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Studying Expression Changes of Genes Involved in Direct Regeneration Pathway in Piarom Dates

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ABSTRACT

Date palm (*Phoenix dactylifera* L) as a monocotyledonous plant of the Palmaceae family are one of the most important economic and strategic products in southern of Iran. Accordingly, in this study, direct branching was investigated to prevent possible abnormalities of indirect proliferation using embryogenic callus. The expression of genes involved in regeneration were investigated between mother plant, one-year-old offshoot attached to mother, and seedlings developed by tissue culture and one-year-old micro-samples in a glass. These samples were investigated using two target genes compared to the control gene beta actin. They were studied in Murashige and Skoog (MS) medium with differential hormonal combinations of IAA, Zeatin, 2IP, BAP, and NAA. The best hormonal treatment compound that led to branching was 3 mg/L of IAA, 4mg/L of Kin, and 4 mg/L of Zeatin. For studying the relationships in controlling changes of expression genes involved in regeneration process of leaf samples in mother plant isolated from offshoot, 3-year-old offshoot leaves still connected to the mother plant, and the regenerated and non-regenerated subsamples were evaluated in the presence of proton symporter *DUR3* and XTH genes. The results of assessing gene expression showed an increase in expression of both *DUR3* and XTH genes. XTH gene was equally expressed as beta actin gene and DUR3 gene was expressed two times more than the control gene in the regenerated subsample. Since, the regenerated sample was considered an immature sample; expression of these genes was lower than adult sections. Expression of the genes was decreased in the non-regenerated embryo sample (XTH gene was expressed by 5.5 unit and DUR3 gene was expressed by 062 unit) showing that this degeneration had higher efficiency in plants regeneration growth stages. Considering the direct regeneration can probably prevent abnormalities caused by tissue culture, and two genes (DUR3 and XTH) in the synthesis pathway make the same compounds that play a controlling role in increasing and reducing expression on each other.

Keywords: Offshoot, Piarom, Terminal Meristem, Gene Expression, Regeneration

Introduction

Date palm is one of the monocotyledonous plants belonging to the Palmaceae family. This family has 200 genera and 1500 species native to tropical and subtropical regions of Africa and South Asia. One of these species is the standard date palm (*Phoenix dactylifera* L). The date palm tree with the scientific name of *Phoenix dactylifera* has a straight trunk without branching, leading to a final bud; and primary origin of the date plant is unknown. Some scientists have stated that origin of the date plant originally goes back to lands in Asia and the Persian Gulf. Others believe that the primary habitat of dates is in North Africa or the Indian subcontinent. Currently, dates are cultivated and exploited in all five continents globally including 34 countries (Henderson, 2009). Palm is a perennial plant tree with two genera, its propagation is asexual, and the seed is not used for its reproduction because of diversity of traits. Dates are one of the most important tropical products of Central Asian countries, which are considered as a unique fruit due to their composition and properties. They are regarded as one of the important sources of natural sugar, accounting for about 60% of dry weight of dates. Dates are a strategic product regarding their importance in the fields of food, income, environmental protection, development of agricultural sustainability, and employment. The date palm tree grows well in warm, all-sunshine weather conditions and produces dates. Propagation of dates is done traditionally through offshoot. Offshoot deficiency is one of the main obstacles to development of date orchards. Date palm trees produce a limited number of offshoots depending on the type of cultivar (7-15 offshoots), so commercial cultivation using this method is faced with offshoot deficiency. Thus, one of the best ways to reproduce dates is using tissue culture. A large number of palm trees have been destroyed in Arab countries due to disease (Morocco), habitat destruction (Egypt), or War (Iraq).Therefore, expansion of the lost palm trees is difficult due to the mentioned factors. Given the limited number of offshoots, one of the methods used for developing and maintaining date gardens is the tissue culture method. This technique has been used to increase the number of valuable palms (coconut, oily palm ,and dates) and is one of the most promising ways to rapidly increase the palm cohort (Abul-Soad et al., 2010)[2]. For eliminating the obstacles related to increasing the amount of seed and offshoot in dates and supplying the necessary plant materials, there is a need to increase tissue culture of the dates (Aslam et al., 2009) [3]. Typically, propagation of the dates using the tissue culture method includes three ways of embryogenesis, organogenesis, and the use of young flowers (Alkhateeb, 2008).

Plant hormones are organic substances that are not considered as food substances, and are produced by plants regulating physiological processes at low concentrations. Sometimes, synthetically produced substances have the same effects as natural plant hormones that should not be called as plant hormones. The correct term for such compounds having products, such as hormones on the plant is growth regulators. These substances regulate growth by imitating hormones, influencing (synthesizing) hormones, and eliminating, transferring, or altering the site of hormonal influence. It can be said that all hormones are growth regulators, but not all growth regulators are hormones (Sharifi et al., 2010).

The urea-proton symporter (DUR3) gene encodes the components needed for active urea transfer in Saccharomyces cerevisiae that has been isolated and sequenced previously. DUR3 protein profile has characteristics including hydrophilic and hydrophobic regions of internal membrane proteins. The strong negative complementation observed during genetic analysis of the DUR3 location has suggested that the DUR3 product may polymerize to perform its physiological function. DUR3 expression is regulated in a similar way to the other genes in the allantoin pathway. Its high-level expression is dependent on induction and requires functional genes including DAL81 and DAL82) Bossinger, 1975). Xyloglucan endotransglucosylase/hydrolases (XTHs) are classified as the 16-glycoside hydrolase family. They play an essential role in organ growth by modifying xyloglucan chains or catalyzing xyloglucan hydrolysis. Several studies have emphasized the significant contribution of this gene family in regulating expansion of cell wall capability. Over-expression of the Brassica campestris homologue of Arabidopsis XTH9 (bcXTH1) gene can cause increases in stem length in Arabidopsis by expanding the cellular surface, an essential parameter in branching and growth (Vissenberg, 2007). Accordingly, in this study, tissue culture of the dates was investigated using subsamples isolated from the date palm tree. Then, gene expression diversity was studied in the mother palm tree. The separated offshoot, the offshoot, which has not yet been separated from the mother tree, and the regenerated and non-regenerated subsamples were all placed in the culture medium.

Materials and Methods

Plant Materials

In this study, subsamples were prepared from 3-5-year-old offshoots of Piarom cultivar obtained from Hajiabad Garden in Hormozgan Province, southern Iran (Figures. 1 and 2). The leaves and roots around the offshoot were separated (Figure. 3), and a section with up to 10 cm of the meristematic area was available (Figure. 4). This 10-cm section of the offshoot's end including leaves around the meristem, terminal meristem, and lateral meristems was placed in an antioxidant solution containing 150 mg/l of citric acid and 100 mg/L of ascorbic acid for 24-48 h.

Figure 1: Palm tree with offshoots

Figure 2: Offshoots separated from the mother tree

Figure 3: Offshoots separated from the mother tree

Figure 4: Separation of the meristem area

Disinfection of Subsamples

Subsamples used in tissue cultures should be free of any pathogens and foreign contamination. For this purpose, first, the subsamples were dipped into 70% alcohol for a few seconds and then, they were placed in 20% sodium hypochlorite along with 5-8 drops of tween 20 for 15-20 min.

The terminal meristem with two early leaf buds (0.1-0.4 mm of length) was separated from the stem's end using a stereomicroscope and surgery blade, and was placed immediately on the culture medium. The chances of survival are decreased if the terminal meristem is separated. Suppose that the meristem is detached along with the two early leaf buds. In this case, the chances of survival are increased (because of less size of subsample, and also less morphogenesis), but the chances of obtaining virus-free plants are decreased.

Culture Medium

MS (Murashige & Skoog, 1962) medium was used according to the treatment containing 30 g/L of sucrose, 3 g/L of activated charcoal ,and 7 g/L of agar, and growth regulators including IAA, KIN, 2iP, BA ,and NAA were used in different conditions as shown in Table 1.

A	3mg/l IAA,4mg/l KIN
B	4mg/l 2IP, 4mg/l BA, .5 mg/l NAA
C	3mg/l IAA, 4mg/l KIN, 4mg/l zeatin

Table 1: The amount and composition of the used hormones

Investigation of the Studied Genes

For obtaining more information, genetic diversity and expression of the genes were investigated as follows:

For this purpose, DUR3, XTH, and β-Actin genes were selected as controls. Variation in expression of these genes was investigated in a mother palm tree, offshoot attached to mother tree, seedlings derived from direct regeneration, and non-regenerated subsample. XTH Gene, DUR 3 Gene. The β-Actin gene as one of 6 actin isoforms has been identified in humans as the housekeeping gene.

First, 1mL of the samples (about half a microtube) was separated and then, was completely crushed, softened, and placed in the microtube. One mL of RL solution (thyrosel) was used to digest the tissue and it was completely dissolved. Then, it was placed on a shaker and was centrifuged at maximum speed so that, the material in the microtube was divided into two separate phases (Figure. 5). The liquid on the tube was transferred to the next tube, and 0.5 mL of chloroform was added to the tube and was centrifuged (Figure. 6), where the white part is the same as impure RNA, and the yellow part is the same as chloroform and other materials. This white liquid was transferred to the column, was centrifuged for 1 min, and finally, what remained on the column paper was pure RNA. Then, 0.05 mL of RNase was added to the column's center to collect all the RNA in the chamber below the column and then, it was centrifuged for 1 min.

Figure 5: Sample after placing on the shaker

Figure 6: Biphasic and RNA separation

cDNA Synthesis

First, 0.5 mL of RNA was poured into a microtube. Then, 0.1 mL of oligo dT primer was added. The total volume was added by mixing with 0.134 mL of distilled water (distilled twice to prepare sterile distilled water), which were then synthesized according to the CDNA synthesis protocol. For ensuring about quality of the synthesized CDNA using electrophoresis gel, quality of the extracted RNA was assessed, and assurance was achieved regarding bonding on the gene.

The Use of Real-Time Device to Investigate Expression of Genes

Regarding gene expression, the researchers are looking for a set of target genes to evaluate expression of the mentioned genes; according to the literature review, it was found that some genes play a crucial role in biosynthesis and metabolic pathways that these genes may be expressed in parts of the plant in particular and not expressed in other parts of the plant. Considering that different samples are evaluated in terms of age and target tissue, they may have distinct gene expression patterns, so DUR3 and XTH genes were selected, and βActin gene was chosen as a reference gene. Some genes, such as βActin or gluconate dehydrogenase (GADH) do not change gene expression under stress and other environmental conditions, and the number of copies is constant and they are expressed anyway.

Results and Discussion

Subsamples of lateral buds, primary leaves, and terminal meristem including 20 glasses for lateral buds and 10 glasses were allocated for terminal meristem in 2 media labeled as A and B media. All the glasses were placed in an incubator with a temperature of 2±27 ˚C and they were kept in dark for 3 months and were sub-cultured every month. Then, they were transferred to the incubator with 16/8 h of light-dark cycle at the same temperature; the first changes were observed after about 6 months (Figure. 7). Then, 6 glasses were transferred to C culture medium (Figure.8).

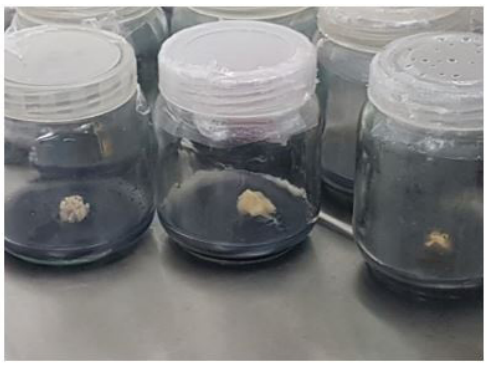

Figure 7: Observing the first changes after 6 months

Figure 8: Transferring to C culture medium

The Results of Tissue Culture

As shown in Table 1, the terminal meristem was located in A medium containing 3 mg of IAA and 4 mg of KIN. The average fresh weight of callus was equal to 3.23g with an average standard error of 0.266, and an average standard deviation of 8.23. Then, terminal meristem was placed in B medium containing 4 mg of 2IP, 4 mg of BA ,and 0.5 mg of NAA. Mean fresh callus weight was equal to 3.7g, with an average standard error of 0.283, and an average standard deviation of 7.63. Lateral buds were placed in A medium containing 3 mg of IAA and 4 mg of KIN, with a mean fresh callus weight of 4.3g, an average standard error of 0.212, and an average standard deviation of 4.91. Lateral buds were obtained in B medium containing 4 mg of IP, 4 mg of BA, and 0.5 mg of NAA, with a mean fresh callus weight of 4.51 g, an average standard error of 0.178, and an average standard deviation of 3.95

Terminal meristem	A	3.23	0.266	8.23
Terminal meristem	B	3.7	0.283	7.63
Lateral buds	A	4.3	0.212	4.91
Lateral buds	B	4.51	0.178	3.95

Table 1: Results obtained from statistical analysis of callus weight produced from the terminal meristem and lateral buds in A and B media

As presented in Table 2, the terminal meristem was located in A medium with 3 mg of IAA and 4 mg of KIN, and mean volume of callus was equal to 0.042 cm3, with standard error of 0.016, and standard deviation of 3.88. The terminal meristem was placed in B medium with 4 mg of IP, 4 mg of BA, and 0.5 mg of NAA, an average callus volume of 0.064 cm^3, an average standard error of 0.0023, and an average standard deviation of 3.64. Lateral buds were located in A medium with 3 mg of IAA and 4 mg of KIN, with an average callus volume of 0.07 cm3, an average standard error of 0.0023, and an average standard deviation of 3.32. Then, lateral buds were exposed to B culture medium containing 4 mg of 2IP, 4 mg of BA and 0.5 mg of NAA where mean callus volume was equal to 0.073 cm3 with mean standard error of 0.006, and mean standard deviation of 0.906, respectively.

Sample	Media	Mean of callus volume (cm^3)	Mean of standard error	Mean of standard deviation
Terminal meristem	A	0.042	0.016	3.88
Terminal meristem	B	0.064	0.0023	3.64
Lateral buds	A	0.07	0.0023	3.32
Lateral buds	B	0.073	0.0006	0.906

Table 2: Results obtained from statistical analysis of callus volume produced from the terminal meristem and lateral bud in A and B culture media

As shown in Table 3, the terminal meristem was placed in A culture medium containing 3 mg of IAA and 4 mg of KIN, with a mean callus size of 0.723 mm2, a mean standard error of 0.029, and a mean standard deviation of 4.096. Then, terminal meristem was cultured in B culture medium containing 4 mg of 2IP, 4 mg of BA and 0.5 mg of NAA with a mean weight of 0.77 mm^2, a mean standard error of 0.023, and a mean standard deviation of 3.023. Lateral buds were placed in A culture medium containing 3 mg of

IAA and 4 mg of KIN, with a mean callus size of 0.836 mm^2, a mean standard error of 0.029, and a mean standard deviation of 3.12. Following that, lateral buds were placed in B culture medium containing 4 mg of 2IP, 4 mg of BA, and 0.5 mg of NAA, with a mean callus size of 0.893 mm^2, a mean standard error of 0.050, and a mean standard deviation of 5.55.

Mean of standard deviation	Mean of standard error	Mean of callus size (mm^2)	Media	Sample
4.096	0.029	0.723	A	Terminal meristem
3.023	0.023	0.77	B	Terminal meristem
3.12	0.029	0.836	A	Lateral buds
5.55	0.050	0.893	B	Lateral buds

Table 3: Results obtained from statistical analysis of callus size produced from the terminal meristem and lateral bud in A and B culture media

For investigating changes in gene expression, DUR3 and XTH genes of the βActin gene were used as control or reference. The samples were evaluated to study changes in gene expression including mother A sample, 3-year-old offshoot attached to mother plant B, regenerated C sample ,and finally non-regenerated subsample with embryo and D sample.

As shown in the diagram of gene expression, XTH gene in A, B, and C samples had an expression equal to one unit similar to that of the reference gene compared to the reference gene, suggesting that in the mother sample, 3-year-old offshoot, and regenerated sample, cellular surface area and cell wall range were increased, which is directly related to the increase in expression changes of this gene. This gene expression was decreased by 0.5 units in the C sample compared to the reference gene and other subsamples. The non-regenerated embryonic sample had less power to increase cell level compared to the others. Therefore, the rate of changes in its expression was decreased compared to the other samples. Still, in this sample, this gene was expressed indicating that this subsample has had the power to be regenerated in the appropriate environment. The function of this gene has been also reported in the regenerated subsamples, maturing seedlings, and mature plants. Because this gene plays a crucial role in biosynthesis pathway of essential compounds that increase cell wall levels, causing plant growth. Function of this gene is increased in seedlings in in-vitro environment when the seedling is maturing and is expressed in the whole plant and as long as the plant is able to produce the product.

Expression of DUR3 gene in A, B, and C samples was increased by 2 units and compared to the reference gene, the gene had a one-unit increase in its expression level. Expression of this gene was increased by one unit compared to the XTH gene. This gene is located downstream of the XTH gene and plays a crucial role in branching and regeneration. Increase in expression of this gene is directly associated with increase in XTH gene expression , as observed in all 3 subsamples of the mother plant, regenerated subsample, and 3-year-old offshoot. The expression for this gene was obtained as 0.06 IDs in the subsample with non-regenerated embryos. In fact, the expression of this gene was decreased compared to the reference gene. Compared to the other 3 subsamples, its expression in this subsample had a decreasing trend, and the decrease in gene expression in this subsample is directly related to regeneration. The function of this gene in the regenerated samples indicated that the plant mechanism for growth and branching is directly associated with the increase in expression level of XTH gene.

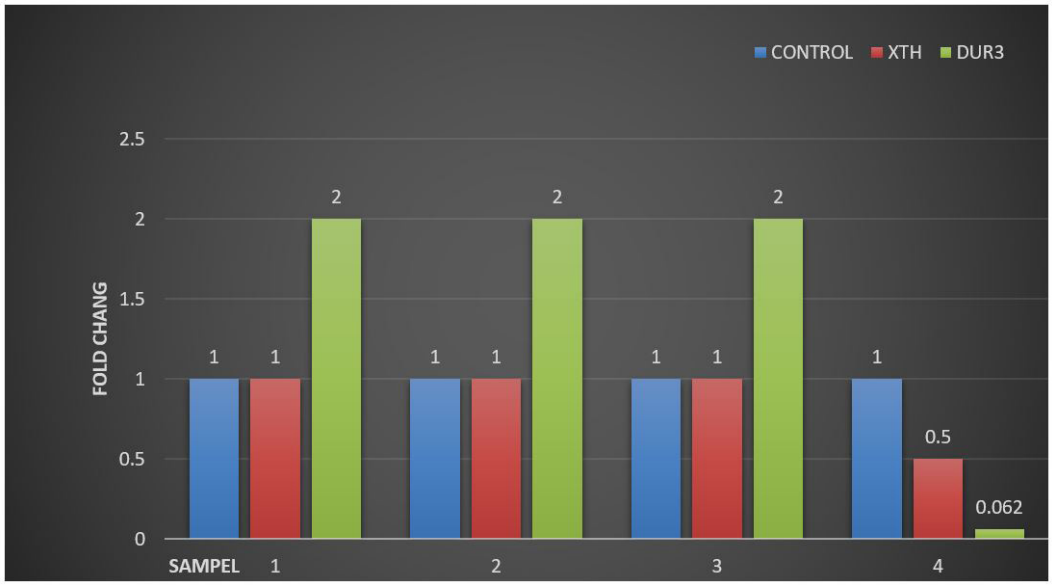

Figure 9: Comparison of gene expression with control gene

The results of assessing gene expression in 4 subsamples of dates including 3-year-old offshoot, maternal plant, non-regenerated embryo, and generated embryo showed that the plant's effects including growth and regeneration are under the control of each series of the studied genes. The gene network between these two genes (DUR3 and XTH) is an essential gene pathway in the subsamples' regeneration and growth. These two genes (DUR3 and XTH) also play a crucial role in biosynthesis and metabolism pathways in the date palm plants. The two genes (DUR3and XTH) in the synthesis pathway make the same compounds that play a controlling role in increasing and reducing expression on each other. Essential proteins synthesized by these two genes as the useful final product in regeneration were active in all 3 subsamples. However, no effect of this activity was observed in the non-regenerated embryo

Discussion

Dates are one of the most critical strategic plants in Iran, and a large part of the lands in south of the country is under cultivation of this crop. More than 50 types of dates are produced in Iran, and even many experts believe that this number is only a part of the main statistics. Researchers also believe that species of dates can be cultivated solely to prepare medicines through extraction of their secondary metabolites and also their low dietary intake. Therefore, in this study, tissue culture of the Piarom date cultivar was investigated, and 10 offshoots were selected for this purpose. In this method, organogenesis protocol was used, according to this method; the offshoot was chosen first and was separated from the mother plant, then the offshoot was prepared, and the leaves and extra parts were removed until reaching the meristem area. Then, the terminal area of the offshoot was washed with sterile water. Next, the subsamples were separated, were disinfected with fungicide for 10 min, and were cleaned with distilled water for 20 min in sodium chloride 20%. Finally, they were washed two to three times with sterile water and were placed in a suitable medium according to the disinfection protocol introduced in the study by Othmani et al., (2009) [6].

For preventing occurrence of browning in subsamples, they were placed in an antioxidant solution containing 100mg of Ascorbic acid and 150 mg of citric acid to avoid release of phenolic compounds in the subsamples, as described by Abahmane et al., (2007) [7]. The selected medium was MS medium, which is consistent with the medium used in the study by Al Khateeb (2006) [1] and the necessary nutrients and salts were provided for the subsamples. The induction phase of the samples was done in dark for 3 - 6 months and then, they were transferred to the new environment with 16/8 h of light-dark cycle at 27-±2˚C; according to the literature (Anjarne et al., 2005)[8] .A culture medium contained a hormonal combination of 3 mg/L of (IAA) and 4 mg/L of KIN and B culture medium contained a hormonal combination of 4 mg/L of 2IP, 4 mg/L of BA, and 0.5 mg/L of NAA, and changes were observed in the media after about 6-8 months. Changes in B culture medium with higher levels of cytokinin were more significant. In the next step, the subsamples were transferred to C culture medium with a hormonal combination of 3 mg/L of IAA, 4 mg/L of KIN ,and 4 mg/L of Zeatin, according to the procedure proposed by Amin et al.,(2001)[9].

Bossinger et al., (2012) [5] in a study on terminal meristem germination of dates showed that DUR3 gene expression plays a crucial role in germination percentage in this plant. When the expression of this gene changes due to environmental or chemical stress and the expression is decreased, the time required for development of the first buds is increased compared to the expected time also, this gene expression has a key and useful role in development of leaves resulting from offshoot culture. Increased expression of DUR3 gene compared to regular expression of this gene has a significant effect on the number and time of emergence of leaflets obtained from stubble culture. Also, Boeke et al., (2008) showed that the DUR3 gene played a crucial role in the plant's regeneration stage. This gene has the least amount of expression in non-regenerative plant samples. It has been also reported that there is an increased expression in the plants, which are regenerating and completely regenerated. Cooper et al., (2014) demonstrated that the DUR3 gene interacts with XTH gene in the gene pathway, and can be influenced by the gene expression pattern in a gene complex.

The increase and decrease in expression of both XTH and DUR3 genes have the same gene expression algorithm in the date palm plant. DUR3 gene expression as an important gene in germination induction signaling pathway in date palm plants showed that in the regenerated and mature samples, this gene expression was increased, and in the non-regenerated samples, the expression of this gene was decreased compared to the other subsamples. Expression of this gene also has a constant pattern in the other studied plants.

Cantarel et al., (2009)[11] claimed that XTH gene plays a crucial role in spread of cell walls in plants. Studies have shown that this gene has maximum expression in growing and matured plants. Van Sandt et al., (2007) [10] indicated that XTH gene in a one-year-old date offshoot had lower expression levels than a 5-year-old offshoot. This gene interacts functionally with DUR3 gene family. The expression of this gene can influence germination rate and increase regeneration in plants. XTH and DUR3 gene networks play a key role in two main plant processes including induction of germination and regeneration as well as leaf emergence (Fry et al., 2012) [5].

Conclusion

Considering that direct regeneration can probably prevent abnormalities caused by tissue culture, tissue culture treatment by disinfection in A culture medium containing 3 mg/L of IAA and 4mg/L of KIN and then transferring subsamples in B culture medium containing 4mg/L of 2IP, 4mg/L of BBA ,and 0.5 mg/L of NAA and again transferring the subsamples to C culture medium containing 3mg/L of IAA, 4 mg/L of KIN ,and 4mg/L of Zeatin ultimately led to regeneration.

The results of assessing gene expression in 4 date subsamples including 3-year-old offshoots, maternal plant, non-regenerated, and generated embryos showed that the effects regarding growth and regeneration in the plant are under the control of each series of the studied genes and the gene network between these two genes (DUR3 and XTH) as one of the most critical gene pathways in regeneration and growth of the subsamples is involved. These two genes (DUR3 and XTH) also play an essential role in biosynthesis and metabolism pathways in date palm plants.

The two genes (DUR3 and XTH) in the synthesis pathway make the same compounds that play a controlling role in increasing and reducing expression on each other. Essential proteins synthesized by these two genes as the final product, effective in regeneration were active in all 3 subsamples. However, no effect of this activity was observed in non-regenerated embryos.

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