A Study on Normal and Nubbin Productivity of Some Mango Trees in Relation to Quantification of Expressed Gene Levels

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ABSTRACT

The present investigation was carried out during 2015 and 2016 seasons on three Egyptian mango cultivars (*Mangifera indica* L.) named Ewais (normal & nubbin), Sedeq and Zebda grown at a private orchard in Idko region under Beheira governorate conditions. The trees were selected to study normal and nubbin fruits of some mango trees productivity and quantification of expressed gene levels as well as trying to make better understanding of embryo abortion phenomenon in mango. Real-time qPCR was used to detect the relative amounts of mRNA of four different genes. Fruit physical characteristics of Ewais (nubbin) cultivar, i e fruit length, width and pulp, stone and peel weights, as well as yield were lower significantly than normal fruits, while its pulp/ stone ratio gave the highest values, in both seasons. The chemical characteristics of Ewais (nubbin), i e TSS, TSS/ acidity, reducing sugars, total sugars and pectin methyl esterase were significantly higher than normal fruits, while acidity, vitamin C and peroxidase were the lowest values. Results showed that the four selected ESTs were differentially expressed in the tissues of normal, aborted embryos and leaves, however, the 4 ESTs (*MaAGA, MaAP2, MaERF,* and *MaMADS*) were upregulated in normal embryo tissues compared to aborted embryos of "Ewais". Also the expression levels in the leaves tissues were upregulated in Sedeq and Zebda varieties compared to Ewais leaves. The results of total protein (SDS-PAGE) explained that Zebda and Sedeq cultivars, which had normal fruits gave the highest several bands in both embryos and leaves tissues than Ewais cultivar which had nubbin fruits.

Keyword: Mango*, Mangifera indica*, Real-time qPCR, Total protein (SDS-PAGE),Productivity.

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NTRODUCTION

Mango (*Mangifera indica* L.) is a globally important fruit crop of the tropics and subtropics, particularly in Egypt, where it is considered the 'king of fruits' due to its delicate, sweet flavor and nutritional value. The total fruiting area of mango trees was about 88510 hectare that produced about 927350 ton at the year of 2014 (Arab Agricultural Statistics Yearbook, 2015). Only perfect (hermaphrodite) flowers are able to set fruit, however, the number of perfect flowers per inflorescence varies between cultivars or is variable from year to year, depending on the location of the inflorescence in the tree (Singh and Arora, 1965 and Singh, 2005).

Embryo abortion, which hinders normal fruit development, seems to play an important role in induction of the abscission process at early stages of fruit development (Mukherjee, 1953). Earlier studies and observations had focused on the physiological and anatomical aspects of mango embryo abortion, and the molecular basis of this disorder is unknown. In this regard, according to Bustin (2002), Real-time PCR differs from classical PCR by the measurement of the amplified PCR product at each cycle throughout the PCR reaction. In practice, a video camera records the light emitted by a fluorochrome incorporated into the newly synthesized PCR product. Thus, real-time PCR allows the amplification to be followed in real-time during the exponential phase of the run and thus allows the amount of starting material to be determined precisely.

It is necessary to identify the genes that are differentially expressed in aborted tissue to explore the molecular basis of embryo abortion in mango. Furthermore, He *et al*. (2012) used a PCR-based subtractive hybridization technique to isolate differentially expressed genes from normal and aborted embryo tissues to characterize the molecular aspects that probably play role in embryo abortion events. However, Choudhury *et al.* (2012)employed (SDS-PAGE 10%) markers to make an assay to systematically investigate the role of an AGAMOUS MADS-box transcription factor in regulation of fruit ripening and floral reproductive organ development in banana cultivar Giant governor. They reported that single distinct protein band with an approximate molecular mass near 27-kDa was obtained in the elution fractions from both flower and fruit specific DNA-protein complexes.

The aims of the present study are to determine normal and nubbin productivity of some mango trees in relation to quantification of expressed gene levels. In addition to understand better embryo abortion phenomenon in some mango trees through obtaining the role of some ESTs genes that implicated in embryo abortion using RT-qPCR technique.

MATERIALS AND METHODS

The present investigation was carried out during two successive seasons 2015 and 2016 on three Egyptian mango cultivars (*Mangifera indica* L.*)* named Ewais (normal & nubbin), Sedeq and Zebda grown in a private orchard in Idko region under Beheira governorate conditions. The experimental trees were about eight years old, budded on Sukkari rootstock, planted on sandy soil at 3 X 5 m For the present study, twenty trees were selected as uniform as possible in vigor (i e 4 accessions x 5 replicates = 20 trees) and under regular horticultural practices including: fertilization, pruning as well as pest and disease control. Flooding system was used for irrigation.

1. Trees productivity

In order to determine mango trees productivity, number of normal and nubbin fruits/ tree was counted and the yield in Kg was calculated by multiplying

number of fruits per tree X the average of fruit weight. Additionally, fruit physical and chemical characteristics were determined in samples collected randomly from each replicate(5 fruits) at the harvest date in 30 August for Ewais, 20 August for Sedeq and 25 July for Zebda in 2015 and 2016 seasons respectively, and directly transported to the laboratoryof the Faculty of Agriculture, Alexandria University.

A. Fruit physical characteristics

Average fruit length and diameter (cm) were measured for each individual fruit using a Vernier caliper and fruit length /diameter ratio was calculated. Fruit, pulp and peel weight (g) were determined for each cultivar. Also, pulp / stone ratio was estimated in the fruit samples by weighing the stone and pulp then the fruit pulp / stone ratios were calculated.

B. Fruit chemical characteristics

In another sample of five fruits, juice from each replicate was used to determine the percentage of TSS by hand refractometer. Vitamin C was determined in 1 ml fruit juice by titration with 2,6-dichlorophenolindophenol blue dye as mg ascorbic acid/100ml juice according to the AOAC (1995). Five ml from the obtained juice were used to determine the titratable acidity as g citric acid /100 ml juice according to the method described by Chen and Mellenthin (1981). The total reducing sugars % was determined according to the method of Malik and Singh (1980). The reducing sugars were determined by the Nelson arsenate-molybdate colorimetric method (Dubois *et al.*, 1956)

The peroxidase activity in fruits pulp was estimated using the method of Thomas *et al.* (1981). According to Matsuno and Uritani (1972) and Carvalho *et al.*(2015),enzyme activity was reported as enzymatic units (U) and is defined as the amount of enzyme that produces a change in absorbance of 1.0/min under the assay conditions at 25°C. Extraction of pectin methyl esterase (PME) was as described by Lazan *et al.* (1989). Pectin esterase activity was assayed using a modification of the method of Lazan *et al.* (1995). The assay mixture (25.5 ml) comprising Ca 1% (w/v) pectin containing 2.5 m mol $CaCl_2$; and enzyme extract (0.5 ml) was titrated (25°C)with0.01M NaOH at pH7.5for 15 min.

Statistical analysis

The data obtained throughout this study were statistically analyzed by the analysis of variance as a randomized complete block design as explained by using the software package SAS 9.13 (Service Pack 4, 2002-2003). The means compared using the LSD test as differences at *P = 0.05* are considered significant.

2. Quantification of expressed gene levels

Leaves from the three cultivars and fresh fruits with aborted embryos from Ewais (nubbin) and normal fruits from all cultivars were collected after 15 days of fruit set in 2016 to use as target samples. Then after 30 days of fruit set normal fruits and leaves were collected from all cultivars for calibrating the gene expression.

A. Real-time quantitative RT-qPCR

Nucleic acid extraction protocol modified from Llanes *et al*. (2002) was applied. Gene expression using Real-time qPCR was used to detect the relative amounts of mRNA of four genes; the selected ESTs were AGAMOUS protein (MaAGA), MADS box protein (MaMADS), AP2D domain class transcription factor (MaAP2) and ethylene response factor (MaEFR). Results were normalized to gene *Actin* were designed based on the sequence of Actin from mango asreference gene according to He *et al*. (2012). Primers are illustrated in Table 1.

One step Real-time RT-qPCR analysis using 2x SensiFAST SYBR® One-Step Kit master mix (BIOLINE) according to the manufacturer's instructions. The amplification program proceeded at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec; annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec. The reaction was performed using a Rotor-Gene 6000 (QIAGEN, ABI System, USA).Data analysis using relative expression ratio was accurately quantified and calculated as described by Livak and Schmittgen (2001).

B. Total protein biochemical (SDS-PAGE) markers

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) was carried out using the discontinuous buffer system as described by Sambrook *et al*. (1989). After the run was completed, the gel was stained as described by Hames and Rickwood (1990).

Table 1. Sequences of primers for Real-time quantitative PCR (RT-qPCR).

Primers	Accession No.	Nucleotide sequence 5' to 3` (F=Forward, R=Reverse)	Reference
MaAGA	JK513220	F:AGAGATGGGGAGAGGAAAAATT R:CGGCTGGAGAAGACAATGAG	He *et al.* (2012)
MaMADS	JK513593	F:GGGGGTTTCCTGTTCTCTCTT R:CACCATGGCTACACCTTTGG	
MaAP2	JK513603	F:GGGCTCTCTCTTTGCTTTCTCT R:CCGCTGCCTTATTCCTCTGTA	
MaERF	JK513364	F:GCATACGTGGAGATAAAGCCA R:CCATAAATCAGCCGAGTAACAGT	

RESULTS AND DISCUSSION

1. Trees productivity

A. Fruit physical characteristics

The data presented in Table 2 cleared that Zebda cultivar had significantly higher yield per tree than the other cultivars, while Ewais (nubbin) cultivar had lowest yield in both seasons. Additionally Sedeq cultivar had significantly heavier fruit, pulp,stone and peel weight than the other cultivars in both seasons. Meanwhile, Ewais (nubbin) cultivar had lowest values in both seasons. In addition, Ewais (nubbin) recorded

the highest significant pulp/ stone ratio as compared to the other cultivars, while Zebda had the lowest total pulp/ stone ratio. In both seasons, Sedeq cultivar had significantly longer fruits length than the other cultivars in both seasons, while Ewais (nubbin) cultivar had the lowest fruit length and width. No significant difference was found between Zebda and Sedeq cultivars in fruit width in the first season, while Sedeq cultivar had higher fruit width than Zebda.

It was noted that the variations in fruit physical characteristics (normal or nubbin) and among the studied cultivars could be due to the variability in the genetic structures. The observed data concurred with those mentioned by Shaban and Ibrahim (2009) whereas, trees of all evaluated cultivars produced higher significant yield of normal fruit than nubbin fruit. Also, Shaban (2005), Boshra (2009), El-Khawaga and Maklad (2013) and Elsheshetawy *et al.*(2016), who measured the physical properties of six cultivars from Sharkia and Ismailia region and Ibrahim *et al.* (2016), who studied genetic diversity on some polyembryonic& monoembryonic mango genotypes using morphological and molecular markers**.**

B. Fruit chemical characteristics

The results in Table 3 indicated that nubbin and normal Ewais had significantly higher juice reducing and total sugars and TSS percentage TSS / acidity ratio as compared to the other cultivars in both seasons, while the remained cultivars had the lowest ones. In addition, Zebda and Sedeq cultivars had significantly higher juice acidity as compared to the other cultivars in both seasons. Sedeq cultivar had significantly higher juice vitamin C, while Ewais (nubbin) cultivar the lowest ones. Zebda cultivar had the significantly higher peroxidase activity (POD) than the other cultivars, while Ewais (nubbin) cultivar the lowest one. On contrary, Ewais (nubbin) cultivar had the significantly higher pectin methyl esterase (PME) than the other cultivars, while Zebda had cultivar the lowest one.

These outcomes were in harmony with those of Hamdard *et al.* (2004) and Anjum *et al.* (2014), who evaluated chemical contents of eight mango cultivars. It was clearly obtained that the variations in the chemical characteristics like total sugars, reducing sugars, TSS, acidity, the ratio between TSS / acidity, vitamin C, peroxidase activity and pectin methyl esterase of fruit (normal or nubbin) and among the studied cultivars could be due to the variability in the genetic structures.

Table 2. Yield and fruit physical characteristics of three mango cultivars Ewais (normal and nubbin), Sedeq and Zebda during 2015 and 2016 seasons.

Cultivars (accessions)	Yield (Kg/ tree)		Fruit weight(g)		Pulp weight (g)		Stone weight (g)		Peel weight (g)		Pulp/ Stone ratio		Fruit length (cm)		Fruit width (cm)	
	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016
Ewais (Normal)	61.62	56.64	251.58	247.40	184.02	182.50	34.78	32.58	33.68	32.54	5.34	5.64	10.76	10.60	6.00	5.82
*Ewais (Nubbin)	2.73	2.78	78.42	76.92	55.84	55.50	5.68	5.30	16.92	16.14	12.68	10.60	6.30	6.50	4.42	4.48
Sedeq	51.18	54.40	534.94	537.66	414.38	421.78	54.24	52.94	66.36	62.92	7.78	8.00	15.58	15.68	7.94	8.10
Zebda	89.70	83.86	380.20	377.06	268.02	273.24	51.32	49.18	60.84	54.62	5.30	5.60	12.72	12.28	8.02	7.36
LSD 0.05	6.11	4.77	22.26	19.52	21.319	19.32	3.57	2.34	4.86	3.29	4.28	0.82	0.73	0.72	0.39	0.21

*: Normal fruits of Ewais (Nubbin) was excluded from the yield

Table 3. Fruit chemical characteristics of three mango cultivars Ewais (normal and nubbin), Sedeq and Zebda during 2015 and 2016 seasons.

Cultivars (accessions)	Total sugars (%)		Reducing sugars (%)		TSS (%)		Acidity (%)		TSS/acidity ratio		Vitamin C (mg/100 ml juice)		POD (U/gm)		PME (U/ ml)	
	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016	2015	2016
Ewais (Normal)	11.94	13.46	6.66	7.10	19.00	20.20	0.69	0.60	28.36	35.68	58.70	62.20	1.62	1.61	133.72	133.22
Ewais (Nubbin)	12.58	13.90	7.36	7.06	20.50	22.00	0.60	0.52	34.66	43.60	39.96	45.54	0.66	0.64	158.46	157.66
Sedeq	9.92	9.40	5.02	5.32	15.80	15.00	0.75	0.64	21.32	23.78	68.60	67.48	2.78	2.77	99.32	98.52
Zebda	9.78	10.10	5.14	5.14	14.18	15.50	0.77	0.74	18.54	21.34	57.56	59.66	3.48	3.42	79.22	77.80
LSD 0.05	1.19	0.88	0.63	0.90	2.10	2.21	0.13	0.16	6.67	9.38	5.11	6.31	0.05	0.04	3.225	3.51

2. Quantification of expressed gene levels
A. Real-time quantitative PCR (RT-qPCR)

The data representing the relative expression ratio was accurately quantified as in Figures (1-4) and calculated according to the algorithm $2^{(-\Delta\Delta Ct)}$ and outcomes are listed in Table (4).The results explained that Zebda and Sedeq cultivars, which had normal fruits had the highest ratio (fold) of AGAMOUS (MaAGA), MADS box (MaMADS), AP2 domain (MaAP2) and ethylene response factor (MaERF) protein than the other cultivar (Ewais), which had a nubbin fruits (aborted) in both of embryos and leaves tissues.

Table 4. Gene expression of mango embryos from Zebda, Sedeq and Ewais (normal and nubbin) cultivars and leaves using RT-qPCR.

Target Genes	Cultivars	Embryos 2^(-ΔΔct) (fold)	Leaves 2^(-ΔΔct) (fold)
MaAGA	Ewais	0.03	0.71
	Sedeq	1.43	5.76
	Zebda	1.82	22.5
MaMADS	Ewais	0.03	0.42
	Sedeq	1.09	1.92
	Zebda	2.04	20.5
MaAP2	Ewais	0.04	0.04
	Sedeq	1.28	0.48
	Zebda	2.55	0.63
MaERF	Ewais	0.04	1.50
	Sedeq	1.42	2.10
	Zebda	2.43	9.51

These findings referred to increasing in gene expression of AGAMOUS in the cultivars, which had normal fruit than the aborted one and illuminated the role of AGAMOUS in fruit development and floral organ identity which controlled by diverse families of homeotic transcription factors, the floral homeotic gene AGAMOUS (AG) is a group C gene and it encodes a MADS box transcription factor as explained by Yanofsky *et al.*(1990). These findings pointed to expanding in gene expression of MADS box in the cultivars, which had normal fruit than the aborted one and represented the role of MADS box in fruit as master regulators of flowering transition and development of flowers, fruits, and seeds as recorded by Ma *et al.* (2011) and Hemming and Trevaskis (2011). Also, Genome-wide studies suggest that most *MADS*-box genes are expressed in a variety of organs and cell types at different developmental stages as reported by Rijpkema *et al.* (2007). Loss-of-function mutations in multiple *MADS*-box genes can convert floral organs to leaf-like structures, and ectopic expression of *MADS*-box genes can convert leaves into floral organs, illustrating the critical role *MADS*-box genes play during floral development as mentioned by Trevaskis *et al.*(2007).

These outputs referred to the increasing in gene expression of AP2D domain class transcription factor (MaAP2) in the cultivars, which had normal fruit than the aborted one and represented the role of AP2D domain transcription factor in fruit development and ripening. However, the deduced amino acid sequences of ERFs comprise a conserved DNA-binding ERF/AP2 domain (ranging from 58 to 59 amino acids), which is characteristic of the plant ERF gene family as described by Liu *et al.* (2010). The 17 ERFs share a highly conserved ERF/AP2 domain with ERF proteins of other plants, proving that the 17 ERFsencode the ERF family TF in the mango fruit, and the cDNA sequences obtained from the RNA-seq are credible according to Hoang *et al.* (2015).

These outputs referred to the increasing in gene expression of ethylene response factor (MaEFR) in the cultivars which had normal fruit than the aborted one

and represented the role of ethylene response factor (MaEFR) in fruit development and ripening. Moreover, Aharoni and O'Connell (2002) isolated a plant specific transcription activation factor associated with ethylene and abscisic acid response (ERF) from strawberry and suggested its involvement in late achene development. High expression of this gene suggested that it is one of the key transcription factors involved in multitude of activities. Also, ethylene response factor (MaERF) may play an important role in cell resistance to infections and degradation as illuminated by Hoang *et al.* (2015).

B. Total protein biochemical (SDS-PAGE) markers

1. Embryos

The SDS-PAGE 15% image of total protein electrophoresis in embryos for the studied mango cultivars were marked and figured in Figure (5-A).The results showed that each cultivar had several numbers of bands were arranged in a descending order: 8, 7, 5 and 3 bands for Zebda, Sedeq, Ewais (normal) and Ewais (nubbin) cultivars, respectively. These results defined that the highest numerous and intensity bands were (8 bands) in Zebda cultivar followed by (7 and 5 bands) in Sedeq and Ewais (normal), respectively. While the lowest multiples were (3 bands) in Ewais (nubbin) cultivar. The bands were with an approximate molecular mass ranged from (28-kDa to 74-KDa) were obtained in the elution fractions of protein from embryos.

2. Leaves

The SDS-PAGE 15% image of total protein electrophoresis in leaves for the studied mango cultivars were marked and figured in Figure (5-B). The results showed that each cultivar had several numbers of bands arranged in a descending order: 6, 5 and 4 bands for Zebda, Sedeq and Ewais cultivars, respectively. These results defined that the highest numerous and strengthens bands were (6 bands) in Zebda cultivar followed by (5 bands) in Sedeq. While lowest multiples were (4 bands) in Ewais cultivar. The bands with an approximate molecular mass ranging from (27-kDa to73-KDa) were obtained in the elution fractions of protein from leaves.

The results explained that the Zebda and Sedeq cultivars which had normal fruits had the highest several bands than Ewais cultivar which had nubbin (aborted) fruits, these were supported to the above described results where, there were high genes expression for all the experimented ESTs primers, especially in cultivars which had the normal fruits and that is reflected on the protein electrophoresis with more bands essentially in normal fruits.

These results agreed with other investigator as He *et al.* (2012) who evaluated the gene expression using subtractive suppression hybridization to investigate the differentially expressed genes involved in embryo abortion of mango fruit cv. 'Jinhuang'. Seven candidate genes from different categories were selected for semi-quantitative PCR analysis, and their possible functions in embryo abortion. They found that many transcriptionally regulated genes differentially accumulated in 2 types of mango embryo. However, 4 ESTs (*MaAGA, MaAP2, MaERF, and MaMADS*) were upregulated in normal embryo tissues compared to aborted embryos.
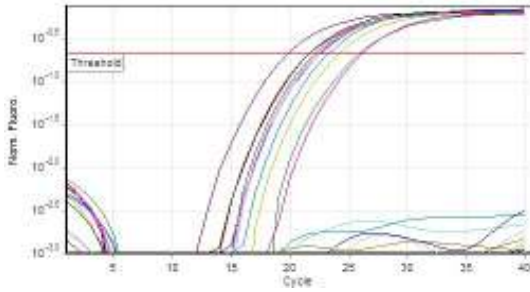
Figure 1. Amplification plot of fluorescent signal versus cycle number measured during quantitative amplification of AGAMOUS protein (MaAGA) by RT-qPCR.
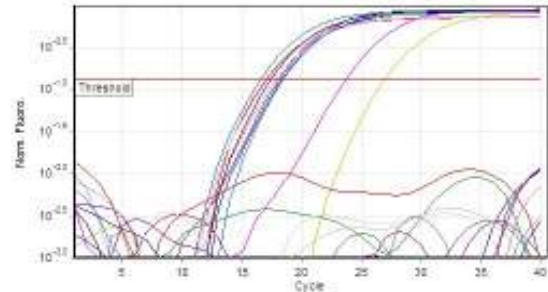

Figure 2. Amplification plot of fluorescent signal versus cycle number measured during quantitative amplification of MADS box protein (MaMADS) by RT-qPCR.
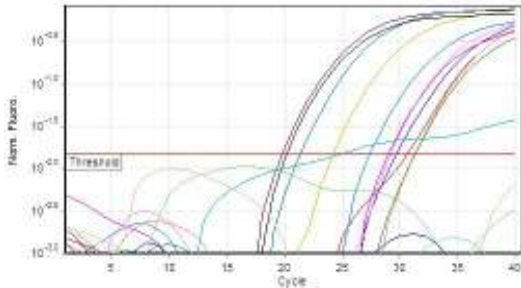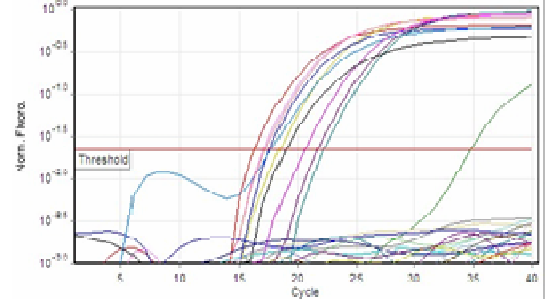

Figure 3. Amplification plot of fluorescent signal versus cycle number measured during quantitative amplification of AP2D domain class transcription factor protein (MaAP2D) by RT-qPCR.

Figure 4. Amplification plot of fluorescent signal versus cycle number measured during quantitative amplification of ERF ethylene response factor protein (MaERF) by RT-qPCR.
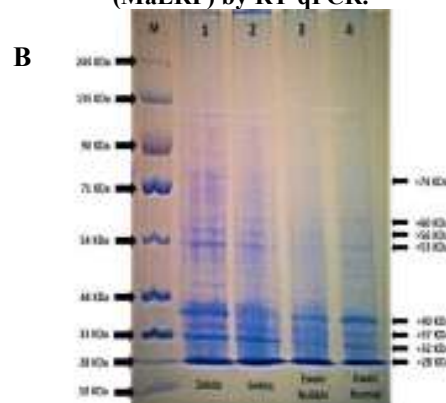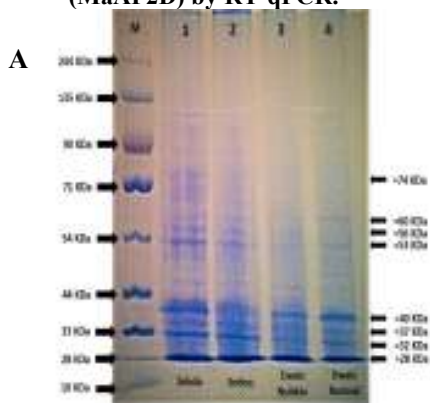

Figure (5 A&B). SDS-PAGE of (A) total protein of mango embryos Zebda , Sedeq and Ewais (normal and nubbin) cultivars, (B) total protein of leaves from Zebda, Sedeq and Ewais (normal and nubbin) cultivars. The bands were calibrated with molecular marker ranging from 10 to 244 kDa.

CONCLUSION

This study suggests that relative quantification data provide basic ESTs and a step forward in our understanding of gene function and regulatory pathways belonged to embryo abortion in some mango cultivars.

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دراسة على إنتاجية الثمار الطبيعية والفص لبعض أشجار المانجو وعلاقتها بمستويات التعبير الجينى كميا

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أجريت الدراسة الحالية خلال موسمي ٢٠١٥ و ٢٠١٦ على ثلاثة أصناف مانجو مصرية هى عويس (طبيعى، فص) وصديق وزبدة مزروعة في بستان خاص بمنطقة إدكو، محافظة البحيرة. الأشجار تم إختيارها لدراسة إنتاجية بعض أشجار المانجو لثمار طبيعية و فص (مجهضة الجنين) و أيضاً التقدير الكمي لمستويات التعبير الجيني لفهم أفضل لظاهرة إجهاض الجنين في المانجو. إستخدمت تقنية الوقت المحدد كمياً لتفاعل البلمرة المتسلسل (RT-qPCR) لتتبع الكميات النسبية للحمض النووي الريبوزي الرسول (mRNA) لأربعة جينات مختلفة.كان المحصول والخصائص الفيزيائية للثمار الفص مثل طول الثمرة، عرضها، وزنها، وزن اللب، وزن البذرة، وزن القشرة أقل معنوياً من الثمار الطبيعية ، بينما كانت نسبة لب الثمرة / البذرة الأعلى معنوياً في كلا الموسمين. الصفات الكيميائية للثمار الفص مثل (المواد الصلبة الذائبة الكلية، المواد الصلبة الذائبة الكلية / الحموضة ،السكريات المختزلة ، السكريات الكلية و إنزيم البكتين مثيل إيستريز) كانت الأعلى معنوياً من الثمار الطبيعية، بينما كانت الأقل معنوياً في قيم (الحموضة، فيتامين سي و إنزيم البيروكسيديز)وقد أظهرت النتائج أن الجينات الخاصة بالأربعة بوادئ المختارةMaERF,MAAP2 ,MaMADS ,MaAGA كانت مختلفة في التعبير الجيني في أنسجة الأجنة (الطبيعية ، المجهضة) و الأوراق . علاوة على ذلك أظهرت الجينات الخاصة بالأربعة بوادئ أعلى تعبيراً جينيا في الثمار الطبيعية عن الثمار الفص لصنف العويس، وقد أظهرت أيضاً مستويات التعبير الجيني في أنسجة الأوراق صورة عالية للتعبير الجينى في صنفي الصديق والزبدة مقارنة بصنف العويس. كذلك أوضحت نتائج تحليل البروتين الكلي بالهجرة الكهربائية لفصل حزم الأوزان الجزيئية للبروتينات المختلفة أن صنفي الزبدة و الصديق اللذان لديهما ثمار طبيعية كانت الأعلى في عدد الحزم المفصولة أكثر من صنف العويس الذي لديه ثمار فص وذلك في أنسجة الأجنة (الطبيعية ، الفص) والأوراق.