l for 24 h did not make marigold mutate. The insufficient concentration and soaking period of marigold seed in EtBr induced mutation because marigold seed cannot absorb EtBr into the seed. It does not affect the mutation as can be seen in the unchanged phenotype (height) relative to the control group.

Conclusion and Suggestions

The lethal concentration (LD_{50}) of EtBr in each of 24 h, 48 h and 72 h treatments of marigold plant was determined. Determination of LD_{50} value for any mutagen is necessary to produce maximum viable mutants with minimum damage to the plant. The LD_{50} concentration levels based on reduction in survival after treatment with different soaking periods of EtBr were 806.62 mg/l for 24 hr, 623.81 mg/l for 48 hr and 392.76 mg/l for 72 hr. In addition, considering the highest R^2 value, it was found that the optimum concentration level and soaking periods based on reduction in survival and growth parameters were 623.81 mg/l for 48 hr which yielded maximum variability with minimum numbers of undesirable mutation. Hence, this was considered the optimum EtBr concentration level and soaking periods for marigold seed.

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