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Heat Shock Enhancement Salicylic Acid Biosynthesis in Callus of *Calendula officinalis* L.

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Abstract

Heat shock, short and long term, had a smooth positive influence to callus exposed to this shock. Fresh weights of non-heat shocked callus were approximately 2.0 gm. After treatment the first increase of weight recorded after 21 days and the second increase of weight registered at 42 days. The results indicate that both LT (Long term) and ST (Short term) heat shock enhanced salicylic acid biosynthesis. The content of salicylic acid was more than 15-folds compared with its content in the non-treated callus as HPLC measurements proved. This binary increase is an interesting medical value. This study revealed that heat shock, reflected positively on callus biological activity, proteins and biosynthesis of salicylic acid.

Keywords: Salicylic acid, heat shock, *Calendula officinalis* L., callus.

INTRODUCTION

Many reports mentioned that heat shock of plant tissue culture is well-characterized as stress condition induced by a rapid increase in temperature up to 10℃ or more above normal limit [20]. The sudden shift in temperature directs transcriptional and translational capacity away from the normal pattern of protein synthesis and formation of a small number of specific proteins referred to as heat shock protein HSP [22]. Other study pointed out the increase of protein building up in cucumber seedlings due to heat shock proteins [24]. Additionally, heat shock enhanced divisions of cell suspension and sustained callus primordia formation in sunflower, *Helianthus annuus* L. [28]. When the magnitude and duration of stress exceeds a threshold, cells are irreversibly damage or die [27]. In plant tissue culture, response for heat shock (HS) reflected at level of HSP induction, protein breakdown, modification protein localization and appearance of acquired thermo tolerance [21]. *Calendula officinalis* L. (Marigold) plants are used for ornamental, medicinal and culinary purposes [12], belong to the Asteraceae family [7]. Many chemotypes of this plant species are important in folk medicines [23]. The positive effects of thermal treatment on many plant species, and their metabolic processes enhancing by building up new proteins [17]. Salicylic acid (SA) present in many plants, including Marigold plant [8]. Salicylic acid (ortho-hydroxybenzoic acid) is a phenolic compound present as a free phenolic acid and as a conjugate form in plants. The best-known natural SA derivative is salicin (ß-glucoside salicylic alcohol), synthesized in the chloroplast in response to pathogen attack, then exported to the cytoplasm, where it establishes both local and (SAR) systemic-acquired resistance [3]. This compound has a remarkable role in response of plant to various stress factors [14]. The present manuscript aimed to assess the effect of heat shock in growth of callus and biosynthesis of salicylic acid in heat shocked callus.

MATERIALS AND METHODS

Establishment of Callus Culture

Sterilized leaf explants [13] were cultured on agar-solidified MS medium [25], provided with sucrose addition increased to 32 gL^{-1} and supplemented with 0.1 mgL^{-1} of BAP (6-Benzylaminopurine) for callus induction. Induced callus was subcultured on MS medium supplemented with 1.0 mgL^{-1} of NAA (Naphthaleneacetic acid) and 1.0 mgL^{-1} of BAP for growth [2].

Exposure of Callus to Heat Shock

The different degrees of temperature 35, 40, 45, 50, 55 and 60℃ were applied for two periods, 5 min. which represent the short-term heat shock (STHS) and 10 min. as long-term (LTHS) heat shock [15]. Young induced callus of two months old was used. Five samples of callus (1.0 gm each) were used for each treatment. These samples were each placed in sterile capped test tube stand on rack, then dipped in water bath exposed to the selected degree for 5 min. as STHS and other samples for 10 min. as LTHS. At the end of exposing all samples in test tubes, were removed and directly soaked in a glass beaker containing tap water. Heat shocked callus samples were cultured on agar-solidified MS + 1.0 mgL^{-1} NAA + 1.0 mgL^{-1} BAP medium and incubated at 25 $\pm$ 2℃ in culture room. They were subcultured every three weeks on the same MS medium mentioned above. Fresh weights of callus were measured and recorded.

Determination of Total Proteins

One gram of each heat shocked callus samples was taken, crushed in prior cooled pestle and mortar contain 5 ml of 5% Trichloroacetic acid (TCA). Determination of total protein was detected according to the standard method [19].

Salicylic acid Quantification

This stage was carried out in three steps to obtain free salicylic acid [3], as below.

Preparation of Samples

The standard stock solution was prepared by accurately weighing 100 mg of salicylic acid (BDH-England) dissolved in 100 mL of distilled water. Four hundreds mg of heat shocked callus was transferred to pre-cold pestle and mortar. Then calli tissue was grind in the presence of liquid nitrogen, callus powder was kept in 2.0 ml eppendrof tube, to the latter 1.6 ml of 70% ethanol was added. Specimens were vortexed for 1.0 min., centrifuged at 10000 x*g* for 10 min. at room temperature. Supernatant was

transferred to 15 ml centrifuge tubes. Again 1.6 ml of 90% methanol was added to the remaining pellet, re-vortexed for 1.0 min. for re-extraction. Mixture was centrifuged again under the same conditions. Similarly supernatant was added to the stock supernatant in 15 ml centrifuge tube, The pooled and clear supernatant solutions contain SA.

Extraction of free SA

Two ml samples of supernatants were each placed in 2.0 ml microcentrifuge tubes to evaporate EtOH and MeOH by air currents for 2.0 h. The remaining supernatants were transferred to eppendrof tubes (2.0 ml capacity) and concentrated up to approx. 600 µl by evaporating alcohol as mentioned above. To this aqueous solution 65 µl of 20% of aqueous TCA (w/v) were added to each solution. Then added 650 µl of ethyl acetate and cyclohexane 1:1 (v/v), vortexed for 30 sec., centrifuged for 2.0 min at 10,000 x*g* for phase separation. Transfer the upper organic phase to a new 2.0 ml eppendorf tube, re-extracted the aqueous phase again with 650 µl of ethyl acetate-cyclohexane mixture, centrifuged for 2.0 min. at 10,000 x*g* for phase separation. Evaporate the solvents to dryness for 30-45 min., solubilize the dry residue in 100 µl of 10% aqueous methanol (v/v) containing 0.1% aqueous trifluoracetic acid TFA (v/v), and vortex for 1.0 min.. The samples became ready to assessed by HPLC.

Detection & Quantification of SA by HPLC

Samples of 20 µl were injected in High-performance liquid chromatography (Sykman-2014-Germany). Separation conditions were carried out using C18 column (250 x 4.6 mm, 5 µm) at 30 °C and flow rate 1.0 ml/min. of aqueous MeOH gradient from 10% (v/v) used as a linear fluorometric detection (excitation at 305 nm; emission at 407 nm). Salicylic acid concentrations were quantified by comparing peak area of standard sample with peak area of heat-shocked samples under the same conditions using the standard equation:

$$C_x(\mu g/g) = \frac{Ax \text{ X total volume of extract (ml)}}{RF_x \text{ X } A_{ref} \text{ X sample weight (g)}} \quad [16].$$

$$RF_x = \frac{\text{peak area of carotenoid}_X \text{ (1(µg/ml)}}{\text{peak area of refrence carotenoid (1(µg/ml)}}$$

C_x = concentration of salicylic acid in the callus samples.

A_x = peak area of salicylic acid in the callus samples.

RF_x = response factor of salicylic acid in the callus samples.

A_{ref} = peak area of 1 mg/ml of standard salicylic acid.

RESULTS

Effect of STHS on Callus Biomass

Data showed that friable callus cultures were obtained on MS medium supplemented with 1.0 mgL^{-1} NAA and 0.1 mgL^{-1} BAP. When this callus was exposed to heat shock, the results indicate that this treatment sustained callus growth. Generally, weights of calli samples exposed to STHS (40 °C/5.0 min.) were increased more than the double compared with the weights of the non-treated callus (Table 1).

Table 1: Weights increase of *Calendula officinalis* L. calli exposed to short term heat shock (STHS) grown on agar solidified MS + 1.0 mgL^{-1} NAA + 0.1 mgL^{-1} BAP medium.

Period after exposure (days)	Weight of non-treated callus (gm)	Weight of STHS callus (gm) °C/ 5.0 min.					
		35	40	45	50	55	60
21	2.80	6.34	7.73	6.58	5.83	3.83	3.00
42	15.72	27.02	28.12	26.66	21.88	19.09	10.57

*Values represented the mean of 5 replicates\treatment.

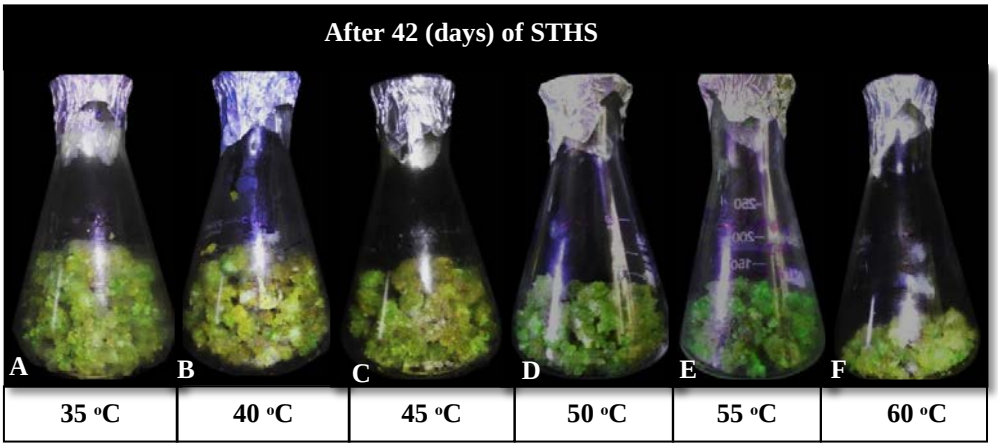

Fig.1: Role of short-term heat shock (STHS) in callus growth of *Calendula officinalis* L. 42 days after exposure cultured on MS + 1.0 mgL^{-1} NAA + 0.1 mgL^{-1} BAP medium.

A, B, C: Increase of callus biomass that restored friable structure and green color.

D, E, F: Weak growth of callus affected by severity of shock, reduction in biomass and change in

It was observed that after 21 days of treatment biomass and weights of callus were increased, as exposed to STHS 35 °C/5.0 min., 40 °C/5.0 min. and 45 °C/5.0 min. (Fig. 1. A, B, C). Symptoms of this stress were apparent through the yellowish color of the peripheral tissue of heat shocked callus. More damage occurred to callus samples exposed to high degree of STHS. Subsequently green structures progressively regained, and enable callus continuing growth. The increasing in weights and biomass of callus was clear at 42 days after exposure. Whiles, it was found that reduction in callus biomass and weights occurred as callus was exposed to 50°C degree (Fig. 1, D), 55 °C (Fig. 1, E) and 60 °C (Fig. 1, F).

Effect of LTHS on Callus Biomass

According to the obtained results (Table 2) callus weight was positively affected by exposure to LTHS. High increase of callus weight was clear at 40 °C/10 min. shock, then callus weights began decreasing with the increasing of LTHS (Table 2). Similar pattern of callus biomass was noted at the second period of weight determination 42 days after exposure.

Observations showed that greatest increase in callus biomass and size obtained at 35, 40 and 45 °C (Fig.2, A, B, C). In this case although the peripheral cells of exposed callus were died as exposed to high temperature of LTHS, but the remaining survived callus cells were regained its full physiological activity through size increase, color and friable structure. In contrast, calli exposed to 50, 55 and 60 °C LTHS were suffered from these treatments. They caused clear loss in calli biomass with unacceptable color (Fig. 2, D, E, F), and many samples of callus which exposed to 60 °C were died.

Protein Content

The results expressed that STHS applications improved total soluble proteins accumulation in treated callus compared with non-treated callus. It was noted that enhancement of protein content was coincided with the increase of callus fresh weight (Fig.3, A). Interestingly, application of LTHS promote protein content more than STHS (Fig.3, B).

STHS Enhancement SA Content

Firstly, salicylic acid standard samples, injected into HPLC their retention time was 3.424. Subsequently, calli extracts of heat shocked and non-treated samples were injected. Generally, the results (Table 3) referred to differences in concentrations of salicylic acid among these calli samples exposed to STHS treatments. Moreover, all treatments increased callus content of salicylic acid many times more than its content in untreated calli. This high increasing start in samples exposed to each of 35 ºC\5.0 min., 40 ºC\5.0 min. and 45 ºC\5.0 min., then they gradually decreased with the increasing of degree (Table 3).

Data devised from curves of HPLC at 407 nm fluorometric analysis detected the retention time of standard sample (Fig.4. A) and non-treated callus (Fig.4. B). The results indicate that STHS supported biosynthesis, induction and accumulation of SA in heat shocked callus samples particularly in each of 35 ºC\5.0 min., 40 ºC\5.0 min. and 45 ºC\5.0 min. treatments as demonstrated (Fig.4. C, D and E). Other heat shocks seems to be less promotive to SA biosynthesis that achieve a lowest level values (Fig.4, F, G, H).

LTHS Enhancement SA Content

Again, the results (Table 4) indicate the positive role of long-term heat shock (LTHS) in the physiological calli behaviors that inducing SA biosynthesis in the heat shocked calli. Its concentrations were measured depending on the peak area assessed by HPLC. It was found that all heat shocked samples were had doubled their SA content compared to the non-treated. Samples that exposed to degrees under 50 °C gave the best outcome SA in calli. Concentration of SA began to increase when callus exposed to 35 °C\10 min. reading the highest concentration 77 µg/g as callus exposed to 40 °C, followed with a slight decrease of SA content in callus treated with 45 °C\10 min.. It was observed that exposing callus at 50 °C\10 min. and over had a little increasing effect in SA content (Table 4).

Measurements of SA was performed in standard solution and non-treated callus (Fig.5. A, B). The long-term heat shock promoting SA content of callus samples exposed to 35 °C\10 min.. This treatment seems to be sufficient to stimulate biosynthesis and accumulation of salicylic acid in callus. This effect was raised in callus shocked by 40 °C\10 min. (Fig. 5. C). The only exception was little enhancement of SA formation in callus happened as temperature raised to 45 °C\10 min. (Fig. 5. D). Salicylic acid biosynthesis began to deterioration and fade in callus that exposed to each of 50 °C\10 min., 55 °C\10 min. and 60 °C\10 min. (Fig. 5. E, F and G) respectively.

Table 2: Weights increase of *Calendula officinalis* L. calli exposed to Long term heat shock (LTHS) grown on agar solidified MS + 1.0 mgL^{-1} NAA + 0.1 mgL^{-1} BAP medium.

Period after exposure (days)	Weight of non-treated callus (gm)	Weight of LTHS callus (gm) ℃ /10 min.					
		35	40	45	50	55	60
21	2.80	4.09	7.91	6.73	5.95	3.98	3.45
42	15.72	21.64	31.99	28.06	25.63	20.73	15.14

*Values represented the mean of 5 replicates\treatment.

Table 3: Enhancement of salicylic acid content in *Calendula officinalis* L. calli exposed to short-term heat shock (STHS) measured by HPLC.

Treatments	SA Standard	Non-treated Callus	Callus Heat Shocked for 5.0 min. (STHS)					
			35 °C	40 °C	45 °C	50 °C	55 °C	60 °C
Conc. µg/g	10.0	5.02	29.02	72.95	56.95	15.42	13.05	12.16
Peak area	7147.510	359.327	2073.933	5213.482	4070.393	1102.245	932.615	869.483

Table 4: Enhancement of salicylic acid content in *Calendula officinalis* L. calli exposed to long-term heat shock (LTHS) measured by HPLC.

Treatments	SA Standard	Non-treated Callus	Callus Heat Shocked for 10 min. (LTHS)					
			35 °C	40 °C	45 °C	50 °C	55 °C	60 °C
Conc. µg/g	100.0	5.02	46.99	77.05	72.13	18.46	9.33	9.15
Peak area	7147.510	359.327	3358.605	5506.811	5155.366	1319.692	666.863	654.359

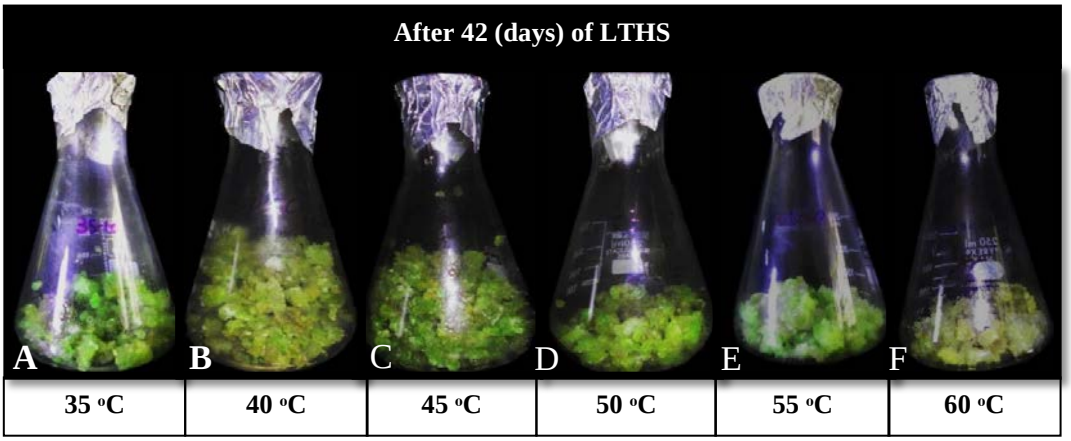

Fig.2: Role of Long-term heat shock (LTHS) in callus growth of *Calendula officinalis* L. 42 days after exposure cultured on MS + 1.0 mgL^{-1} NAA + 0.1 mgL^{-1} BAP medium.

A: Callus restored its friable structure and green color with increasing weights after 42 days of exposure.
B, C: Increase of callus biomass.
D, E, F: Weak growth of callus affected by severity of shock, reduction in biomass and changing in color.

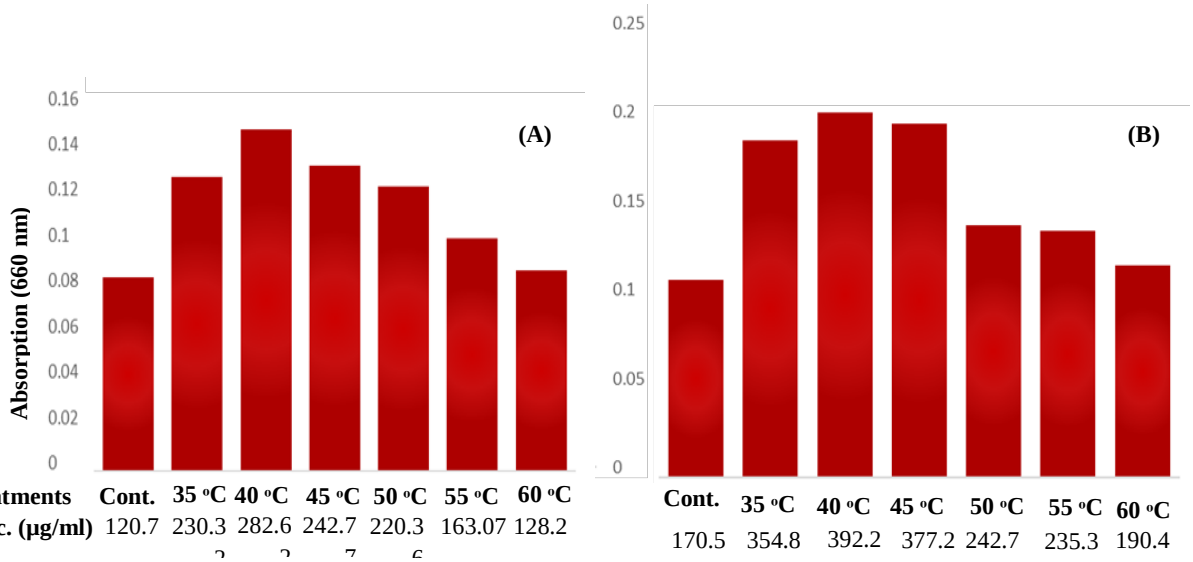

Fig. 3: Protein accumulation in *Calendula officinalis* L. callus 42 days after heat shock exposure.

(A): Callus exposed to STHS.
(B): Callus exposed to LTHS.

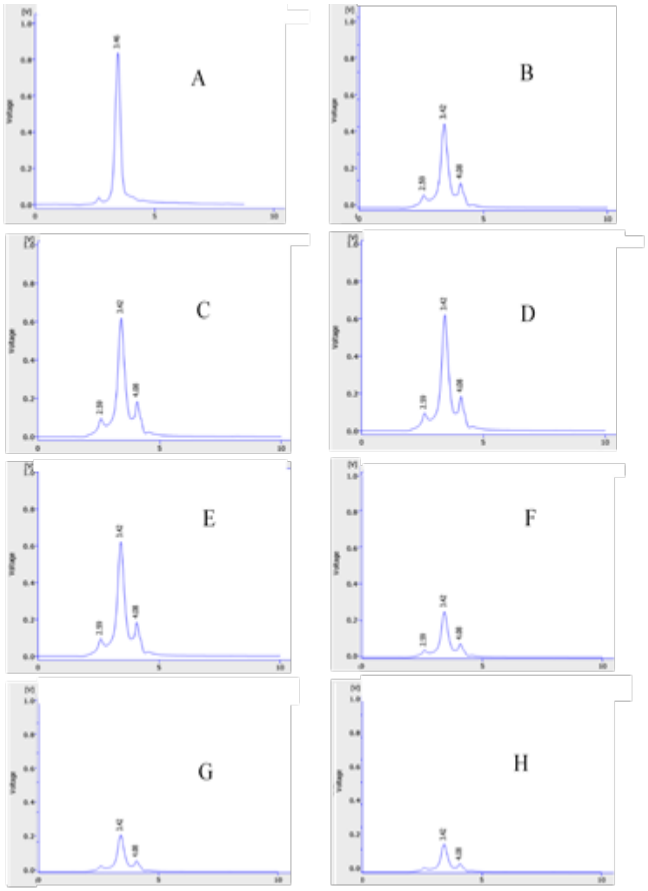

Fig.4: Detection of salicylic acid in *Calendula officinalis* L. callus which exposed to short-term heat shock.

A: A standard solution of Salicylic acid 100 µg/g.
B: Non-treated callus sample.
C, D, E: Callus exposed to smooth level of heat at 35, 40 and 45 ºC respectively.
F, G, H: Callus exposed to high level of heat at 50, 55 and 60ºC respectively.

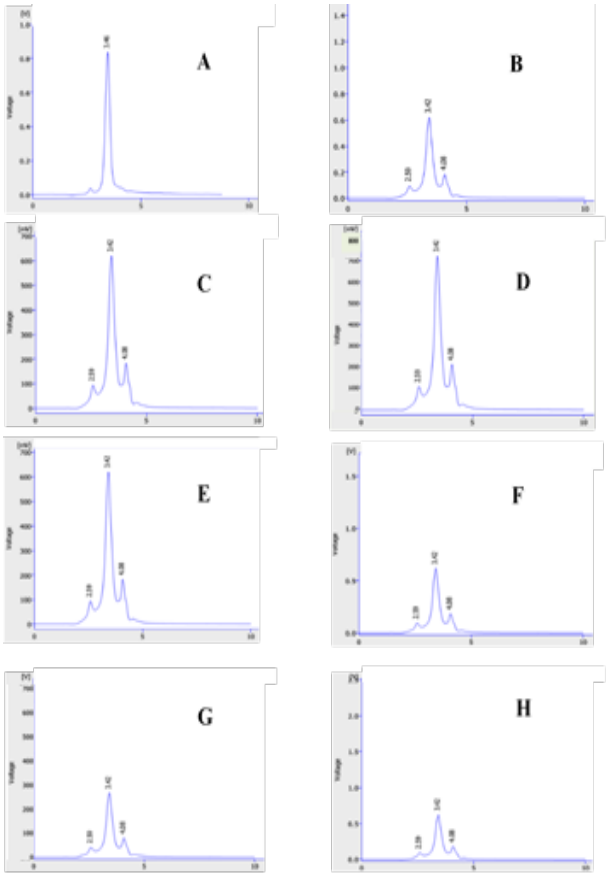

Fig.5: Detection of salicylic acid in *Calendula officinalis* L. callus which exposed to long-term heat shock by HPLC.

A: A standard solution of Salicylic acid 100 µg/g.
B: Non-treated callus sample.
C, D, E: Callus exposed to smooth level of heat at 35, 40 and 45 ºC respectively.
F, G, H: Callus exposed to high level of heat at 50, 55 and 60ºC respectively.

DISCUSSION:

High biomass of callus that heat shocked is one of a critical result had obtained in this study, this response to heat shock (HS) may be attributed to total polyamines such as in rice *Oryza sativa* callus exposed to 45 °C [29]. As well these results are supported by other workers [4] they reported that heat shocks enhanced callus biomass, amounts of DNA, RNA, total proteins, specific activity of thymidylate synthase and dihydrofolate reductase and serine hydroxy methyl transferase enzymes in *Sesamum indicum* callus exposed to 40 °C. Whereas high degrees exceed 45 °C caused sever injury to callus tissue leading to reduction in growth [1]. High temperature may resulted in leaking of water and other organic and inorganic substances from the cells ending in the death of them [30].

The adaptation of plants to heat shock is accompanied by alterations in the level of protein patterns [10]. Heat shock proteins (HSP) were synthesized and their accumulation was affected by heat stress with a very similarity to the pattern of callus biomass increase [6]. The positive effects of heat shock in many plant and stimulation the metabolic pathways in the cells represented in building a new protein in young leaves of barley and sorghum called heat shock proteins [11]. Plant cells respond to heat shock by increasing gene expression, which later encodes the construction of HSP by stimulating of activating HSPs gene groups [9]. Other investigator [5] pointed out that the diversification of these proteins reflects an adaptation to tolerate stress. HSPs have some kind of related roles in regulating a range of effect or components, all of which contribute to survival under abiotic stress. Exposing of soybean and tobacco cells to 39-40 °C stimulates the synthesis of HSPs [32], which might be occurred according to increasing in polypeptides observed in callus derived from explant treated at 47 °C [31]. SA levels were significantly increased at the start of acclimation to heat stress [18], short-term heat shock caused increases in endogenous SA levels, likely as a result of its *de novo* synthesis [26]. However, the initial and substantial increase in endogenous SA following the administration of

short-term heat diminishes over time as occurred with grape plants. Thus, after 24 h the endogenous SA levels in control and heat-shocked plants were essentially the same [34,35]. Other support for high temperature induction of SA synthesis comes from studies with *Arabidopsis thaliana* [11]. At moderately high temperatures, injuries or death only occur after long-term exposure, at very high temperatures, severe cellular injury and even cell death may occur within minutes. Heat stress causes physiological and biochemical changes in plants. Direct injuries due to high temperatures probably include protein denaturation, aggregation, and the increased fluidity of membrane lipids. Indirect or slower heat injuries include the inactivation of enzymes in chloroplasts and mitochondria, the inhibition of protein synthesis, protein degradation and loss of membrane integrity. These injuries eventually lead to starvation, growth inhibition, ion flux reduction, and the production of toxic compounds and ROS [33].

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