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Biochemical and Molecular Characterization of Some Rice Accessions Tolerated To Salt Stress

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Abstract

Rice is considered one of the most important food crops, and it ranks third in terms of strategic nutritional importance after wheat and maize. Given that this strategic crop is sensitive to salt stress, especially in the areas designated for rice cultivation in the valley and delta regions, it was necessary for researchers to develop new lines with good tolerance in addition to, their high yielding through using the original rice cultivar (Giza 178 and its 7 mutants derided from it). These seven mutants were derived by five gamma rays doses; 100, 200, 300, 400 and 500 and characterized with highly rank of stable. Indeed, this investigation succeeded in developing and eliciting a number of promising rice accessions that are tolerant to salt stress and high output using various doses of gamma rays while, highlighting the biochemical, physiological and genetic evidence of this tolerance under Egyptian conditions. Based on this, these promising new lines represent a major scientific leap in the field of breeding and improving rice tolerance to face the risk of soil salinity, which seemed to be increasing in the past few years due to water scarcity needed for irrigation and the fact that Egypt is in an area of water stress. The final results confirmed that the highest rice mutants recorded highly rank of yield components and the other attributes under salinity treatment compared to the standard experiment were mutant 2, followed by mutant 7 and then followed by mutant 3, respectively. Further, the five ISSR primers discovered 2 unique markers considering that these fragments are the taxonomic basis for salt stress tolerance in the five new rice mutants compared to the original variety and this is the biggest progress in this investigation.

***Keywords:* -** Rice, Genetic Analysis, Salinity stress, Biochemical, Physiological Parameters, Mutation.

1. Introduction

Rice is the main food for more than 3.5 billion people worldwide, as this crop represents a major source of food energy because it contains a selection of the most important nutrients necessary for the body. Further, this crop is characterized by its high genetic diversity, which qualifies it to be an important source of all nutritional and medicinal benefits alike **[1].** It is worth noting that the interest of increasing rice crop productivity will have a great impact and will reflect positively on raising the economies, income and well-being of developing countries **[2].** In the past years, a large number of researches and studies dealt with discussing the most important environmental constraints and challenges that impede rice productivity globally and locally, especially the environmental stresses like water and salt stresses. Salt stress leads to devastating damage to the growth and productivity of crops in general and rice in particular. As, it causes damage to all vital organelles and negatively affects all vital, biochemical and physiological processes, which leads to a decline in germination, growth and elongation of cells **[3].** As for the rice crop, salt and water stresses causes great sterility in the early stages of growth and grain filling, and causes complete atrophy in the case of excessively high soil salinity and irrigation water, which destroys the final output by no less than 80% **[4].** A large number of papers and studies have also proven that water and salt stresses had the greatest impact in destroying the productivity of a large number of grass crops of national and strategic nutritional dimension, such as rice, canola, wheat, barley, maize, sorghum and sunflower **[5-14].** However, the science of plant breeding and improvement, especially through using different doses of gamma rays to create superior mutations for increasing salt stress tolerance in rice plants had a great impact in this regard **[15].** In the same context, it is noted that the problem of salt stress and the increase in the toxicity of heavy elements in the soil, as well as the increase in the salinity of irrigation water, began to worsen in the past ten years. The reason for this is due to the low level of water needed for agriculture and drinking, because the Arab Republic of Egypt is located in the area of global water poverty, especially after the construction of the Grand

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Ethiopian Renaissance Dam. And the low water level in the soil definitely leads to an exacerbation of the level of salts, especially in the surface soil layers due to the lack of leaching process, and this of course negatively affects the cultivation of summer crops such as rice, cane and maize, and impedes the cultivation of winter crops such as wheat, barley and fava beans **[16].** A large group of research and studies tended to find out the mechanisms and systems responsible for the tolerance of plants to salt stress, but only a few of them shed light on breeding to develop strains of rice that are tolerant to salt stress **[16- 18].** Also, water and salt stressors are two sides of the same coin, because each of them is associated with the other and causes it, especially in tropical, subtropical and arid regions, which impedes agricultural production processes and delays the course of economic development in such areas Among the countries in the world most exposed to this environmental danger are African countries, especially Egypt **[18].** Further, this confirms the utmost importance of discussing this serious environmental challenge and trying to contain these environmental problems and reduce their devastating effects, which destroy agricultural output and the expected national return, especially in developing countries whose overall economy depends on agricultural production. Rice plant is classified as medium tolerant to salt stress and the degree of response to tolerance varies according to the growth stage. Further, the tolerance class is a complex physiological attribute besides, its inheritance is controlled by a large number of genes and it takes several segregation generations to reach genetic stability **[19].** The process of salt stress tolerance is an aggregate process of each growth stage tolerance. This is directly reflected in the final rice output under saline soil conditions and the extent of the plant's ability to modify its genetic, physiological and biochemical properties to keep pace with this serious environmental obstacle. On this basis, the process of genetic improvement of rice tolerance to environmental stresses such as salinity and water stress will be very feasible if the breeding technique is used by creating highly productive, more stable and tolerant mutations with climate change conditions **[15].** Among the successful mechanisms in this regard is the creation of new mutations with the aim of enriching the base of genetic track, which will be the locomotive in choosing the best traits and lines tolerant to salt stress, after determining the extent of success of mutant accessions in bringing about biological, physiological, genetic and biochemical changes that qualify it for the desired level of tolerance **[20].** Hence, if we talk about the most scientific victories in the field of breeding and improving field crops to face environmental stresses such as salt stress, we note that breeding with mutations had the largest and pivotal role in changing the traditional genetic path and making a quantum leap in modifying the genes responsible for bringing about tolerance and resistance. Further, the traits of tolerance or resistance to salt stress, it is transferred to sensitive genotypes by traditional breeding technique represented in crossing among new lines that are mutagenic and tolerant, which have become genetically stable with sensitive varieties. Then, tolerance or resistance attribute is followed through the different segregation generation in order to reach complete genetic stability and produce high-tolerance rice accessions to salt stress as well as its high yielding. After what has been presented, the aim of this study can be briefly summarized in knowing the genetic, physiological and biochemical responses of a promising group of rice accessions that have been induced using a distinct dose of gamma rays, which has already succeeded in reaching a safe and acceptable level of salt stress tolerance in rice. Therefore, these new genotypes will in the future be high-tolerance rice varieties for salt stress besides, their high yielding because rice is considered one of the most important strategic field crops, which represents food security for the vast majority of people besides, its distinguished nutritional value.

2. Materials & Methods

2.1. . Background

This work used (Giza 178 rice variety) which has excellent morphological and physiological traits that qualify it to be highly yielding and distinguished in other agro-morphological characters. As well, its biochemical and physiological traits make it resistant to salinity stress. Therefore, this variety is an excellent experimental material that can be used in this investigation.

2.2. Field evaluation:-

The Giza 178 rice seeds used in the recent investigation were originally performed from Rice research & Training Center, Agriculture Research Centre. Five hundred pure seeds of Giza 178 cultivar were subjected to gamma irradiation treatments at dosages of 100, 200, 300, 400 and 500 Gy using the Co source at the National Center for Radiation Research and Technology, Nasr City and Cairo, Egypt in 2010 season (M0). The irradiated materials of all doses were grown and series of selections among the mutant population under normal soil conditions in Sakha location in Kafr El-Sheikh Governorate and this process was carried out during 2011-2018 seasons (M1-M8) to produce the selected seven mutant rice lines and all plants have reached full genetic stability at the eighth generation (M8).

2.3. Sowing and Treatments:-

This investigation was conducted in two experiments, the first one was conducted under normal soil conditions (Sakha city) in Kafr El-Sheikh, Governorate and the second treatment was under saline soil conditions (El-Sirw city) in Damietta governorate, Egypt during 2019 and 2020 seasons using the original rice cultivar (Giza 178) besides, 7 mutant lines derived from it and selected from M8 generation as follows. The chemical analysis of both soil types were shown in table (1) as follows:-

Table 1. Chemical Classification of normal and both levels of salinity soils during two growing seasons

Characteristics	Normal Soil at (Sakha city)	Saline Soil at (El-Sirw city)
EC (dS/m)	2.76	10.18
pH (1:2.5)	7.62	8.73
TDS mg/ litre (ppm)	523	6213.32-6455.19
Ca++	1.97	15.21
Mg++	1.46	14.42
Na+	9.54	62.11-66.30
K+	0.39	0.36
$CO3^{--}$	0.15	0.17
$HCO3^{-}$	4.06	1.82
Cl^{-}	10.59	27.94
$SO4^{-}$	4.03	17.89
Texture	Clay	Clay

2.4. Studied traits

Sixty plants were used for determined all attributes under the study of each treatment (normal and Saline conditions), with 20 plants for each replicate. Each experiment included three replicates, and the eight rice genotypes were grown in a randomized complete block design.

2.4.1. Yield, physiological and biochemical Traits;-

1):- 1000-grain weight (gm)
2):- Grain yield/plant (gm)
3):-Proline Content.
4):- Glycine betaine Content.
5):- Trehalose Content.
6):- Osmotic adjustment.

The proline content was determined from a standard curve and calculated on a fresh basis is as follows: [(µg proline / ml C m1 toluence) / 115.5 µg / µ mole] / [(g sample/5)] = µ moles proline / g of fresh weight material where the results were average values at least 3-4 samples for each species, according to **[21, 22]** while glycine betaine and trehalose contents were carried out according to **[23].**

2.4.2. Molecular Depiction

Total genomic DNA of all samples was extracted from green rice leaves using Qiagen DNeasy Plant Minikit following the protocol of the manufacturer (Qiagen Inc, Valencia, CA, USA). The quality of the extracted DNA was assessed on agarose gel electrophoresis. PCR was performed using 5 preselected ISSR primers based on their ability to generate reproducible and informative amplification patterns. Amplification reactions were carried out in Biometra T One Thermal Cycler (Analytik Jena, Jena, Germany). PCR amplification was performed in 25 µl reaction mix which contained 20–30 ng DNA template, 10 pmol of each primer, 2.5 µl of 2 mM Thermo dNTPs, 5 µl of 5X Promega Green GoTaq Flexi Reaction Buffer, 2.5 µl of 25mM Promega MgCl2, and 0.125 µl of 5U/µl Promega GoTaq Flexi DNA polymerase. The reaction was assembled on ice, and amplification was performed at certain conditions as follows: an initial denaturing step at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, annealing at 50 °C for 1 min, an extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. The PCR products were assessed on 1.6% agarose gel **[24-26].** The banding profile of ISSR were scored using the Labimage program, and the polymorphism percentage was estimated as follows Percent of polymorphism = (number of polymorphic bands/total number of bands) × 100, **(Table 2).**

Table 2. Band variation and polymorphism percentage in 8 ric accessions using 5 ISSR primers

No.	ISSR Primers	Sequence
1	98 A	5` CAC ACA CAC ACA AG 3`
2	ISSR-11	5'-ACACACACACACACACYA-3'
3	ISSR-13	5'-AGAGAGAGAGAGAGAGYT-3'
4	HB-12	5`CAC CAC CAC GC 3`
5	HB-13	5´ GAG GAG GAG C 3`

Data was scored for computer analysis on the basis of the presence or absence of the amplified products for each primer. Pairwise components of the eight rice genotypes based on the presence or absence of unique and shared polymorphic products, were used to determine similarity coefficients according to **[27].** The similarity coefficients were then used to construct dendrograms, using the un weighted pair group method with arithmetic averages (UPGMA) employing the SAHN (sequential, agglomerative, hierarchical, and nested clustering) from the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 1.80 (Applied Biostatistics Program). All calculated data of all traits under evaluating in two seasons for both treatments were analysis using the formula by **[28].**

3. Results & Discussion

3.1. Analysis of Variance

There is no doubt that the factors of climate change have contributed significantly to the most dangerous events and the greatest impact on the bad impact on global agricultural plans and agricultural growth paths in developing countries, especially the Arab Republic of Egypt. Moreover, the excessive change in the global thermal system has already led to the occurrence of biochemical, physiological and morphological changes that had the greatest advantage in reducing the global and Egyptian agricultural area alike. This has already negatively affected the economies of countries and their growth programs, especially countries that depend entirely or almost entirely on agricultural production. So, thousands of papers and studies were launched that would try to protect plants and crops, especially strategic crops, from the devastating effects of climate change factors, such as high levels of salinity in irrigation water and soil, as well as the risk of water stress, especially for countries that suffer from limited water, such as the Arab Republic of Egypt. In the same context, severe water stress, as its damage was previously discussed, does not only affect the productivity of field crops and the vegetation surface in general, but also leads to an increase in the rank of salinity percentages in the surface layers of the soil, especially those close to the sea coast in the valley and delta regions. This actually destroys crop productivity by not less than 80% **[4-6].** For all this, this study was launched with the aim of genetically improving the rice crop to withstand salt stress by using different doses of gamma rays under Egyptian conditions. The main focus of such research and studies was in an attempt to devise modern lines of the most important crops for human and animal food, especially the rice crop, given that it represents the main food or meal for the majority of the world's population. Further, these new genotypes must be characterized by their tolerance of environmental stresses that are not conducive to growth and productivity, such as salt stress, the pressure of which has begun to increase in the past ten years due to the decline in water rates required for agriculture and the desertification of a large sector of land allocated to agricultural production in countries known for their water poverty, especially Egypt. Also, its high yielding and genetic and environmental stability under various conditions, it should eventually be strategic rice lines that are resistant to a large number of diseases as well. On its way to be rice varieties grown under local conditions and meet the needs of both the producer and the consumer. This is the real and nominal goal in this study, which if achieved will be a major scientific leap in the path of genetic improvement of rice to meet and bear abiotic stress, especially salt stress. Therefore, the physiological nature of rice classifies it as a sensitive crop to salt stress, and its growth is severely affected by the high salinity rank of the soil and irrigation water, as previously mentioned **[8-11].** The following is a detailed explanation of the process of genetic improvement and breeding in the rice crop in order to develop rice accessions that are highly tolerant to salt stress using mutations.

Data presented in tables (3 & 4) revealed that highly significant differences were observed among all rice accessions (the original variety Giza 178 and their seven mutant Lines) for all studied attributes under both conditions during the two growing seasons (2019 & 2020). Also, coefficient of variance percentages was low for all studied characters under normal and salinity conditions in both growing seasons except osmotic adjustment trait for the same treatments. It is worth mentioning that the analysis of variance test is one of the most accurate and important tests to ascertain the degree of significant difference between all genotypes under study. This is the entrance and prerequisite for conducting the analysis in both environments. On this basis, this statistical step has proven that the seven rice mutants differ from the original variety descended from it in all biochemical, genetic and physiological attributes, and even outperformed it, especially in the final economic output. These new rice genotypes proved to be highly tolerant to salt stress compared to the original variety Giza 178 after calculating and estimating all studied traits under saline soil treatment compared the normal soil conditions. This remarkable superiority may refer to a group of alleles and genes that were formed and activated by different doses of gamma rays, as those genes contributed to enhancing the additive gene effect and its fruitful role for increasing the degree of salt stress tolerance in these new promising mutants. Accordingly, these new rice lines will be the actual nucleus for producing modern rice varieties that are highly tolerant to salt stress and high in final output after continuing to cultivate these accessions for several segregation generations to reach full genetic stability, and after making sure that containing all other quantitative traits such as resistance to water stress, diseases resistance and promising quality characteristics.

Table 3. Analysis of variance for all studied traits of the eight rice accessions under normal conditions (Sakha location) in the two growing seasons

S.O.V	D.F	Seasons	1000-grain weight (gm)	Grain yield/plant (gm)	Proline Content.	Glycine betaine Content.	Trehalose Content.	Osmotic adjustment.
Genotypes	7	2019	23.45**	41.02**	11.56**	27.68**	12.96**	19.33**
		2020	17.66**	36.72**	10.07**	21.15**	36.88**	8.92**
Replicates	2	2019	13.82**	12.79**	54.21**	60.04**	27.55**	78.55**
		2020	19.42**	28.11**	60.18**	48.77**	31.05**	93.71**
Error	14	2019	1.55	1.93	1.79	1.35	1.52	1.68
		2020	1.17	1.86	1.45	1.59	1.66	1.34
C.V. %		2019	3.89	1.98	2.12	1.81	2.41	154.30
		2020	3.36	1.95	1.92	1.96	2.49	133.05

Table 4. Analysis of variance for all studied traits of the eight rice accessions under normal conditions (El-Sirw location) in the two growing seasons

S.O.V	D.F	Seasons	1000-grain weight (gm)	Grain yield/plant (gm)	Proline Content.	Glycine betaine Content.	Trehalose Content.	Osmotic adjustment.
Genotypes	7	2019	21.44**	49.03**	10.94**	25.19**	10.33**	27.12**
		2020	15.61**	38.13**	13.72**	19.33**	42.17**	10.13**
Replicates	2	2019	18.60**	16.72**	58.19**	54.23**	21.05**	82.06**
		2020	23.05**	34.51**	73.55**	39.84**	33.04**	89.03**
Error	14	2019	1.48	1.39	1.38	1.29	1.78	1.55
		2020	1.14	1.68	1.71	1.64	1.83	1.28
C.V. %		2019	4.52	1.93	1.48	1.47	1.76	234.90

3.2. Field evaluation

This study succeeded in eliciting new genotypes of rice with high tolerance to salt stress and its final crop as well, through genetic improvement of the Giza 178 rice variety using different doses of gamma radiation. Also, there is no doubt that the different plant breeding programs aim to improve plant tolerance to environmental and biotic stresses as well as its high yield. Among the environmental stresses is the high salinity of soil and irrigation water, especially after the degree of water scarcity that the Egyptian state suffers from which began to worsen, especially after the construction of the Grand Ethiopian Renaissance Dam. Therefore, it was necessary for the majority of researchers to develop scientific methods and techniques in order to devise modern rice lines that are tolerant to salt stress besides, their high yielding as well. Further, one of the most important methods of breeding and improving crops is breeding with mutations to produce mutant plant strains with promising genetic mutations that are superior to the original varieties descended from them, in all genetic, physiological, biochemical and molecular attributes, which are positive changes that the plant resorts to resist and tolerant to environmental stresses, especially salt stress and this will be discussed in detail in this regard after presenting the most important results and values of the seven new rice mutations under salt stress conditions compared to the natural experiment. Results of mean values viewed in tables (5 & 6) revealed that the seven rice mutant lines had actually outperformed the original cultivar (Giza 178) in all studied traits under salinity experiment compared to the standard treatment over the two growing seasons and were exhibited highly trend of salinity stress tolerance in this context. For the normal treatment, the rice mutants (2, 3, 6) were recorded the highest mean values (33.82, 34.52, 34.69 gm) for 1000-grain weight trait, the mutants (3, 6, 7) recorded (75.98, 74.28, 80.67 gm) for grain yield/plant, the mutants (1, 4) revealed (80.57, 81.38) for proline content, the mutants (4, 5) detected (70.31, 83.0) for glycine betaine content, the rice mutants (5, 6, 7) were recorded (62.52, 58.54, 70.18) for trehalose content and the mutants (1, 2, 3, 4, 6, 7) were achieved (0.99, 0.84, 0.74, 0.80, 0.79, 0.65) for osmotic adjustment, respectively. While under saline experiment, the values were (29.58, 29.81 gm) of the rice mutants (2, 6) for 1000-grain weight, the values were (74.18, 72.34 gm) of the mutants (2, 7) for grain yield/plant, the values were (99.62, 90.14, 102.03, 88.09) of the rice mutants; (1, 3, 4, 5) for proline content, the values were (86.55, 90.13, 97.04, 88.40) of the rice mutants; (2, 4, 5, 6) for glycine betaine content, the values were (83.39, 92.23, 86.58, 103.36) of the rice mutants; (3, 4, 6, 7) for trehalose content and the values were (0.40, 0.59, 0.49, 0.62, 0.35, 0.43, 0.49) of the seven rice mutants for osmotic adjustment, respectively. Therefore, the rice genotypes mentioned above were achieved the highest rank of salinity stress tolerance under saline experiment compared to the standard treatment. The technique of breeding by mutations, especially by using different doses of gamma rays, succeeded in bringing about physiological and biochemical changes in a positive direction and the final result was elicitation of seven rice accessions tolerant to salt stress under saline soil conditions compared to natural soil conditions **[29, 30].** Hence, results of a large number of studies and papers have shown that plants and field crops may resort to some biochemical changes in order to contain or reduce the negative effects of environmental stresses, especially water and salt stresses and to transform the condition from sensitive to tolerant or resistant **[30].** These changes serve as a lifeline that plants resort to when exposed to environmental stresses, especially salt stress. This type of physiological change is a vital and extremely important type of simulation with difficult environmental conditions. In terms of the most prominent biochemical changes, the increase in the proportion of proline content in the leaves of plants comes to the fore. It is considered one of the most important hormonal modifications to salt stress tolerance in a large number of crops such as rice, wheat, barley, sorghum, maize, cotton, flax, canola and sunflower **[30-38].** Further, this investigation produced seven highly productive and salt-stress-tolerant rice mutants after confirming their ability to increase their content of organic acids related to salt stress tolerance such as proline, glycine betaine, and trehalose contents under saline conditions compared to the standard experiment **[31, 32].** It is worth noting that some studies have shown that rice, wheat and barley are strategic crops that have the superior ability to produce organic acids and reducing sugars that reduce osmotic pressure and adjust it to the modified osmotic pressure. Among these organic compounds, proline comes in first place **[32].** Also, glycine betaine content is considered one of the most famous and strongest organic acids produced by plants to face environmental stresses such as high salt stress in the soil and irrigation water, and its production process increases in the initial plant growth stages such as seedling germination until flowering and fruit ripening, especially during exposure to environmental stress **[10 , 13].** Also, trehalose content helps to raise the degree of tolerance of rice, wheat and barley to salt stress, and this has been proven in a large number of genetic and physiological studies with the aim of improving field crops to face the most severe environmental stresses such as high soil salinity, irrigation water, toxicity of heavy elements and water stress **[13].** Among the most prominent achievements of this study is that the seven rice mutants had already outperformed the original variety (Giza 178) descended from it in all the traits under study and proved beyond any doubt that these are tolerant to salt stress through the values of yielding, physiological and biochemical traits. As, those improved genotypes gave the highest values of proline, glycine betaine, trehalose contents, grain yield/plant and 1000- grain weight under saline soil conditions compared to the natural experiment **[33, 34].** Also, these improved genotypes were able to adjust their osmotic pressure to the level that achieves endurance and simulates salt stress conditions in what is known as osmotic adjustment **[35, 36].** Accordingly, the new lines of mutant rice have already succeeded in producing a positive case of genetic, physiological and biochemical improvement of the tolerant rice variety Giza 178. The seven promising rice accessions were already distinguished for their salt stress tolerance and high yield. Also, they represented the kernel for producing tolerant local rice varieties after being cultivated in several regions and different environments to ensure their genetic stability.

Table 5. Mean Performance and Combined Analysis for All Studied Traits of the eight rice accessions under normal conditions (Sakha location) in the two growing seasons

Traits / Genotypes	1000-grain weight (gm)			Grain yield/plant (gm)			Proline Content.			Glycine betaine Content.			Trehalose Content.			Osmotic adjustment.		
	2019	**2020**	**Mean**	**2019**	**2020**	**Mean**	**2019**	**2020**	**Mean**	**2019**	**2020**	**Mean**	**2019**	**2020**	**Mean**	**2019**	**2020**	**Mean**
Giza 178	27.43	25.98	26.70	54.32	55.09	54.70	34.45	37.12	35.78	42.15	39.18	40.66	28.14	31.09	29.61	1.04	1.02	1.03
Mutant 1	30.15	31.05	30.60	64.71	65.72	65.21	83.23	77.92	**80.57**	51.08	53.12	52.10	32.55	30.08	31.31	0.97	1.01	**0.99**
Mutant 2	33.62	34.02	**33.82**	83.04	82.22	82.63	55.93	53.71	54.82	53.45	55.04	54.24	46.12	47.22	46.67	0.88	0.81	**0.84**
Mutant 3	35.07	33.97	**34.52**	77.82	74.15	75.98	72.14	69.88	71.01	60.03	62.01	61.02	52.07	54.03	53.05	0.71	0.78	**0.74**
Mutant 4	30.88	31.52	31.20	59.03	61.03	60.03	79.54	83.22	**81.38**	72.18	68.44	**70.31**	57.23	59.02	58.12	0.76	0.84	**0.80**
Mutant 5	32.89	33.04	32.96	64.15	66.05	65.10	68.13	65.79	66.96	81.95	84.05	**83.0**	61.03	64.02	**62.52**	0.98	1.05	1.01
Mutant 6	34.19	35.19	**34.69**	75.43	73.14	74.28	65.32	66.18	65.75	80.63	79.23	79.93	59.45	57.63	**58.54**	0.74	0.84	**0.79**
Mutant 7	31.60	32.16	31.88	81.03	80.32	80.67	45.96	47.77	46.86	69.73	72.18	70.95	71.04	69.33	**70.18**	0.64	0.66	**0.65**
Mean	31.97	32.11	32.04	69.94	69.71	69.82	63.08	62.69	62.89	63.90	64.15	64.02	50.95	51.55	51.25	0.84	0.87	0.85
LSD at 5%	1.79	1.55	1.67	2.00	1.96	1.98	1.92	1.73	1.82	1.67	1.81	1.74	1.77	1.85	1.81	1.86	1.66	1.76
LSD at 1%	2.66	2.31	2.48	2.97	2.92	2.94	2.86	2.57	2.71	2.48	2.70	2.59	2.64	2.76	2.70	2.77	2.48	2.62

Table 6. Mean Performance and Combined Analysis for All Studied Traits of the eight rice accessions under normal conditions (El-Sirw location) in the two growing seasons

Traits / Genotypes	1000-grain weight (gm)			Grain yield/plant (gm)			Proline Content.			Glycine betaine Content.			Trehalose Content.			Osmotic adjustment.		
	2019	**2020**	**Mean**	**2019**	**2020**	**Mean**	**2019**	**2020**	**Mean**	**2019**	**2020**	**Mean**	**2019**	**2020**	**Mean**	**2019**	**2020**	**Mean**
Giza 178	22.18	19.72	20.95	48.03	46.93	47.48	55.14	52.06	53.60	48.23	42.56	45.39	51.71	49.33	50.52	0.88	0.67	0.77
Mutant 1	27.13	28.02	27.57	55.13	57.02	56.07	97.22	102.03	**99.62**	66.13	67.04	66.58	61.03	70.18	65.60	0.39	0.42	**0.40**
Mutant 2	30.02	29.14	**29.58**	75.14	73.22	**74.18**	63.04	68.01	65.52	81.07	92.03	**86.55**	55.93	59.73	57.83	0.60	0.58	**0.59**
Mutant 3	27.12	28.26	27.69	61.08	63.73	62.40	89.04	91.24	**90.14**	72.06	75.66	73.86	81.45	85.33	**83.39**	0.47	0.51	**0.49**
Mutant 4	26.05	24.08	25.06	50.04	48.13	49.08	104.02	100.05	**102.03**	88.11	92.15	**90.13**	96.23	88.23	**92.23**	0.71	0.54	**0.62**
Mutant 5	25.33	27.04	26.18	57.11	60.02	58.56	87.13	89.05	**88.09**	96.02	98.07	**97.04**	74.23	78.41	76.32	0.32	0.39	**0.35**
Mutant 6	29.51	30.12	**29.81**	68.14	66.05	67.09	77.18	80.12	78.65	86.74	90.06	**88.40**	83.12	90.04	**86.58**	0.42	0.44	**0.43**
Mutant 7	27.83	26.08	26.95	73.16	71.52	**72.34**	59.03	63.14	61.08	77.12	81.03	79.07	102.54	104.18	**103.36**	0.51	0.48	**0.49**
Mean	26.89	26.55	26.72	60.97	60.82	60.90	78.97	80.71	79.84	76.93	79.82	78.37	75.78	78.17	76.97	0.53	0.50	0.51
LSD at 5%	1.75	1.53	1.64	1.69	1.86	1.77	1.69	1.88	1.78	1.63	1.84	1.73	1.92	1.94	1.93	1.79	1.62	1.70
LSD at 1%	2.60	2.28	2.44	2.52	2.77	2.64	2.51	2.80	2.65	2.43	2.74	2.58	2.85	2.89	2.87	2.66	2.42	2.54

3.3. Molecular Characterization

The five ISSR primers used for comparing among the eight rice genotypes namely; 98 A, ISSR-11, ISSR-13, HB-12 and HB-13 exhibited a total of 33 fragments, 11 of them were monomorphic. In addition, 22 bands were found to be polymorphic, with 66.66 % (polymorphism) including two distinct bands as presented in (Table 7; Fig.1). The average numbers of polymorphic ISSR markers were 4.4 fragments for each primer. Polymorphic bands number ranged from 3 to 7 and molecular size ranging from 165 to 1117 bp, respectively. The highest number of total bands were observed by primer ISSR-11 (9), followed by ISSR-13 (7), followed by the primers; HB-12 and HB-13 (6) and followed by 98 A primer (5), respectively. Also, the highest number of polymorphic bands were showed in primer ISSR-11 (7), followed by ISSR-13 primer (5), followed by the primer HB-12 (4) and followed by the primers; 98 A and HB-13 (3), respectively. In the same regard, the highest polymorphism % was observed in primers; ISSR-11 (77.77 %) and ISSR-13 (71.42 %), respectively. Further, primers 98 A and ISSR-11 showed one unique band for each one of them. While, the rest primers namely; ISSR-13, HB-12 and 13 recorded no unique band in this investigation. This is considered a specific genetic marker and extremely important in distinguishing between sensitive or intermediate genotypes from those that are tolerant to salt stress.

Table 7. Band variation and polymorphism percentage in the eight rice genotypes using 5 ISSR primers

No.	ISSR primers	T.B	M.B	P.B	U.B	P %	R.S (bp)	Sequence
1	98 A	5	2	3	1	60.0%	978-165	5` CAC ACA CAC ACA AG 3`
2	ISSR-11	**9**	2	**7**	1	77.77.0%	1019-172	5'-ACACACACACACACACYA-3'
3	ISSR-13	**7**	2	**5**	0	71.42%	1117-196	5'-AGAGAGAGAGAGAGAGYT-3'
4	HB-12	6	2	4	0	66.66%	1050-188	5`CAC CAC CAC GC 3`
5	HB-13	6	3	3	0	50.0%	1016-173	5´ GAG GAG GAG C 3`
Total		33	11	22	2	66.66%	1036-894	

T.B: Total bands, M.B: Monomorphic bands, P.B: Polymorphic bands, U.B or P.S.M: Unique bands or positive specific marker, P%: Polymorphism percentage and R.S (bp): Range size

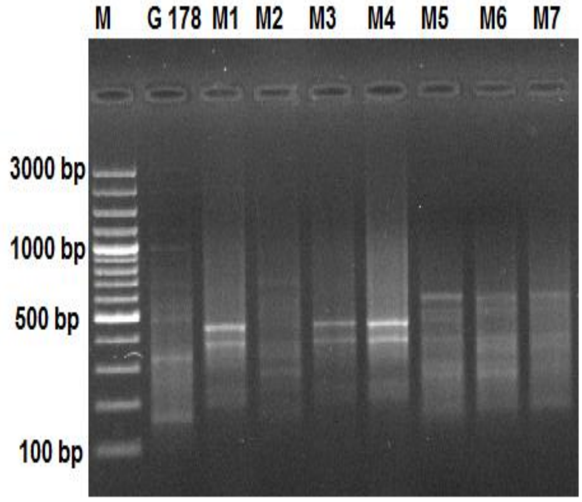
Data presented in (Table 8) exhibited (28) pairwise comparisons to debate the genetic relationships between the eight rice entries revealed in terms of genetic similarity. The genetic similarity values ranged from (0.862 to 0.310) with an average of (0.586). Where, the highest level of genetic similarity was (0.862) among (Mutant 1 & Mutant 4). While that, the lowest rank of similarity was (0.310) was observed between (Giza 178 & Mutant 1), respectively. The rest genetic similarity values show values ranged from low to high in this regard. Results of cluster analysis or phylogenetic tree presented in (Fig. 2) divided all rice accessions into two main cluster. Where, the cluster number one included the mutants; (1, 3 and 4). While, cluster II contained two sub-cluster. The sub-cluster one included mutant 2 only. Regardless, the sub-cluster number two included the cultivar Giza 178 as well as the mutants (5, 6 & 7).

Molecular genetic markers is considered one of the most important branches of genetics which has achieved great success in identifying genes and hereditary factors related to increasing yield and resistance to environmental and biological stresses. Further, determining their location on chromosomes, clarifying whether are dominant or recessive. In this regard, the current study focused on the use of a selection of molecular markers in order to differentiate between the seven high-tolerance rice mutants to salt stress and the original variety (Giza 178) which descended from it by plant breeding with mutations through the use of different doses of gamma rays. Also, these markers were able to identify the most important fragments responsible for salt stress tolerance in the seven rice accessions, especially **ISSR-11 and 13** which came in the first place of polymorphism % where the values were 77.77% and 71.42%, respectively in this regard.

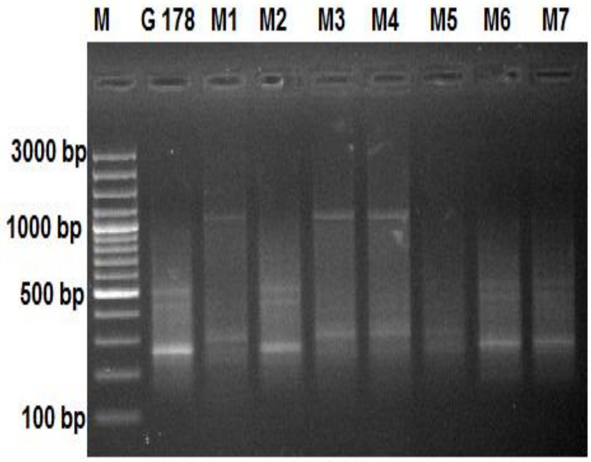
Table 8. Genetic similarity % in the eight rice genotypes using 5 ISSR Primers

Genetic Similarity	Giza 178	Mutant 1	Mutant 2	Mutant 3	Mutant 4	Mutant 5	Mutant 6	Mutant 7
Giza 178	1.0							
Mutant 1	0.310	1.0						
Mutant 2	0.590	0.423	1.0					
Mutant 3	0.346	0.850	0.416	1.0				
Mutant 4	0.392	0.862	0.407	0.809	1.0			
Mutant 5	0.521	0.423	0.416	0.416	0.407	1.0		
Mutant 6	0.727	0.333	0.480	0.370	0.366	0.608	1.0	
Mutant 7	0.789	0.333	0.571	0.320	0.370	0.571	0.714	1.0

Further, one of the most prominent achievements of this investigation is finding of two specific markers for salt stress tolerance in new rice mutants through using 98 A and ISSR-11 primers **[37-41].** After all that was presented in this regard at some length, it can be said that this study was able to achieve great progress in the path of genetic improvement of rice to tolerate salt stress through devising five rice mutants that are tolerant to high salinity levels. These new lines will contribute to enriching the genetic library of the Egyptian rice crop. Also, this will be done by crossing it with other local varieties that are sensitive to this dangerous environmental factor to transfer tolerance genes. This step is considered one of the most important scientific steps in the future. Further, this confirms that the methods of plant breeding with mutations have become one of the most effective plant breeding methods for genetic improvement in strategic crops to face both environmental and biotic stresses. This is what this investigation proved with a certain scientific fact that does not bear any room for doubt. In the same context, although traditional plant breeding programs are very important in developing new entries that have improved genetic traits in all crops, this stand helpless in front of the time factor, as it takes a large number of years that may reach fifteen years to produce a complete, genetically stable variety with all quantitative and descriptive traits needed by plant breeders. That is why scientists resorted to modern plant breeding programs, especially genetic engineering and genetic transfer programs, which had a great impact in improving field crops and raising their productivity and resistance to diseases and water and salt stressors. As well as, molecular genetic markers, which succeeded in identifying the genetic factors responsible for environmental and biotic stresses tolerance, but at the molecular level **[29, 30].**

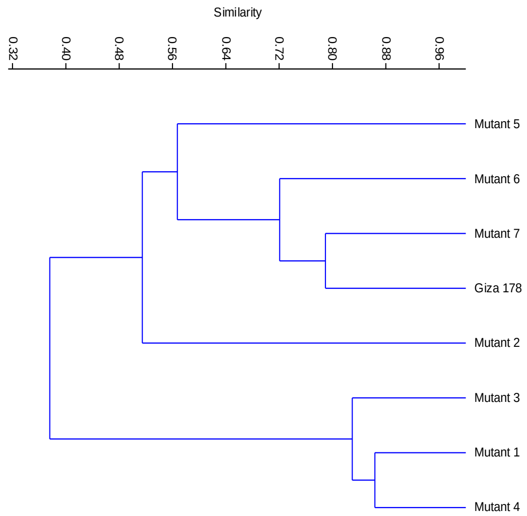


ISSR-11



ISSR-13

Fig 1. The inter-simple sequence repeat (ISSR) amplification pattern obtained in the eight *rice accessions* namely 1: original cultivar (Giza 178), 2: Mutant 1, 3: Mutant 2, 4: Mutant 3, 5: Mutant 4, 6Mutant 5, 7: Mutant 6 and 8: Mutant 7 using 5 ISSR primers, respectively



UPGMA

Nei-Li's similarity coefficient

Fig. 2: Dendrogram representing the genetic relationship between the eight rice genotypes using UPGMA cluster analysis of Nei-Li's similarity coefficient generated from the 5 ISSR primers

4. Conclusion

The current study showed the fruitful and positive role of breeding by mutations in elicitation of seven rice lines tolerant to salt stress were developed from the original tolerant variety Giza 178 using different gamma rays doses. Moreover, the seven new rice lines outperformed of the original cultivar (Giza 178) descended from it in all yielding, physiological and biochemical attributes under salt stress experiment compared to the natural conditions. Also, molecular markers technique using five primers namely; 98 A, ISSR-11, ISSR-13, HB-12 and HB-13 succeeded in identifying the genetic evidence at the molecular level responsible for salt stress tolerance in the aforementioned new genotypes and this is considered a major scientific leap in this regard.

Conflict of interest

There is no conflict of interest.

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