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Improvement of Strawberry *(Fragaria x ananassa)* through Induced Mutations and *in vitro* Culture

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Abstract **– In this study, the effects of different EMS concentrations (0, 0.2, 0.4, 0.6 and 0.8%) and durations (60, 90 and 120 minutes) on plant development both *in vitro* conditions have been investigated using tissue-cultured-meristems. Furthermore, different plant growth regulators at meristem growth and development (MS+BAP at 1.00 mg.L^{-1} + IAA at 1.00 mg.L^{-1}), proliferation (MS+TDZ at 1.50 mg.L^{-1} + IAA at 1.00 mg.L^{-1}) and rooting stages (MS+AC at 5.00 g.L^{-1}) on three different strawberry cultivars (Osmanli, Camarosa and Festival) have been examined.**

As a result, explant survival rates, number of shoots, number of roots, root length and number of leaves decreased when EMS concentrations and durations were increased. For explant survival rate, a maximum of 0.4% EMS concentration with 60 minutes; and for multiplication and rooting, a maximum of 0.2% EMS concentration with 60 – 90 minutes were determined as the best treatments. While the 'Camarosa' was determined to be the most sensitive cultivar, the 'Festival' the most tolerant cultivar to the EMS doses.

Keywords **– *Ethyl methane sulphonate* (EMS), *in vitro* mutagenesis, chemical mutagen, induced mutations, *Fragaria x ananassa***

Abbreviations Used - EMS: ethyl methane sulphonate MS: Murashige and Skoog, BAP: Benzylaminopurine, IAA: Indole acetic acid, TDZ: Thidiazuron; AC: activated charcoal.

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I. INTRODUCTION

Ethly Methane Sulphonate (EMS) is the most commonly used chemical potent mutagen under *in vitro* and *in vivo* conditions [1]. Various morphological variations were produced via EMS treatments [2]. Furthermore, EMS has widely been applied to various plant species, such as soybean [3], banana [4, 5], loquat[6].

Tissue culture has been extensively used not only in clonal propagation but also in plant breeding studies [7]. Tissue culture increases the efficiency of mutagenic treatments for variation induction, handling of large populations and using ready selection methods and rapid cloning of selected variants [8, 9, 10, 11, 12]. Therefore, tissue-culture-induced-mutations have been extensively studied as a source of plant improvement [13].Moreover, *in vitro* techniques are the best mutation breeding tools, particularly, for strawberries which is variety to vegetative propagation.

To increase the genetic diversity of strawberries, somaclonal variation and cross breeding techniques have widely been reported; however, studies related to *in vitro* mutation on strawberries are very limited. Several studies have recently been accomplished to investigate mutagenic treatments on strawberry plants. In these studies, gamma rays and EMS combinations were obtained strawberry mutant lines [14]. On *in vivo* conditions, EMS treatments to runner [15]; EMS treatments to in vitro roots [16]; and responses of different varieties to gamma rays were investigated [17]. Researchers were determined to LD_{50} values 55 Gy and 64.6 Gy for 'Toyonaka' and 'Akihime' cultivars, respectively [17].

The physical and chemical mutagen doses used in mutagen studies vary depending on species and varieties. While the mutagenic efficiency increased with increase in concentration of mutagen, physical damage is getting increased [18]. Therefore, LD_{50} dose should be determined for each variety in breeding programs.

The aims of this study were to determine the optimum EMS treatments for inducing mutations on selected strawberry cultivars (Osmanli, Camarosa, Festival) for plant development. The use of chemicals for mutagenesis on strawberry tissues *(meristems)* on these selected cultivars has not previously been reported. Hence, this study will be the first stage aiming at expanding the gene pool of strawberries.

II. MATERIALS AND METHODS

A. EMS Treatment to Meristems

The 'Osmanli, Festival and Camarosa' strawberry cultivars were used as test plants and runner tips from primer runners collected in May were used as explants. The runner tips were surface sterilized by keeping in 96% alcohol for about 10 seconds under sterile conditions and two step sterilization processes were carried out. The explants were exposed to 15% sodium hypochlorite for 15 minutes, the outer leaves were removed and the shoot apices were treated with 5% sodium hypochlorite for five minutes to ensure complete surface sterilization. Following sterilization processes, all explants were inoculated on media [19, 20]. Murashige and Skoog[21](MS) medium with 3% sucrose and 0.2% Phytagel was used. Meristems were isolated under binocular microscope to possess one or two leaves primordia and were cultured on MS medium for four weeks. 1 mg L^{-1} BAP and 1 mg L^{-1} IAA combinations were tested for meristem growth and development stage. Developed meristems were treated with previously set EMS concentrations and treatment times given in Table 1.

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Number of initial EMS treated materials was calculated according to Predieri and Virgillo[22] and 180 explants were used for each treatment. Water based solution containing 1 M of EMS along with 1% (v/v) DMSO as a carrier agent was prepared in deionized water and then diluted with 0.1 M phosphate buffer (pH 7) using filter sterilization under aseptic condition to give working solution (0.2, 0.4, 0.6, 0.8%) of mutagen. Sterile deionized water was also prepared as controls. Meristems were submerged in mutagen solutions and controls as 1 mL meristem for different periods of 60, 90 and 120 minutes for each concentration. Subsequently, meristems were rinsed three times with sterile water and dried.

Eventually, the EMS treated explants were inoculated in 100 ml jars (six explants/jar) consisting of EMS supplemented with 1.50 $mg.L^{-1}$ TDZ and 1.00 $mg.L^{-1}$ IAA combination. Explants were kept in four subcultures (M1V1-M1V4) proliferation media for four weeks (Table 2) and then they were transferred into rooting media containing 5 $g.L^{-1}$activated charcoal. Rooted plantlets were then transferred into the growing media containing sterile peat and perlite and were kept in full automatic growth chambers for acclimatization process.

Cultures were kept under a controlled environment at 25°C±1°C with 16 h photoperiod amended with cool white florescent light of 3000 µmol $s^{-1}m^{-2}$ for 6 months to allow further regeneration variants.

Table 1. EMS treatments and numbers

Treatment Numbers	EMS Treatments (Duration / Concentration)
Control	Water soaked
1	60 min / 0.2 %
2	90 min / 0.2 %
3	120 min / 0.2 %
4	60 min / 0.4 %
5	90 min / 0.4 %
6	120 min / 0.4 %
7	60 min / 0.6 %
8	90 min / 0.6 %
9	120 min / 0.6 %
10	60 min / 0.8 %
11	90 min / 0.8 %
12	120 min / 0.8 %

B. Observations Recorded

In each stage, data for different criteria was collected as follows; as in the proliferation stage, survival rate and number of shoots per explant (M1V1-M1V4); in the rooting stage, average number of roots, root length and number of leaves.

C. Data Analysis

The treatments were arranged in randomized parcels design. The data was analyzed using the Statistical Analysis System (SAS) software program, version 9.0 [23]by ANOVA and treatment means were statistically compared with using LSD test 5% of error probability.

III. Results and Discussion

A. Effect of EMS-induced Mutagenesis on Survival Rate

The effect of EMS treatments on explant survival rate was given in Fig. 1. While varying among strawberry cultivars, explant survival rates ranged from 75.00% to 87.22% for 0.2% and 0.4% EMS concentrations with 60 and 90 minute treatments. However, for 0.6% EMS concentration, the survival rate of explants was determined in only 60 minutes. As shown in Figure 1, the reaction of cultivars to different EMS concentrations varied as the highest explant survival rate was identified for *'Festival'* cultivar at 0.2% EMS treatment. Moreover, 0.2% EMS concentration with 60 minute treatment reduced explant survival rates by 25.17% on 'Osmanli', 36.30% on 'Camarosa' and 20.00% on 'Festival' cultivars as compared to the control treatment (Table 3). Similarly, 0.2% EMS concentration with 90 minute treatment also reduced explant survival rates by 41.96% on 'Osmanli', 64.96% on 'Camarosa' and 35.55% on 'Festival' cultivars as compared to control treatment (Table 3). Therefore, explants obtained from 'Festival' cultivar were determined to be more tolerant to the EMS treatments than 'Osmanli' and 'Camarosa' cultivars.

Data obtained from all tested cultivars used in our study has shown that increasing EMS concentrations and treatment times have reduced explant survival rates[24, 5, 14, 25]. In general, research datas were determined that the highest EMS concentration should be 0.4% and the longest duration time should be 60 minutes for tested strawberry cultivars.

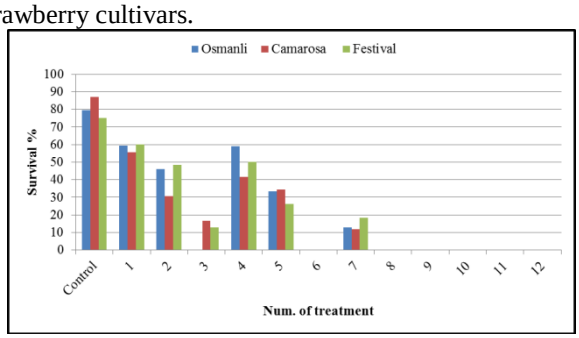

Fig. 1.Effect of *in vitro* EMS treatments on explant survival rate for different strawberry cultivars.

In this regard, in induced mutations in strawberries, the followings have been identified in previous studies; gamma rays + EMS 7 µM [14]; concentrations and durations 0.25% and 0.50% EMS / 12 h in rice varieties [26]; 0.4% EMS / 3 h in oak seeds [25]; 200 mM EMS / 30 min in banana shoot tips [5]; and 0.3% EMS / 2 h in loquat varieties [6]. It was also determined in our study that each strawberry cultivar had different sensitivity to the EMS treatment (cultivars 'Festival' was tolerant, 'Osmanli' was moderately tolerant and 'Camarosa' was sensitive) [27].

Table 2. Protocol designed to select mutated lines of strawberry cultivars 'Osmanli', 'Festival' and 'Camarosa' via stepwise *in vitro* technique.

Stage (1)	Stage (2)	Stage (3)	Stage (4)	Stage (5)	Stage (6)	Stage (7)
Meristem growth and development stage.	Multiplication phase-1 EMS treatments	Multiplication phase-2	Multiplication phase-3	Multiplication phase-4	Rooting phase	Acclimitization phase
MS+ BAP at 1.00 $mg.L^{-1}$ + IAA at 1.00 $mg.L^{-1}$	MS + TDZ at 1.50 $mg.L^{-1}$ + IAA at 1.00 $mg.L^{-1}$	MS + TDZ at 1.50 $mg.L^{-1}$ + IAA at 1.00 $mg.L^{-1}$	MS + TDZ at 1.50 $mg.L^{-1}$ + IAA at 1.00 $mg.L^{-1}$	MS + TDZ at 1.50 $mg.L^{-1}$ + IAA at 1.00 $mg.L^{-1}$	MS + AC at 5.00 $g.L^{-1}$	
	M1V1	**M1V2**	**M1V3**	**M1V4**	**M1V4**	

Furthermore, Basha [25] reported that *'variety x concentration'* interactions are effective on EMS treatments. Zhang [17] reported that reaction of strawberry varieties to Co^{60} physical mutagen also varied; hence, they findings were also as in harmony with what our data revealed.

B. Effect of EMS-induced Mutagenesis on Shoot Numbers

The effect of EMS treatments on shoot numbers per explant from M1V1 to M1V4 was given in Table 4. For three strawberry cultivars tested, increased EMS concentrations and duration times reduced the number of shoots. In M1V4 stage, the highest number of shoots was detected by 0.2% EMS concentration with 60 and 90 minute treatments. While the number of shoots for control treatment was 18.33, 20.67 and 20.00 for *'Osmanli, Camarosa and Festival'* cultivars, 0.2% EMS concentration with 30 minute treatment resulted in 4.00, 3.67 and 3.33 shoots for *'Osmanli, Camarosa and Festival'* cultivars, respectively. The EMS concentrations higher than 0.4% and all tested duration times did not produce any satisfactory results in terms of the number of shoots.

Consequently, increasedEMS concentrations significantly suppressed the number of *in vitro* shoots. From M1V1 to M1V4 subcultures, the highest number of shoots was detected by the lowest EMS concentration and shortest duration times (0.2% / 60 and 90 minute) for three cultivars. There was an explicit multiplication capacity difference among the tested cultivars. On this issue, Bhat[15]; reported that increased EMS concentrations and durations on strawberries reduced the runner numbers and EMS concentrations higher than 0.4% and more treatment times inhibited *in vivo* runner production. As similar to our findings on strawberries; another study also reported that EMS concentrations and durations were effective on average number of shoots per explant and survival rate on banana *in vitro* mutagenesis [5]. While effects of treatments vary among cultivars, the highest reproduction was detected in *'Rastali'* cultivar with 200 mM for 30 minute [5]. However, in our study, while there were significant differences in survival rates among strawberry cultivars, there were no apparent variations with respect to number of *in vitro* shoots.

C. *Effect of EMS-induced mutagenesis on number of roots, root length and number of leaves*

As a result of rooting in M1V4 subculture containing 5 g L^{-1} activated charcoal, data for number of roots, root length and number of leaves were given in Table 5. With regard to number of roots, all three strawberry cultivars tested generated the best roots at 0.2% and 0.4% EMS concentration with 60 and 90 minute treatments. While varying with cultivars in EMS treatments, the number of roots varied from 5.00 to 8.51. For all cultivars, the longest roots and the highest number of leaves were obtained from control treatments; however, explants treated with 0.2% and 0.4% EMS concentrations for 60 and 90 minutes showed satisfactory level of explant development.

When *in vitro* plant growth was examined in explants treated with EMS, depending on increments in EMS concentration and duration time, number of roots, root length and number of leaves reduced and *in vitro* plant growth declined by higher than 0.4% EMS concentration / 90 minute treatment. In contrary to our findings; Deepika[2] reported that EMS treatment on cluster beans increased the root and shoot lengths. Similar to our findings, however, Ramchander[28].reported that increased EMS doses on rice varieties reduced shoot length, root length and vigor index. In addition, Basha[25].reported that EMS treatments higher than 0.8% on oak seeds dramatically suppressed the root growth; Murthy [29] stated that 0.4% EMS treatment on mulberry was advantageous in terms of leaf and plant sizes, and accordingly, Bhat[16]indicated that 0.1% EMS / 90 minute treatment on strawberry was effective on *in vitro* rooting.

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Table 3. Effect of EMS treatments on explant survival numbers

Treatment No	Number of explants per treatment	Number of survival explants			*% change as compared to control *		
		Osmanli	Camarosa	Festival	Osmanli	Camarosa	Festival
Control	180	143	157	135	0.00	0.00	0.00
1	180	107	100	108	-25.17	-36.31	-20.00
2	180	83	55	87	-41.96	-64.97	-35.56
3	180	0	30	23	-100.00	-45.45	-73.56
4	180	106	75	90	-25.87	-52.23	-33.33
5	180	60	62	47	-58.04	-60.51	-65.19
6	180	0	0	0	-100.00	-100.00	-100.00
7	180	23	21	33	-83.92	-86.62	-75.56
8	180	0	0	0	-100.00	-100.00	-100.00
9	180	0	0	0	-100.00	-100.00	-100.00
10	180	0	0	0	-100.00	-100.00	-100.00
11	180	0	0	0	-100.00	-100.00	-100.00
12	180	0	0	0	-100.00	-100.00	-100.00

*% change as compared to control= (EMS-treated explant –Control explant/Control)x100

Table 4. Effect of *in vitro* EMS treatments on explant multiplication capacity for different strawberry cultivars.

Treatment	No. of shoots (shoots/explant)											
	M1V1			M1V2			M1V3			M1V4		
	Osmanli	Camarosa	Festival	Osmanli	Camarosa	Festival	Osmanli	Camarosa	Festival	Osmanli	Camarosa	Festival
Control*	5.00 a	7.33 a	5.33 a	5.67 a	7.33 a	9.00 a	13.33 a	15.67 a	20.33 a	18.33 a	20.67 a	20.00 a
1	2.00 b	2.33 b	3.00 b	2.67 b	3.33 b	4.00 b	4.00 b	4.00 b	5.00 b	3.33 bc	4.67 b	4.33 b
2	1.33 c	2.00 bc	1.67 c	2.33 bc	2.67 b	3.67 b	3.00 bc	2.33 c	5.33 b	4.00 b	3.67 bc	3.33 b
3	-	1.00 cd	1.33 c	-	2.33 b	1.67 c	-	-	-	-	-	-
4	2.00 b	2.33 b	1.33 c	1.67 bc	2.33 b	1.33 c	2.33 bc	2.00 c	3.00 bc	-	2.67 bc	2.67 b
5	1.00 c	1.33 bcd	1.00 c	1.33 c	1.67 b	0.67 c	1.67 c	1.33 c	2.00 c	1.67 c	1.00 c	2.33 b
6	-	-	-	-	-	-	-	-	-	-	-	-
7	1.00 c	0.67 d	-	1.33 c	2.33 b	-	1.33 c	1.33 c	-	1.67 c	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-
LSD $_{\%5}$	0.429	1.153	1.084	1.242	1.807	1.134	1.747	1.227	2.454	1.682	2.28	4.75

*Control treatment: Untreated EMS (water soaked)
Means followed by the same letter are not significantly different at the 5% level by LSD.

Table 5. Effect of *in vitro* EMS treatments on number of average roots, root length and number of leaves for different strawberry cultivars.

Treatment	No. of roots (root/plant)			Root length (cm)			No. of leaves (leaf/plant)		
	Osmanli	Camarosa	Festival	Osmanli	Camarosa	Festival	Osmanli	Camarosa	Festival
Control*	9.33 a	11.08 a	12.50 a	6.33	7.50 a	6.83	10.20 a	12.33 a	13.76 a
1	6.33 bc	8.51 b	7.17 bc	4.67	7.17 ab	6.90	5.50 b	8.33 b	7.37 b
2	5.00 c	5.60 c	7.27 bc	4.67	6.27 bc	6.50	3.40 c	7.67 b	6.83 b
3	-	-	-	-	-	-	-	-	-
4	5.67 bc	5.33 c	6.20 c	5.33	5.50 cd	6.23	4.67 bc	5.83 b	7.03 b
5	7.00 b	5.00 c	7.87 b	4.67	5.17 d	5.13	3.87 bc	5.93 b	6.68 b
6	-	-	-	-	-	-	-	-	-
7	6.00 bc	-	-	4.67	-	-	4.17 bc	-	-
8	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-
LSD $_{\%5}$	1.927	2.00	1.375	NS	0.924	NS	1.698	3.977	2.198

*Control treatment: Untreated EMS (water soaked)
Means followed by the same letter are not significantly different at the 5% level by LSD.

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