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Mutagenesis of *Dunaliella salina*

A.A TAMMAM[1], M.M. ALLAM AND M. OSMAN†
Botany Department, Faculty of Science, Alexandria, University, Alexandria, Egypt
†*Genetic Engineering, Biotechnology Institute, Minoufiya University, Egypt*
[1]Corresponding author's e-mail: amltammam@yahoo.com

ABSTRACT

Dunaliella salina were grown in MH culture medium under controlled condition of light and temperature. *Dunaliella salina* were exposed to different doses of gamma irradiation (100,180, 330, 430 gray) by cobalt 60-Source. Exposure of *Dunaliella salina* to various doses of gamma rays revealed a production of new four mutants with a great variation in their nucleotide which leads to their alteration in the pattern of gene expression. The peptide mapping reveal a great variation in the peptides between the wild type and mutants. Analysis peroxidase isozymes reveal that there is a variation in isozyme pattern. Gamma ray project was not only resulted in a marked variation in nucleotide between the wild and their mutants but also between the mutant themselves.

Key Words: Mutagenesis; *Dunaliella salina*; Temperature

INTRODUCTION

Most plant can tolerate much doses of radiation higher than animals (Hluchovsky & Srb, 1963). The blue green algae, in particular, tolerate various doses of ionization radiation (Krous, 1969). It has long been known, that the nucleus is much more sensitive to radiation injury than the cytoplasm, and the chromosomes are among the earliest cellular structures affected by radiation (Read, 1959).

Derks *et al.* (1992) represented that effects of gamma rays on the viability, cell division frequency and chloroplast DNA integrity of *Lycopersicon peruvianum* protoplasts through damage to the nuclear DNA (but not inhibit the functioning chloroplast DNA). Their results showed that identification of chloroplast DNA fragmentation was found only after irradiation and the proportion of linear chloroplast DNA increased with increasing gamma doses.

Schevehenko *et al.* (1982) reported that the repair of tranferming DNA is performed with the participation of DNA polymerase and poly nucleotide ligase which function in the cell free extract of the blue green alga, *Aracystic nidulaus*.

Shabanova (1972) reported that the low doses of gamma radiation on chlorella with 50.75 rad/day induce a statistically significant increase of visible mutation.

Meenakshi and Donat (1996) studied the effect of UV radiation on the cyanobacterium, *Aulosira fertilissima* and reported that a substantial decrease in protein band occurred after 2 h of UVB exposure. The data also indicated the destruction of the monomers as well as the high molecular weight linker proteins.

Allam *et al.* (2002) represented that exposure of *Anabaena oryzae* to relatively high doses of gamma irradiation (170 gray up 420 gray) led to alteration in gene expression and this confirmed by changes in both protein and DNA banding patterns, and hence, the development of two new stable mutants one of them has antibacterial activity.

Other authors have suggested an important role of protein synthesis in the induction of resistance to high doses of gamma rays, UV oxidative stress (H_2O_2) by postulating that newly synthesized proteins called "heat shock proteins" help cells to defend themselves against stress (Mitchell & Morrison, 1982; Sharp *et al.,* 1984; Chrisman *et al.,* 1985).

In this study, we tried to produce new stable mutagenic populations of *Dunaliella salina* by exposing to critical doses of gamma rays and to what extent can these mutation changed the gene expression in these mutants.

MATERIALS AND METHODS

Culture of *Dunalliella salina* (obtained from Algal Collection of Botany Department, Faculty of Science, University of Alexandria) were grown in MH medium (Johnson *et al.,* 1968) as modified by Loeblish (1982). Both irradiated and control algal cultures were grown in 500 mL conical flasks in a culture room under controlled conditions of light (3500 lux) and temperature ($25C^0 \pm 1$) and 12 h photoperiod. After the 7^{th} generation of the treated *Dunaliella salina* to different doses of gamma irradiation (100, 180, 330, 430 gray) by cobalt 60-Source the treated and untreated cells cultured and harvested at the exponential phase to estimate PCR DNA, peptide mapping and peroxidase isozyme.

Extraction, purification and PCR amplification of DNA. DNA was extracted according to Neilan (1995). PCR reaction was performed in a total volume of 100µl, containing 50ng of chromosomal DNA dissolved in TE (Tris EDTA) buffer (pH 8) (Sambrook, *et al.*, 1989) and 200 ng of each of the two primers (MA1 and MA2) were

added. The reaction mixture was overlaid with a minute drops of light mineral oil (Sigma). The amplification was performed using a thermal cycler (Perkin Elmer PCR system 2400) programmed for 1 cycle at 95°C for 5 min and 40 cycles of 40 sec at 94°C, 1 min at 37°C and 2 min at 72°C, and a final extension at 72°C for 12 min.

Primer code	Sequences
	5'----------3'
MA1	GGGGATCCGTAGTCATATGCTTGTCTC
MA2	CGGAATTCCTTCTGCAGGTTCACC

Digestion of the PCR products. Once the size of the PCR products had been identified, 5µl of each reaction were digested with BstUl restriction enzymes under appropriate conditions. PCR products were digested for 3h in a 30-µl volume. Digestion was stopped using loading buffer solution (Sambrook, *et al.*, 1989) and kept at -20°C.

RFLP analyses. Digestion of the DNA PCR products from the species of *Dunaliella* were analyed by electrophoresis in 1.5% agarose gels with 0.5x TBE buffer (pH 8) and detected by staining with ethidium bromide solution (stock solution of 10 mg/ml in distilled water) for 30 min. and analyed to determine the RFLP profile of the species. Gels were then photographed under UV light.

Trypsin treatment and peptide mapping. Peptide mapping were prepared according to Judd (1990). Extracted protein sample (mg/ml) were treated with trypsin (Cooper Biomediat) in a ratio of µg/0.207 unit, sample were then incubated at 37^0c for 1 hr. After which, samples were heated to stop reaction and then mixed with sample buffer and submitted native PAGE and SDS. PAGE technique using the discontinuous buffer system as described by laemmli (1970) and modified by Hames and Rickwood (1990).

Peroxidase. *Dunaliella salina* pellets was homogenized in an ice cold porcelin mortor in 1.5 cm^{-3} of 5 mM Tris Hcl buffer pH (7.2) containing 0.25 M sucrose and 1 mM $MgCl_2$. The homogenates were centrifuged for 7 min at 9000 g and the supernatants collected corresponded to soluble enzyme fraction.

Electrophoresis. Isoform of peroxidase were separated by electrophoresis using 12.5% SDS. Ployacrylamide gel stabilized by 43% glycerol as described by Taklil *et al.* (1999). Gel were stained for peroxidase isoforms by incubation in 0.1 M acetate buffer (pH 5.0) containing 0.1 (m/v) benzidine and 0.01% hydrogen peroxide.

Estimation of similarity coefficient

$$\text{Similarity coefficient} = \frac{2a}{2a+b+c} \quad (0 \rightarrow 1)$$

Where a: number of similar bands in both gels.
b: number of marker bands in first gel.
C: number of marker bands in second gel.

It assumes values from zero for nil similarity or complete dissimilarity to unity for complete similarity.

The coefficient ($0 \rightarrow 1$) has been expressed as a percentage (0% - 100%). The coincidence of czekanowskis coefficient actually represents the extent of similarity between the two operational taxonomic units (Out's) rather than others (Czekanowski, 1913).

RESULTS AND DISCUSSION

Fig. 1 and Table I showed that the total of 17 DNA nucleotide base were detected by performing PCR reaction using two primers MA_1 and MA_2 followed by a digestion with UE restriction enzyme. Exposure of *Dunaliella salina* to various doses of gamma rays (50, 100, 180, 230 & 430 gray) revealed production of four new mutants with a great variation in their PCR. They markedly differed in the pattern of PCR nucleotides for example in the 1st and 2nd treatments absence of three nucleotides than control at 806.49, 364.08, 267.58 k bp, respectively and developed one newly nucleotide at 718 k. base pair with average of similarity 33%. The high doses of gamma rays (180, 230, 430 gray; Table I) resulted in the appearance of a newly nucleotides. The 936.65 k bp with average of similarity 57.4 appeared in 180 gray. The 718.4 k bp with average of similarity 57.14 appeared with 230 gray. While at highest doses, two newly nucleotides were synthesized with 718.4 and 579.6 k bp with average of similarity 25% in comparison with control. This confirmed the ability of various doses of gamma ray in the alteration of DNA base sequence of *Dunaliella salina*. Such alterations led to omitting some RE (Restriction enzyme) recognition sites and generation sites and generation of new band patterns which consequently leads to their alteration in the pattern of gene expression. Moreover, the gamma ray project resulted not only a marked variation in PCR between the wild and their mutants but also between the mutants them selves. In mutant one, a newly nucleotide at 579.6 k bp, mutants No. 2 one nucleotide at 930.6 k bp, mutant No. 3 one nucleotide at 718.4 k bp and finally in M_4 two nucleotides at 718 and 579.6 k bp. Schevchenko *et al.* (1982) recorded that the repair of transforming DNA is performed with the

Table I. RFLP pattern of PCR product DNA using the restriction enzyme UI

Base pair	Gamma dose						
	50	100	180		230	430	Control (Untreated)
1031	0	0	0	1	0	0	0
936.651	0	0	1	1	0	0	0
806.499	0	0	0	1	0	0	1
718.496	0	0	0	1	1	1	0
579.664	1	1	0	1	0	1	0
511.436	1	1	1	1	1	1	1
364.086	0	0	0	1	1	0	1
267.583	0	0	1	1	0	0	1
200	0	0	0	1	0	0	0
100	0	0	0	1	0	0	0
80	0	0	0	1	0	0	0

Fig. 1. PCR, RFLP DNA banding pattern of *Dunaliella salina* digested by BstUI, lane 1,2,3,5,6 treated organism by different doses of gamma radiation (50, 100, 180, 330, 430 gray), Lane 7 (control) and Lane 4 (marker)

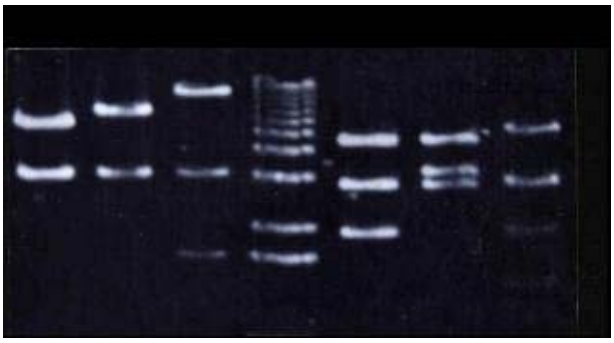
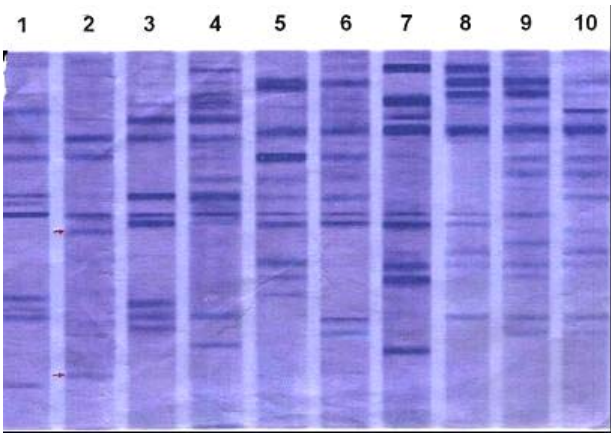


Fig. 2. Alkaline native polyacrylamode gel electrophoresis (PAGE) of *Dunaliella salina* proteins and four mutants resulted from different doses of gamma radiation



Lane 1. Wild type (zero time), Lane 2. Wild type (1 hour of trypsin treatment), Lane 3. Mutant 1 (zero time), Lane 4. Mutant 1 (1 hour of trypsin treatment), Lane 5. Mutant 2 (zero time), Lane 6. Mutant 2 (1 hour of trypsin treatment), Lane 7. Mutant 3 (zero time), Lane 8. Mutant 3 (1 hour of trypsin treatment), Lane 9. Mutant 4 (zero time) Lane 10. Mutant 4 (1 hour of trypsin treatment)

participation of DNA polymerase and polynucleotide ligase with function in the cell free extract of the green alga, *Aracystic nidulaus.* Schabonova (1972) reported the storage of irradiated material in active state showed the wave like changes in the mutability level of *Chlorella* sp.c.

On the study of the effect of different doses of gamma rays (100, 180, 230, 430 gray) on peptide bands development, the Fig. 2 represented that a pronounced differences in migration position of peptides, number and band intensity. These differences change with or without the time change and also with the change of the gamma doses. For example at zero time the number of major bonds in control (wild type) at zero time was nine while five peptides were appeared after one hour of trypsin treatment.

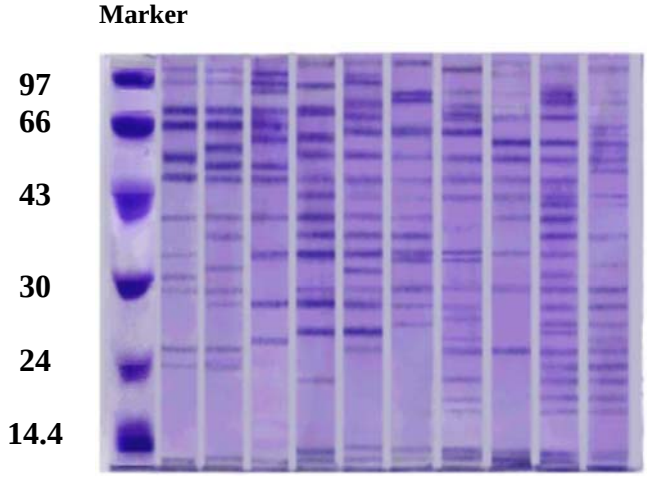
It is obvious that two major bands (indicated by arrows) of here are not present in the pattern of wild type (untreated). The treatment of *Dunaliella salina* with Gamma radiation with (100, 180, 230 & 430 gray) resulted in the appearance of four different electrophoretic patterns (Lanes 3, 5, 7 and 9. Since in wild type the number of major peptide bands was nine in mutant are, twelve in mutant 2, nine in mutant 3 and twelve in mutant 4. Although, the number of major peptides in wild type and mutant one and three was similar, the migration position and band's intensity were different this explain that the mutants have different electrophoretic patterns.

The treatment of mutants proteins with trypsin resulted in appearance of 13, 9, 9 and 10 peptide bands mutant 1, 2, 3 and 4, respectively. These bands are different than those of control one. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) patterns of the same protein samples of *Dunaliella salina* and its mutants are shown in Fig. 3.

The wild type pattern showed that appearance of 13 peptide bands having molecular mass ranged from < 14.4 KDa and > 97 KDa of these four major bands of molecular weight 86, 80, 72 and 64 KDa were present. In mutant one pattern, 13 peptide bands were also appeared. Of these four peptides are not present in the wild type pattern. This is due to the synthesis of such peptides. They have molecular mass of 98, 62, 28 and 26 KDa (Lane 3) with an average of similarity 61.5% of the wild type. In mutant two pattern, seven peptides were synthesized of molecular masses 82, 64, 43, 36, 30, 26 and 12 KDa with an average of similarity 45.7% of the wild type. In mutant three seven peptides were synthesized 94. 43, 24, 20, 18, 16 and 12 KDa with an average of similarity 36.5% of the wild type and mutant four nine peptide bands were synthesis with an average of similarity 38%. These were peptides of molecular masses 74, 70, 52, 40, 26, 24, 22 20 and 15 KDa.

On the other hand, the trypsin treatment of mutant proteins revealed that four different electrophoretic peptide maps were appeared on the gel. This means that the mutants are completely different in protein structure. For example the trypsin treatment after one hour resulted in appearance four peptide bands of molecular mass similarity 45% of the wild type in mutant one in mutant two five peptides were appeared on the gel of molecular mass 100, 52, 48, 15 and 14.4 KDa with average of similarity 42% of the wild type. In mutant three, five peptides appeared on the gel of 100, 52, 48, 15 and 14.4 KDa with an average of similarity 42% of the wild type. In mutant four, four peptide bands appeared on the gel of molecular mass 60, 58, 30 and 23 KDa with an average of similarity 47% of the wild type. The data show the great variation between the wild type type and the mutant and also between the mutants. The newly synthesized peptides were markedly higher in mutant four than the other mutants. Surprisingly, it is more sensitive mutant under salinity condition *Paper II). This confirms the mutagenesis program of *Dunaliella saline* when exposed

Fig. 3. Sodium dodecyl sulphate polyacryl amide gel electrophoresis (SDS – PAGE) of wild type of *Dunaliella salina* proteins and four mutants resulted from different doses of gamma radiation



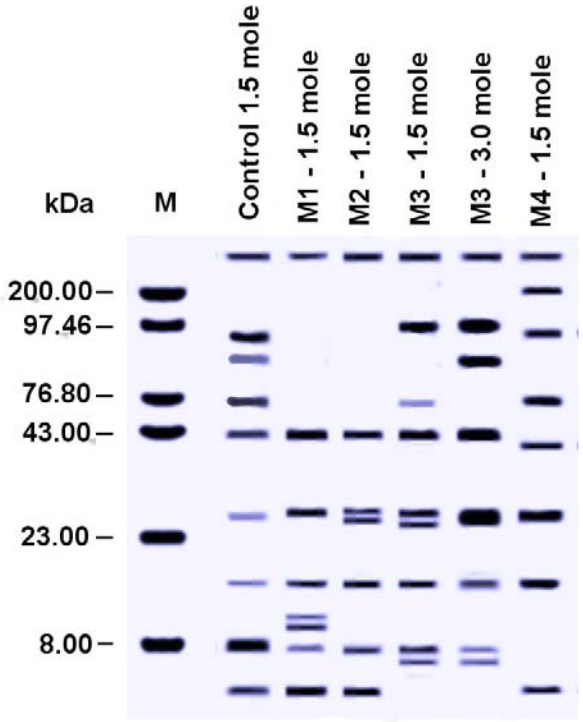
Lane 1. Wild type (zero time), Lane 2. Wild type (1 hour of trypsin treatment), Lane 3. Mutant 1 (zero time), Lane 4. Mutant 1 (1 hour of trypsin treatment), Lane 5. Mutant 2 (zero time), Lane 6. Mutant 2 (1 hour of trypsin treatment), Lane 7. Mutant 3 (zero time), Lane 8. Mutant 3 (1 hour of trypsin treatment), Lane 9. Mutant 4 (zero time), Lane 10. Mutant 4 (1 hour of trypsin treatment)

to the various doses of gamma radiation. Meanakshi and Donat (1996) recorded a gradual decrease in low molecular masses peptide, phycobiliprotein, monomers and high molecular mass linker proteins of *Aulosira fertilisina* (blue green alga) with uv exposure. Sinha *et al*. (1995) have been reported the same results in Anabeana sp. and *Nostoc carmium*.

Another important confirmation for our mutagensis program of *Dunaliella salina* is the criteria of peroxidase isozyme. The data indicated the big differences in the isozymes and their molecular weights in mutants when compared with wild type.

For example the appearance of 2 isoforms of molecular masses 15,12 KDa in mutant one with average of salinity 53%, one isoform band of 20 KDa in mutant

Fig. 4. Isoenzyme pattern of acid soluble peroxidase in *Dunaliella salina* wild type and its 4 mutant after gamma ray exposure (100, 180, 330, 430 grays)



number two with average of similarity 75.14% , one isoform of 76.8 KDa and two isoforms of 20 and 5 KDa respectively in mutant 3 with average of similarity 37.5% and finally one isoform band at very high molecular mass 150 KDa with an average of similarity 61.54 in mutant 4. This again confirm the major differences between the wild type and their mutants four the hand among mutants themselves from one other hand. Additionally the synthesis of 150 KDa only in the mutant 4 indicated this big variations consequently one importance of this breeding program.

Increase of peroxidase activity accompanied with synthesis of new isoforms, which may be consequence of cell specific gene expression (Brune *et al.,* 1994), are

Table II. Similarity matrices for sodium dodecyl sulphate polyacrylamide gel of *Dunaliella salina* (wild type) and four mutants resulted from different doses of gamma radiation

	Wild type 0.5 hr	**Wild type 0.5 hr**	**Mut. 1 0.5 hr**	**Mut. 1 1 hr**	**Mut. 2 0.5 hr**	**Mut. 2 1 hr**	**Mut. 3 0.5 hr**	**Mut. 3 1 hr**	**Mut. 4 0.5 hr**	**Mut. 4 1 hr**
Wild type 0.5 hr										
Wild type 1 hr	73%	\\\								
Mut 1 0.5 hr	61%	61.5%	\\\							
Mut 1 1 hr	58%	45%	58%	\\\						
Mut 2 0.5 hr	45%	38%	36%	66%	\\\					
Mut 2.1 hr	68%	38%	33%	45%	70%	\\\				
Mut 3.05 hr	36%	32%	27%	43%	38%	62%	\\\			
Mut. 3 1 hr	55%	42%	55%	71%	58%	53%	51%	\\\		
Mut 4 0.5 hr	38%	40%	35%	61%	61%	63%	59%	64%	\\\	
Mut 4 1 hr	69%	47%	53%	42%	36%	41%	60%	48%	69%	\\\

Table III. Similarity matrices between control and four mutants between mutants itself according the production of peroxidase isoenzyme of *Dunaliella salina* after gamma ray exposure

	Wild type	Mutant 1 (100 grays)	Mutant 2 (180 grays)	Mutant 3 (330 grays)	Mutant 4 (450 grays)
Wild type					
Mutant 1 (100 grays)	53				
Mutant 2 (180 grays)	57.14	76.92			
Mutant 2 (180 grays)	37.5	40	46.15		
Mutant 2 (180 grays)	61.54	57.14	57.15	53.3	

directed to protect the cell against oxidative damage. (Garcia *et al.,* 1996).

Peroxidase participate in a variety of plant defense mechanisms (Moerschbacher, 1992) in which H_2O_2 is often supplied by an oxidative burst, a common event in defense responses (Lamb & Dixon, 1997).

Finally, according to the data of RAPD-PCR, peptide mapping and isozymic pattern the four. *Dunaliella salina* mutant are genetically different. To follow up, we studied the physiological responses of the wild type and their mutants when grown under some drastic environmental conditions (we start with salinity conditions).

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