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ROLE OF HEAT SHOCK IN BUILDING UP PROTEINS *HSP70, HSP90*, INCREASING ANTHOCYANINS CONTENT IN CALLUS OF STRAWBERRY *FRAGARIA ANANASSA* DUCH

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ABSTRACT

This study was conducted in the genetic applications laboratories of University of Mosul, College of Education for Pure Sciences. Heat shock proteins HSPs were constructed for increase of withstand long-term physical shocks, and production of resistant to heat strawberry plants, The results of the study showed the production of callus cultures after two months of cultivation on Murashige and Skoog (MS) medium + 1.0, 2.0, 3.0 and 4.0 mg/L of 2, 4 – Dichlorophenxy Acetic Acid (2,4-D) + 6 Benzyl adenine (BA) (1.0 mg/L). Also, a-Naphthalene Acetic Acid (NAA) was used in the same previous concentrations with BA, and the medium MS + 4.0 mg/L of 2 ,4-D + 1.0 mg/L of BA outperformed the rest of the concentrations in inducing callus, after 16 days of culture. The callus was yellow-brown color. One-month-old callus samples were exposed to short term heat shock represented by 10 minutes, and the long term which is 20 minutes at temperatures of 35, 40, 45 and 50°C. The result indicates an increasing of fresh weights, protein, and anthocyanin. The results concluded that the 45°C for 30 min shock encouraged also protein content at the 30-day age stage, while anthocyanin pigment decreased in the same period. The current study succeeded in detecting the expression of heat shock genes *HSP70* and *HSP90* using RT- PCR, the results demonstrated an increase in gene activity with an increase in the severity of heat shock when callus cultures were exposed to the shock 45°C / 30 min.

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INTRODUCTION

*Fragaria ananas*sa Duch is a perennial herbaceous fruit plant, fruits are distinguished by their distinctive taste and antioxidant capacity resulting from high levels of anthocyanins therapeutic and nutritional properties (Husaini and Neri, 2016). The reason for the increase in global consumption of the very high fruit of strawberry is because of its anti-bacterial, anti-fungal and it contains compounds such as carotenoids, vitamins, phenols, flavonoids, and glutathione, as it is believed to reduce the incidence of cancer diseases, and it also resulted in the increased release of many secondary metabolites from this plant (Fierascu *et al*, 2020).

Strawberry cultivation has spread in a wide range geographical regions in the world, extending from tropical and subtropical regions to cold regions, making it adaptable to a wide range of climatic conditions, as it cultivated in 80 countries (FAO,

2016). There are many studies that confirm the role of heat shock proteins in the treatment of some diseases that affect strawberry, (Turichek and Peres, 2009) Exposing plant to 44°C for 4 hours or 48°C. for two hours resulted in formation of special proteins known as heat shock proteins (HSPs). These proteins are found in the cytosol, chloroplasts, and mitochondria, which protect the plant during stress (Wang *et al*., 2004). Interestingly there are five groups of these proteins: *HSP100s, HSP90s, HSP70s, HSP60s*, and small heat shock proteins (sHSPs) (Krish, 2003). Exposure of callus to heat shock led to change in gene expression. moreover, the treated callus become more tolerant to heat shock treatment many studies reported the encouragement the synthesis of a new type of proteins called heat shock proteins. Additionally, these proteins support the cells ability to resist the effect of heat shock. Heat shock proteins (HSPs) function as molecular chaperones, successfully involved in the quality and type control of protein within living cells to maintain cellular homeostasis under environmental stress condition (waters, 2013) Heat shock of plant cells contributed to improving the plant species through changes in the fluidity of cell membranes and the accompanying changes at the molecular level. The expression of genes related to the regulation of the transcription process and synthesis of various proteins (Berz *et al*.,2019). Also increase the biomass of callus and chlorophyll in the chickpea plant (Al-Taee and Rasheed, 2022). And enhanced nucleic acide, proteins content in stem callus of *Sesamum indicum* L (AL-Taee *et al*., 2013).

MATERIALS AND METHODS

Production of sterile seedlings

Seeds of the Albion variety of *Fragaria anana*ssa Duch obtained from the local markets were sterilized by immersing them in 70% ethyl alcohol for two minutes, followed by immersing in 6% sodium hypochlorate solution (NaOCl 6%) for 10 minutes. Then seeds were washed with sterile distilled water three times at a rate of two minutes. (Abdullah *et al*., 2023; Pavalovic *et a*l., 2010), Sterilized seeds were dried by placing them on sterile filter paper and they were planted on the surface of agar solidified MSO (Murashige and Skoog,1962) medium in glass bottles and incubated in the growth room condition (8.h light /16.h dark, intensity 2500 lux, 25±2 ° C).

Callus induction

Leaves explant of 2 cm^2 and stems fragment of 2 cm length were prepared and separated from sterilized Strawberry seedlings at two months old and placed in 100 ml glass bottles containing 30 ml solid MS medium containing 2,4-Dichlorophenoxyacetic acid(2,4-D) and benzyl adenine (BA) as below.

MSO Control

MS + 1.0 mg L^{-1} 2,4-D +1.0 mgL^{-1} BA

MS + 2.0 mg L^{-1} 2,4-D +1.0 mg L^{-1} BA

MS + 3.0 mg L^{-1} 2,4-D +1.0 mg L^{-1} BA

MS + 4.0 mg L^{-1} 2,4-D +1.0 mg L^{-1} BA

Also, another set of induction media were tested as below.

MSO Control
MS + 1.0 mg L^{-1} (NAA) +1.0 mg L^{-1} BA
MS + 2.0 mg L^{-1} NAA +1.0 mg L^{-1} BA
MS + 3.0 mg L^{-1} NAA +1.0 mg L^{-1} BA
MS + 4.0 mg L^{-1} NAA +1.0 mg L^{-1} BA

(Sirijan *et al*, 2019)

Exposing callus to heat shock (short and long term)

Callus samples (one gram of leaf callus) were taken in empty, sterile glass bottles 30 ml with a tight lid. Their bases were immersed in a water bath and the callus samples were exposed to different temperatures 35, 40, 45, 50°C for 10 minutes, this represents the short-term shock. Another group of calluses were subjected to the same previous temperatures for 20 minutes which represent the long-term heat shock (Hong, *et al*., 2003). The exposed callus samples and the control (unexposed) samples were grown on MS induction medium + 4.0 mgL^{-1} 2,4-D + 1.0 mgL^{-1}. BA and incubated in growth room under same condition mentioned previously Data were recorded after 15 and 30 days.

Determination of total proteins

One gram of each heat shocked callus samples was taken, crushed in prior cooled pastel and mortal contain 5.0 ml of 5% Trichloroacetic acid (TCA) Determination of total protein was detected according to the standard method (Lowry *et al*., 1951).

Detection of the expression of heat shock protein *HSP70* and *HSP90* genes in callus exposed to shock

One gram of leaf callus was subjected to heat shock at 45°C for 30 minutes with the aim of stimulating heat shock protein genes. Results were taken on 30 days after exposure. DNA was isolated from exposing callus according the manufacturer instructions. The extracted genomic DNA was stored at -20°C for the downstream applications (Muhammad *et al*., 2022). The reverse transcription PCR test was used to determine the complementary DNA strand (cDNA) by adopting mRNA as the genetic material. Isolated from callus, the cDNA reverse amplification product serves as a basis for a second step in determining the number of copies of the reporter DNA resulting from the expression of the target gene (Larion *et., al,* 2005).

Real-Time-PCR

To quantitatively test the level of gene expression, the primers specific for the heat shock genes *HSP70* and *HSP90* were used, along with the primers for the housekeeping genes The forward and reverse primers for the genes were designed based on the sequences of the nitrogenous bases. The complete coding sequence was obtained from the gene bank database at the National Center for Biotechnology Information. (Ibrahim *et al*., 2023; ALshatty and ALkero, 2024), Table (1).

How to calculate gene expression rate

Calculation of the gene expression rate of the long-term heat shock genes *HSP70* and *HSP90* was calculated based on the CT value of the aforementioned genes with the standard housekeeping gene for both control and exposed samples using the following formula (Haimes *et al*., 2013).

1- ΔCT control=CT target-CT control

CT control represents the number of mRNA cycles for the standard gene
CT target represents the number of mRNA cycles for HSP70 and HSP90 genes.

2- Equation of ΔCT for the exposed sample relative to ΔCT for the control sample using the following law
ΔΔCT=ΔCT target-ΔCT control

3- The gene expression value is calculated according to the formula below:
Gene Expression folding = $2^{\Delta\Delta CT}$

Table (1): Specialized primers for the *HSP70* and *HSP90* genes and primers for the housekeeping genes used in RT-PCR reactions.

Gene	primer	primer sequence
HSP70	F	AATCTCTTGGTAGTTTCCGTCT
HSP70	R	TGGCATCTCTCTTCTCCTTGT
HSP90	F	CTTCATCCACATTATCCCCGAC
HSP90	R	GAAACCAACACCAAACTGACCA
Housekeeping	F	GACTGGAATGGTTAAGGCTGGA
Housekeeping	R	GATTCCGTGTTCGATGGGT

Housekeeping gene: Beta.H.K gene

RESULTS AND DISCUSSION

Producing axenic seedlings

The sterilized seeds began their germination after eleventh day of their cultivation on agar solidified MSO medium in conditions of complete darkness. Then transferred to photoperiod conditions of 16 hours light/8 hours darkness. The germination rate was 70%, Their heights reached 3-4 cm. Stems were bearing several subcotyledonous leaves.

Induction of callus from leaves and stem explants

Data obtained Table (2) indicate the ability of leaves explant to induce callus on MS medium supplemented with 1.0 mg L^{-1} BA and different levels of 2,4-D, Induction rate was 90% in MS medium contained 1.0 mgL^{-1} of BA + 4.0 mgL^{-1} of 2,4-D.

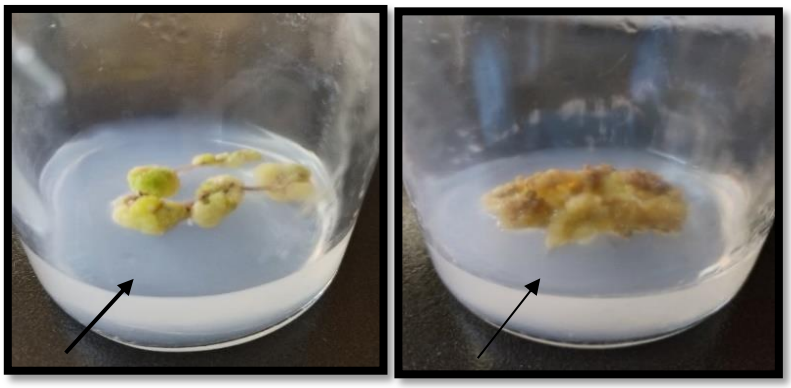

Figure (1): callus Induction from leaf and stem explants of strawberry *Fragaria ananassa* seedling (A) stem callus 8 weeks old (B) leaf callus 8 weeks old

The leaves began to form callus 16 days after clatur, and their callus was characterized by a brittle consistency and a yellow-brown color Figure (1-A) while stem explant showed a weak response in callus induction. The first signs of induction

developed after 28 days, their callus was characterized of compact texture and yellow in color Figure (1-B).

Table (2): Callus induction from stems and leaves explant of *Fragaria anana*ssa Duch grown on solid MS medium supplemented with 1.0 mgl^{-1} BA and different concentrations of 2,4-D

Media	Explant*	number of induced explants	Induction period (Day)	Induction %
MSO (Control)	Stem	0	0	0.0
	Leaf	0	0	0.0
1.0 2,4-D	Stem	28	30	56
	Leaf	35	20	70
2.0 2,4-D	Stem	32	26	64
	Leaf	40	18	80
3.0 2,4-D	Stem	38	24	76
	Leaf	44	18	88
4.0 2,4-D	Stem	40	22	80
	Leaf	45	16	90

* No. of cultured explants in each treatment was 50

Data Table (3) indicated the ability of hypocotyl leaves to induce callus when grown on agar solidified MS medium supplemented with different concentrations of BA and NAA, the medium MS + 1.0 mg L^{-1} of BA + 4.0 mgL^{-1} of NAA was significantly superior to the rest media. The induction rate recorded 80%, The leaves explant began to form callus 22 days after planting Table (3). The callus was yellow-brown color and brittle texture, while stems of explants required more time to induce callus at the same medium and the callus was corn pact texture and yellow color.

Table (3): Callus induction from stems and leaves explants of Fragaria ananassa Duch grown on solid MS medium supplemented with 1.0mg of BA and various concentrations of NAA.

Media	Explant *	responded number	Induction period (Day)	Induction Rat %
MSO (Control)	Stem	0	0	0.0
	Leaf	0	0	0.0
1.0 NAA	Stem	30	36	60
	Leaf	33	24	66
2.0 NAA	Stem	30	33	60
	Leaf	35	25	70
3.0 NAA	Stem	35	30	70
	Leaf	39	24	78
4.0 NAA	Stem	37	29	74
	Leaf	40	22	80

* No. of cultured explants in each treatment was 50

The superiority of leaves explants for callus induction may be due to many genetic or non-genetic influences, such as the type of explant, its age, as well as its internal content of plant hormones (Feher, 2019). In addition, adding 2,4-D and NAA

to MS nutrient medium with graded concentrations of the cytokinin's BA had a prominent role in the induction of callus from leaves and stems explant of strawberry (Aziz *et al*., 2015). The combination of cytokinin (BA) and auxin (2,4 -D) and (NAA) produced better results compared to the use of auxin alone. The combination of 1 mg/l 2 ,4-D and 0.5 mg/l BA produced maximum callogenesis percentage 100% and minimum days for callus induction (Chom boon and Lohasupthawee ,2022) Cytokinin play an important role in increasing the construction of RNA, proteins, and enzymes inside the cell and thus encouraging the process of cell division. The reason may attribute to the structural nature of BA, which linked to its side chain by three double bonds, which encouraged its superiority in activity over other growth regulators. The side bonds increase the effectiveness and activity of the compound in cell division, expansion in size, and differentiation, Growth and development accelerate the effect of the most important cytokinin required in propagation process of many plant species (George *et al* , 2008) .The results of the current study are consistent with what many investigation have indicated the induction of callus from leaves and stems explant of strawberry in MS medium supplemented with graded concentrations of... BA, 2,4-D and NAA separately (Sirijan *et al* ,2019;Chung and Ouyang,2021; Akter et al , 2024 ; Banerjee *et al*, 2023 ; Irshad *et al*, 2023) .

The role of exposing callus to short- and long-term heat shock on the fresh weights of callus anthocyanin level and protein content

The results achieved Table (4) pointed out that there is an increase in the fresh weights of callus at 15- and 30-days post treatment, respectively. In other words, the increase of the callus mass is directly proportional to the time period when growing on the induction medium MS + 1.0 mgL^{-1} BA + 4.0 mgL^{-1} 2,4-D and showed that the temperature 45 °C led to an increase of callus mass over the rest of the treatments in the short-term heat shock, with an increase in the concentration of anthocyanin pigment at the 15-day old and decrease at the 30-day old, while the protein content increase was slight at the two age Table (4).

Table (4): The role of exposing callus of *Fragaria ananassa* Duch leaves to short-term (10 minutes) heat shock on its fresh weights, protein content, and anthocyanin levels at two different age stages.

	15 (Days)			30 (Days)		
Temperature (Cº)	Fresh weight (gm)	Proteins (mg/g)	Anthocyanins (mg/g)	Fresh weight (gm)	Proteins (mg/g)	Anthocyanins (mg/g)
35/10	1.20±0.40	1.25±0.42	2.44±0.35	1.60±0.61	1.70±0.50	1.40±0.20
40/10	1.35±0.36	1.30±0.44	3.25±0.36	1.85±0.20	1.84±0.32	1.30±0.53
45/10	1.70±0.50	1.55±0.16	4.37±0.44	2.60±0.62	2.30±0.26	1.10±0.26
50/10	1.29±0.40	1.33±0.35	4.68±0.10	1.75±0.26	1.42±0.20	0.90±0.53
Control	1.40±0.44	1.40±0.44	2.40±0.36	1.90±0.32	1.50±0.62	2.03±0.29

The results of Table (5) showed an increase in the fresh weight of callus in the two age stages, specifically at 45 °C. Whereas callus exposure to high heat shock (50Cº/20min) led to the cessation of growth subsequently decreased callus fresh. The data from the same table confirms a slight increase in the level of proteins in the callus

exposed to shock at levels of 35Cº/40/45, when the limits of these levels were exceeded, the protein content of callus began to decrease, as did the anthocyanin pigment. In general, all types of heat shock outperformed the comparison.

Table (5): Role of exposing leaf callus of *Fragaria ananassa* Duch to long-term (20 minutes) heat shock on callus fresh weights, protein content and anthocyanin levels at two age stages.

	15 (Days)			30 (Days)		
Temperature (Cº)	fresh weight (gm)	Proteins (mg/g)	Anthocyanins (mg/g)	fresh weight (gm)	Proteins (mg/g)	Anthocyanins (mg/g)
35/20	1.50±0.62	1.50±0.50	2.60±0.62	1.60±0.61	1.85±0.20	3.73±0.44
40/20	1.60±0.20	1.65±0.20	3.80±0.62	1.85±0.20	1.99±0.50	3.21±0.36
45/20	1.84±0.31	2.00±0.62	4.20±0.62	2.20±0.20	2.45±0.35	2.95±0.50
50/20	1.50±0.61	1.67±0.70	4.90±0.62	1.90±0.44	1.60±0.44	2.24±0.53
control	1.40±0.78	1.50±0.44	3.00±0.87	1.85±0.20	1.50±0.56	2.03±0.29

Exposing callus to each short- and long-term heat shock increase the fresh weight, protein content, and anthocyanin pigment, this is what many researchers have concluded (AL-Taee, 2013). The increase in fresh weight and protein in callus as exposed to short and long heat shock, resulted in an increase in the production of heat shock proteins *HSP70* and *HSP90*. Cells respond to stresses by increasing their genetic expressions that encode for the construction of these proteins due to their stimulation of heat shock factors (HSFs), which are known for their prominent role in activating the *HSP70* and *HSP90* gene group. (Al-Taee and Rasheed, 2022). One of the indicators that explain the construction of heat shock proteins (HSPs) is that they stimulated gene expression activity by activating a group of heat shock protein genes, especially at temperatures of 45°C, as they provided protection for the callus from damage, restored its damaged cells, and protected the structure of the newly created proteins from damage (Liu *et al*., 2019). Exposing callus to a temperature of 50 degrees resulted in a decrease in the rates of fresh weights, protein content, and anthocyanin pigment. The reason may be due to exceeding the optimum level for enzyme activities and changing the three- and four-dimensional structure of the protein, therefore cells weren't divided, this will reflect on both low fresh weights and expression *HSP* genes. A study (Brown *et al*. 2016) indicated that when exposing the shellac to a temperature of 48°C, it leads to the lack of formation of heat shock proteins, which leads to the death of the plant.

Activity of *(HSP70*) and (*HSP90*) genes in the callus

Molecular biology data confirmed gene expression in callus exposed to shock. The heat shock genes *HSP70* and *HSP90*, responsible for activating the heat shock genes, were selected as evidenced by RT-PCR tests the revealed the activity of heat shock proteins. moreover, the results Table (6) confirmed an increase of gene expression heat shock *HSP70* and *HSP90* genes in callus exposed to shock compared with control callus.

Table (6): Number of CT cycles of reporter DNA mRNA for heat shock genes *HSP70* and *HSP90* and the standard gene Beta.H.K.gene

Genes	Treatment	(CT) number of cycles
HSP70	Not exposed	35.849
HSP70	Exposed	30.618
HSP90	Not exposed	35.668
HSP90	Exposed	32.480
Beta.H.K.gene	Not exposed	32.392
Beta.H.K.gene	Exposed	32.834

The obtained results Table (7) pointed out to an increase in the activity of *HSP70* gene in heat shock 45°C / 30 min in comparison with the rest of the treatments and control.

Table (7): levels of gene expression as a function of RT-PCR

CT (*HSP70*) – CT (Control)	-5.231
CT (*HSP90*) – CT (Control)	-3.188
CT (Beta.H.K) – CT (Control)	0.442
$\Delta\mathrm{CT}\ (HSP70) - \Delta\mathrm{CT}(\mathrm{control})$	-5.673
$\Delta\mathrm{CT}\ (HSP90) - \Delta\mathrm{CT}(\mathrm{control})$	-3.632
Gene Expreession *HSP70*	51.02
Gene Expreession *HSP90*	12.38

The symbol CT value and the gene expression level of the heat shock HSP*70* and *HSP90* genes and the control genes. An increase in gene expression of the heat shock genes compared to the control genes is observed as a result of the formation of heat shock proteins in the exposed callus in order to protect it from damage and restore its damaged cells to their physiological activity. This What other researcher confirmed (Al-Taee and Rasheed, 2022; Kesici *et al*, 2020).

CONCLUSIONS

The main conclusion of this investigation that heat shock stress increase callus fresh weigh. Also caused a beneficial increase of proteins and anthocyanin pigment level which is very important in industrial applications.

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CONFLICT OF INTEREST

The authors state that there are no conflicts of interest with the publication of this work.

دور الصدمة الحرارية في بناء بروتينات HSP70 و HSP90 والانثوسيانين في كالس نبات الفراولة *Fragaria ananassa* Duch

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الخلاصة

أجريت هذه الدراسة في مختبرات التطبيقات الوراثية التابعة لجامعة الموصل كلية التربية للعلوم الصرفة / قسم علوم الحياة. الهدف الرئيسي للدراسة هو تحفيز بناء بروتينات الصدمة الحرارية HSPs لزيادة تحمل الصدمات الفيزيائية طويلة المدى وإنتاج نباتات الفراولة المقاومة للحرارة ، وبينت نتائج الدراسة انتاج مزارع كالس الفراولة من قطع أوراقه بعد شهرين من زراعتها على الوسط الصلب(Murashige and Skoog) MS + 1.0 ، 2.0 ، 3.0 و 4.0 ملغم/لتر من (2 , 4 – Dichlorophenxy Acetic Acid) (2,4–D) +(6–Benzyl adenine) BA)(1.0 ملغم/لتر وكذلك استخدام (a–Naphthalene Acetic Acid) (NAA) بنفس التراكيز السابقة متداخلا مع BA ، واظهر الوسط MS + 4.0 ملغم/لتر من 2 ,4–D + 1.0 ملغم/لتر من BA تفوقا عن بقية التراكيز في استحثاث الكالس بعد 16 يوما من الزراعة ، واتصف الكالس بلونه الأصفر المائل الى الاسمرار ، عرضت عينات الكالس بعمر شهر للصدمة الحرارية قصيرة المدى 10 دقيقة والطويلة المدى 20 دقيقة بدرجات الحرارة 45،40،35 و50 م ° وأظهرت نتائج الكشف عن تأثيرها في الاوزان الرطبة والمحتوى البروتيني وصبغة الانثوسيانبن بعد 15 و 30 يوما" ، واستنتجت الدراسة ان الصدمة 45 م ° شجعت حصول زيادة ملحوظة في الاوزان الرطبة للكالس والمحتوى البروتيني في المرحلة العمرية 30 يوما بينما انخفضت صبغة الانثوسيانين في المدة نفسها. وأكدت الدراسة الحالية في الكشف عن تعبير جينات الصدمة الحرارية *HSP70* و *HSP90* باستخدام التفاعل التسلسلي لانزيم البلمرة RT–PCR الذي برهنت نتائجه زيادة نشاط الجين مع زيادة شدة الصدمة الحرارية عند تعريض مزارع الكالس للصدمة 45م° ولمدة 30 دقيقة.

الكلمات المفتاحية: الصدمة الحرارية، استحثاث الكالس، وسط MS, *HSP70 HSP90*، محتوى البروتين والانثوسيانين.

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