Effect of Pollen Treatments on Fruit Yield and Gene Expression in Offshoot and Tissue Culture Date Palms (Phoenix dactylifera L. cv. Barhi)

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Abstract

The low performance of date palm cv. Barhi resulting from tissue culturing is one of the main challenges in the production of these plants. On the other hand, the final yield of the plant depends on various metabolic and biochemical factors which are caused by gene expression. The plant reacts to environmental factors to survive in different growth and environmental conditions through gene expression. This experiment was conducted to investigate the relationship between the expression of certain genes before and up to two weeks after pollination with the yield of off-shoot and tissue culture of Phoenix dactylifera L. cv. Barhi (10-year-old). Off-shoot and tissue-culture date palms were pollinated with Red Ghanami pollen, Green Ghanami pollen, and Green Ghanami + Red Ghanami pollen (50:50) based on a factorial design in randomized complete blocks with three replications. To this end, the relative expression levels of Histone acetyltransferase HAC1-like (LOC103717600) (HAC 1), Arginine N-methyltransferase 6.1 (LOC103716582) (ANM), TIME FOR COFFEE-like (LOC103716450) (TFC), Homeobox-leucine zipper protein HOX9-like (LOC103703962) (HOX 9), MADS-box transcription factor 2-like (LOC103702602) (GLO 1), and MADS-box transcription factor 16 (LOC103701267) (DEF 1) genes were examined using qRT-PCR method in 3 times: (1) Before pollination, (2) 1 week after pollination, and (3) 2 weeks after pollination. The fruit yield was measured at 180 days after pollination (the end of the experiment). The results showed that the tissue culture palms had lower yield but higher relative genes expression in all pollen treatments compared to off-shoot palms. Off-shoot date palm pollinated with Red Ghanami pollen (5.93 tons/ha) showed the highest yield while the lowest yield was recorded in tissue culture date palm pollinated with Green Ghanami pollen (2.09 tons/ha). The relative expression of the six studied genes significantly increased in all treatments two weeks after pollination. Two weeks after all investigated treatments, the GLO gene had the highest expression, while the HAC gene showed the lowest relative expression. The relationship between the yield and the genes expression in the three studied times showed that the HOX gene had no considerable effect on flowering and yield. The ANM and TFC genes expression before pollination exhibited a positive relationship, whereas the expression of HAC, DEF, and GLO genes had a negative relationship with 1 week after pollination. Two weeks after pollination, the expression of the ANM gene revealed a significant positive relationship with the final yield of the date palm. Moreover, the correlation results showed the prominent role of the genes in different stages of growth and yield of date palms by influencing the expression of each other. In general, it is possible to improve the yield of the tissue culture date palm by affecting the expression of the genes in specific stages based on their function.

1. Introduction

Phoenix dactylifera L. is a known species in the Phoenix genus (Hazzouri et al., 2015), which is native to tropical and subtropical regions of Africa and South Asia (Almaaty et al., 2022). Date palm is the only species of this family that can produce off-shoot (Johnson, 2011). Date palms are perennial and dioecious plants with 2n = 36 (Naqvi et al., 2021). In 2019, Iran produced nearly 19% of the world's dates (99,255,327 ton), scoring the second rank after Egypt (FAO, 2021). This plant can be propagated by off-shoot. But the number of off-shoot is small (Othmani et al., 2020). The resulting progeny in the propagation of the plant by tissue culturing method shows some differences from the mother plant (Shair et al., 2016). Studies conducted by genetic markers showed differences between off-shoot and tissue culture date palms. This dissimilarity can be due to gene expression changes (Shair et al., 2016; Mirani et al., 2022). The date palm-specific inter-retrotransposon amplified polymorphism (IRAP) and miniature inverted-repeat transposable element (MITE) markers were applied to identify the somaclonal genetical changes of date palm plants obtained from tissue culturing. The results showed some somaclonal variations due to the activation of DNA transposons and retrotransposons in tissue culture plants

(Mirani et al., 2020). The molecular genetic analysis of abnormal tissue culture trees was achieved by Isoenzyme profiles (Azeqour et al., 2002) and RAPD analyzes (Saker et al., 2000). These strategies confirmed some phenotypic differences between tissue culture and off-shoot date palm plants, but they could not detect abnormalities of tissue culture date palm that appeared only in early and mature stages. AFLP analysis of normal and abnormal date palm cv. Barhi also did not show any specific DNA marker band patterns for the abnormal phenotype of low level of fruit production (Gurevich et al., 2004). Jaligot et al. (2000) used two complementary methods to examine the relationship between mantled somaclonal type and possible changes in DNA methylation level of Elaeis guineensis Jacq populations resulting from somatic embryogenesis. The measurement of the relative amount of 5-methyl-deoxycytidine through HPLC showed that the total methylation in the DNA of the unnaturally regenerated plant leaves was 2.5% less than that of natural plants (20.8% vs. 22%, respectively). These researchers also used the Sssl-methyl allyl acceptance test based on the enzymatic saturation of methyl groups in the CG site which showed similar results. Therefore, a positive correlation can be concluded between DNA methylation and mantled somaclonal type and yield in oil palm (Jaligot et al., 2000). DNA methylation changes of deoxycytidine (dC) are involved in gene regulation at the transcription level, specially, in differentiation/ dedifferentiation processes (Chen et al., 2022). The in vitro somatic embryogenesis protocol in date palms involved treatment with various growth regulators which may affect DNA methylation levels (Mirani et al., 2020; Jaligot et al., 2000). Based on Hadi et al. (2015) and Abd-Elhaleem et al. (2020), one of the characteristics of tissue culture date plants is the formation of parthenocarps, which significantly decreased the yield compared to off-shoot plants.

In most of the date palm cultivation areas in Khuzestan province (Dezful, Ahvaz, Abadan and Shadgan cities), the pollination of the. tissue culture and off-shoot *Phoenix dactylifera* L. cv. Barhi is done with Green, Red Ghanami pollen grains and their combination. Off-shoot date palm cv. Barhi pollinated with these pollen grains have been produced normal fruits, while parthenocarpic fruits was produced in the Barhi tissue culture date palm pollinated with the same pollen grains. Inaddition, Although the existing reports had shown the gene expression changes and somaclonal variations in the tissue culture plants compared to the parent plants, none of them addressed the involvement of each gene in the selected time which affects the final yield of the product. Therefore, this study aimed to investigate the effect of differtent pollen treatments (Green Ghanami, Red Ghanami and combination of 50% Green Ghanami and 50% Red Ghanami pollen grains) on the final fruit yield of offshoot and tissue-culture date palms cv Barhi.

Assitionally, this experiment was conducted to investigate the relationship between the expression of certain genes before and up to two weeks after pollination with the yield of off-shoot and tissue culture of Phoenix dactylifera L. cv. Barhi (10-year-old).

2. Material and Methods

2.1. Research methods and plant materials

Experiments were carried out on off-shoot and tissue culture date palm cv. Barhi and pollinating varieties of Green Ghanami and Red Ghanami. The experiments were conducted in the Department of Horticultural Sciences, Faculty of Agriculture, Shahid Chamran University, Ahvaz, and Tropical and Subtropical Fruits Research Institute, Umm al-Tamir, based on a factorial design in randomized complete blocks with 3 replications. The 10-year-old off-shoot and tissue-culture date palm cv. Barhi was selected and marked in the research farm of the Tropical and

Subtropical Fruits Research Institute. The effect of pollen grains on the yield was determined for each female palm using 3 spaths. To this end, each of the three fully ripe spaths of each female palm (off-shoot and tissue culture) were manually pollinated with Red Ghanami pollen, Green Ghanami pollen, and Green Ghanami + Red Ghanami pollen (50–50). For this purpose, the male spaths were separated from the date palm after ripening of the pollen grains (a little before the natural opening of the spaths), followed by immediate pollination. To create isolated conditions, the pollinated spaths were placed in cloth bags until the end of the experiment. During the experiment, date palms were irrigated once every two weeks.

2.2. Yeild evaluation

The final fruit yield was determined after pollinating off-shoot and tissue culture date palms with Red Ghanami pollen, Green Ghanami pollen, and a mixture of Green Ghanami and Red Ghanami pollen (50–50). The percentage of normal fruit set and production of multi-carple parthenocarp fruits were calculated 5 weeks after the pollination. For this purpose, 5 strands from each marked cluster were randomly selected and counted; the numbers of normal and parthenocarp fruits (single, double or triple seedless fruits) were also recorded. Finally, the percentage of normal fruit set and parthenocarp fruits were calculated by determining the ratios of the number of normal fruits/total number of flower primordia and number of fruit parthenocarps/total number of flower primordia, respectively. The fruit yield was obtained by calculating the percentage of fruit set in the average number of palms spaths in the number of palms available per hectare and reported based on tons/ha (Mohammadi et al., 2017).

2.3. Molecular studies

The expression of selected genes in control samples of off-shoot and tissue culture date palm cv. Barhi was determined by the Rael time PCR technique. The flower tissue of each treatment was sampled (1) before pollination, (2) 1 week after pollination, and (3) 2 weeks after pollination. The samples were quickly transferred to liquid nitrogen and preserved in a freezer at -80 ℃ until RNA extraction.

Then, RNAtotal was extracted from the prepared samples (according to the company's kit protocol). The DNAseI kit was used to remove DNA contamination according to the manufacturer's protocol. The primer design was based on the NCBI database on mRNA sequence of the available data. PCR reaction was performed by the designed primers to confirm the specific amplification of the selected gene. The qRT-PCR was used to verify the amplification of the selected gene using designed primers to compare the expression of target genes in off-shoot and tissue culture date palms. In this method, all data were normalized with the reference gene as an internal control. The changes in the gene expression in off-shoot and tissue culture date palm were measured.

2.3.1. Extraction of total RNA and determining the quantity and quality of RNA samples

The total RNA was extracted from date palm flower tissue using a Korean GeneAll kit according to the manufacturer’s instructions. The quantity and quality of the extracted RNA were checked by ND-1000 nanodrop spectrophotometry (BioRad, USA) at 260 and 280 nm and OD260/OD280 and OD260/OD230 ratios, respectively to investigate polyphenol, polysaccharide, protein, and metabolite contamination (Green and Sambrook, 2020). To check the accuracy of the electrophoretically extracted RNA, 1% agarose gel and a size marker were used as a control to estimate the RNA concentration (Badai et al., 2020).

2.3.2. cDNA synthesis

The synthesis of cDNA from RNA is also known as reverse transcription. cDNA was synthesized according to the NEB kit (England). First, 0.5 ml microtubes were prepared for the experiment. Then, the necessary materials (10 µl Baffet Mix buffer (2x), 2 µl Enzyme Mix, 3 µl RNA sample, and 15 µl DEPS water with the final volume of 20 µl) were added to the microtubes. Afterward, the microtubes were transferred to the PCR. The following temperature conditions were applied to the kit, 25 ℃ for 10 min, 47 ℃ for 60 min (reverse transcription), and 85 ℃ for cDNA synthesis for 5 min (inactivation of reverse transcription by heat treatment). The samples were then kept at -20 ℃.

After cDNA synthesis, nanodrop spectrophotometry and gel electrophoresis were used to ensure the performance of the cDNA synthesis kit and determine the quantity and quality of the synthesized cDNA. After the completion of PCR, the cDNA samples were diluted in the same way at a concentration of 100 µg/µl. The samples were then stored in a refrigerator at -20 ℃ until the tests.

2.3.3. Design of primers relevant to the selected genes and actin gene (internal control) for gene expression analysis

Examination of the RNA-Seq transcriptome of normal and abnormal flower oil dates revealed the involvement of different pathway genes in flower abnormalities, including hormone response, DNA replication and repair, chromatin reconstruction, and those involved in DNA methylation. No direct interaction was seen between different genes which experienced expression changes. Therefore, flower abnormality is a significant disorder which is not specific to the changes in the expression of a specific gene.

As the transcriptome results of edible dates were not available, differential genes were identified based on the transcriptome results of oil dates in normal and abnormal conditions (Shearman et al., 2013). Based on the KASS results of the edible date genome against the KEGG database, the genes involved in the most important pathways were considered primary candidate genes. The primary candidate genes included blastx and blasted against the edible date genome whose homologues were found in the edible genome. The primer design was based on the mRNA sequence of the available date studies in the NCBI database. The CDS sequences of each gene were first given to Primer premier 6 software to design the primers. Then, the quality of reverse primers was checked using Beacon Designer service (www.premierbiosoft.com/qOligo). Those with the best conditions were selected and synthesized by Biomagic Gene (BMG) Company. The primers were diluted according to their respective formula and kept at -20°C. The list of primers and their abbreviations and sequences can be found in Table 1.

Table 1
Designed forward and reverse primers related to studied genes and actin gene

Abbreviation	Primer		Nucleotide sequences (5' → 3')	Primer length	Annealing temperature	% GC
Actin	Actin-1	F	TAGTGGTCGCACAACAGGTAT	21	59.1	47.6
		R	CGCTCAGCCGTGGTAGTAA	19	59.49	57.9
HAC 1	Histone acetyltransferase HAC1-like (LOC103717600)	F	ATTGCTGTCTACTTATGGTGTCTG	24	58.58	41.7
		R	CGGAGTAAGGATGTGCTTGTC	21	58.73	52.4
ANM	Arginine N-methyltransferase 6.1 (LOC103716582)	F	CACCAGTTACAGATAGCGAGAGG	23	60.24	52.2
		R	TGACAGTGTAGCAATCCACATACT	24	59.78	41.7
TFC	TIME FOR COFFEE-like (LOC103716450)	F	TCAAGCACGGTCTCCTCAAG	20	59.68	55
		R	ACCAGCCATCATTAGAGCAGAA	22	59.49	45.5
HOX 9	HOX9 Homeobox-leucine zipper protein HOX9-like (LOC103703962)	F	TCTGCCACAAACCGCTCTG	19	60.6	57.9
		R	AACATACTGCCTTGCCATTGC	21	59.79	47.6
GLO 1	MADS-box transcription factor 2-like (LOC103702602)	F	AGAGATTGCTGGAAGAGGAGAAC	23	59.8	47.8
		R	ATGGAAGGCTAGTGGCATCTG	21	59.86	52.4
DEF 1	MADS-box transcription factor 16 (LOC103701267)	F	CCACGCAGACGGATACTTACAA	22	60.42	50
		R	TCGGCTGAACACGGAATGC	19	61.03	57.9

a

After diluting the primers, PCR of all designed primers was performed along with one of the cDNA samples to confirm the specific amplification and achieve the exact temperature of the desired gene amplification. In this step, three temperatures of 56, 58, and 60°C were considered for the Annealing step. Upon confirming the amplification of the desired gene using the designed primers and determining the exact temperature of the amplification for each gene, qRT-PCR was performed to compare the expression of the target gene in off-shoot and tissue culture date palm cv. Barhi. According to the electrophoresis results, the suitable temperature for the qRT-PCR reaction for Actine-1, HAC1, ANM, TFC, and HOX9 primers is 56, 58, and 60°C for DEF1 primer 58 and 60, and 56°C for the GLO1 primer, respectively (Fig. 1).

2.3.4. Quantitative real-time PCR (qRT-PCR)

QRT-PCR reactions (ABI StepOne) were used to investigate the relative expression level of the target genes and compare them in the female type of date palm cv. Barhi, male cultivars, and also before and after the pollination. This reaction was performed using Ampliqon SYBR green Master Mix High ROX. The actin gene was used as a reference and internal control gene with stable expression. Basic solutions were prepared to prevent errors and

waste of time and speed up the operation. All the steps and the addition of cDNA to the special strips of the ABI PRISM 7500 machine were carried out on ice. QRT-PCR reaction mixture (12.5 µl) was prepared for each sample using 6.25 µl of SYBR Premix Ex Taq (2X), 0.5 µl of forward primer (10 µM), 0.5 µl of reverse primer (10 µM), 2 µl of sample cDNA, and 3.25 µl nuclease-free sterilized distilled H2O. The reaction temperature cycle included 1 cycle of the initial annealing step at 95°C for 15 min, 40 cycles of the annealing step at 95°C for 15 s, annealing at 56–60°C for 30 s, amplification at 72°C for 30 s, and one cycle of final amplification at 72°C for 30 s. Using the results of qRT-PCR, the relative expression level of each gene was calculated using $2^{-\Delta C_T} = \left[2^{-(C_{TGenestudied} - C_{TInternalcontrolgene})}\right]$. The fold change of genes was also obtained by $2^{-\Delta\Delta C_T}$ (Schmittgen and Livak, 2008).

2.4. Statistical Analysis

Statistical analysis and data correlation were carried out using SAS 9.3 software. The yield was analyzed based on the factorial design in complete randomized blocks; while the average values were compared based on Tukey's test. The yield and the relative expression of the gene were plotted using GraphPad Prism 8.3 software, whereas the heat map graph was drawn using CIMminer online software. Values were reported as mean ± SE. The significance in Gene fold change was calculated based on the t-test.

3. Results and discussion

3.1. Final fruit yield

According to Figure 2, the highest fruit yield was obtained in off-shoot date palms pollinated with Red Ghanami pollen (5.93 tons/ha). The lowest fruit yield was recorded in tissue culture date palm pollinated with Green Ghanami pollen (2.09 tons/ha). No significant difference was detected between the fruit yield of off-shoot date palm pollinated with Green Ghanami pollen (3.69 tons/ha), off-shoot date palm pollinated with Red Ghanami pollen + green Ghanami (4.33 tons/ha), tissue culture date palm pollinated with Red Ghanami pollen (3.74 tons/ha) and tissue culture date palm pollinated with Red Ghanami and Green Ghanami pollen (50:50) (3.26 tons/ha).

The effect of the pollination variety on fruit set and fruit yield was reported by different researchers (Omar and El-Ashry, 2015; Mohammadi et al., 2017; Shahsavar et al., 2022). The effect of the pollen grain type and the cv. Barhi female date palm, the percentage of fruiting and parthenocarpy can be attributed to the difference in genetic diversity, pollen grain survival, pollen tube growth, fertilization, and compatibility between different types of pollen and female trees which is responsible for the germination ability of pollen grains (Jain et al., 2012; Zaid and Dewet, 2003).

Lower fruit set percentage and higher parthenocarpy percentage in tissue culture date palms compared to off-shoot date palms were also reported in previous studies (Cohen et al., 2004; McCubbin et al., 2004). Based on some reports, the type of pollen variety and the number of times of pollination did not cause satisfactory fruit set in the date fruit obtained from tissue culture Nabt Saif date palm as other endogenous factors are responsible for such abnormality in tissue culture Nabt Saif palms (Awad, 2006). According to Shabana and Al Sunbol (2006), the growth and development of date fruits with and without seeds are regulated by endogenous hormones such as auxin and gibberellin and environmental factors. Pollen grains, immature seeds, and ovary tissues deliver

endogenous auxins and gibberellins which promote cell division and enlargement and stimulate ovary cells to produce more auxins, thereby, controlling cell size and the number of date fruits (Liu et al., 2018).

Lack of seeds due to the lack of endogenous hormones as a result of the expression change of the gene involved in the post-transcriptional products of the genes reduces the development of ovaries (Li et al., 2017) leads to small dates without seeds with low-quality features, also known as "Shees" (Khalil et al., 2021). Abd-Alaal et al. (1982) reported that exogenous auxin and gibberellin foliar spraying on the flowers of cv. Khadrawi date palm caused the growth of parthenocarp dates. In general, the process of fruit formation in higher plants involves many hormones produced in the ovule as a result of fertilization (Gorguet et al., 2005). In the absence of fertilization, the growth and development of the fruit either do not occur or are drastically delayed, probably due to insufficient internal hormones (Hadi et al., 2015). The developing seeds also produce hormones that cause changes in the ovary walls, leading to the formation of normal fruits (Dauelsberg et al., 2011). However, lack of fertilization in some species results in the formation of parthenocarpic fruits (Gorguet et al., 2005, Hamza et al., 2016).

2. Molecular studies

The expression of selected genes was investigated in the control samples of off-shoot and tissue culture date palm cv. Barhi by real-time polymerase chain reaction technique (RT-PCR). The relative expression of genes involved in pollination, fertilization, and date fruit formation was determined. Six important genes, including Histone acetyltransferase HAC1-like (LOC103717600) (HAC 1), Arginine N-methyltransferase 6.1 (LOC103716582) (ANM), TIME FOR COFFEE-like (LOC103716450) (TFC), Homeobox-leucine zipper protein HOX9-like (LOC103703962) (HOX 9), MADS-box transcription factor 2-like (LOC103702602) (GLO 1), and MADS-box transcription factor 16 (LOC103701267) (DEF 1) were selected. The expression of HAC, ANM, TFC, HOX, and GLO genes was significantly higher in the tissue culture plants than in off-shoot ones before pollination. In other words, the expression level of all studied genes before pollination was higher in tissue culture plants than in off-shoot plants (Figure 3). Meanwhile, the highest fruit set and yield (Figure 2) and the lowest parthenocarpy (data not shown) were observed in off-shoot plants.

The relative changes in the expression of these genes were analyzed in flowers of off-shoot (O) and tissue culture (T) date palm cv. Barhi before pollination (control) and after pollination (1 and 2 weeks) with cv. Green Ghanami (G), Red Ghanami (R), and a mixture of Green Ghanami and Red Ghanami pollen (GR). The experiment aimed to examine the expression level of genes involved in the adaptation, fertilization, and formation of normal date fruits in cv. Barhi treated with Green and Red Ghanami pollens during pollination, fertilization, and fruit set. In addition, the expression of 6 selected genes was examined before pollination to 2 weeks after pollination to ensure the growth of the pollen tube and the fertilization of the reproductive nucleus of the pollen grain with the ovule of the Barhi cultivar. The results are shown in Figure 3 based on the type of pollination treatment (GO, RO, GRO, GT, RT, and GRT). In line with the the qRT-PCR results, an increasing trend can be seen in the expression of the six studied genes (HAC 1, ANM, TFC, HOX 9, GLO 1, and DEF 1) over time. The expression of these genes significantly increased 2 weeks after pollination when compared to before pollination in all the treatments.

The changes in the relative expression of the six studied genes green GO- pollinated off-shoot date palm cv. Borhi showed a rise in the expression of HAC, ANM, TFC, DEF, and GLO genes one and two weeks after the pollination. The expression of HOX gene showed no significant change before and one week after pollination; but increased significantly two weeks after pollination (Figure 4). Among studied genes, DEF (182.21) showed the highest increase in the relative expression 2 weeks after pollination of off-shoot date palm cv. with green Ghanami pollen

(GO). It is worth noting that the treatment with Green Ghanami pollen led to the lowest yield in off-shoot date palms compared to the two treatments with Red Ghanami pollen and a mixture of Green Ghanami and Red Ghanami pollen (Figure 2).

Figure 4 showed a significant increase in the relative expression of HAC, HOX, and GLO genes in off-shoot date palm cv. Barhi pollinated with Red Ghanami pollen (RO) 2 weeks after pollination. The expression of HOX gene exhibited the highest enhancement two weeks after pollination compared to other studied genes. Meanwhile, the relative expression of ANM, TFC, and DEF genes showed an ascending trend followed by a decline. Red Ghanami-pollinated off-shoot date palm showed the highest fruiting percentage and yield compared to other treatments due to the increase in the expression of HAC, ANM, TFC and GLO genes and a decrease in HOX expression 1 week after pollination compared to before-pollination state. We can make this statement considering that the growth of vegetative pollen tube and reaching the reproductive nucleus to the ovule take a maximum of one week. On the other hand, 2 weeks after pollination (the time required for ovary growth and early fruit growth), the relative expression changes of the examined genes indicate their effective role in this fruiting process.

The off-shoot date palm cv. Berhi pollinated with a mixture of Green Ghanamy and Red Ghanami pollen (Figure 4) shows an increasing trend of the relative expression for HAC, ANM, and TFC genes 1 week after pollination compared to before pollination, this trend, however, became descending 2 weeks after pollination. The relative expression of DEF and HOX genes first decreased followed by a significant increasing trend. GLO gene expression showed a mild increasing trend in this treatment. So that the highest relative gene expression of this treatment belongs to TFC (2.75) and HOX (2.31) 1 week after pollination and HOX (14.78) 2 weeks after pollination

Changes in the relative expression of HAC in tissue culture date palm pollinated with Green Ghanamy treatment showed no significant difference. The relative expression of GLO gene, however, significantly increased. No significant difference was observed between the relative expression of ANM gene before and 1 week after pollination, while it significantly increased 2 weeks after pollination. Although the relative expression of TFC gene increased after 1 week of pollination, it showed a decreasing trend 2 weeks after pollination. The expression of DEF and HOX first exhibited a decreasing trend followed by an increasing one. The HOX gene had the highest relative expression compared to other studied genes in 2 weeks after pollination of the tissue culture date palm cv. Berhi pollinated with Green Ghanami (Figure 4).

Examination of the relative expression of some genes in the RT treatment indicated the increasing pattern of HAC and GLO genes, while ANM, TFC, and DEF showed a decreasing trend followed by an ascending one 2 weeks after the pollination. Unlike other genes, HOX expression first increased followed by a decline. The highest and lowest relative gene expression in all three investigated times belonged to HOX and HAC, respectively (Figure4).

The increasing trend of relative expression of HAC, ANM, TFC, DEF, HOX, and GLO genes in GRT treatment (Figure 4) is clearly visible. HOX gene had the highest gene expression compared to other genes 2 weeks after pollination. One week after pollination, the highest and lowest relative gene expression levels were detected in TFC (57.10) and HAC (0.13), respectively. TIME FOR COFFEE (TFC) is a circadian regulator contributing to regulating the flowering time. Mutation of this gene leads to late flowering, plastochron defects, and diverse anatomical phenotypes (Sanchez-Villarreal et al., 2013).

The relative expression of each examined genes was separately investigated for each pollination treatment compared to non-pollinated off-shoot and tissue culture date palms (before pollination). The t-test results are

shown in Table 2. The aim is to separately investigate the changes in the relative expression of selected genes 1 and 2 weeks after pollination in treated off-shoot and tissue culture plants. Concerning the HAC gene, no statistical difference was observed between the gene expression of RO treatment 1 week after pollination and GRO treatment 2 weeks after pollination with the gene expression condition before pollination in off-shoot date palm. In the case of GLO, no statistical difference was recorded between its expression in the GRO- pollinated off-shoot palm before pollination and 1 week after that. In other studied genes, the pollination of off-shoot date palm led to a significant difference in their relative expression compared to pre-pollination condition. In off-shoot date palm, HOX gene showed the highest relative expression in all three pollination treatments 2 weeks after pollination. The relative expression of HOX 1 week after pollination with Red Ghanami and Green Ghanami + Red Ghanami (50:50) exhibited a significant decreasing trend (-0.45 and -1.06, respectively) compared to pre-pollination conditions, but it increased by 98.85% and 95.13% 2 weeks after pollination, respectively.

In tissue culture date palm, the relative expression of HAC in the pollinated palms showed no significant difference compared to the pre-pollination state (Green Ghanami pollen (1 and 2 weeks after pollination), Red Ghanami (1 and 2 weeks after pollination), and Green Ghanami + Red Ghanami (50:50) 1 week after pollination). A significant increase was detected for the Green Ghanami + Red Ghanami (50:50) treatment 2 weeks after pollination. No statistically significant difference was observed in the relative expression of ANM gene in tissue culture date palm before pollination and 1 and 2 weeks after pollination with Green Ghanami and Red Ghanami. However, the tissue culture date palm pollinated with Ghanami pollen + Red Ghanami pollen (50-50) exhibited a significant increase in the expression of the mentioned gene1 and 2 weeks after pollination.

Concerning the relative expression of GLO gene in the tissue culture date palm, no significant difference was detected before pollination and 1 week after that for Green Ghanamy treatment. A remarkable rise was, however, observed 2 weeks after pollination. The relative expression of HOX in tissue culture date palm significantly rose after pollination with Green Ghanamy 1 week after pollination (-2.47) and Red Ghanamy 2 weeks after pollination (-0.77) (Table 2). Based on Table 2, the highest relative increase in the expression of all the investigated genes in tissue culture date palm occurred 2 weeks after pollination with Green Ghanami + Red Ghanami (50:50), suggesting the decisive high role of these genes in the formation and growth of the natural date fruit. On the other hand, the HOX gene showed the highest relative expression (159.94) compared to other studied genes before pollination, indicating the especial role of this gene in fertilization and the date palm fruit set.

Table 2: Gene fold change of each treatment compared to non-pollinated off-shoot and tissue culture *Phoenix dactylifera* L. cv. Barhi

Date palm cv. Barhi	Pollen type	Sampling time	Relative gene expression changes					
			HAC	ANM	TFC	DEF	HOX	GLO
Off-shoot	Before pollination		1	1	1	1	1	1
	Green Ghanami	1 week after pollination	1.02**	1.3*	1.55*	1.50*	0.87**	1.90**
		2 weeks after pollination	2.41**	6.76**	30.49**	55.39**	15.18**	28.28**
	Red Ghanami	1 week after pollination	1.05n.s	1.34**	3.94**	3.78**	-0.45**	2.61*
		2 weeks after pollination	1.06*	1.24**	2.04**	2.20*	39.42**	2.97*
	Green Ghanami + Red Ghanami (50:50)	1 week after pollination	1.09**	1.25**	1.64**	0.79*	-1.06**	1.36n.s
		2 weeks after pollination	1.01n.s	1.09**	1.50*	1.14**	21.77**	1.57*
Tissue culture	Before pollination		1	1	1	1	1	1
	Green Ghanami	1 week after pollination	1.01n.s	1 n.s	1.95*	0.67**	-2.47**	1.30n.s
		2 weeks after pollination	1.01n.s	1.17n.s	1.41**	1.69**	10.17**	1.91*
	Red Ghanami	1 week after pollination	1 n.s	0.96n.s	0.59*	0.56**	5.41**	1.27*
		2 weeks after pollination	1.04n.s	0.98n.s	0.82**	1.13*	-0.77**	1.55*
	Green Ghanami + Red Ghanami (50:50)	1 week after pollination	1.01n.s	6.41**	17.63**	0.86*	5.96*	2.11**
		2 weeks after pollination	3.03**	24.40**	46.60**	37.06**	159.94**	30.85**

HAC 1: Histone acetyltransferase HAC1-like (LOC103717600); ANM: Arginine N-methyltransferase 6.1 (LOC103716582); TFC: TIME FOR COFFEE-like (LOC103716450); DEF 1: MADS-box transcription factor 16

(LOC103701267); HOX 9: Homeobox-leucine zipper protein HOX9-like (LOC103703962); GLO 1: MADS-box transcription factor 2-like (LOC103702602).

n.s, * and ** indicate non-significance, significance at 5% and 1%, respectively.

Aberrant HAHB-10 expression in Arabidopsis causes phenotypic abnormalities, such as dark cotyledons, flattened leaves, reduced life cycles, and accelerated flowering (Rueda et al., 2005). HD-Zip (HOX) proteins are plant-unique transcription factors characterized by a homeodomain and a leucine zipper motif. Despite their structural similarities, HD-Zip proteins participate in diverse processes including stress responses ranging from morphogenesis to growth. Genetic analyses have shown that their function relies on the activation of downstream target genes, most of which remain unknown. The downstream processes will be key to understanding the function of this important class of transcription factors (Elhiti and Stasolla, 2009).

All studied genes increased in line with each other over time after the pollination treatment, implying the effective and direct role of HAC 1, ANM, TFC, HOX 9, GLO 1, and DEF 1 in fruit set of all pollinated off-shoot and tissue culture date palm cv. Barhi. The extent of changes in the expression of each gene was investigated in all six pollination treatments (GO, RO, GRO, GT, RT, and GRT) based on their color intensity and illustrated as a heat map using CIMminer online software (Figure 5). Red color shows the high expression level while the low expression level is depicted by green color. The intermediate colors indicate a moderate expression level. The expression of studied genes increased in the flowers of pollinated off-shoot and tissue culture date palm cv. Barhi (Green Ghanami, Red Ghanami pollen, and a 50:50 mixture of both) 1 and 2 weeks after pollination compared to pre-pollination state.

On the other hand, according to the cluster results in Figure 5, the expression of ANM and HOX genes had a direct and similar relationship with the formation of date fruit. Meanwhile, HAC 1, TFC, GLO 1, and DEF 1 genes are similarly involved in fertilization and fruit formation. The cluster results of the different heat map treatments showed the similar expression trends of the studied genes in GOT3, ROT2, GROT2, GTT2, and GRTT2 treatments. The process of gene expression and their changes in RTT3, GOT2, GRTT3, GTT3, RTT2, ROT3, and GROT3 treatments were also identical.

Shair et al. (2016) carried out a molecular analysis on normal flowers of off-shoot and abnormal flowers of tissue culture cv. Barhi date palm using RAPD marker to investigate genetic diversity. They reported a low level of gene diversity between normal and abnormal flowers of off-shoot and tissue culture date palm cv. Barhi. Moreover, they stated that flower abnormalities in tissue culture cv. Barhi may be due to epigenetic changes. They also reported no significant changes in DNA level of tissue culture samples in different growth stages. On the other hand, some researchers considered the tissue culture method an important factor in genetic diversity between off-shoot and tissue culture plants. Al Kaabi et al. (2007) examined the genetic stability of seedlings of some tissue culture date varieties using AFLP markers. They report more genetic diversity of date palms obtained from tissue culture samples through embryogenesis (0.6%) compared to organogenesis (0.038%) than the mother plant (off-shoot). They also proposed the early identification and diagnosis of off-type plants to prevent the incidence and proliferation of these abnormalities. Mirani et al. (2022) also observed no significant difference in the genetic diversity of off-shoot and tissue culture date palm cv. Dedhi.

One method for propagation of date palms is callus and somatic embryogenesis which may show abnormalities in their flowers, called "mantled." The abnormal flowers create the second complete axis of the stamen instead of the stamens (Alwee et al. 2006), which does not grow properly and can only be recognized when the palms start to flower after 2 to 3 years of cultivation. Eeuwens et al. (2002) conducted a detailed study to determine which tissue culture conditions lead to defective flowers. This disorder was more common in susceptible genotypes when short subculture periods were used and high cytokinin/low auxin was added. This disorder does not seem to be related to endogenous cytokinin levels (Jones et al. 1995). As this abnormality is usually a homeotic change, changes in the homeotic genes which determine the sex of the floral organ participating in fertilization should be considered (Alwee et al. 2006). These genes are members of the MADS-box transcription factor family.

On the other hand, regarding the regularity of this change, an epigenetic change is the basis of this abnormality rather than a genetic one. Although some genetic changes were found, these changes were not related to the incidence of morphological abnormalities (Matthes et al. 2001). Random DNA methylation changes were found among regenerated palms, which were not associated with this abnormality (Jaligot et al. 2002).

Long-term changes in DNA expression have been increasingly documented during plant growth. Changes in flowering ability (vernalization) and ontogenetic have been studied from adolescence to maturation. In this case, both DNA methylation and changes in histones are involved. Changes have been reported in DNA methylation and histone in many cases of tissue culture plants. Such changes are the basis for the production of various abnormalities in date palm (Smulders and De Klerk, 2011; Jullien and Berger 2010; Johannes et al. 2009).

Post-translational modifications of histone N-terminus play an essential role in maintaining chromatin structure and function. One of these modifications is histone acetylation, which is involved in many biological processes (Liu et al., 2004). Histone acetylation is an important post-translational modification associated with gene activation. In Arabidopsis, the histone acetyltransferase AtHAC1 is homologous to p300/CREB binding proteins (cAMP-responsive element-binding proteins), which are major histone acetyltransferases involved in many physiological processes, including proliferation, differentiation, and apoptosis (Cheung et al., 2000; Strahl and Allis, 2000). Damage to AtHAC1 causes pleiotropic growth abnormalities, including delayed flowering, primary root shortening, and reduced fertility (Deng et al., 2007). Accordingly, HAC1 is probably an acetyltransferase agent and thus helps to regulate flowering time.

On the other hand, Deng et al. (2007) showed that HAC1 affects flowering by influencing the expression of MADS-box genes. Accordingly, HAC1 may regulate the expression of the MADS-box transcriptional repressor. Histone methylation in lysine and arginine is one of the most complex and important covalent modifications of eukaryotic organisms (Bannister and Kouzarides, 2005). Protein arginine methyltransferases (PRMTs) catalyze the transfer of methyl groups from S-adenosyl-L-methionine (SAM) to guanidine nitrogen atoms of arginine residues. Moreover, all active PRMTs catalyze the formation of O-monomethyl arginine (MMA). Several histone H3 lysine methyltransferases were identified in Arabidopsis thaliana with prominent roles in gene silencing and growth regulation (Kim et al., 2005; Zhao et al., 2005). It was shown that Arabidopsis protein arginine methyltransferase 5 (AtPRMT5) symmetrically demethylates histone H4R3 (Pei et al., 2007; Wang et al., 2007). Niu et al. (2007) showed that arginine methyltransferases (PRMTs) genes significantly regulate flowering and growth. Protein arginine methyltransferase 6 (PRMT6) is a type of PRMT which participates in epigenetic regulation of gene expression, development and differentiation, DNA repair, cell proliferation, and senescence, DNA methylation, mitosis, activation of nuclear transreceptors, and cell signaling (Gupta et al., 2021).

Teo et al. (2019) and Shah et al. (2022) also mentioned the role of MADS-box in the flowering and transition from the vegetative to the reproductive phase. MIKC-type MADS-box genes are involved in plant development from vegetative growth to reproduction and various stress responses (Agarwal and Khurana, 2019). According to the ABCDE model, MADS-box genes of type M (conserved MADS domain) are involved in plant reproduction, especially in the growth and function of female gametophytes, embryos, and endosperms. In addition, MIKC-type MADS-box genes are involved in meristem differentiation, flowering, determination of flower organogenesis, and fruit development (Callens et al., 2018).

Examining the changes in the expression of the studied genes in this experiment showed an increasing trend in the expression of GLO for all treatments from the pre-pollination time to 2 weeks after pollination. The HAC gene exhibited the lowest relative expression compared to other studied genes in all pollination treatments 2 weeks after pollination. The greatest changes in the relative expression of all genes were observed in tissue culture date palm pollinated with Green Ghanamy + Red Ghanamy pollen (50-50) treatment. HOX gene had the highest relative expression in all treatments except the tissue culture date palm pollinated with Red Ghanamy pollen treatment compared to other genes before pollination and 1 and 2 weeks after pollination (Figures 2 and 3 and Table 2). All these changes indicate the decisive role of these genes in the regulation of flowering, fertility, and, finally, the formation of normal fruit and the somaclonal. The somaclonal and epigenetic changes in tissue culture date palms through regulation of the transcription of these genes caused phenotypic changes, fertilization, fruit formation, and reduced yield.

3.3. Relationship between function and gene expression

The correlation results between the yield and expression levels of HAC 1, ANM, TFC, HOX 9, GLO 1, and DEF 1 genes before pollination and 1 and 2 weeks after pollination are listed in Table 3. This table is presented to determine the correlation between gene expression changes and performance. According to the purpose of the paper, finding the precise influence of genes in inoculation and early fruit formation can affect the final yield of the tissue culture date palm cv. Barhi. According to Table 3, a significant positive relationship can be detected between the final yield of date palm and the expression level of ANM (0.98) and TFC (0.95) genes at the $P \geq 1\%$. Niu et al. (2007) showed the significant role of arginine methyltransferase (ANM) gene in the epigenetic regulation of gene expression, flowering and growth time. The mutation in the expression of this gene delays flowering (Gupta et al., 2021). TIME FOR COFFEE (TFC) is a circadian regulator with a key role in the regulation of flowering time whose mutation causes late flowering, plastochron defects, and diverse anatomical phenotypes (Sanchez-Villarreal et al., 2013). According to the positive relationship shown in Table 3, increasing the expression of ANM and TFC genes before pollination in date palm tissue culture plants can significantly affect the final yield. At the same time, no correlation was recorded between the expression changes of HAC, DEF, HOX, and GLO genes, suggesting that the expression level of these genes in the growth and development stage had no impact on the fruit yield of date palm cv. Barhi.

Also, the examination of the yield of date palm and the expression of the studied genes 1 week after pollination indicates a significant negative correlation between the yield and the expression of HAC (-0.54), DEF (-0.70), and GLO (-0.67) genes. In other words, the yield will significantly increase by reducing the expression of these genes 1 week after pollination. However, the expression of ANM, TFC, and HOX genes 1 week after pollination showed no significant effect on the yield (Table 3). Any damage to AtHAC1 gene causes pleiotropic developmental abnormalities, including delayed flowering, primary root shortening, and reduced fertility (Deng et al., 2007). In

addition, Callens et al. (2018) mentioned the role of MADS-box genes (DEF and GLO) in flowering, organogenesis, and fruit growth.

According to Table 3, only the expression level of ANM had a significant positive correlation with the final yield of the product at the level of 5% (0.53) 2 weeks after pollination. In other words, the expression level of the ANM gene is effective before pollination and 2 weeks after pollination due to its significant role in flowering. In other words, the ANM protein was effective in regulating flowering (before pollination), after fertilization, and during the initial growth of the fruit (2 weeks after pollination). Histone methylation at lysine and arginine is one of the most complex and important covalent modifications in the eukaryotic organisms (Bannister and Kouzarides, 2005). Protein arginine methyltransferase (ANM) catalyzes the transfer of methyl groups from S-adenosyl-L-methionine (SAM) to guanidine nitrogen atoms of arginine residues. All active ANMs catalyze the O-monomethylarginine (MMA). In Arabidopsis thaliana, several histone H3 lysine methyltransferases have been identified with important roles in gene silencing and growth regulation which confirm the results of this experiment (Jackson et al., 2002; Kim et al., 2005; Zhao et al., 2005). Recently, A. thaliana protein arginine methyltransferase 5 (AtANM5) was shown to symmetrically demethylate histone H4R3 (Pei et al., 2007; Wang et al., 2007). Therefore, it seems that the epigenetic changes and methylation of HAC 1, ANM, TFC, HOX 9, GLO 1, and DEF 1 in tissue culture date palm decreased the yield of the final product compared to off-shoot date palms (Figure 1). Noteworthy, Table 3 shows the expression of HOX gene in all 3 investigated stages which showed no significant correlation with the yield of date palm. Rueda et al. (2005) reported that aberrant expression of HOX-10 in Arabidopsis leads to various phenotypic abnormalities, including dark cotyledons, flattened leaves, shortened life cycles, and accelerated flowering. In other words, it influenced various phenotypic characteristics considering the high expression levels of this gene in off-shoot and tissue culture date palms. The increase in its expression in tissue culture date palm led to significant phenotypic changes compared to off-shoot samples while causing no significant effect on the final yield, flowering, and inoculation.

Table 3: Correlation between the yield of off-shoot and tissue culture *Phoenix dactylifera* L. cv. Barhi with the expression of the investigated genes in Before pollination, 1 and 2 weeks after pollination.

Yield	HAC	ANM	TFC	DEF	HOX	GLO
Before pollination	$0.293^{n.s}$	0.958^{**}	0.980^{**}	$-0.093^{n.s}$	$0.531^{n.s}$	$-0.331^{n.s}$
1 week after pollination	-0.546^{*}	$-0.045^{n.s}$	$-0.124^{n.s}$	-0.708^{**}	$-0.338^{n.s}$	-0.673^{**}
2 weeks after pollination	$-0.159^{n.s}$	0.538^{*}	$0.360^{n.s}$	$0.015^{n.s}$	$-0.194^{n.s}$	$-0.235^{n.s}$

HAC 1: Histone acetyltransferase HAC1-like (LOC103717600); ANM: Arginine N-methyltransferase 6.1 (LOC103716582); TFC: TIME FOR COFFEE-like (LOC103716450); DEF 1: MADS-box transcription factor 16 (LOC103701267); HOX 9: Homeobox-leucine zipper protein HOX9-like (LOC103703962); GLO 1: MADS-box transcription factor 2-like (LOC103702602).

n.s, * and ** indicate non-significance, significance at 5% and 1%, respectively.

Table 4 shows the relationship between the expression level of HAC 1, ANM, TFC, HOX 9, GLO 1, and DEF 1 before pollination and 1 and 2 weeks after pollination. Accordingly, a positive relationship can be seen between the expression level of ANM and TFC genes (0.97) (P≥5%) and HAC and HOX genes (0.79) (P≥5%) before pollination. Table 3 also suggests a significant positive relationship between the expression level of ANM and TFC genes with

the final yield. According to Tables 3 and 4, there will be a significant increase in yield of off-shoot and tissue culture date palms by increasing the expression of ANM and TFC genes before pollination which directly affect each other.

The relationship between the expression levels of the examined genes 1 week after pollination is shown in Table 3. Accordingly, a difference can be seen between the expression of TFC and DEF (0.48), DEF and HOX (0.49), TFC and GLO (0.47), and HOX and GLO (0.54) 1 week after pollination at P≥5%. A significant positive correlation was also recorded between the expression of HAC and DEF (0.78), HAC and GLO (0.84), and DEF and GLO (0.94) at P≥1%. Table 4 indicated that the expression level of the HAC has a significant and direct relationship with the expression of ANM (0.54), TFC (0.49), DEF (0.64), and GLO (0.61) in 2 weeks after pollination. On the other hand, ANM gene expression showed a significant positive relationship with TFC (0.77) 2 weeks after pollination. Moreover, a significant positive relationship was detected between TFC and DEF (0.47) and DEF and GLO (0.64) 2 weeks after pollination (Table 4). Post-translational modifications of histone N-terminal play an essential role in maintaining chromatin structure and function. One of these modifications is histone acetylation which is involved in many biological processes (Liu et al., 2004). Therefore, histone acetylation is a significant post-translational modification associated with gene activation. In Arabidopsis, the histone acetyltransferase AtHAC1 is homologous to p300/CREB binding proteins (cAMP-responsive element-binding proteins), which are major histone acetyltransferases in many physiological processes, including proliferation, differentiation, and apoptosis (Cheung et al., 2000; Strahl and Allis, 2000). Based on Deng et al. (2007), HAC1 is probably an acetyltransferase agent, thus, helps to regulate flowering time. On the other hand, Deng et al. (2007) showed that HAC1 affects flowering by influencing the expression of MADS-box genes and preventing their transcription.

Table 4: Correlation between the expression of the investigated genes in Before pollination, 1 and 2 weeks after pollination.

Sampling time		HAC	ANM	TFC	DEF	HOX	GLO
Before pollination	HAC	1					
	ANM	0.158 n.s	1				
	TFC	0.278 n.s	0.978**	1			
	DEF	0.521 n.s	-0.301 n.s	-0.098 n.s	1		
	HOX	0.798*	0.516 n.s	0.521 n.s	-0.083 n.s	1	
	GLO	0.154 n.s	-0.304 n.s	-0.205 n.s	0.518 n.s	-0.192 n.s	1
1 week after pollination	HAC	1					
	ANM	0.220 n.s	1				
	TFC	0.297 n.s	0.407 n.s	1			
	DEF	0.780**	0.206 n.s	0.487*	1		
	HOX	0.400 n.s	0.352 n.s	-0.036 n.s	0.493*	1	
	GLO	0.844**	0.398 n.s	0.476*	0.946**	0.549*	1
2 weeks after pollination	HAC	1					
	ANM	0.547*	1				
	TFC	0.490*	0.775**	1			
	DEF	0.640**	0.408 n.s	0.473*	1		
	HOX	-0.081 n.s	-0.010 n.s	-0.112 n.s	-0.342 n.s	1	
	GLO	0.618**	0.424 n.s	0.368 n.s	0.648**	0.068 n.s	1

HAC 1: Histone acetyltransferase HAC1-like (LOC103717600); ANM: Arginine N-methyltransferase 6.1 (LOC103716582); TFC: TIME FOR COFFEE-like (LOC103716450); DEF 1: MADS-box transcription factor 16 (LOC103701267); HOX 9: Homeobox-leucine zipper protein HOX9-like (LOC103703962); GLO 1: MADS-box transcription factor 2-like (LOC103702602).

n.s, * and ** indicate non-significance, significance at 5% and 1%, respectively.

HD-Zip proteins (HOX 9) are plant-unique transcription factors represented by a homeodomain and a leucine zipper motif. Despite these structural similarities, HD-Zip proteins participate in diverse and sometimes overlapping events, from stress responses to morphogenesis and development (Shao et al., 2018; Sharif et al., 2021). Genetic analyses have shown that their function depends on the activation of downstream target genes, most of which remain unknown. Elucidation of these downstream events will be a key step to understanding the

role of this important class of transcription factors (Elhiti and Stasolla, 2009). Teo et al. (2019) and Shah et al. (2022) addressed the role of MADS-box (DEF 1 and GLO 1) in the process of flowering and transition from the vegetative to the reproductive phase. MIKC-type MADS-box genes are involved in plant development from vegetative growth to reproduction and function in various stress responses (Agarwal and Khurana, 2019). Based on the ABCDE model, M-type MADS-box genes (conserved MADS domain) contribute to plant reproduction, especially in the development and function of female gametophytes, embryos, and endosperm (Callens et al., 2018). According to the results, the expression of the studied genes affected the other and finally led to phototypic and functional characteristics of tissue culture plants compared to off-shoot ones. Each gene in each growth stage has a special function which can improve the functional characteristics of the date palm.

4. Conclusion

The formation of parthenocarp fruit in tissue culture date palm is one of the most important factors in reducing the productivity of the palm grove and, as a result, the dissatisfaction of the farmers. The extent of the cultivation of these plants in gardens worldwide and the lack of sufficient crop production motivated the researchers to investigate the relationship between the expression of some genes involved in flowering and fertilization with the final yield of the product. In this study, the effects of 3 dominant pollen treatments (Green Ghanami, Red Ghanami, and a mixture of Green and Red Ghanami) were examined on the final product yield of off-shoot and tissue culture date palm cv. Barhi considering the correlations with the expression of HAC, ANM, TFC, DEF, HOX, and GLO genes before pollination and 1 and 2 weeks after that. The results showed a decline in the yield of tissue culture date palm compared to off-shoot in all pollen treatments, while the gene expression was increased. Pollination with Red Ghanami pollen and a mixture of Red and Green Ghanami pollen resulted in the highest fruit yield in both off-shoot and tissue culture palms. Despite the involvement of the HOX9 gene in the phenotypic differences of tissue culture and off-shoot plants, it did not directly affect flowering and the final yield. The role of these genes depends on their contribution to different biochemical and metabolic factors at certain times of flowering (before pollination), fertilization (1 week after pollination), or fruit set (2 weeks after pollination). Therefore, the highest yield can be achieved in tissue culture and off-shoot date palm cv. Barhi upon regulating ANM and TFC genes expression before pollination, HAC, DEF, and GLO 1 week after pollination, and ANM 2 weeks after pollination.

Declarations

Disclosure statement

No potential conflict of interest was reported by the authors.

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Figures

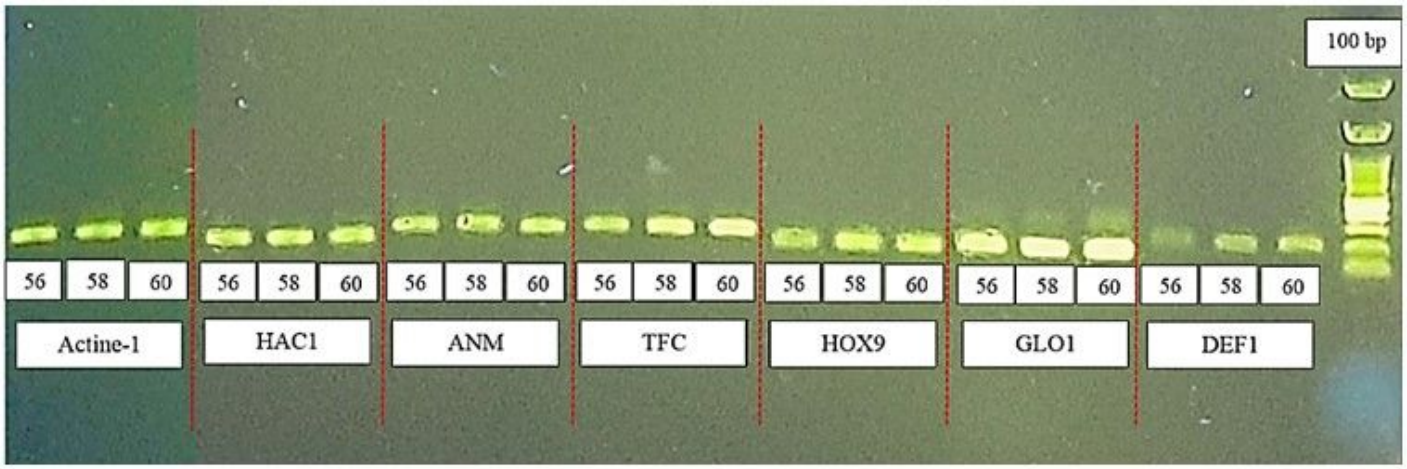

Figure 1

Electrophoresis of primer efficiency and determination of the appropriate time in qRT-PCR reaction of the studied genes in this experiment. HAC 1: Histone acetyltransferase HAC1-like (LOC103717600); ANM: Arginine N-methyltransferase 6.1 (LOC103716582); TFC: TIME FOR COFFEE-like (LOC103716450); HOX 9: Homeobox-leucine zipper protein HOX9-like (LOC103703962); GLO 1: MADS-box transcription factor 2-like (LOC103702602); DEF 1: MADS-box transcription factor 16 (LOC103701267).

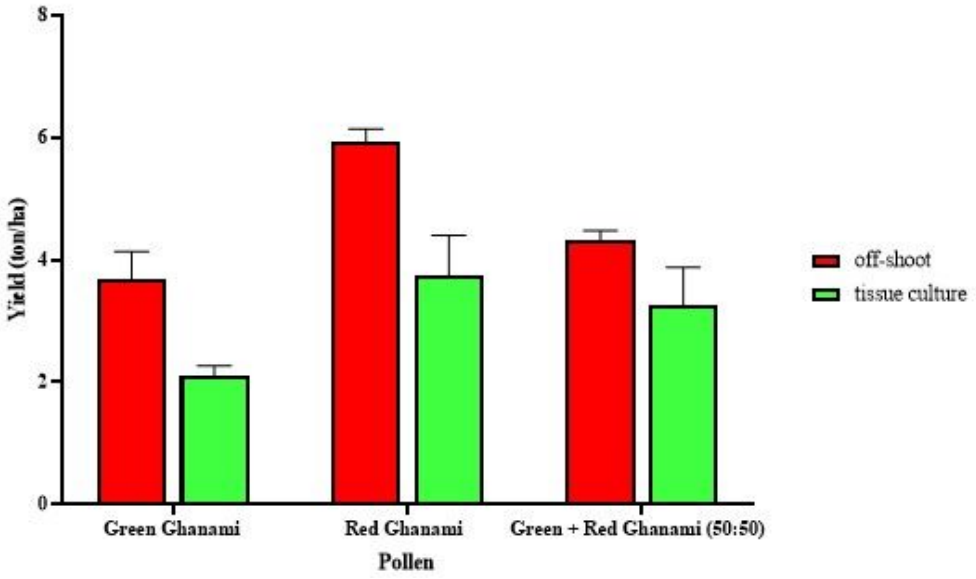

Figure 2

The effect of date palm type cv. Barhi and pollen type on fruit yield (150 days after pollination).

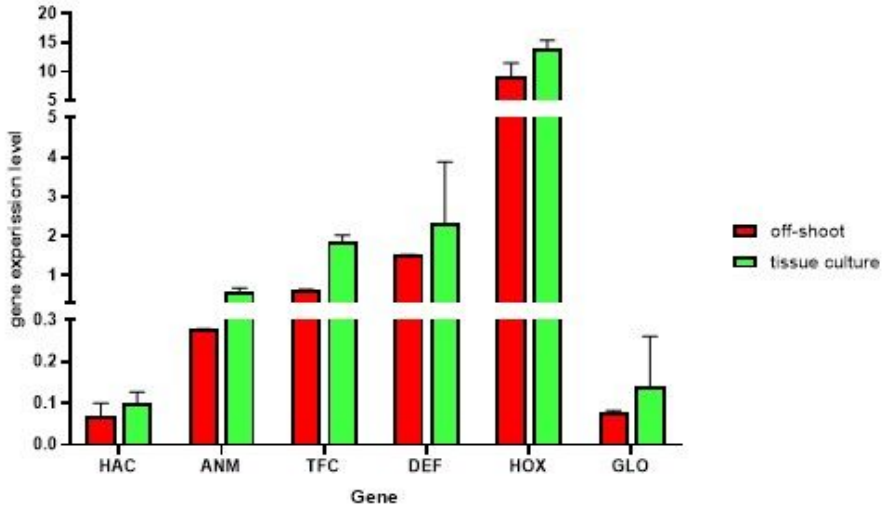

Figure 3

Comparison of the expression level of the studied genes in off-shoot and tissue culture *Phoenix dactylifera* L. cv. Barhi before pollination.

HAC 1: Histone acetyltransferase HAC1-like (LOC103717600); ANM: Arginine N-methyltransferase 6.1 (LOC103716582); TFC: TIME FOR COFFEE-like (LOC103716450); DEF 1: MADS-box transcription factor 16 (LOC103701267); HOX 9: Homeobox-leucine zipper protein HOX9-like (LOC103703962); GLO 1: MADS-box transcription factor 2-like (LOC103702602).

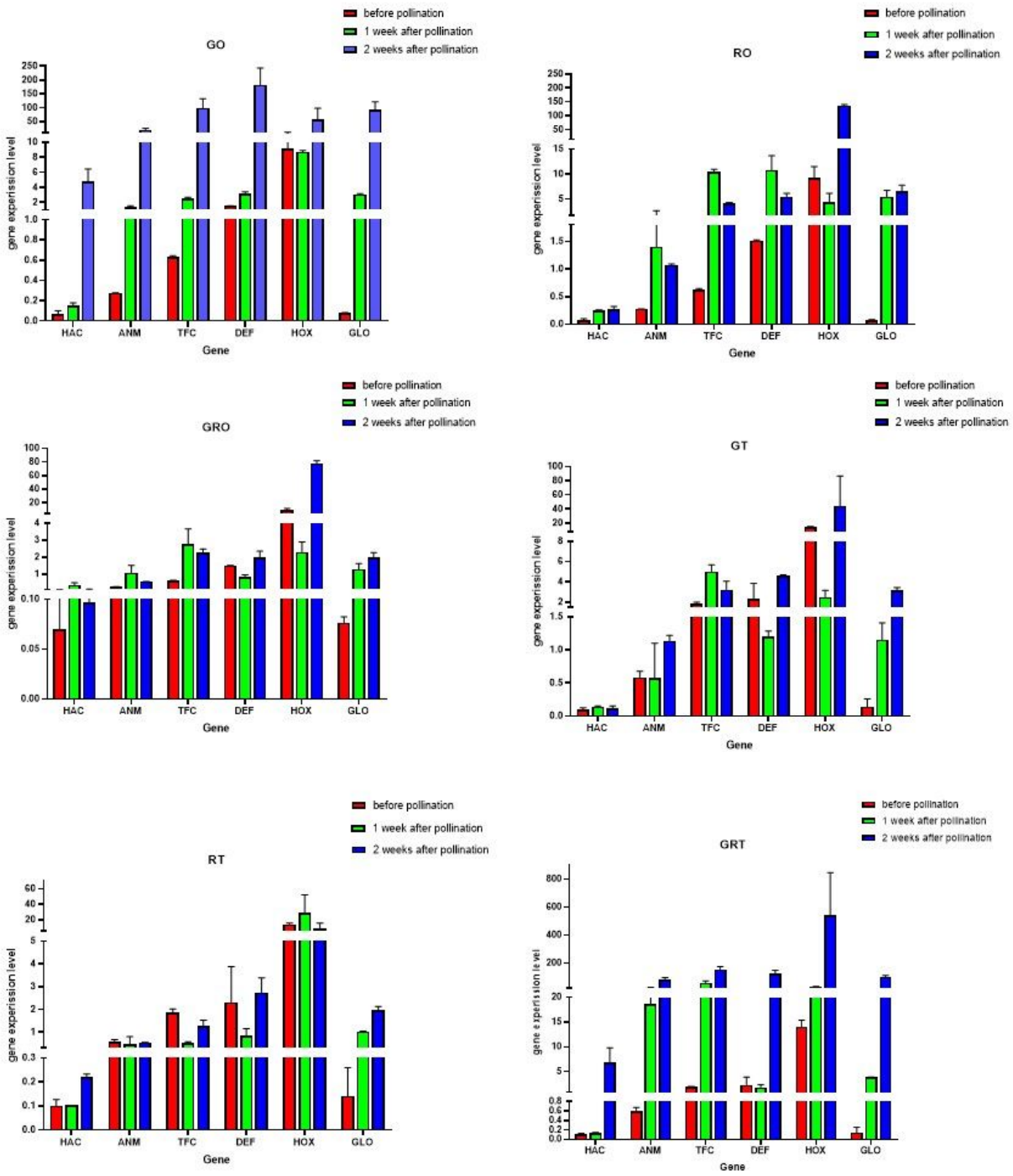

Figure 4

The expression level of studied genes in off-shoot *Phoenix dactylifera* L. cv. Barhi pollinated with Green Ghanami (GO), Red Ghanami (RO) and Green Ghanami + Red Ghanami (50:50) (GRO) as well as tissue culture *Phoenix dactylifera* L. cv. Barhi pollinated with Green Ghanami (GT), Red Ghanami (RT) and Green Ghanami + Red Ghanami (50:50) (GRT).

HAC 1: Histone acetyltransferase HAC1-like (LOC103717600); ANM: Arginine N-methyltransferase 6.1 (LOC103716582); TFC: TIME FOR COFFEE-like (LOC103716450); DEF 1: MADS-box transcription factor 16

(LOC103701267); HOX 9: Homeobox-leucine zipper protein HOX9-like (LOC103703962); GLO 1: MADS-box transcription factor 2-like (LOC103702602).

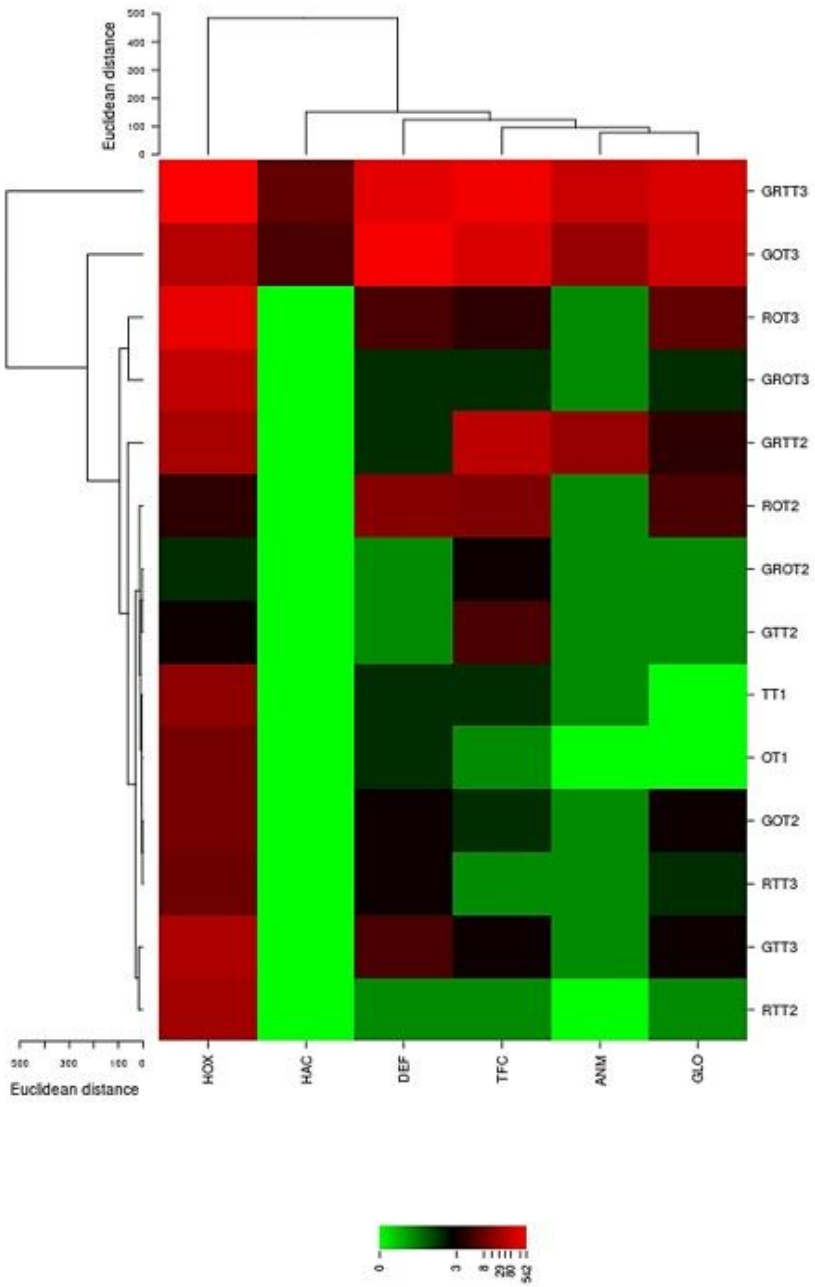

Figure 5

Heat map of studied genes. The cluster is based on the correlation between the expression level of genes and treatments. G = Green Ghanami pollen, R = Red Ghanami pollen, GR = Green Ghanami + Red Ghanami pollen (50:50), O = off-shoot date palm, T = Tissue culture date palm. T1 = before pollination, T2 = 1 week after pollination, T3 = 2 week after pollination. HAC 1: Histone acetyltransferase HAC1-like (LOC103717600); ANM: Arginine N-methyltransferase 6.1 (LOC103716582); TFC: TIME FOR COFFEE-like (LOC103716450); DEF 1: MADS-

box transcription factor 16 (LOC103701267); HOX 9: Homeobox-leucine zipper protein HOX9-like (LOC103703962); GLO 1: MADS-box transcription factor 2-like (LOC103702602).