Evaluation of gene expression and genetic polymorphism of four genetic structures of *Vicia faba* L. under the influence salinity stress

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ABSTRACT:

A field experiment for the winter season (2022-2023) was carried out in the field of the Department of Crops / Faculty of Agriculture - Al-Qasim Green University, to find out the estimation of gene expression and genetic distancing using cluster analysis The four genetic structures of beans under stress of different salinity levels, the experiment included two factors according to factor experiments, the first factor is four genetic structures (local V1, Turkish V2, Spanish V3, Dutch V4) while the second factor included three levels of soil salinity (2, 6 and 9 decemens). The results indicated that salt stress had an effect on gene expression and the highest gene expression in the Turkish and Spanish varieties showed the highest values in the amount of gene expression of the study genes at the salt level 6 and 9 decimensem-1, while the local and Dutch varieties gave a lower amount of gene expression compared to the control gene. The results of the data analysis also showed that the highest Euclidean distance based on quantitative indicators was 1111 between the Turkish genotype V2 and the Spanish genotype V3 V3, while the Euclidean distance between the V3 genotype and the V4 genotype was (237.51), which is the lowest distance calculated. Based on PCR molecular indices, the highest Euclidean distance was 2.24 between local composition V1 and Dutch V4, while the lowest Occilidental distance of 1 was between Spanish composition V3 and Dutch V4.

Keywords: Broad bean - Salinity Stress - Genetic dimensions - Gene expression

1- Introduction

Broad bean *Vicia faba* L is one of the most important leguminous crops and the most widespread in the world, due to their high nutritional importance and are grown in various countries with the aim of obtaining juicy green pods and dry seeds that are used as a basic food after grass crops for humans and as animal feed, as well as beans are a staple food for the population of poor countries, especially North African countries and the countries of the Middle East for its high content of proteins 25-35% [1] In a statistic for the World Food Organization in 2021, it confirmed that the volume of global production of legumes amounted to 88.38 million tons [2]. The total production of this crop at the level of Iraq reached 9190 tons of dry and soft beans for the year 2020 [3]. Genetic differences provide a continuous source of variation, which is the basis for the selection of plants that excel in their productive characteristics, and to find genetic kinship between parents and sons , which can be done through the use of molecular indicators, which is one of the most important indicators of modern RNA that depends on polymerase chain reaction (PCR) technology. It is an effective and powerful tool for studying genetic diversity in living organisms, detecting genetic kinship relationships, analyzing, and finding the fingerprint[4]. In addition to the ease of application, analysis of their results and classification of genetic populations, but it is an expensive method or misreading of the asymmetric as homologous in the event of a mutation at the initiator site. The efficiency of the election depends on the existence of genetic variations, that is, whenever the genetic variation increases in the community or clan, the election can be held and vice versa, and that the election does not

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lead to genetic changes, but can be used to create a new society that differs from the characteristics of the society from which he was elected [5] The study of genetic diversity relied for long periods on phenotypic indicators to show variation between individuals, which depend on quantitative traits, which are important for plant breeders, because it is the oldest, easiest, least expensive and complex method and reveals important qualities for plant breeders, which are necessary in the taxonomic study of plants, evaluation of genetic structures and diagnosis of superior ones in the field. The study of genetic diversity within species, i.e. the structures of the same species, is important for identifying it, and estimating the proximity or genetic distance between genetic structures, which are of great importance in breeding programs and the selection of parents with the desired characteristics of the breeder [6] Plant breeders usually put all their attention to improve the yield and its components for any crop through breeding programs by adopting the selection that saves time, effort and costs, where the adoption of the selection guide technique is resorted to, through which the trade-off is made to choose the composition with a high yield and the most suitable for environmental conditions, and that the electoral guide that includes a number of qualities, including the characteristics of the yield, is one of the best means to improve the performance of the variety and is more feasible than direct selection of seed yield [7] The current slowdown in production is a cause for concern and the development of saline stress tolerance genotypes is imperative in order to maintain global food security, so the study aimed to estimate the genetic dimension of the four genotypes of beans and evaluate the performance of the four structures under underdeveloped levels of salt stress.

3-MATERIALS AND METHODS

The first experiment was carried out in the laboratory in the first season (2021-2022) for the purpose of molecular study to estimate gene expression and genetic Distance based on PCR indicators, while the second experiment was carried out in the field in the second season (2022-2023) for the purpose of estimating genetic dimensions based on quantitative characteristics.

3-1- Laboratory experiment:

method of work:

The use of genes of the *Unigene* family as an indicator for screening genetic structures, as these genes are genes that carry salt stresses in the bean crop.

1. Seeds were grown in pots in the laboratory and subjected to saline stress in a saline medium 14 days after germination and genes were detected after the appropriate 28-hour exposure period that induces gene expression.
2. Then the vegetative parts were taken directly and sent to the molecular analysis laboratories at the College of Education for Girls at the University of Kufa to detect the genes in the leaves with the Real Time PCR device.
3. Through the results, genetic variations are studied at the molecular level of taxa based on the largest gene expression to withstand stress.

3-1-1 Preparation of plant samples:

leaves were washed several times with sterile distilled water, to clean them of dust and plankton, then wiped with medical cotton dipped in alcohol at a concentration of 70% for the purpose of sterilization, then cut into small pieces (1 cm 2) using sharp scissors clean sterilized, and the pieces were placed in his eyelid ceramic (ceramic mortar) to which liquid nitrogen was added very carefully to avoid the damage caused by nitrogen when it came into contact with the skin or any of the visible organs of the body, then the

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samples were ground well until they turned into powder and it is ready for extraction.

Table (1): Shows the prefixes used in estimating the relative amount of gene expression of Unigene genes in the bean plant

gene name	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Encoded protein
Unigene072411	ACATCATAACTCGCACAGCA	CTGCAACATCAGTGCCAAGT	alpha/beta fold hydrolase
Unigene047182	TGGTAGGATGTAAGCACTGAAGC	GAGAATGGAGATGTAGATCACGCT	zinc ion-binding protein
Unigene012043	ACACCACCACCACGATCATC	CGGCGACTGAGGTTCTGAAG	late embryo-genesis abundant protein
House.Keeping 18srRNA	AGGTTCGTAGTGAACCGCTG	TTCGTTTACTCAGGGGGTGC	

Solutions used to analyze the relative amount of gene expression:

The solutions shown in Table (2) were used as components of the reaction of the Real-time PCR device as follows:

Table (2) Components of Master Mix Real-time PCR Reaction

Master Mix	1 Rxn (µl)	27 Rxn (µl)
1- Go Taq master mix SYBr Green kit	12.5	412.5
A- 100 mM KCl		
B- 40 mM Tris – HCl (pH 8.4)		
C- 0.4 mM dNTPs		
D- U/ µl I Taq DNA polymerase		
E- mM $MgCl_2$		
2- Primer Mix (F + R + D.W)	2.5	82.5
3- Distilled water (D.W)	7.5	247.5
	22.5	742.5

1.
2. 9 tubes containing cDNA were prepared according to the tubes of the study classes, each tube contains (7.5 µl) of cDNA by 3 repeaters and then distributed to the repeaters (2.5µl) of cDNA in the (plate

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tube) for the reaction and inserted into a device.

3. A special plate was prepared for the real-time PCR device, marked showing the varieties, and a map similar to the plate placed in the reaction for the real time was drawn, and each tube of the reaction plate contains 25 µl of the reaction material: (cDNA 2.5 µl + M.Mix SYBr Green 22.5 µl) or (M.MixBlank SYBr Green 22.5 µl).
4. SYBer Green Master mix prepared and added in the Mix-ready tube (412.5 µl) of Go Taq master mix SYBr Green kit.
5. Primer mix is prepared by taking 82.5 µl from it, adding it to the collector tube and placing the tube in the centrifuge for 6 seconds and quickly (Xg 12.000).
6. (247.5 µl) of D.W distilled water was added to the collector tube and then placed in the centrifuge for 6 seconds at speed (12.000 Xg).
7. Prepare two types of control treatment Blank Master Mix by calculating 3 repeaters for each type and complete the volume with water as in the interaction of tables (3) and (4) as follows:

Table (3): Components of cDNA depleted control treatment reaction

B (-cDNA)	1Rxn (µl)	3Rxn (µl)
- Go Taq master mix SYBr Green PCR	12.5	37.5
- Primer mix	2.5	7.5
- D.W	10	30
	25	75

Table (4) Components of the Reaction of the Incomplete Control Coefficient

B (-Primer)	1Rxn (µl)	3Rxn (µl)
- Go Taq master mix SYBr Green PCR	12.5	37.5
- cDNA	2.5	7.5
- D.W	10	30
	25	75

8. The contents of the Master mix were distributed (22.5 µl) for each tube of the Real Time Plate after mixing well with the Micro centrifuge for 4 seconds and quickly (12.000 Xg).
9. (2.5 µl) of cDNA per tube except for cDNA control treatment (B-cDNA) and by 3 replicates per class were distributed on the Plate of the PCR Real Time reaction to complete the volume in the reaction plate tubes to (25 µl).
10. The contents of the two Blank parameters are distributed by map on their location in the Plate interaction dish.
11. Cover the plate with a transparent cover called Sealing sheet and glue well and avoid touching the surface of the transparent by hand.
12. Plate mode interaction in the Real Time PCR device and monitors the interaction through the calculator screen associated with the real time device and equipped with a special program (CFX Manager

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software) and using the following program:

N	Stage	temperature	Duration	number of courses
1	Pre – Denaturation	94	5 min	1
2	Denaturation	94	45 sec	40
3	Annealing	55	30 sec	
4	Extension	72	1 min	
5	Final extension	72	5 min	1

13. Adjust the program by selecting the program by Master, and determine the samples by Plate set up, in which plate appears, and by shading the sample according to the map and writing the name of the sample to be compared with House. Keeping 18srRNA specify the protocol, names and colors for each sample.
14. The device was monitored until the temperature of the lid reaches 103 °C and the temperature of Block to 72 °C and this process lasted for 2.43 hours.
15. Charts then start to appear starting with the appearance of the Threshold.
16. The readings were automatically taken to the Genex statistical program for comparison, quantification and data preservation of gene expression of genes used for the varieties under study [16].

3-2 Statistical analysis (analysis of gene expression results)

The relative amount of gene expression was analyzed according to the program for analyzing the results of gene expression extracted by the Real time PCR device, as gene expression was analyzed according to the statistical program (Genex) associated with the (CFX Manager Software) program for the Real time PCR device, as after the end of the device's work period, we click on the Analysis icon, which shows the values of (CT) and with it the values of (SD) Standard deviation (standard deviation).

3-3 Field Experiment

The experiment was carried out in the field according to the experiments of the worker in the fields of the Faculty of Agriculture, Al-Qasim Green University. The field was divided into three replicates according to the design of the complete randomized sectors (RCBD) each repeater includes 12 experimental units, so the total number of experimental units is 36 units, the experiment included two factors The first factor includes four genetic structures of beans (local, Spanish, Turkish and Dutch code V1, V2 , 3V, V4 respectively, and the second factor included three concentrations of soil salinity, a treatment compared to the concentration of 2 decemens (S0) and the second level with a concentration of 6 Desmens (S1) As for the

third level, its concentration was 9 ds m^{-1} (S2), and the planting process took place on 1/11/2022.

3-2-1 Finding genetic relationships between breeds :

The results were analyzed according to the pre-prepared characterization tables using the PAST biostatistical program version 62.1 [10] as follows:

- **a.** Genetic distances created the Euclidean distances matrix using the above program among the breeds under study.
- **b.** Drawing a genetic kinship tree diagram Dendrogram Drawing a tree diagram for breeds using the above program and according to the Euclidean dimensions between these individuals in an even unweighted group method with the arithmetic average (UPGMA) Unweighted pair group method with arithmetic average in order to show the distribution of these strains into groups or clusters Clusters main and secondary depending on the distance between them and the shape of the genetic kinship tree Dendrogram scheme.
- **c.** Analysis (PCA) Principal Component The two-dimensional main component analysis diagram of the strains under study was drawn using the above program for the purpose of confirming the results of the genetic tree diagram, and the common variation of the 10 main components of the phenotypic and molecular indicators that were used in the study was found.

4- RESULTS AND DISCUSSION

4-1 Detection of the gene Unigene072411 for study varieties under saline stress conditions on agarose gel at a concentration of 1%

The main objective of using the gene *Unigene072411* and amplifying it using conventional PCR technology is to stabilize the appropriate experimental conditions and evaluate the efficiency of the reaction, and this gene is an endogenous control element that maintains the integrity of cells and prevents protein metamorphosis of the studied samples [11].

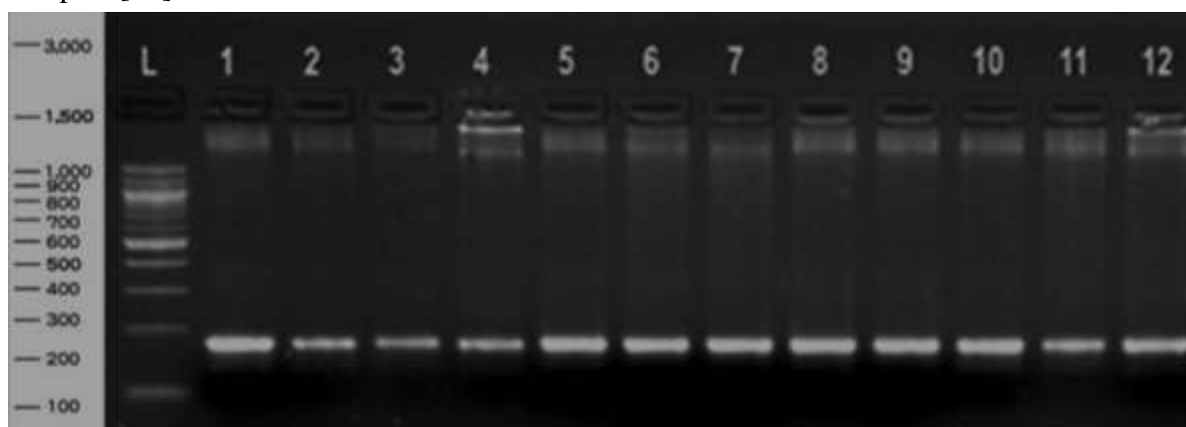

Figure 1: Relay of Real Time PCR reaction product of gene Unigene072411 to the genotypes of beans under salt stress conditions on agarose gel at a concentration of 1%

After installing the appropriate conditions for interaction and migrating samples for all bean plants that underwent the same conditions in agarose gel, a clear main package appeared in all studied models with a molecular weight of 180 bp (Figure 1) depending on the volumetric index used with a molecular weight of 100bp, and this indicates the success of the

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amplification process of the gene Unigene072411 and that the interaction conditions are suitable for gene amplification and Unigene047182 and Unigene012043, and therefore the genetic structures of beans varied among themselves in tolerance to salinity levels and in the ability to express The Unigene072411 gene is one of the most important salt-bearing genes for the bean plant.

4-2 Detection of *Unigene047182* Gene for Study Varieties Under Saline Stress Conditions on agarose Gel at 1% Concentration

After creating the reaction conditions QPCR, the gene *Unigene047182* was used to study the gene expression of the salt-tolerance trait based on the same method used with the gene *Unigene072411* and after amplifying the gene *Unigene047182*, the results of the data analysis by ordinary PCR technique were observed when the reaction product was migrated and in the presence of the 100bp type graduated volumetric index in the agarose gel for the genetic structures of the salt-tolerant bean plant, there was a clear self-contained molecular weight beam of 140 bp representing the gene *Unigene047182*. This shows that the gene *Unigene047182* with a molecular weight of 140 bp has an important role in plant tolerance to high salinity Figure (2). Salt tolerance in any plant is closely related to the presence of highly expressed salt-tolerant genes under salinity conditions, note [12]. The type of salt tolerance mechanism that has developed in the plant depends on the type of salt-tolerant gene and the level of salinity. Excess expression of the salt-tolerant gene has been shown to increase plant growth against salt stress [13].

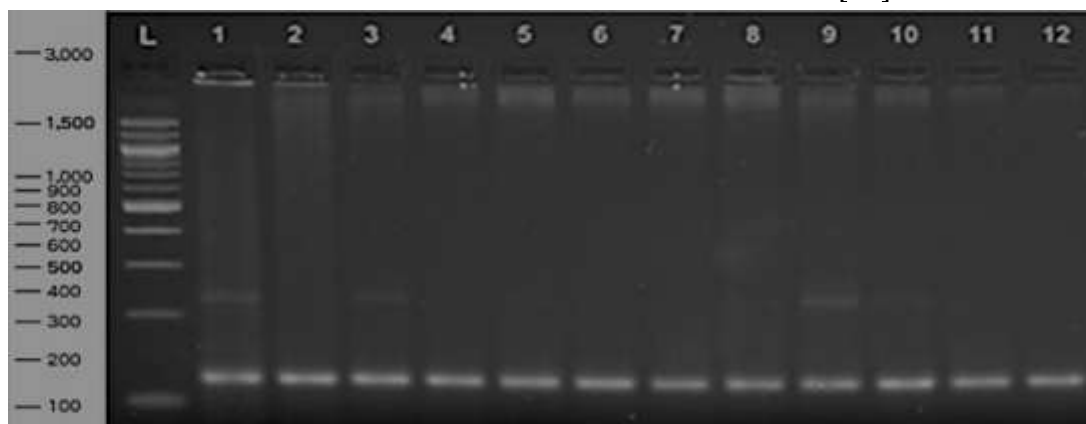

Figure 2: Relay of Real Time PCR reaction product of gene *Unigene047182* to the genotypes of beans under saline stress conditions on agarose gel at a concentration of 1%

Figure (3) shows the appearance of the gene *Unigene012043* in all genotypes cultured from beans (local, Turkish, Spanish and Dutch) at salt concentrations 4 and 9 ds m^{-1}, which indicates that the gene *Unigene012043* with molecular weight 220 bp expressed itself when detected by PCR technique and that The plant had the ability to tolerate high salt stress because it possessed the gene *Unigene012043* and increased gene expression in the presence of saline stress.

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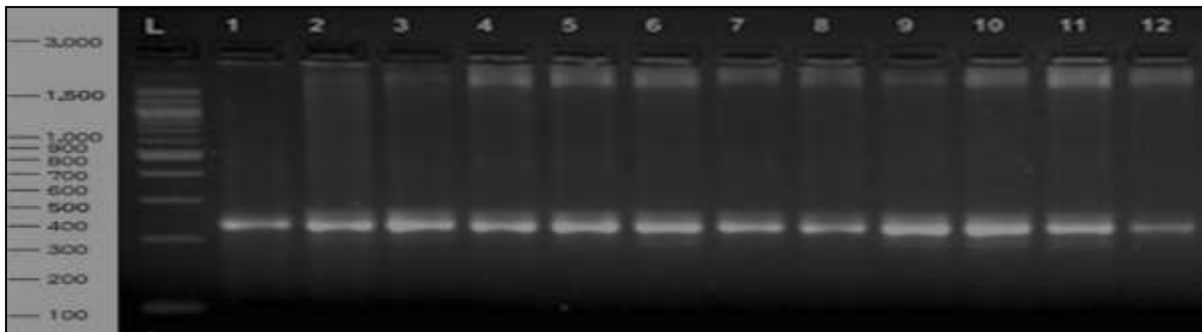

Figure 3: Relay of Real Time PCR reaction product of gene Unigene012043 to genotypes of legumes studied under saline stress conditions on agarose gel at a concentration of 1%.

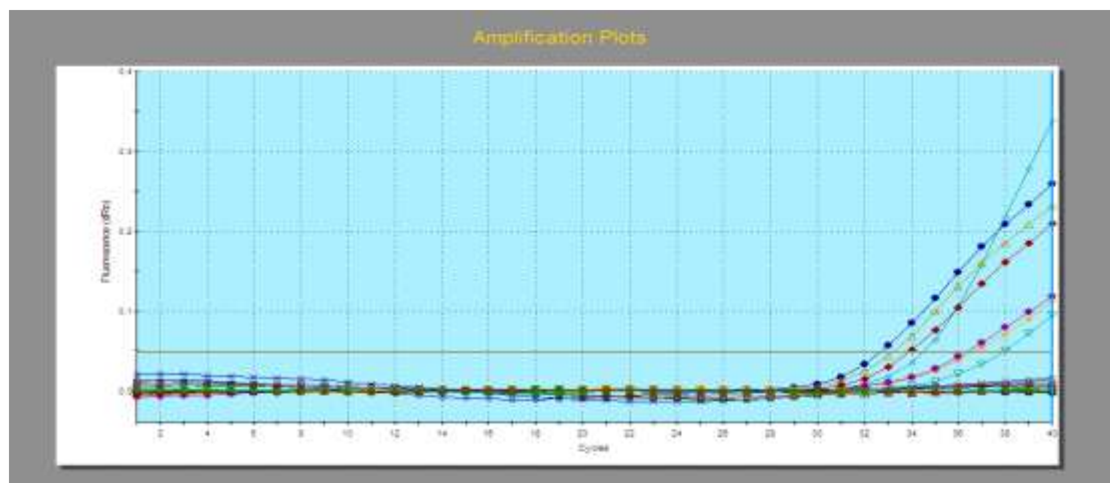

Figure 4: Real Time PCR reaction product of the gene Unigene072411 for genotypes of beans under saline stress conditions

4-3 Detection of Unigene012043 Gene for Study Varieties under Saline Stress Conditions on agarose Gel at 1% Concentration

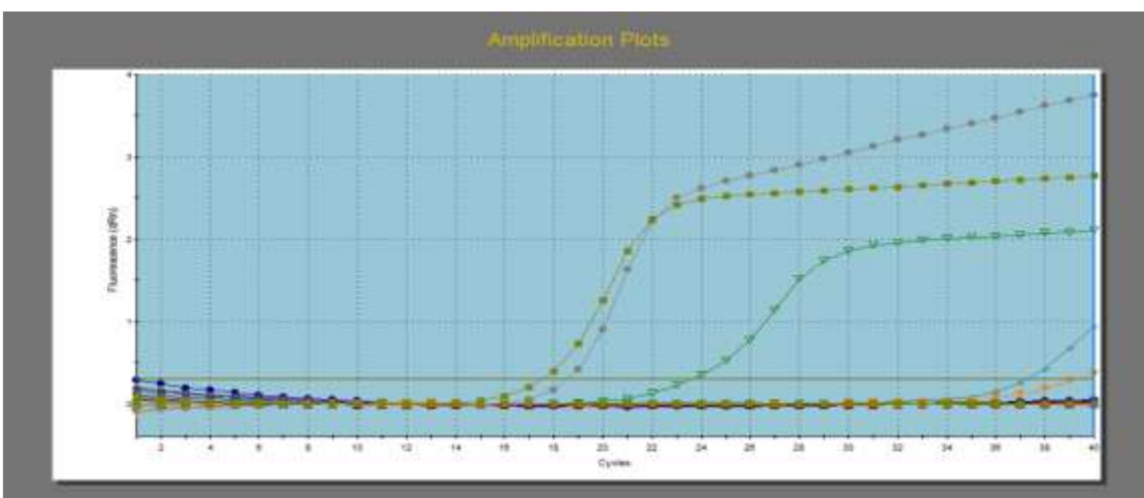

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Figure 5: Real Time PCR reaction product of the gene Unigene047182 for genotypes of beans under saline stress conditions.

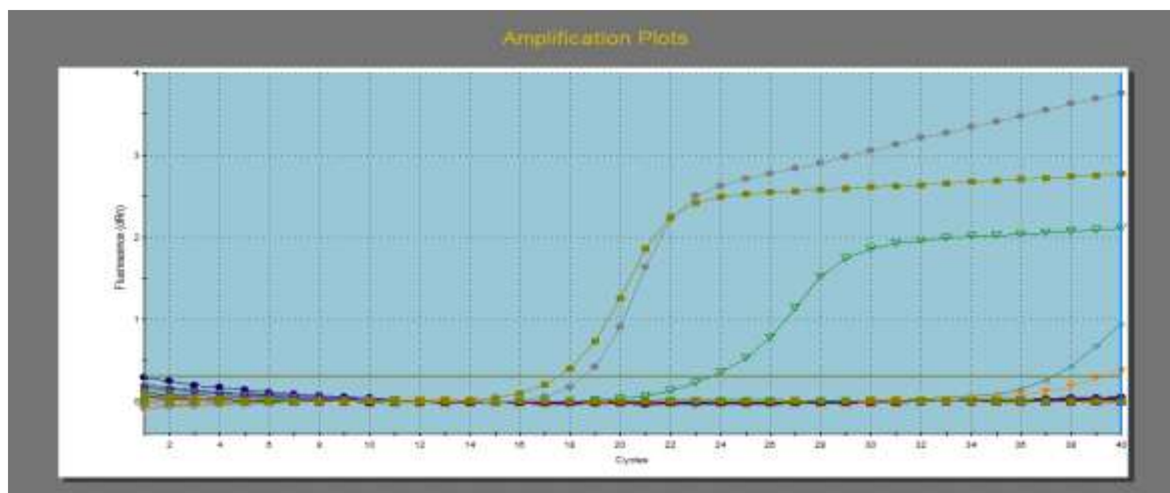

Figure 6: Real Time PCR reaction product of gene Unigene012043 for genotypes of beans under conditions of salt stress

As shown in Figures (4, 5 and 6), the results of the data analysis showed that saline stress had an effect on gene expression and the highest gene expression in the Turkish and Spanish varieties showed the highest values in the amount of gene expression of the study genes at the salt level 6 and 9 ds m^{-1} , while the local and Dutch varieties gave less gene expression compared to the control gene. The expression of any gene depends on the type of gene, the level of salinity and the amount of gene separated through the screening and selection cycle.[14] In a study on understanding the mechanisms of gene expression, pointed out [12] that the value of gene expression depends on the degree of environmental stress, the higher the salinity, the greater the gene expression because the amount of expression of any gene is the interaction between the environment and genetics or the so-called gene environmental interaction (Gene Environmental interaction). Therefore, the investigation of any gene associated with salinity tolerance must be accompanied by an assessment of its gene expression and determination of the salt level that it effect on the expression. That is, whenever the gene expression is high for a certain range, the plant tolerance to salinity is high, and this depends on the extent of the gene's tolerance to the level of salinity, and this is confirmed by the results of this study, where the gene expression was high at the third salt level (9 ds m^{-1}) in the Turkish, Spanish and Dutch genetic structure, as the current study showed that the salinity tolerance genes in the bean plant are different in the degree of tolerance to salinity and that the mechanisms of carrying salinity in the plant are multiple and vary from one plant to another, which determines The degree of tolerance to salinity of the plant. Studies have shown clear variations in their possession of genetic factors that enable them to tolerate saline tension. [15] pointed out that there are three main aspects at the level of gene expression that control the tolerance to saline stress: ion balance, damage control and growth regulation, and these factors must be controlled by the regulation of three classes of functional genes: transport, cell defense, detoxification, metabolism, energy and photosynthesis.

4-4 Genetic dimension values depending on the average of the measured traits;

4-4-1 Calculation of the Euclidean distance:

The results of the study presented in Table (5) show that the highest Euclidean

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distance between the genetic structures of the bean plant was 1111 between the Turkish genotype V2 and the Spanish genotype V3, while the Euclidean distance between the genetic type V3 and the genetic type V4 was (237.51), which is the lowest distance calculated.

Table (5): Euclidean distance between breeds depending on the average of the measured traits.

G	V1	V2	V3	V4
V1	0			
V2	397.1	0		
V3	714.2	1111	0	
V4	478.4	874.88	237.51	0

4-4-2 Genetic kinship tree between breeds depending on the average of the measured traits

Figure (7) shows the four cluster analysis of the genetic structures of the bean plant by studying the Euclidean dimension between them based on all the rates of the measured traits common to them, as the genetic structures were divided into two main groups, A and B. Group A included the genetic structures V3 and V4, who gave the lowest genetic distance between them amounted to (248), followed by the genetic structures V1 and V2 in the second group B, as they were given a genetic dimension of (397), while the maximum genetic dimension was Between V3 and V2 it was (800). Figure (1) shows the four cluster analysis of the genetic structures of the bean plant by studying the Euclidean distance between them based on all the rates of the measured traits common to them, as the genetic structures were divided into two main groups, A and B. Group A included the genetic structures V3 and V4, who gave the lowest genetic distance between them amounted to (248), followed by the genetic structures V1 and V2 in the second group B, as they were given a genetic dimension of (397), while the maximum genetic dimension was Between V3 and V2 it was (800).

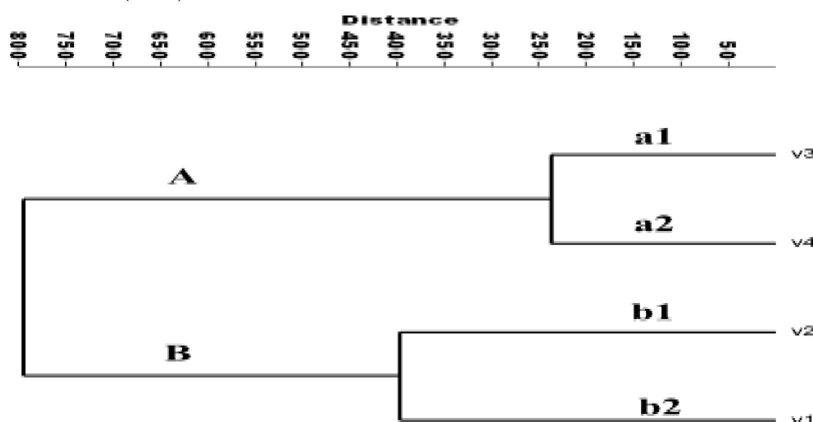

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Figure 7: Dendrogram kinship tree by UPGMA method and depending on average of the measured traits

4-4-3 Principal Component Analysis

Figure 8 shows the distribution of genotypes at the two orthogonal levels, which represent the two highest levels of genetic variation among the individuals studied. The distribution shows the matching of the groups formed for the genetic kinship tree that was drawn based on the average of the traits measured for it, which shows the divergence of the V3 genotype, followed by the relative V4 from the V1 and V2 genotypes in group B.

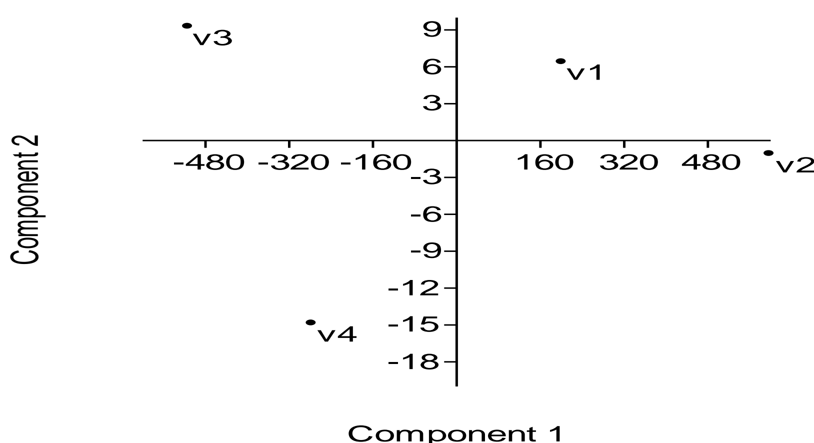

Figure 8: Distribution of four genotypes of beans at the orthogonal level according to the PCA Principal Component Analysis based on the average of the measured traits.

4-4-4 Genetic distance values based on QPCR indicators

The results shown in Table (6) indicate that the highest Euclidean distance between the genotypes of the bean plant was 2.24 between the local structure V1 and the Dutch V4, while the results of the same table showed that the lowest Euclidean distance of 1 was between the Spanish structure V3 and the Dutch V4. The results of the current study showed that the Euclidean distance between genetic structures was high.

Table (6): Euclidean Dimensions Using QPCR Indicators between Four Genotypes of Beans

0	V1	V2	V3	V4
V1	0			
V2	1.4	0		
V3	2.00	1.43	0	
V4	2.24	1.73	1	0

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4-4-

4 Genetic kinship tree between breeds based on QPCR indicators

Figure (9) shows the cluster analysis scheme based on the results of 6 reaction prefixes that the four genetic structures of the beans grown in Iraq were distributed in two main groups A and B. The first group A, which included the Spanish genetic type V3 and the Dutch V4, which had the genetic dimension in between them 1, and the genetic distance between the two main groups was 1.96, the second group B included the Turkish genetic type V2 and local V1 and the genetic distance between them 1.42**.**

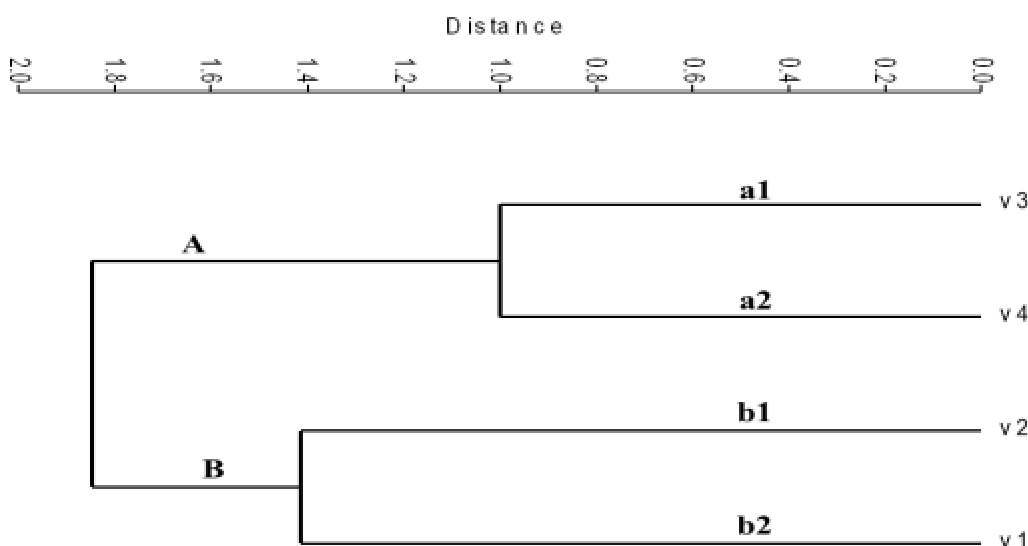

Figure 9: Dendrogram of four legumes genetic structures according to the UPGMA method based on the results of three prefixes used to estimate the relative amount of gene expression of the Unigene gene in the bean plant

4-4-5 Results of the analysis of the main component of the genetic structures based on the indicators of QPCR

The results of the Principal Component Analysis(PCA) in Figure (9) showed a clear match with the results of the genetic kinship tree shown in Figure (10), as the distribution results showed the conformity of the groups formed for the genetic kinship tree based on QPCR indicators, which show their distribution in two main groups, the first group included the genetic structure V4 and V3, while the second group included the genetic structures V1 and V2.

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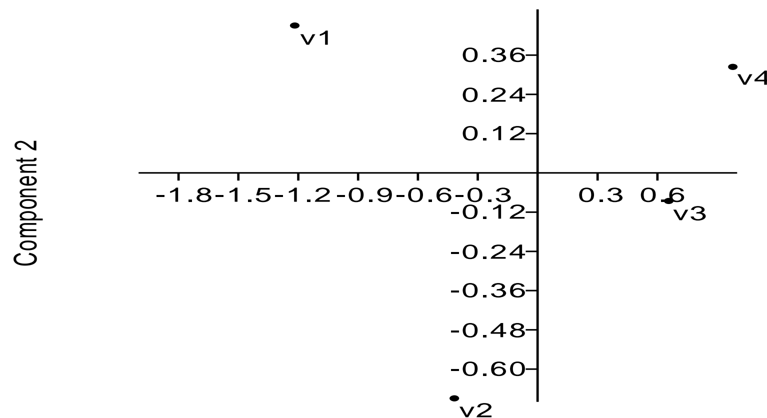

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Figure 10: Distribution of four genotypes of beans at the orthogonal level according to PCA (Principal Component Analysis based on the results of QPCR indicators).

CONCLUSIONS:

The results of the analysis of variance showed that saline stress had an effect on gene expression and showed the highest gene expression in the Turkish and Spanish varieties, which achieved the highest values in the amount of gene expression of the study genes at the salt level 6 and 9 decimensem-1, and the gene Unigene012043 had the ability to tolerate high salt stress in the studied genotypes.

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