Effect of some stress factors on the genetic stability of the sugar cane *Saccharum officinarum* L. in *vitro*

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Abstract

The current study was conducted in the tissue culture laboratory at the Marine science Center University of Basrah. The objective of this study was to enhance the salt tolerance of two varieties of sugar cane, CO331and CP72-2086, by studying the intersection between three levels of salinity (0,120 and 150 mmol) and four doses of radiation (0,10,20,30 Gy) using gamma rays, the effect of the pretreatment with Ethyl Methane Sulfonate (EMS) for 2.5 hours on salt tolerance of sugarcane callus was studied by cultivating callus treated with this compound in saline media containing the lethal dose (180 mmol) and higher than it (200 mmol). In addition to the control treatment, the results showed the differentiation of the plants from the callus that was exposed to different treatments resulted in plants with genetic variation when compared with the parent plant using the RAPD technique and four random primers. This variation ranged from 52.63% using the OPA-01 primer to 67.85% using the OPH-04 primer in cultivar CO331.While the genetic variation of cultivar CP72-2086 ranged from 29.41% using the primer OPA-01 to 69.23% using the primer OPH-04. What became clear from the results was that treating the callus with a radiation dose of 20 Gy had an effective effect in increasing the salt tolerance of the callus and the resulting plants when grown in the salt medium of 150-millimolar sodium chloride. Also, the treatment with the EMS compound improved the salt tolerance of the plant in the media containing the lethal dose for both the two types.

Keywords: sugar cane, Tissue culture, gamma rays, (EMS), genetic variation.

*The study is taken from the first researcher's doctoral dissertation.

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Introduction

One of the perennial tropical plants in the Poaceae family is sugarcane *Saccharum officinarum* L. It is one of the more salt-sensitive plants, with a critical salinity limit of 1.7 dS.m-1 for growth and economic output. Up until it hits this limit, growth is significantly impacted. At a salinity level of eight dS.m-1, the consequent loss is greater than 50% (Mass, 1986). Salt stress has an effect on the sugarcane plant's growth and development because it interferes with biochemical processes. The ideal strategy to create novel hybrids that can tolerate salinity is to create salinity-tolerant types, but at the moment, this goal is challenging to accomplish due to even with the aid of plant genetic engineering tools, it is challenging to transmit the salinity-tolerant trait since it is genetically complex and is regulated by numerous genes (Flower, 2004; Munns and Tester, 2008). Alternative technologies must be used to achieve this goal, such as plant tissue culture technology, which aims to increase salt tolerance. Mutagens can have an impact on plant breeding methods since they depend on genetic mutations, crossbreeding, and selection, and because genes are what cause the emergence of genetic features. the genetic structure of an organism or part of it, leading to the emergence of a new trait that did not previously exist, as well as the fact that the incidence of mutations spontaneously occurring in nature is extremely rare and can be caused industrially. Numerous studies have demonstrated that utilizing mutagens as radiation improved the sugarcane plant's induced callus tolerance to salt stress. (Patade *et.al*, 2008, Patade and Suprasana, 2009; Nikam *et al*., 2015). Additionally, the use of some chemicals, such as the direct mutagen ethyl methane sulphonate (EMS), which alters the chemical structure of nucleotides and the order of nitrogenous bases in the DNA strand, is used to alter the genetic structure of plants (Green *et al*., 2003). Several mutations in Arabidopsis were created using this substance (Salinas and Sanchez, 2004), and salt-tolerant sugarcane was created using it (Purnamaningsih and Hutami, 2016).

Aim of study:

A- Production of salt-tolerant sugarcane plants using gamma rays and EMS.
B- Investigating the progeny's genetic compatibility with the mother plant using RAPD technology.

Materials and Methods:

The current study was conducted in the tissue culture laboratory of the Marine Sciences Center using two varieties of sugar cane, one local CO331 and the other American CP72-2086 obtained from the General Company for Sugar Industry in Missan. In this study, a nutritional medium composed of a group of salts MS (Murashige & Skoog, 1962) obtained from (ZAS) company Zist Arman Sabz was used at a concentration of 4.6 $g.l^{-1}$. Several concentrations of pure sodium chloride salt were used and were added to the media during preparation, which are (0, 120,150, and 200 mmol)

The degree of electrical conductivity (EC) of the media was measured by an EC-meter (Sartorius), callus was induction from both types using Growth regulator Dichlorophenoxy acetic acid (2,4-D). The abundant callus from the study of Al-Aradi *et al.* (2020) was adopted in conducting the experiments, Panel (1a&b). An approximate amount of 150 ± 10 mg of callus irradiated with gamma rays emitted from the Europium-152 source was cultivated in doses (0, 10, 20, 30 Gy) in media supplemented with sodium chloride salt, at concentrations (0, 120, 150 mmol) and the callus irradiation process was performed. In one of the laboratories of the Directorate of Radioactive Waste Handling/ Atomic Energy Commission/ Tuwaitha/ Baghdad Panel (2). In the other experiment, ethyl methane sulfonate (EMS) was used at a concentration of 0.5%, and this compound was prepared by dissolving it in chloroform at a rate of (200 µl. ml-1) Because it is rapidly decomposed by heat, it was subjected to a sterilization process by filtering using a filter number 0.20 µm before treating the callus with it (Schalet ,1978), after which the callus was immersed in the prepared solution for 2.5 hours, then cultured in a media containing sodium chloride salt at concentrations (0, 180, 200 mmol). A completely randomized design (CRD) was used, and the experiments were factorial, as ten replicates were used for each treatment.

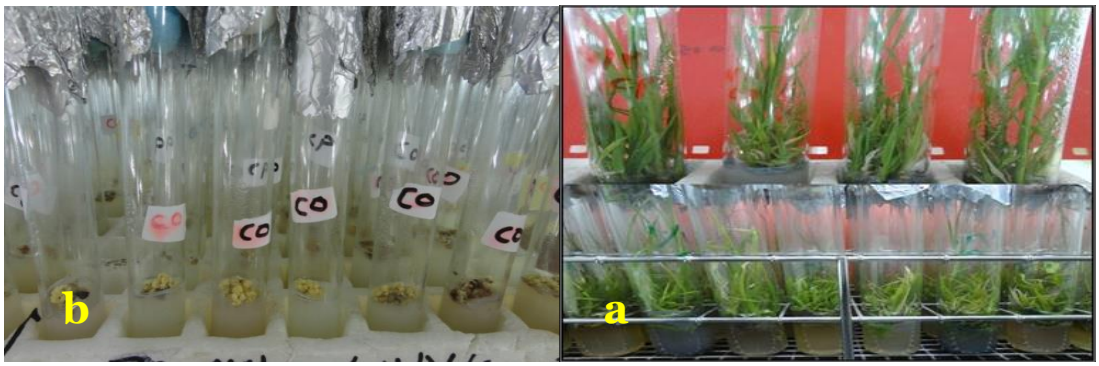

Figure 1: a/ Callus propagation in saline media, b/Sugarcane plant growing from callus.

Figure (2): Irradiation of callus using EU-152 radioactive source.

Genetic fingerprint test:

Before conducting the DNA test, leaf samples were dried using freeze-drying technology. Using a lyophilizer (Edwords Pirani-501) with a temperature of (-26) C° , Random Amplifid Polymorphic DNA (RAPD) was used to determine the genetic fingerprint, study genetic variation, and extract DNA in accordance with the method of Weigand *et al*. (1993) used for this study.

OPA-01=5'-CAGGCCCTTC-3'
OPH-04=5'-GGAAGTCGCC-3'
OPH-05=5'-AGTCGTCCCC- 3'
OPH-19 =5'-CTGACCAGCC- 3'

As for the program that was used to perform the process of amplifying the extracted DNA, it is as shown in Table (1).

Table 1: The program used in RAPD technology.

Stag	Step		Time	Cycle
1	1	Denaturation 94c°	1 min	1
2	1	Denaturation 94c°	1 min	40
	2	Annaling 37 c°	1.5 min	
	3	Extension 72c°	2 min	
3	1	Extension 72c°	10 min	1

It was done using the size index (Marker) of size (100 bp). The UVI-SOFT application was used to estimate the sizes of the DNA fragments. The following equation was used to determine the percentage of genetic variation: % of genetic variance = (number of divergent bands/number of total bands) x 100

Results and Discussion

Exposing sugarcane callus to an appropriate dose of gamma rays and then cultivating it in salt media increases its salt tolerance, thus increasing its division rate and becoming the dominant cells and they can be considered cells selected to withstand salt stress (Figure, 3). After the selected callus is multiplied to tolerate salinity through repeated replanting, it is transformed into complete plants that have the ability to grow in salt media. It is clear from the results that gamma rays have a role in improving the salt tolerance of sugarcane plants by improving the characteristics of vegetative growth, especially increasing the number of branches growing from the callus and increasing the length of the root when it interacts with salt levels after planting them in the rooting medium.

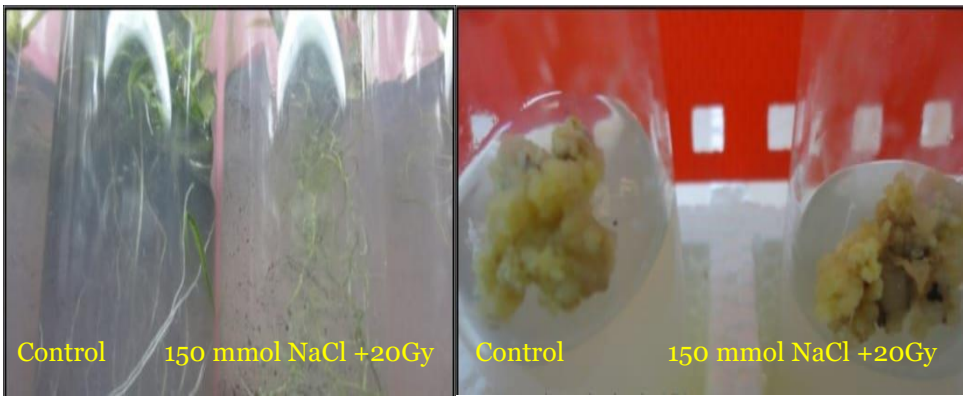

Figure (3): Growth of callus and root exposed to 20 Gy in 150 mM saline.

Salinity levels caused a significant decline in all growth indicators for calluses treated with the EMS compound for 2.5 hours. The reason may be due to the high salt concentrations in the growth medium, as these concentrations were higher than the lethal dose, which is 180 mM sodium chloride, which caused tissue death. Callus not treated with this compound. The decrease in growth indicators as a result of treating the callus with the EMS compound is due to the occurrence of dormancy in the callus that lasted for 16 weeks of cultivation in saline media. After this period of growth in the high-salt media had passed, the callus began to grow and multiply rapidly, and it was replanted in a new nutrient medium. It contains Benzyl adenine (BA) at a rate of 0.5 $g.L^{-1}$ and Kinetin at a rate of 1 $g.L^{-1}$. It was noted that the callus turned to purple and then to green (Panel 4), after which adventitious branches began to appear.

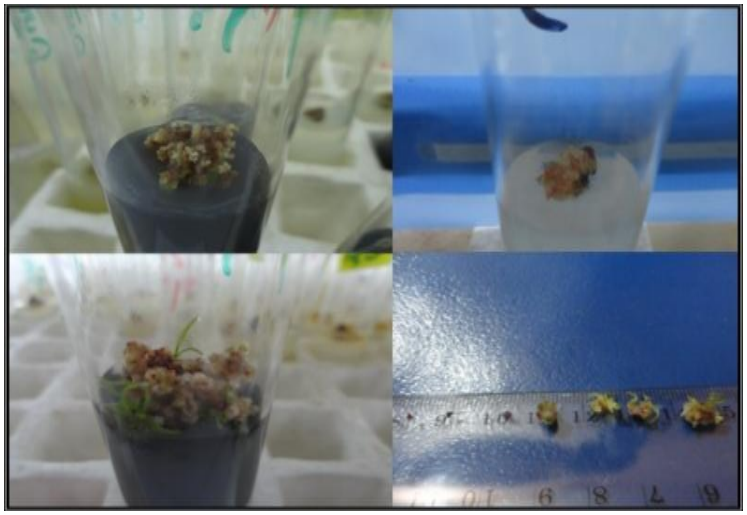

Figure (4): The callus treated with EMS compound multiplies for 2.5 hours, and it is noted that the callus turns purple.

Genetic match of plants resulting from tissue culture with the mother plant by the effect of different treatments (DNA Finger Printing).

1-Use the OPA-01 primer:

The genetic variance across plants as a result of several OPA-01 primer treatments on the mother plant and cultivar CO331 is depicted in Figure (5) and table (2). The percentage of genetic variation reached 100% when compared with the mother plant in the tissue culture plants produced without any treatment, whereas the treatment produced by treating the callus with the EMS compound and then cultivating it in media devoid of sodium chloride salt produced a percentage of variance of 85.71% when compared to the mother plant. The treatment, which included 20 Gy of gamma radiation and 120 mmolar NaCl, resulted in 33.33% genetic variation in the plants. The data in the table clearly show that the offspring produced using the OPA-01 primer from cultivar CO331 and the mother plant had 52.63% genetic variation.

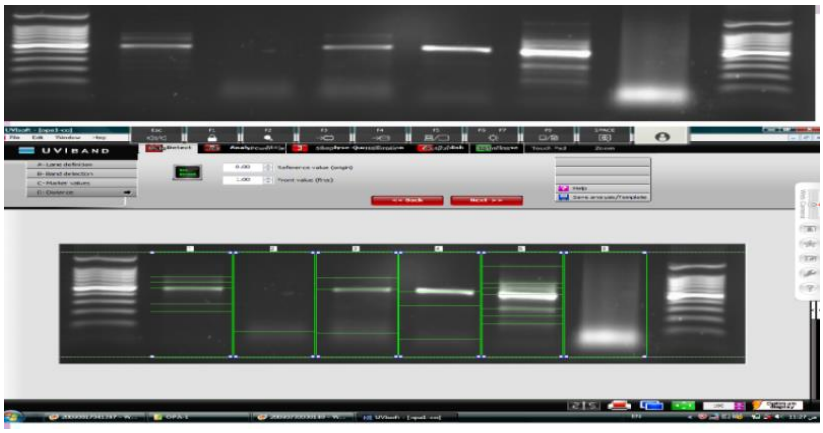

Figure 5: results of PCR-RAPD analysis using the primer OPA-01 in sugarcane plant CO331.

*The numbers at the top of the board from left to right 1-6 refer to the same number contained in the attached table.

Table 2: Genetic variation between plants resulting from the effect of different treatments and the mother plant using the OPA-01 primer of sugar cane cultivar CO331.

Treatment	Total number of bands	Number of differentiated bands	genetic variation %
1- The mother plant from the field	5		
2- The plant produced *in vitro*	1	1	100
3- 120 mmol NaCl and 20 Gy - gamma rays	3	1	33.33
4-150 mmol NaCl and 20 Gy-	3	2	66.66

gamma rays			
5- EMS Zero	7	6	85.71
6-200 EMS	0	0	0
Total	19	10	52.36

With the genetic diversity in cultivar CP72-2086 brought on by the use of the primer OPA-01, it is obvious that the majority of treatments did not produce distinct bands. This suggests that the majority of the sequences identified in cultivar CO331's DNA strand are absent from cultivar CP72-2086.

2- Use of the OPH-19 primer:

Table 3 and Figure 6 show the genetic variation between the plants as a result of the treatments and the mother plant of cultivar CO331 using the OPH-19 primer. The amount of genetic variation in the mother plant was thus 60%, whereas the lowest amount was 33.33% after treatment with 120 mmol sodium chloride and 20 Gy gamma rays, and the highest amount was 85.71% in plants resulting from callus treated with EMS compound and grown in the nutrient medium containing sodium chloride salt. It was revealed that this resulted in an average genetic variation for strains of 80%.

Table 3: Genetic variation between plants resulting from the effect of different treatments and the mother plant using the OPH-19 primer of sugarcane cultivar CO331.

Treatment	Total number of bands	Number of differentiated bands	genetic variation %
1- The mother plant from the field	2		
2-The plant produced *in vitro*	5	3	60
3- 120 mmol NaCl and 20 Gy -gamma rays	3	1	33.33
4- 150 mmol NaCl and 20 Gy -gamma rays	7	5	71.42
5-Zero EMS	6	4	66.66
6- 200 EMS	7	6	85.71
Total	30	19	63.33

*The numbers at the lowest of the board from left to right 1-6 refer to the same number contained in the attached table.

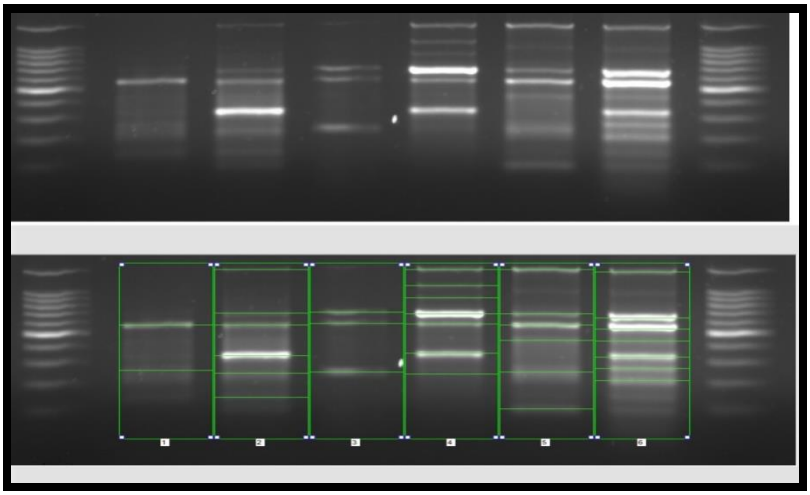

Figure 6: Results of the PCR-RAPD analysis using the OPH-19 primer in sugarcane plant CO331.

3- Use of OPH-04 Primer:

Using the OPH-04 Primer, Figure (7) and Table (4) display the genetic variance between the progeny produced from cultivar CO331 and the mother plant. The amount of genomic variation with this Primer was 75% because there were three different bands. The effect of the EMS therapy produced the largest percentage of genetic variation, which amounted to 100%, while the effect of the treatment using 150 mmol sodium chloride and 20 Gy-gamma rays produced the lowest percentage of variation, which amounted to 66.66%. Given that the primer OPH-04 produced 28 bands, including 19 unique bands, as shown in the aforementioned table, the percentage of genetic diversity between strains resulting from variation of CO331 using the primer OPH-04 is 67.85%.

Figure 7: results of PCR-RAPD analysis using the primer OPA-04 in sugarcane plant CO331.

*The numbers at the top of the board from left to right 1-6 refer to the same number contained in the attached table.

Table 4: Genetic variation between plants resulting from the effect of different treatments and the mother plant using the OPH-04 primer of sugar cane cultivar CO331.

Treatment	Total number of bundles	Number of differentiated bundles	genetic variation %
1- The mother plant from the field	5		
2-The plant produced *in vitro*	4	3	75.00
3- 120 mmol NaCl and 20 Gy -gamma rays	7	6	85.71
4- 150 mmol NaCl and 20 Gy -gamma rays	3	2	66.66
5-Zero EMS	5	5	100
6- 200 EMS	4	3	75.00
Total	28	19	67.85

4- Using the primer OPH-04:

Figure (8) and Table (5) display the genetic diversity of the strains descended from cultivar CP72-2086. The effect of the treatment (150 mmol sodium chloride and 20 Gy -gamma rays) and the effect of the treatment (10 Gy -gamma rays) both produced percentages of genetic variation that were 100%, while the lowest percentage of variation was recorded when the treatment (20 Gy -gamma rays) reached 60%, leading to the total number of genetic variations being 26 bands, including 18 unique bands, and a genetic variation rate of 69.23% were obtained using the OPH-04 Primer and the cultivar CP72-2086.

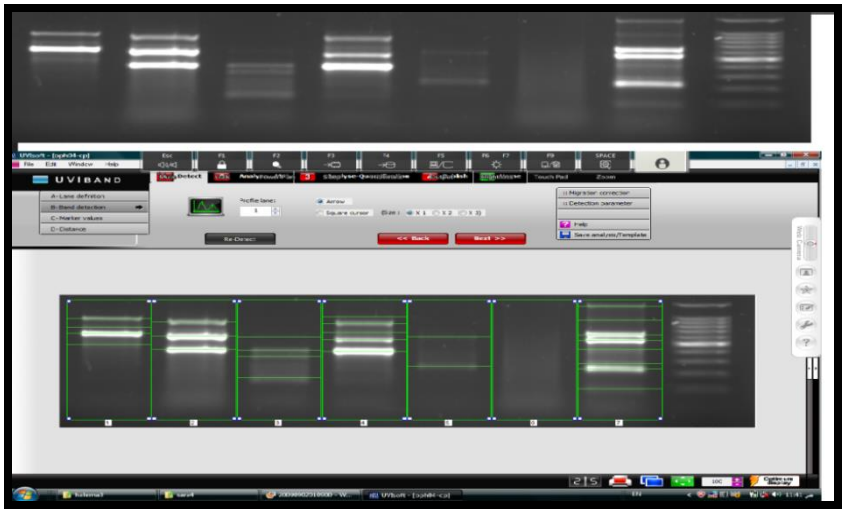

Figure 8: results of PCR-RAPD analysis using the primer OPH-04 in sugarcane cultivar CP72-2086.

*The numbers at the top of the board from left to right 1-7 refer to the same number contained in the attached table.

Table 5: Genetic variation between plants resulting from the effect of different treatments and the mother plant using the OPH-04 primer of sugarcane cultivar CP72-2086.

Treatment	Total number of bundles	Number of differentiated bundles	genetic variation %
1- The mother plant from the field	4		
2- The plant produced *in vitro*	3	3	100
3- 10 Gy -gamma rays	4	4	100
4-20 Gy -gamma rays	5	3	60
5- 150 mmol NaCl and 20 gamma rays	2	2	100
6-Zero composite EMS	0	0	0
7- 200 EMS	8	6	75
Total	26	18	69.23

5- Use OPH-05 primer:

The genetic variety of the strains produced by cultivar CO331 when grown in vitro is evident in Figure (9) and table (6), and it is evident that the mother plant could be identified by five distinct bands.

Figure 9: Results of PCR-RAPD analysis using OPH-05 primer in sugarcane plant CO331.

While the plant grown in vitro produced three bands, including two differentiated bands, resulting in a genetic variance percentage of 66.66%, the highest percentage of genetic variance reached 80% as a result of treatment with EMS compound in an empty medium of sodium chloride salt and treatment with 150 mmolar sodium chloride and 20 Gy -gamma rays.

Table 6: Genetic variation between plants resulting from the effect of different treatments and the mother plant using the OPH-05 initiator of sugar cane cultivar CO331.

Treatment	Total number of band	Number of differentiated band	genetic variation %
1-Ladder			
2- The mother plant from the field	5		
3-The plant produced *in vitro*	3	2	66.66
4- 120 mmol NaCl and 20 Gy -gamma rays	0	0	0
5- 150 mmol NaCl and 20 Gy -gamma rays	5	4	80
6-Zero EMS	5	4	80
7- 200 EMS	4	3	75.00
Total	22	13	59.09

With 13 distinct bands. included, this primer generates a total of 22 bands, with a genetic variation rate of 59.09%. The genetic diversity of the strains arising from cultivar CP72-2086 when cultivated in vitro using the OPH-05 primer is depicted in Figure (10) and table (7).

When compared to the treatment with EMS in 200 mmol saline, which produced five bands, including three separate bands with a genetic variation rate of 60%, only the two dosages of 10 Gy and 20 Gy supplied each of them one bands that was genetically identical to the mother plant and had a 0% genetic variation rate. Thus, 10 bands, including three different bands, were created from CP72-2086 using the OPH-05 primer, and the degree of variance reached 30%.

Figure 10: results of PCR-RAPD analysis using the primer OPH-05 in sugarcane cultivar CP72-2086.

Table (8) shows that the OPH-19 primer produced the most total bands for both cultivars, while the OPA-01 primer for CO331 and the OPH-05 primer for CP72-2086 produced the least total bands for both cultivars, giving only 19 and 10 bands, respectively. The OPH-04 primer produced the highest percentage of genetic variation, reaching 67.85 and 69.23 for cultivars CO331 and CP72-2086, respectively.

Table 7: Genetic variation between plants resulting from the effect of different treatments and the mother plant using the OPH-05 primer of sugarcane cultivar CP72-2086.

Treatment	Total number of band	Number of differentiated band	genetic variation %
1- The mother plant from the field	3		
2-The plant produced *in vitro*	0	0	0
3- 10 Gy -gamma rays	1	0	0
4-20 Gy -gamma rays	1	0	0
5- 150 mmol NaCl and 20 non-gamma rays	0	0	0
6-200 EMS	5	3	60
Total	10	3	30

Table 8: Comparison between the products of random primers OPA-01, OPH-19, OPH-04 and OPH05 for two varieties of sugarcane CO331 and CP72-2086.

	CP72-2068			CO331		
Primers	genetic variation %	The number of differentiated bands	The number of total bands	genetic variation %	The number of differentiated bands	The number of total band
OPA-01	/	/		52.63	10	19
OPH-19	/	/		63.33	19	30
OPH-04	69.23	18	26	67.85	19	28
OPH-05	30	3	10	59.09	13	22

Tables 2 through 7 clearly show that one of the most effective technologies for identifying genetic variation among plant species, cultivars, and even treatments is RAPD technology. Its ease of use comes from the ability to use more than one primer to distinguish between the various and related DNA strand sequences that are present. The method of isolating DNA (Bands) according to their molecular weight is made easier by electrophoresis. This is in line with the findings of other studies that showed how well RAPD technology can identify the degree of variation between plants grown in vitro and the mother plant from which they were harvested (Geisteira *et al*., 2002; Bennici *et al*., 2004).

The findings of the present investigation also corroborated Prammanee and Pitakpolrat's (2001) finding that six strains were obtained from successive sugar cane cup F36-819 cultures. One of the divergent bundles was discovered in a strain after the

RAPD indicator was employed to determine genomic diversity. The sequence of these DNA fragments was discovered to be identical to the sequences in the genes responsible for the plant's salt tolerance, having a molecular weight of 995 base pairs. Different primer associations with DNA plants or the Deletion or addition of a base or many nitrogenous bases that are present in the DNA could be the cause of the dissimilar bits of DNA strand. When cultured in vitro to detect somatic changes, molecular molecules in the agarose gel using the electrophoresis device could activate some genes (Gene On) or silence other genes (Gene Off), leading to a change in one or more of the traits present in the plant. (Kunert *et al*., 2003) As a result, it is possible to identify the genetic differences between varieties or even within the same variety.

Using the RAPD indicator, Saker *et al*. (2000) discovered 4% genetic diversity across 70 plants produced from date palm tissue cultures. Callus cultures chosen to endure salt and water stress using gamma rays obtained various genetic modifications thanks to the employment of plant tissue culture technologies and the development of mutations outside the body. is extremely sensitive to identifying the traits of stress-tolerant plants for in vitro cultivation of sugar cane. Nikam *et al*. (2015) evaluated the salt tolerance of sugarcane embryonic callus by irradiating it with radiation (from 10 to 80 Gy) and then exposing it to NaCl (0, 50, 100, 150, 200, and 250 mmol L).

While it was found that calluses exposed to 20 Gy of radiation and developed in saline solution produced proline and glycin betaine, increasing the concentration of sodium chloride resulted in lower development and greater membrane damage. Under NaCl stress, the survival percentage of irradiation callus was 50–60%, whereas the survival rate of plants was 80–85%. He stated that mutations brought on by radiation presented a potent means of enhancing genetic diversity in sugarcane. For in vitro cultivation of sugar cane, this approach was particularly sensitive in identifying the traits of stress-tolerant plants. The same outcomes as those discovered by Wang *et al*. (2007) when utilizing the RAPD index to identify genetic alterations in gamma-exposed sweet potato callus. In a study he conducted in Alsaffar (2022), he demonstrated how RAPD-PCR genetic markers were utilized to analyze relationships between six wheat genotypes and genetic variation in wheat plants (140).

DNA fragments with an average of 6.7 distinct bands per primer, were generated by four random primers 85 pieces (44.64%) of the six genotypes were polymorphic. RAPD analysis can be used to describe and categorize wheat genotypes. Future efforts to produce wheat producing offspring will benefit from these discoveries. As shown by Moghaieb *et al*. (2011), the existence of genetic similarity between two cultivars of date palms cultivated histologically, One of which is unknown and the other is the Ferhi cultivar, and the plants resulting from their in vitro offspring, when using the RAPD indicator and ten random primers, and each primer resulted in one bundle of 200–2600 integrated bases. The results showed that there was genetic variation between the unknown cultivar and the Ferhi cultivar, which amounted to 36.2 and 37.8%, respectively, when compared with the parent plant.

Conclusion

Traditional plant breeding methods are considered slow methods, especially for sugarcane plants, because the plant reproduces vegetative and flowering rarely occurs due to its needs for light requirements, especially the length of the day and the number of days the plant is exposed to light. Therefore, the best way to develop salt-tolerant varieties is to use plant tissue culture technology that aims to improve salt tolerance, in which cells that are resistant to salt stress are selected in vitro.

The current study showed that the technology of plant tissue culture has led to variations in plants resulting from re-differentiation of cells or cultured callus tissue, and these variations can be considered sources of new species in genetic improvement of plants and obtaining plants that are tolerant of environmental stress in a relatively short period through selection. Given that plant-breeding methods depend on genetic mutations, hybridization, and selection, and that genes are the factors responsible for the emergence of hereditary traits, the study showed that the mutagens used changed the genetic variations of the sugarcane plant, or part of it. It was also concluded that exposing the callus induced from the sugarcane plant to gamma rays and an ethyl Methanesulfonate leads to genetic variations.

Study of genetic stability using the RAPD technique revealed the presence of genetic variation between the plantlet resulting from the different treatments and the mother plant, there was a difference between the two types of sugarcane plants in this regard, and the best result was obtained using the primer OPH-19 in both varieties.

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أثر بعض عوامل الإجهاد في الثبات الوراثي لنبات قصب السكر *Saccharum officinarum* L المزروع نسيجيا

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المستخلص

أُجريت الدراسة الحالية في مختبر زراعة الأنسجة التابع لمركز علوم البحار/ جامعة البصرة، وذلك بهدف زيادة التحمل الملحي لصنفين من قصب السكر CO331 و CP72 2086- درس تأثير التداخل بين ثلاثة مستويات من الملوحة (120,0 و150 ملي مولار) مع أربعة جرع من الإشعاع وهي (30,20,10,0 غري) باستخدام أشعة جاما، ودُرس تأثير المعاملة الأولية بمركب أثيل ميثان سلفونيت EMS لمدة (2,5 ساعة) في التحمل الملحي لكالس نبات قصب السكر عن طريق زراعة الكالس المعامل بهذا المركب في أوساط ملحية حاوية على الجرعة القاتلة Lethal dose (180 مليمولار) والأعلى منها (200 مليمولار) بالإضافة إلى معاملة المقارنة، بينت النتائج إن إخلاف النبيتات من الكالس الذي عرض إلى معاملات مختلفة نتج عنه نباتات تمتلك تباين وراثي عند مقارنتها مع النبات الأم باستخدام تقنية الـ RAPD وأربعة بادئات عشوائية وقد تراوح هذا التباين 52.63% باستخدام البادئ OPA-01 إلى 67.85 % باستخدام البادئ OPH-04 في الصنف CO33، في حين تراوح التباين الوراثي للصنف CP72-2086 من 29.41 % باستخدام البادئ OPA-01 إلى 69.23 % باستخدام البادئ OPH-04 كما اتضح من النتائج أن معاملة الكالس بجرعة الإشعاع 20 غري كان له تأثير فعال في زيادة التحمل الملحي للكالس والنبيتات الناتجة عند زراعتها في الوسط الملحي 150 مليمولار من كلوريد الصوديوم، كما إن المعاملة بمركب EMS حسن من التحمل الملحي للنبات في الأوساط الحاوية على التركيز القاتل ولكلا الصنفين.

الكلمات المفتاحية: قصب السكر، زراعة الأنسجة، أشعة كاما، EMS، تباين وراثي.

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