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POSSIBLE IMPROVEMENT TOWARDS SALT TOLERANCE IN EMS MUTATED STRAWBERRY (*Fragaria* x *ananassa* Duth.) FESTIVAL CULTIVAR

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ABSTRACT

The present study was established to enhance NaCl tolerance in strawberry (*Fragaria x ananassa* Dutch) 'Festival' cultivar which is grown widely in Iraq and it is sensitive to salt stress, Leaf explants were treated with Ethyl Methane Sulfonate (EMS) to induce genetic variation and then used for callus induction and shoot regeneration under NaCl stress. Different concentrations of NaCl (0.0, 10, 25, 35 or 45 mM) were dissolved with irrigation water and added to the soil. Response to NaCl was evaluated after three months. Callus cultures were initiated on Murashige and Skoog (MS) medium supplemented with different concentrations of α-naphthalene acetic acid (NAA) and 6- benzylaminopurine (BAP), 4 mg/L^{-1} NAA+1 mg/L^{-1} BAP. The regenerated shoots were inoculated on shoot induction medium containing 2 mg/L^{1} BAP + 0.5 mg/L^{-1} NAA then transferred to MS medium supplemented with IBA 1.0 mg/L^{-1} for rooting. Morphological parameters, number of leaves, plant height, vegetative area, number of flowers, number of fruits and weight of fruits were investigated compared with *in vivo* plants. Genetic variation based on RAPD- PCR technique was used to detect variability. The results showed a high reduction in shoots regeneration and rooting in response to increasing NaCl. Morphological characteristics decreased in response to increase NaCl concentrations as well. However, *in vitro* regenerated plants derived from leaf discs treated previously with EMS, exhibited better performance in these parameters than *in vivo* plants at high concentrations of NaCl. Using eight RAPD primers a total number of 15 unique bands resulted after EMS treatment in Festival, while 3 unique bands appeared under NaCl stress. This study revealed that EMS as a chemical mutagen is a promising in enhancing NaCl tolerance.

Key words: Strawberry, Festival, EMS, NaCl, RAPD, *In vitro*, *In vivo*.

INTRODUCTION

Strawberry (*Fragari* x *ananassa* Duth.) is a member of Rosaceae family which is considered as essential plant that provides basic fruit. Strawberries have been grown worldwide since fruits are nutritious and rich vitamin C, flavonoids, ellagic acid and autocianidin (Suvalaxmi *et al*., 2015). Phytochemicals and antioxidants present in strawberry fruits decrease the hazard of cardiovascular events disease and tumorogenesis (Hannum, 2004). USDA (1999) pointed out that fruits of strawberry have nutritious properties which essential for the health of human being. Food and Agriculture Organization (FAO) (2007) reported that the total world yield of strawberry fruit in 2005 reached 3.9 million tons. These advantages of strawberry fruits have attracted researchers to develop new cultivars with desirable traits (Mercado and Quesada, 2007).

Strawberry in Iraq is cultivated in some provinces; however, the cost of production is high compared with neighbouring countries due to low plant yield. Reduction in productivity in Iraq is mainly due to salt build up concentration in soil or using saline irrigation water (Hanna, 1982). Strawberry is considered highly salt-sensitive plant and, salinity effects on its growth and fruit yield reflecting negative effects on root development and water uptake (Albert, 2009). Increasing electrical conductivity (EC) from 1 to 2.5 could decrease the strawberry yield by 50% (John, 1999). Many studies have pointed that salt causes approximately 70% decline in plants productivity (Mantri *et al*., 2012).

Strawberry cultivars are heterozygosity and thus some cultivars adapt to environment and tolerate stresses (Emarah, 2008). There are numerous studies state the link between changes in genomic DNA patterns after the exposure of plants to various stresses. Ethyl Methane Sulfonate (EMS) was used to induce mutations in *in vitro* cultures then multiplication of the mutants preferably under salt stress. Shoot tips in many plant species were treated with EMS which led to creating phenotypic and genetic variation (Phillips, 2008). *In vitro* mutagenesis may produce salt tolerant plants derived from selected cell lines (Lu *et al*., 2007). Variation has been observed in

strawberry leaf colour and other phenotypic traits as well (EL-Sawy, 2007). Therefore, the current work is an attempt to increase strawberry tolerance to NaCl stress after inducing variation using EMS in *in vitro* cultures then screening and selection for NaCl tolerance at the cellular level. Regenerated plants may exhibit NaCl tolerance which can be manifested at morphological level and using DNA markers.

MATERIALS AND METHODS

Plant material and experimental conditions: Strawberry (*Fragaria* x *ananassa* Dutch.) c Festival cultivar was kindly supplied by Horticulture Research Dept. Hort. Office, Ministry of Agric. and Iraq. Plants were uniform in size; leaf explants were dissected from juvenile shoots.

EMS treatment and surface sterilization: Healthy leaf explants of strawberry were dissected carefully; leaves were washed with tap water for 5 min then soaking in distilled water supplemented with 0.1% of EMS for 1.5 hrs in attempt to induce mutation following (Bhat *et al*., 2015). Treated leaves with EMS were rinsed with tap water 5 times. Then the explants were transferred to a laminar air flow cabinet where cut into 1.0 cm^2 discs before rinsing with 4% aqueous solution of sodium hypochlorite for 20 min. One drop of tween-20 was added to the solution. Leaf discs were washed three times in sterile distilled water before inoculation to the culture medium.

Culture medium: Standard MS medium was used, and then, 30 g sucrose was added. The pH was adjusted to 5.7-5.8 then, 7 g agar was added to the medium. MS medium was supplemented with 4 mg/L^{-l} NAA + 1$mg.L^{1}$ BAP to initiate calli. The volume was completed to one litre and then sterilized by autoclaving at 1.04 $kg.cm^{-2}$ pressure and 121 ºC for 20 min. Leaf discs were inoculated into vials containing 10 ml of dispensed medium. Vials were kept at a growth room under dark conditions at 25 ± 2°C. After a period of five weeks, callus initiation was noticed.

Shoots and roots regeneration and NaCl treatments: A quantity of 100 mg of callus from festival cultivar was placed on MS medium supplemented with 2mg/L^{-1} BAP + 0.5mg/L^{-1} NAA provided with different concentrations of NaCl (0, 10, 25, 35 or 45mM) for shoot regeneration. After shoots formation, each micro shoot 5-6 cm long was transferred into MS medium supplemented with 1.0mg/L^{-1} IBA for rooting. Four weeks later, root formation was observed. Regenerated plantlets were lifted from MS medium; roots were washed with distilled water and then transferred into pots filled with sterilized peat moss, then kept for two weeks in a growth room at 25+ 2 ºC and 16/8 light/dark photoperiod, 2000 Lux light intensity. Plantlets were transferred to black plastic pots 15 cm in diameter containing peat moss 50%, horticultural perlite 25% and Sand 25%. Pots were kept in a greenhouse at 26 ± 2 °C during the day and 15± 3 °C at night. Pots were irrigated with distilled water supplied with different concentrations of NaCl (0, 10, 25, 35 or 45 mM).

Vegetative and yield parameters: Plants exposed to saline treatments, were harvested after three months of treatment. The mean of four replicates for treatment was calculated for plants height, number of leaves, vegetative area, number of flowers and fruits weight. Height was measured using a ruler from soil surface to the top of shoots. A digital camera (Apple Iphone 4s, Finland) was used to photograph the plants. Image. J software (Image J 1.45< imagej.en.softonic.com>) was used to estimate the total vegetative area in cm^2.

Molecular Parameters

DNA extraction: The DNA was extracted from fresh leaf tissues and used as templates for eight RAPD reactions to investigate the variation between strawberry samples in response to different NaCl treatments. Hamorabi Genotech kit (supplied by Genetic Engineering and Biotechnology Institute, University of Baghdad) was used for DNA extraction with slight modifications by adding using PVP to bind the phenolic compounds. The DNA pellet was suspended in 20µl of rehydration buffer and stored at -20 ºC. Nano drop (2000C, Thermo Scientific, USA) was used to estimate the quality and purity of extracted DNA.

RAPD PCR technique: RAPD markers were used to detect the possible genetic variation between *in vivo* plants and *in vitro* plantlets and between treated and untreated samples in response to salt treatments. Eight oligonucleotide primers were used in this study
(OPK01: 5'TGCCGAGCTG'3; OPK02: 5'GTGAGGC GTC'3; OPK03: 5'CCCTACCGA C3'; OPK04: 5'TCG-TTCCGCA3'; OPK05: 5'CACCTT-TCCC 3';
B104: 5'GGGCAATGAT 3'; OPA16: 5'AGCCAGCGA A 3'; OPA17: 5'GACCGCTTGT 3').

PCR reactions were carried out in 25 µl volumes containing 2µl of 34 ng/$µl^{-1}$ genomic DNA, 1 µl oligoprimer, DNA master mix (GoTaq@ G2 Green

Master Mix 2X, Promega). The thermal cycler was programmed with an initial step of 5 min at 94 °C that was followed by 35 repeated cycles for 1 min at 94 °C, an annealing step of 1 min at 36 °C and an elongation step of 2 min at 72 °C and finally, a 7 min extension at 72 °C. Ladder plus/M1071 contains 3000 bp was used. Amplification products were separated on 1.5% agarose with EtBr stain, diluted with 100 ml of 10x TBE (Dongsheng Biotech). PCR products were visualized on UV light and photographed using a gel documentation system (Spectroline TE 3123/F, Slimline™ Series, USA).

Experimental design and statistical analysis: A completely randomized design (CRD) with four replicates arranged as a factorial experiment was used. Least significant difference (LSD) at P ≤ 0.05 was used to compare between means. The Statistical Analysis System- SAS (SAS, 2012) program was used to calculate the differences between means for the studied parameters. ANOVA was used to compare between means at p ≤ 0.05.

RESULTS AND DISCUSSION

Effect of NaCl on shoot and root regeneration: A significant drop in the mean number of shoots / callus piece happened with the increase of NaCl levels supplemented to the culture medium (table 1). The highest number of regenerated shoots recorded 26 shoots at 0.0 mM NaCl, while the callus failed to regenerate shoots at 45 mM of NaCl and the number of shoots was very low at 35 mM which recorded 6 shoots. High NaCl concentrations caused a significant reduction in the percentage of rooting with mean percentages 90, 80, 60, 40 and 30% at 0, 10, 25, 35 and 45 mM respectively (table 1).

The results are in line with Shokaeva *et al*., (2011) who reported that the number of shoots regeneration was decreased when the NaCl concentrations increased in solid medium for the most strawberry cultivars that were under the examination. The results are in line with those of Zhao *et al.,* (2017) who showed that strawberry Sweet Charlie and Benihoppe cultivars exhibited a decrease in their ability to form roots *in vitro*; they found that the rooting rate decreased with increasing NaCl treatment.

High NaCl cause an imbalance in water and minerals absorption, and thus affecting on metabolism process which negatively influenced by enzymes activity (Al-Aubaidi, 2008). On the other hand, osmotic stress may cause ionic imbalance leading to a decline in cell division (Vieiria and Gustavo, 2012). EMS is a chemical mutagen has been used in crop breeding programs when different parts of plants are exposed to this mutagen, it disturbs the metabolic activities ultimately may resistance in plant (Salim *et al*., 2009). The pathway of biosynthetic activities might illustrate those effects, different stresses such as salinity cause accumulation in detoxification of reactive oxygen species (ROS) that enhance antioxidant compounds as defense machinery. The strawberry leaf treated with EMS might simply create salt tolerant variation (Liu *et al*., 2006). There were variations in rooting resulted after mutagenesis used (Saba and Mirza, 2002). Moreover, delivered callus from leaf and anther *in vitro* of strawberry cultivars, mutagenesis used for salt tolerance showed that the percentage of regeneration declined by 10 to 15% compared with the control treatment (Liu, 2006).

Table 1: The effect of NaCl on number of shoots regenerated from strawberry cv. Festival calli after eight weeks in MS medium supplemented with the combination of 2 mg/L^{-1} BAP + 0.5 mg/L^{-1} NAA and the effect on the percentage of rooting after six weeks of shoots transfer on MS medium supplemented with 1.0 mg/L^{-1} IBA.

NaCl (mM)	Mean No. Shoots	Rooting %
0	26 ± 1.02	90
10	18 ± 0.77	80
25	9 ± 0.36	60
35	6 ± 0.14	40
45	1 ± 0.05	30
Mean	12 ± 0.75	60
LSD (P≤ 0.05)	4.06*	12.463*

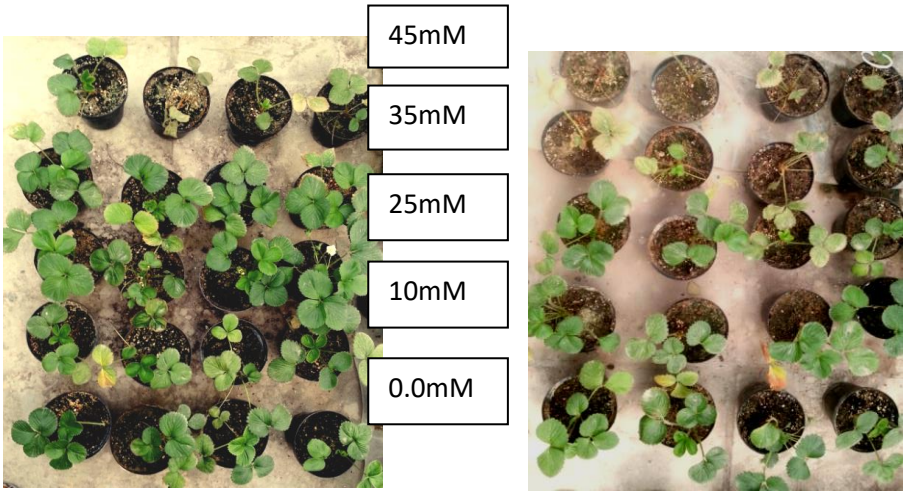
Analysis of morphological parameters: Table 2 shows that after increasing NaCl, the number of leaves, plant height and vegetative area exhibited a different response between in *vitro* and *in vivo* plants (Figure 1). The increasing in NaCl concentrations 0.0, 10, 25, 35 or 45 mM showed a significant decrease in these parameters. However, *in vitro* plants showed better response than *in vivo* ones in all studied parameters. *In vitro* Festival cv. recorded 6.25 leaves, 10.75 cm of plant height and 122.20 cm^2 vegetative areas at 45 mM NaCl. However, *in vivo* plants, recorded 3.75, 10.00 cm and 61.58 cm^2 at 45 mM NaCl at 45 mM NaCl respectively.

The results are in line with the results of Alnayef (2012) who demonstrated that the leaves

area and the number of leaves in strawberry cultivars negatively reduced in response to increasing salinity stress. The present results are similar to those of Karim *et al*., (2015) who observed that *in vitro* regenerated plants from calli exhibited somaclonal variations resulted in wide differences in plant characters such as plant height and number of leaves compared to mother plants. Biswas *et al.,* (2009) demonstrated that vegetative area and number of runners were greater in plants regenerated in *in vitro* ones than those plants propagated in the field.

Table 2: Effect of NaCl on number of leaves, plant height and vegetative area in strawberry plants grown *in vivo* and *in vitro* after three months.

NaCl (mM)	*In vivo*			*In vivo*		
	No. leaves/plant	Plant height cm	Vegetative area cm^2	No. leaves /pant	Plant height cm	Vegetative area cm^2
0	8.00	13.00	168.54	6.25	14.07	166.90
10	8.00	13.00	155.16	7.00	13.12	149.34
25	8.00	13.50	132.73	6.50	12.75	119.31
35	7.25	13.12	124.75	5.50	11.00	86.80
45	6.25	10.75	122.20	3.75	10.00	61.58
Mean	7.50	12.67	140.68	5.80	12.19	116.78
LSD 0.05	**1.31***	**2.02***	**57.53***	**1.28***	**1.58***	**40.80***



Festival *in vitro* **Festival *in vivo***

Figure 1: Improvement in morphological parameters in Festival cultivar that mutagenized with EMS in *in vitro* and subjected to NaCl stress compared with *in vivo* grown plants after three months.

Effect of NaCl in yield parameters: *In vitro* and *in vivo* plants supplemented with different concentrations of NaCl were conducted to evaluate flowering and fruiting parameters in response to NaCl stress. The results showed that the mean number of flowers, the mean number of fruits and the mean of fruit weights exhibited differences of decrease between *in vitro* and *in vivo* plants. *In vitro* regenerated plants produced 7.00 flowers, 5.10 fruits and 7.12 g of fruit weight while *in vivo* plants recorded 5.95 flowers, 4.25 fruits and 5.81g fruit weight respectively (table 3). All studying yield parameters showed a significant decrease in response to increasing NaCl concentrations. However, *in vitro* plants showed better performance than in *in vivo* plants. *In vitro* regenerated plants recoded 4.75 flowers, 4.0 fruits and 3.63 g fruit weights while plants derived *in vivo* displayed 2.75, 1.75 and 1.28 g at 45 mM NaCl respectively.

The results are in agreement with those of Alnayef (2012) who regenerated strawberry plants *in vitro* that exhibited a significant improvement in fruit weights at 40 mM NaCl compared with control plants and imposed significant differences between investigated strawberry cultivars. In addition, results are in line with the results of Biswas *et al*., (2009) who pointed out that straw-

berry *in vitro* derived plants showed higher number of flowers, fruits and the fruit weights compared to control treatment in response to salt stress. Karim *et al*., (2015) stated that *in vitro* somaclonal variations improved the morphological parameters such as number of fruits per plant and the mean number of fruit weights in comparison with mother plant.

NaCl damages leaf metabolism in susceptible species, photosynthesis is decreased, and carbohydrate formation is inadequate, this leads to reduced strawberry fruit yield (Saied *et al*., 2005). A reduction in leaf area of strawberry in response to salinity stress usually resulted from reducing cell division and photosynthetic activity which affected negatively on the yield (Yilmaz and Kina, 2008). Therefore, maintenance of a large vegetative area during salt stress may result from treatment with EMS as shown in this study (table 2). This is critical to plant yield and translocation of photosynthetic to the fruit. A large vegetative area has beneficial role in the final yield in strawberry (Keutgen and Pawelzik, 2009).

Table 3: Effect of NaCl concentration on number of flowers/plant, number of fruits/plant and fruit weight in strawberry plants grow *in vivo* and *in vitro* after three months.

NaCl (mM)	*In vitro*			*In vivo*		
	No. Flower/plant	**No. Fruit/plant**	**FW. Fruits (g)**	**No. Flower/plant**	**No. Fruit/plant**	**FW. Fruit (g)**
0	11.00	6.50	11.02	9.25	6.50	11.04
10	6.75	5.50	8.74	8.75	6.50	9.61
25	6.50	4.25	7.09	5.25	3.75	8.93
35	6.00	5.25	5.08	3.75	2.75	2.64
45	4.75	4.00	3.63	2.75	1.75	1.28
Mean	7.00	5.10	7.12	5.95	4.25	5.81
LSD 0.05	**1.172***	**1.250***	**0.902***	**1.047***	**1.106***	**0.647***

RAPD analysis: RAPD markers have been used widely to detect the genomic DNA for identification and characterization of genetic diversity in different strawberry cultivars in response to environmental stress conditions. It was apparent from the figure 2 that there was certain variability between treated and untreated plants with EMS strawberry cultivar in respect to eight primers (OPK01, OPK02, OPK03, OPK04, OPK05, B104, OPA16 and OPA17). There were new bands in lanes that regenerated from leaf disc explants treated with EMS compared with those untreated leaf discs. Some bands were absent in lanes of regenerated plants when mutated leaf discs subjected to NaCl stress. Table 4 displays 15 unique bands ranged between 150-1500bp, 15 (150-2000bp) and 3 (290-1000bp).

Results show that banding patterns for EMS treatment of Festival cv. in regard to eight RAPD primers displayed 57 bands with 15 unique bands while the control treatment produced 32 bands with 15 unique bands (table 4). NaCl stress combined with EMS treatment changed the genomic DNA of Festival genotype since it decreased the total of DNA bands to 19 bands with 3 new unique ones. Also, the results displayed different number of bands for some primers used individually in response to EMS or NaCl treatments. Primers OPA16, OPK03, OPA17 and OPK04 produced 10, 8, 5 and 4 bands in EMS treatment compared with 6, 3, 2 and 1 band for control treatment respectively while, all bands of OPK04, OPK05, B104 and OPA16 primers disappeared in response to NaCl treatment (figure 2) and (table 4).

The results are in line with those of Coungiu *et al*. (2000) who tested 31 strawberry cultivars and found that 13 out of these cultivars had consistent bands differed from control. The results are also in agreement with the results of Gaafar and Saker (2006) who found that using RAPD-PCR for identification of different genomic strawberry cultivars to determine and estimate the genetic distances between cultivars and their genetic relationships since the results detected a high level of genetic variability among seven strawberry cultivars. Bhat *et al*. (2015) stated that leaf discs in strawberry treated with EMS which led to change phenotypic traits positively. RAPD was utilized effectively in strawberries to study genetic differences between cultivars (Graham *et al*., 1996). The variation between *in vitro* plants treated with EMS and those untreated ones manifested clearly. EMS may modify G/C to A/T and induces mutation in some DNA sequences. Some

DNA fractions expressed disappearance of the amplification bands which may be occurred due to the absence or preventing of complementary sequence in the RAPD primers (Ibrahim *et al*., 2011). This could be an evidence of the effect of EMS and NaCl stress in creating mutation in plant cell which led to improve salt tolerance in *in vitro* regenerated plant more than *in vivo* ones as presented in this study.

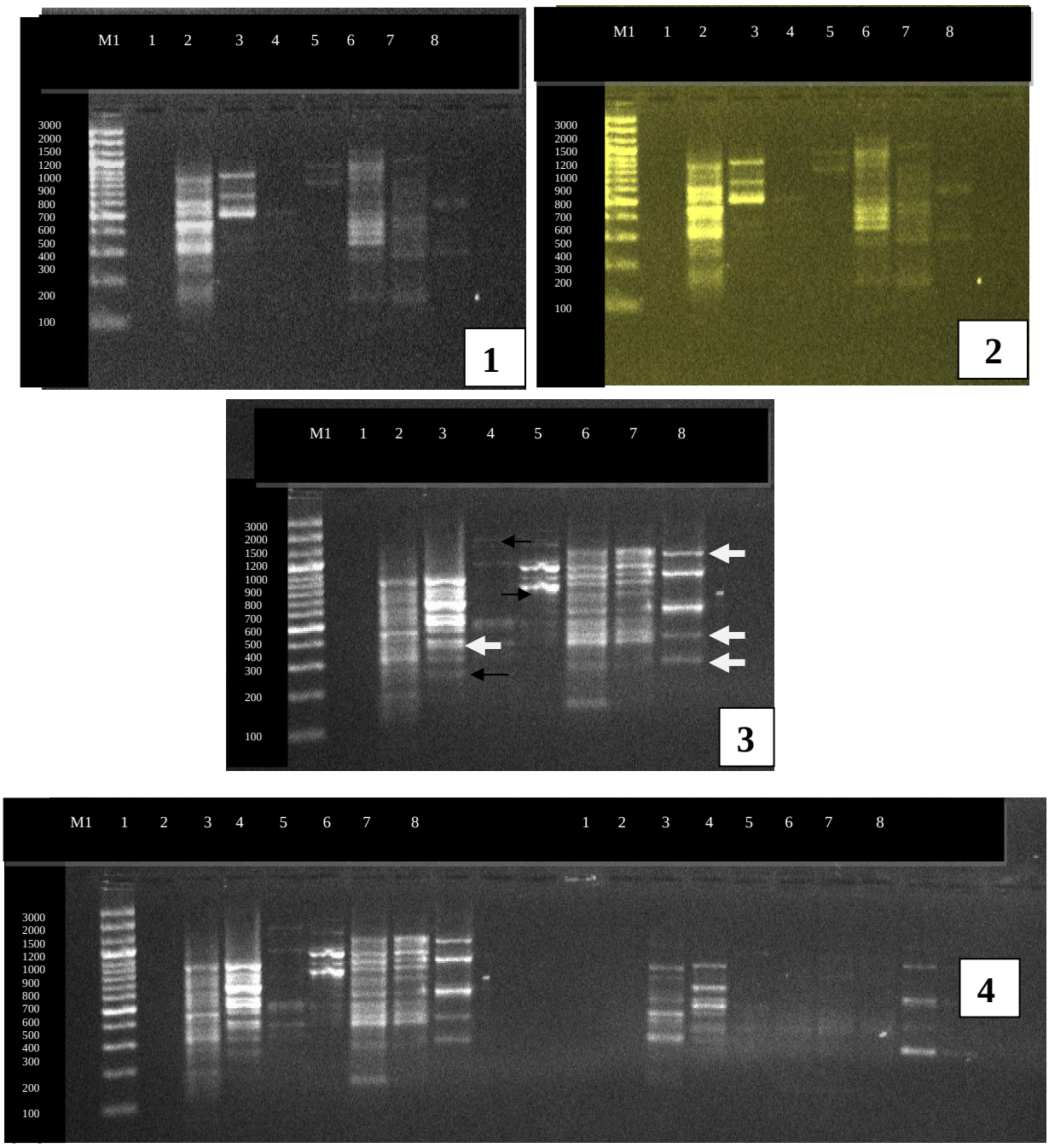


Figure 2: Agarose gel electrophoresis of PAPD-PCR reaction for festival cv. DNA samples using eight random primers as lines numbered (1: OPK01, 2: OPK02, 3: OPK03, 4: OPK04, 5: OPK05, 6: B104, 7: OPA16 and 8: OPA17). Picture 1 is for DNA samples derived from *in vivo* plants. 2: is for DNA samples derived from *in vitro* plants. 3: is for DNA samples derived from *in vitro* plants treated with EMS. 4: to the right is for DNA samples derived from *in vitro* plants treated with EMS+ NaCl. Bands electrophoresis with 1.5% agarose gel at 1hr, 100 V and 1X TBE buffer. Visualization was under U.V light using ethidium bromide. M1: 3 Kb ladder (comparison must be between same primer for different treatments to each sample).

Table 4: Number of fragment bands and unique bands resulted from eight RAPD primers for Festival that treated with EMS or treated with EMS + NaCl compared with control.

Cultivar	Treatment	primer	Fragment size bp	Fragment No.	Unique band
Festival	Control	OPK01	0	0	0
		OPK02	150-1000	8	1
		OPK03	800-1000	3	0
		OPK04	600	1	1
		OPK05	800-1100	2	2
		B104	150- 1500	10	5
		OPA16	150-1200	6	4
		OPA17	300-700	2	0
		Total band		**32**	**15**
		Average		4.00	1.87
	EMS	OPK01	---	0	0
		OPK02	200-1000	11	2
		OPK03	290-1000	8	1
		OPK04	450-2000	4	2
		OPK05	400-1900	7	2
		B104	150-1600	12	5
		OPA16	400-1500	10	2
		OPA17	300-1500	5	1
		Total band		**57**	**15**
		Average		7.12	1.87
	EMS + NaCl	OPK01	-----	-----	
		OPK02	300-1000	8	1
		OPK03	350-1100	7	2
		OPK04	-----	0	0
		OPK05	-----	0	0
		B104	-----	0	0
		OPA16	-----	0	0
		OPA17	290-1200	4	0
		Total band		**19**	**3**
		Average		2.37	0.37

CONCLUSIONS

Callus culture of Festival cultivar was initiated on MS medium supplied with 4 mg/L^{-1} NAA +1mg/L^{-1} BAP for leaf discs explants, shoots regenerated on MS medium supplemented with 2 mg/L^{-1} BAP + 0.5 mg/L^{-1} NAA and 1 mg/L^{-1} BAP was appropriate to initiate roots under NaCl stress in Festival cultivar. In *in vitro* explants regenerated from leaf explants treated with EMS at 0.1 % for 1.5 hrs and grown under NaCl stress (0.0, 10, 25, 35, or 45 mM) showed NaCl tolerance more than untreated ones in terms to morphological parameters (the mean number of leaves, height plants, vegetative area and fresh and dry weight for shoots and roots) in strawberry cultivar compared with *in vivo* untreated plants. The gain in NaCl tolerance of plants regenerated from leaf explants treated with EMS increased strawberry yield compared with those plants propagated in the field. RAPD-PCR technique was an efficient for examine the influencing of EMS on mutation of *in vitro* cultures.

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