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Research Article

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Improvement of CHS genes expression and flavonoids content in callus culture of *Silybum marianum* treated with extracts of yeast and *Aspergillus niger*

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KEY WORDS

Biotic elicitors,
Flavonoids,
Gene expression,
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ABSTRACT

The present study aims to evaluate the impact of extracts of yeast and *Aspergillus niger* as biotic elicitors, on flavonoids accumulation in callus culture of *Silybum marianum*. Also the gene expression levels of Chalcone synthase genes (*CHS1, CHS2* and *CHS3*) which inter in flavonoids formation were evaluated by Quantitative realtime PCR (qRT-PCR) technique. The findings indicated that total flavonoids content got enhanced in response to elicitation by extract of yeast and *A. niger* in callus. Expression analysis of the three *CHS* genes in callus with elicitor treatments showed that biotic elicitors (yeast and *A. niger* extracts) strongly induced their expression. It was noted that the *CHS2* expression was notably enhanced by *A. niger* and yeast extracts with 13.7- and 8.8-fold respectively compared to control. The results indicated that, biotic treatments contribute to the up regulation of *CHS* genes, which in turn, lead to an increase of flavonoids content in callus culture of *S. marianum*.

Introduction

Silybum marianum (L.) is a broadleaf plant belong to family *Asteraceae* (**Abbasi *et al.*, 2010**). Medicinal compounds present in this plant play a vital role in pharmacology, medical and pharmaceutical purposes. Silymarin is the very important gradient in the plant, and composed of many flavonolignans including silybin, isosilybin, silydianin, silychristin, and taxifolin that derived from fruits of this plant (**Godlewska**, ***et al.*, 2020**). Milk thistle is used for various medical aspects, and fruit extract is important to manipulate liver illness as cirrhosis (**Karasawa, *et al.*, 2018**). Silymarin is a component applied to manipulate alcoholic liver disease, viral hepatitis, and toxin-induced liver disorder, and consider powerful in manipulation of hepatocellular carcinoma (**Abenavoli and Milic, 2017 and Marmouzi *et al.*, 2021**). Flavonoids are active metabolite with much impotence in stress defense mechanism (**Wu *et al.*, 2020)**. Also it possesses antioxidant activity to capture free radical and organize pathway in cells signaling (**Zhang *et al.*, 2020**). Chalcone synthase (CHS) enzyme is considering very important enzyme for flavonoids synthesis pathway in plant that induce the formation of chalcones (**Koes *et al.*, 1994**), which consider first step in formation of many flavonoid derivatives, like flavones, flavanols, anthocyanins and glycoside (**Zhang *et al.*, 2017**). CHS is the key enzyme in formation of many active gradients, like rosmarinic acid that has antiviral activity to Japanese encephalitis (**Swarup *et al.*, 2007**). CHS enzymes family helps plants, fungi, and bacteria to produce vital secondary compounds (**Kaur *et al.*, 2020 and Wu *et al.*, 2020**). Chalcones are important enzymes which are vital for the production of flavonolignans in milk thistle and other plants (**Lv *et al.*, 2017**). Plant tissue culture is considered powerful technological tool for the synthesis of many compounds that have medical importance (**Murthy *et al.*, 2014; Dias *et al.*, 2016**). Research effort directed toward the increasing secondary metabolite production with low production cost, that makes plants tissue cultures technologies more commercially effective (**Isah *et al.*, 2018**). Addition of elicitors from natural origin found to enhance the formation of targeted flavonoids by activating plant defense mechanism **(Jiao *et al.*, 2018**). *A. niger* and *A. oryzae*, (Generally considered safe fungi) which applied in fermentations industries like as the manufactures of wines, soy sauces, vinegar, soybean pastes (**Maeda *et al.*, 2016; Ni *et al.*, 2015**). These two fungi are considered vital and important elicitors, which increase induction of many desired

compound *in vivo* or in tissue culture (**Aisyah *et al*., 2015 and Kümmritz *et al*., 2016**). **Hedayati *et al*., (2021)** mentioned that yeast extract can be applied as a powerful elicitor for enhancement alkaloids synthesis in hairy root cultures of *A. belladonna*.

The goal of this work is to evaluate the use of biotic elicitors (yeast and *A. niger* extracts) on flavonoids content in *S. marianum* callus culture and determine the expression level of *CHS* genes (*CHS1*, *CHS2* and *CHS3*) in callus culture compared to control to understand the molecular events due to the addition of yeast and *A. niger* extracts.

Materials and methods

Seeds sterilization and callus formation

The seeds of *S. marianum* were sterilized by soaking them in 70% ethanol for 2 min, then immersion in 40% Clorox with drops of any detergent for 15 min, followed by many rinses with sterilized distilled water. The sterilization process was done in complete aseptic condition to prevent any contamination. Then the sterilized seeds are planted on 1/2 Murashige and Skoog (MS) media (**Murashige and Skoog, 1962**), fortified with 30 g/l sucrose and 10 g/l agar. The culture then placed in 26±2°C in dark to enhance seeds to germinate. The sterilized stems of the seedlings **(Fig. 1)** were cut into 0.5 cm segments then cultured on solid MS medium fortified with 1 mg/l 2, 4-D (2, 4-dichlorophenoxy acetic acid) and 0.5 mg/l BA (6-benzyladenine) to induce callus synthesis.

Elicitors preparation

Preparation of yeast extract

Yeast extract (Sigma) was prepared as described in **Peltonen *et al*., (1997)**. The powder was dissolved in distilled water 1g/l, sterilized before addition to the culture medium at certain ratio (75 µg/ml).

Preparation of *A. niger* extract

A. niger was given from Department of Botany and Microbiology, Al-Azhar University (Biotechnology Lab.). The biotic elicitor prepared according to (**Giridhar and Parimalan 2010**). The fungus was grown in malt extract (10 g/l) in a flask (1000 ml) with 250 ml medium on rotary shaker (130 rpm) at room temp. After 10 days, the fungal suspensions were harvested (pellets), dried and grounded. The fungal dry powder was suspended in distilled water in a solution of concentration of 10 g/l. The solution was then sterilized by autoclaving before adding to the callus culture medium at certain concentration (1.0 ml/50 ml).

Culture conditions

After eight weeks of culturing, the resulted calluses were removed from the stem cuttings and cultured again until efficient amount of callus was obtained.

The callus obtained were cultured on MS media fortified by (1 mg/l, 2,4-D, and 0.5 mg/l BA), then treated with 1.0 ml/50 ml *A. niger* extract and 75 µg/ml yeast extract for six weeks and placed at 26 ±2°C in dark condition. After six weeks, calluses had removed to undergo the following estimations.

Determination of total flavonoids content in callus of *S. marianum*

Aluminum chloride colorimetric method was applied in flavonoids estimation **(Chang *et al.*, 2002)** as prescribed in **(Khalifa *et al.*, 2022)**. Concentrations of flavonoids content were expressed as mg/g d. wt.

RNA extraction, cDNA synthesis and qRT-PCR

The expression level of *CHS* genes which involved in flavonoids synthesis (*CHS1*, *CHS2* and *CHS3*) were evaluated using qRT-PCR. All samples are directly freezing in liquid nitrogen and placed at −86°C until RNA extraction. Briefly, RNA was extracted by RNA isolation kit (Promega, USA). Then estimated using a Pico200 spectrophotometer (Picodrop, UK). cDNA was synthesized using a cDNA synthesis kit (Promega, USA). qRT-PCR was done by the Step One real-time PCR apparatus (Thermo Fisher Scientific, Germany). The methods of cDNA formation and genes quantification were found in **(Yap *et al.*, 2021)**. All *CHS* genes which used in the quantification method and reference gene are listed in **Table (1)**.

Table (1): Primer sequences of *CHS* genes and reference gene

Target genes	Primer Sequences (5′ → 3′)	Product size (bp)	Gen Bank Accession Number
CHS1	**F-TCTTGATTCCCTCGTTGGTC** **R -TCTCAAACAACGGCCTCTCT**	**101**	**JN182805.1**
CHS2	**F -AGGACATTGCGGAAAACAAC** **R -AACGGCCTCTCTGTCTTCAA**	**184**	**JN182806.1**
CHS3	**F -ACCCACCTCATCTTTTGCAC** **R -CATCATGAGGCGTTTGATTG**	**105**	**JN182807.1**
NADH	**F -TTCCGCATTTTGGAAATACC** **R -CCCGTCTTGATTGAAAGGAA**	**134**	**KC589999.1**

Statistical analysis

The data was described as mean of the replicates ± standard error (SE). Difference between treatment for the different measured variables was tested by one-way variance ANOVA (Analysis of variance), followed by Student's t-test and Dunnett's test since significant difference was found

($p < 0.05$). ANOVA of relative genes expression was done based on completely randomized design (CRD) by three replicates using data SAS ver.9.4 software.

Results and Discussion

Impact of biotic elicitors on flavonoids content

It is necessary to choose the most suitable elicitors at appropriate concentration for the accumulation of large-scale metabolic products. In order to determine impact of the addition of yeast and *A. niger* extracts, total flavonoids content was measured. By comparing the data of the measurement of flavonoid contents in callus culture of *S. marianum* in three treatments (**Fig. 2**), it was revealed that the total flavonoids amount in the callus treated with *A. niger* extract is 6.99 fold more than untreated callus (control). Also it was found that flavonoid contents in callus treated with yeast extract is 6.48 fold more than the free treatments **(Table 2)**. Therefore, *A. niger* extract and yeast extract application into callus culture of *S. marianum* showed a valuable effect on flavonoids production. Our results in accordance with (**Akitha Devi *et al.*, 2020**) who stated that the biotic elicitor *A. niger* at 0.1% increased the flavonoid contents in *Glycine max* L. in cell suspension culture by 5.9 folds compared to control.

Fig. (1): In vitro germinated seeds of *S. marianum* on 1/2 MS medium.

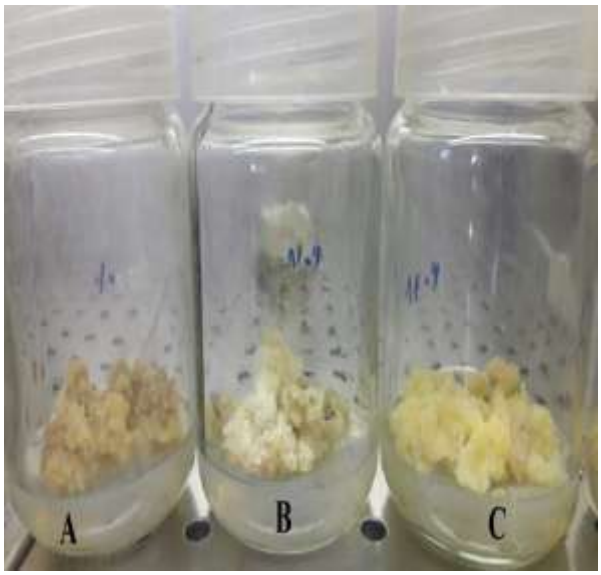

Fig. (2): Callus of *S. marianum* induced on MS medium supplemented with 1 mg/l BA and 0.5 mg/l 2,4-D.(A) control, (B) callus treated with *A. niger* extract, (C) callus treated with yeast extract**.**

Table (2): Total content of flavonoids (mg/g d. wt.) in treated *S. marianum* callus compared to untreated culture

Treatment	Flavonoids contents (mg/g d. wt.)	Folds control
Control	1.18±0.002[c]	-
***A. niger* extract**	8.25±0.008[a]	6.99
Yeast extract	7.65±0.004[ab]	6.48

Mean followed by the same letter are not significantly different at P≤0.05

Differential expression of *CHS* genes in response to biotic elicitors

The abundance of gene transcripts was investigated in the harvested callus after the incubation with yeast and *A. niger* extracts. In general, the expression of *CHS* genes were up regulated in both treatments (**Fig. 3**). The obtained results showed that the transcripts of *CHS2* showed the highest abundance (13.7-fold increase) under the effect of *A. niger*. Moreover, *CHS2* was more elicited with yeast treatment than the *CHS1* and *3* genes (8.8 -fold increase). Thus, the application of elicitor in certain quantity or suitable concentration can enhance and increase formation of vital gradients in cells also minimize the period of accumulation of more important products. In the present work, high relation was observed in *CHS* genes expressions and flavonoids quantity of milk thistle callus, indicating the prominent role of yeast and *A. niger* extracts in *CHS* genes expression and in the formation of flavonoid compounds in comparisons with control (**Fig.3**). Similar finding was observed in milk thistle, since the expressions of the three *CHS* genes have related to flavonoids formation, containing silybin formation (**Lv *et al.*, 2017**).

In this work, over expression of *CHS* genes in *S. marianum* callus was observed in both treatments, which is in accordance with **Alkuwayti *et al.*, (2022)**, who stated that *CHS1*, *CHS2*, and *CHS3* expressions were increased in plant that treated with 40 mL/L *Aloe vera* extract as a foliar spray on *S. marianum* plants. The current results also is agreed with (**Awasthi *et al.*, 2016**), they found a high enhancement in *CHS* gene expressions and total flavonoid contents in *Coleus forskohlii* leaf that treated with Methyl Jasmonate. Other research observed *CHS1*, *2*, and *3* genes in milk thistle presented in the silymarin formation pathway by different approaches (**El-Garhy *et al.*, 2016**).

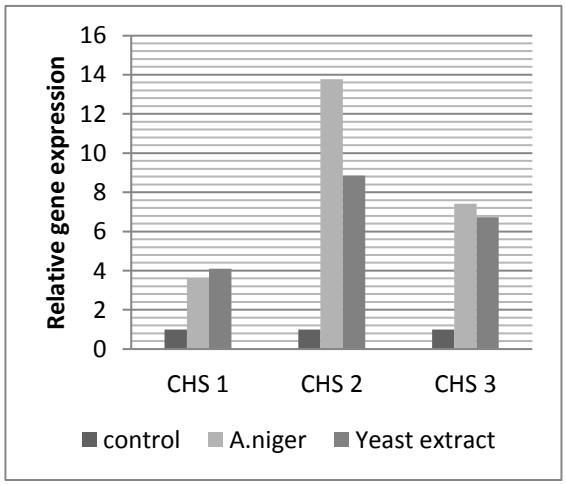

Fig. (3): Expression profile of *CHS* genes using qRT- PCR

The flavonoids production pathway is vital secondary metabolites pathway, and CHS is the first necessary enzyme that induce the synthesis of flavonoids. The abundance of many secondary plant metabolites is highly affected by growing conditions, while stress condition possessing an important effect on the metabolic pathways, leading to formation of associated important natural compounds (**Alkuwayti *et al.*, 2022**). Previous work demonstrated the role of *CHS* in the induction of flavonoids and increase the plant tolerance to drought stress (**Wang *et al.*, 2018**). Our results also indicate that yeast extract and *A. niger* extract is a potential post transcriptional regulators for *CHS* genes expression and flavonoids biosynthesis.

Conclusion

In the current work, the effect of two biotic elicitors, yeast and *A. niger* extracts, on callus culture of milk thistle flavonoids content and *CHS* gene expressions were investigated. The outcomes revealed that the two biotic elicitors improved total flavonoids content, therefore enhancing the medical importance of milk thistle by increasing the biosynthesis of bioactive gradients. Furthermore, gene expression analysis demonstrated up regulation of *CHS* genes. *CHS* genes were found to be significantly induced by yeast and *A. niger* extracts therefore increase in total flavonoids in milk thistle callus culture.

References

Abbasi B.H, Khan N.A, Mahmood T, Ahmad M, Chaudhary M.F, Khan M.A (2010). Shoot regeneration and free-radical scavenging activity in *Silybum marianum* L. Plant Cell, *Tiss. Org. Cult. J*., 101: 371–376.

Abenavoli, L. and Milic, N. (2017). Silymarin for Liver Disease, Chapter 45. In: "Liver Pathophysiology" (Muriel, P. Ed.), Academic Press, p. 621- 631.

Aisyah, S., Gruppen, H., Slager, M., Helmink, B., Vincken, J.P., (2015). Modification of prenylated stilbenoids in peanut (*Arachis hypogaea*) seedlings by the same Fungi that elicited them: the fungus strikes back. *J. Agric. Food Chem*., 63:9260–9268.

Akitha Devi, M. K., Kumar, G. & Giridhar, P. (2020). Effect of biotic and abiotic elicitors on isoflavone biosynthesis during seed development and in suspension cultures of soybean (*Glycine max* L.) 3 Biotech

10:98.https://doi.org/10.1007/s13205-020-2065-1

Alkuwayti, M.A.; Aldayel, M.F.; Yap, Y.-K.; El Sherif, F. (2022). Exogenous Application of Aloe vera Leaf Extract Improves Silybin Content in *Silybum marianum* L. by Up-Regulating Chalcone Synthase Gene. *Agriculture*, 12, 1649. https://doi.org/10.3390/ agriculture12101649.

Awasthi P., Mahajan V., Jamwal V. L., Kapoor N., Rasool S., Bedi Y. S. and Gandhi S. G. (2016). Cloning and expression analysis of chalcone synthase gene from *Coleus forskohlii*. *J. Genet*,. **95**, xx–xx]

Chang, C., Yang, M., Wen, H.& Chern, J. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal*. 10:178–182.

Dias, M.I., Sousa, M.J., Alves, R.C.& Ferreira, I.C., (2016). Exploring plant tissue culture to improve the production of phenolic compounds: a review. *Ind. Crop. Prod.* 82: 9–22.

El-Garhy, H.A., Khattab, S., Moustafa, M., Ali, R.A., Azeiz, A.Z.A., lhalwagi, A.& El Sherif, F.(2016). Silybin content and overexpression of chalcone synthase genes in *Silybum marianum* L. plants under abiotic elicitation. Plant Physiol. Biochem. 108: 191–202.

Giridhar, P. and Parimalan, R. (2010). A biotechnological perspective towards improvement of annatto color production for value addition— the influence of biotic elicitors. Asia Pac J Mol Biol Biotechnol 18:77–79.

Godlewska, K., Biesiada, A., Michalak, I.& Pacyga, P.(2020). The Effect of Botanical Extracts Obtained through Ultrasound-Assisted Extraction on White Head Cabbage (*Brassica oleracea* L. Var. *Capitata* L.) Seedlings Grown under Controlled Conditions. *Sustainability*, 12: 1871.

Hedayati, A., Hemmaty, S., Nourozi, E.& Amirsadeghi, A.(2021). Effect of yeast extract on h6h gene expression and tropane alkaloids production in *Atropa belladonna* L. hairy roots. Russ. *J. Plant Physiol*., 68: 102–109.

Isah, T., Umar, S., Mujib, A., Sharma, M.P., Rajasekharan, P.E., Zafar, N., Frukh, A., (2018). Secondary metabolism of pharmaceuticals in the plant in vitro cultures: strategies, approaches, and limitations to achieving higher yield. *Plant Cell Tissue Organ Cult.*, 132, 239–265.

Jiao, J.; Gai, Q.Y.; Wang, X.; Qin, Q.P.; Wang, Z.Y.; Liu, J.; Fu, Y.J. (**2018).** Chitosan elicitation of *Isatis tinctoria* L. hairy root cultures for enhancing flavonoid productivity and gene expression and related antioxidant activity. *Ind. Crops Prod*., 124: 28–35.

Kaur, R., Aslam, L., Kapoor, N.& Mahajan, R.(2020). Identification and comparative expression analysis of chalcone synthase, flavanone 3-hydroxylase and dihydroflavonol 4-reductase genes in wild pomegranate (*Punica granatum* L.) organs. *Braz. J. Bot.*, 43: 883–896.

Karasawa, M.M.G. and Mohan, C. (2018). Fruits as Prospective Reserves of bioactive Compounds: A Review. Nat. Prod. Bioprospecting, 8: 335–346.

Khalifa, A. M, Abd-ElShafy, E., Abu-Khudir, R and Gaafar, R. M. (2022). Influence of gamma radiation and phenylalanine on secondary metabolites in callus cultures of milk

thistle (*Silybum marianum* L.). *J. Genet. Eng. & Biotechnol*., 20:166.

Koes, R.E., Quattrocchio, F., Mol, J.N.M., (1994). The flavonoid biosynthetic pathway in plants: Function and evolution. BioEssays 16: 123–132.

Kümmritz, S., Louis, M., Haas, C., Oehmichen, F., Gantz, S., Delenk, H., Steudler, S., Bley, T.& Steingroewer, J., (2016). Fungal elicitors combined with a sucrose feed significantly enhance triterpene production of a *Salvia fruticosa* cell suspension. *Appl. Microbiol. Biotechnol.*, 100: 7071–7082

Lv, Y., Gao, S., Xu, S., Du, G., Zhou, J.& Chen, J.(2017). Spatial organization of silybin biosynthesis in milk thistle [*Silybum marianum* (L.) Gaertn]. *Plant J*., 92: 995–1004.

Maeda, H., Sakai, D., Kobayashi, T., Morita, H., Okamoto, A., Takeuchi, M., Kusumoto,K.I., Amano, H., Ishida, H., Yamagata, Y., (2016). Three extracellular dipeptidyl peptidases found in *Aspergillus oryzae* show varying substrate specificities. *Appl. Microbiol. Biotechnol.*, 100: 4947–4958.

Marmouzi, I., A. Bouyahya, S.M. Ezzat, M. El Jemli & Kharbach (2021). The food plant *Silybum marianum* (L.) Gaertn.: phytochemistry, ethnopharmacology and clinical evidence. *J. Ethnopharmacol*., 30 (265): 113303.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol. Plant*., 15: 473–497.

Murthy, H.N., Lee, E.J. & Paek, K.Y., (2014). Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. *Plant Cell Tissue Organ Cult.*, 118: 1–16.

Ni, H., Chen, F., Jiang, Z.D., Cai, M.Y., Yang, Y.F., Xiao, A.F.& Cai, H.N., (2015). Biotransformation of tea catechins using *Aspergillus niger* tannase prepared by solid state fermentation on tea byproduct. *LWT Food Sci. Technol.* 60: 1206–1213.

Peltonen, S.; Mannonem, L. & Karjalainem, R. (1997). Elicitor-induced changes of phenylalanine ammonia-lyase activity in barley cell suspension cultures. *Plant Cell Tissue and Organ Cult*., 50: 185–93.

Swarup V., Ghosh J., Ghosh S., Saxena A. & Basu A. (2007). Antiviral and anti-inflammatory effects of rosmarinic acid in an experimental murine model of *Japanese encephalitis*. Antimicrob. Agents Chemother. 51: 3367–3370.

Wang, Z., Yu, Q., Shen, W., El Mohtar, C.A., Zhao, X.& Gmitter Jr., F.G., (2018). Functional study of CHS gene family members in citrus revealed a novel CHS gene affecting the production of flavonoids. BMC Plant Biol. 18**.**

Wu, X., Zhang, S., Liu, X., Shang, J., Zhang, A., Zhu, Z. & Zha, D. (2020). Chalcone synthase (CHS) family members analysis from eggplant (*Solanum melongena* L.) in the flavonoid biosynthetic pathway and expression patterns in response to heat stress. *PLoS ONE*, 15, e0226537.

Yap, Y.-K.; El-Sherif, F.; Habib, E.S.; Khattab, S. (2021). *Moringa oleifera* Leaf Extract Enhanced Growth, Yield, and Silybin Content While Mitigating Salt-Induced Adverse Effects on the Growth of *Silybum marianum*. Agronomy, 11, 2500.

Zhang, X., Abrahan, C., Colquhoun, T.A. & Liu, C.J., (2017). A proteolytic regulator controlling chalcone synthase stability and flavonoid biosynthesis in arabidopsis. Plant Cell 29: 1157.

Zhang, Q., Yang, W., Liu, J., Liu, H., Lv, Z., Zhang, C., Chen, D.& Jiao, Z. (2020). Identification of Six Flavonoids as Novel Cellular Antioxidants and Their Structure-Activity Relationship. Oxidative Med. *Cell. Longev*.1–12.

تحسين التعبير الجينى ومحتوى الفلافونيدات باستخدام مستخلصات فطرى الخميرة والاسبرجلس نايجرعلى مزارع كالس نبات شوك الجمل

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تهدف الدراسة الحالية الى دراسة تاثير مستخلص فطرى الخميرة والاسبرجلس نايجر،كمسرعات نمو حيوية، على محتوى الفلافونيدات والتعبير الجينى لجينات (*CHS1, CHS2, CHS3*) فى مزارع الكالس الخاصة بنبات شوك الجمل. وقد وجد ان معاملة الكالس بهذه المستخلصات الحيوية ادى الى زيادة محتوى الفلافونيدات اكثر من الكالس الغير معامل بهذه المستخلصات.ايضا التعبير الجينى لهذه الجينات قد زاد ١٣,٧ مرة اكثر باستخدام مستخلص فطر الاسبرجلس نايجر مقارنة بالكالس الغير معامل.بينما زاد التعبير الجينى ٨,٨ مرة اكثر باستخدام مستخلص فطر الخميرة مقارنة بالكالس الغير معامل. وهذا يدل على اهمية استخدامها فى مزارع الكالس لزيادة التعبير الجينى وبالتالى زيادة المواد الفعالة فى الكالس.