IN VITRO PRODUCTION OF SOME TERPENOIDS COMPOUNDS FROM *Nigella sativa* WITH DIFFERENT EXPLANTS TYPE AND PEG CONCENTRATIONS

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ABSTRACT

This experiment was conducted to study the effect of explant and poly ethylene glycol (PEG) on the production of monoterpeniods compounds in the callus of *Nigella sativa*. The explant type had a significant effect on callus induction from the hypocotyl giving a significant increase on fresh and dry weight of 282.8 and 25.25 mg. respectively. as well, as the production of the borneol compound at 26.70 µg.100mg^{-1} dry weight.The callus inducted from the cotyledon has a significant increase of the thujone, α-pinene, camphene and carvacrol compounds at 24.01, 20.58, 13.58 and 23.80 µg.100mg^{-1} dry weight, respectively. The increasing concentrations of PEG resulted in a significant reduction in fresh and dry weight. Also, the addition of PEG stimulated the production of terpeniods with the PEG concentration of 100g.L^{-1} producing the highest amount of borneol and carvacrol at 36.60 and 37.80 µg.100mg^{-1} dry weight. Meanwhile, 150 g.L^{-1} PEG treatment produced the highest amount of thujone, α-pinene, camphene and mycene at 23.29, 21.13, 14.45 and 12.45 µg.100mg^{-1} dry weight, respectively. The interaction between the two factors was significant, with the callus inducted from cotyledon treated with 100 g.L^{-1} of PEG, showed the highest production of thujone, α-pinene and carvacrol at 27.57, 29.33 and 47.20 µg.100mg^{-1} dry weight. Limonene reached 14.96 µg.100mg^{-1} dry weight, at 150g.L^{-1} of PEG treatment in callus inducted from the hypocotyl.

Keywords: plant tissue culture, hypocotyl, cotyledon, poly ethylene glycol.

مجلة العلوم الزراعية العراقية -2018 :49(4):534-540 **نعمة**

إنتاج بعض المركبات التربينية خارج الجسم الحي لنبات الحبة السوداء *Nigella sativa* بإختلاف الجزء النباتي وتراكيز الكلايكول متعدد الأيثلين

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المُستخلص

نُفذت تجربة مختبرية، بهدف دراسة تأثير الجزء النباتي وتركيز الكلايكول متعدد الأثيلين في إنتاج بعض التربينات الأحادية من كالس نبات الحبة السوداء *Nigella sativa*. أثر الجزء النباتي معنوياً وحقق الكالس المُستحث من السويقة الجنينية السفلى زيادة معنوية في الوزن الطري والجاف بلغ 282.8 ، 25.25 ملغم بالتتابع، كما حقق زيادة في إنتاج مركب borneol بلغ 26.70 مايكروغرام. 100ملغم$^{-1}$ وزن جاف. بينما حقق الكالس المُستحث من الورقة الفلقية زيادة معنوية في إنتاج مركبات thujone و α–pinene و camphene و carvacrol بلغ 24.01 و 20.58 و 13.58 و 23.80 مايكروغرام.100ملغم$^{-1}$ وزن جاف بالتتابع. في حين سببت تراكيز الكلايكول متعدد الأثيلين زيادة في إنتاج بعض المركبات التربينية وحقق التركيز 100غم.لتر$^{-1}$ أعلى معدل لإنتاج مركبي borneol و carvacrol بلغ 36.60 و 37.80 مايكروغرام.100ملغم$^{-1}$ وزن جاف بالتتابع. بينما حقق التركيز 150غم.لتر$^{-1}$ أعلى إنتاج لمركبات thujone و α–pinene و camphene وmycene بمعدل بلغ 23.29 و 21.13 و 14.45 و 12.45 مايكروغرام.100ملغم$^{-1}$ وزن جاف بالتتابع. كما أثر التداخل بين عاملي الدراسة معنوياً وكانت معاملة الكالس المُستحث من الورقة الفلقية عند التركيز 100غم.لتر$^{-1}$ من الكلايكول متعدد الأثيلين هي من أظهرت أعلى معدل في إنتاج مركبات thujone و α–pinene و carvacrol بلغ 27.57 و 29.33 و47.20 مايكروغرام.100ملغم$^{-1}$ وزن جاف بالتتابع. ارتفع تركيز limonene عند إضافة 150غم.لتر$^{-1}$ من الكلايكول متعدد الأثيلين، إذ وصل إلى 14.96 مايكروغرام.100ملغم$^{-1}$ وزن جاف للكالس المُستحث من السويقة الجنينية السفلى.

الكلمات المفتاحية: زراعة الأنسجة النباتية، السويقة الجنينية السفلى، الورقة الفلقية، الكلايكول متعدد الأثيلين.

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INTRODUCTION

Nigella sativa L. belongs to the Ranunculaceae family, and it was known for more than 2000 years, It grows in Eastern Europe, the Middle East and West of Asia (24). This lant is known for its medicinal value, due to its high concentration of essential oils (5, 15), from which it derived, its important therapeutic properties such as anti-inflammatory (3, 16), antidiabetic (4), antihypertensive (10), antihistamine (8), antioxidant (21), antitumor (11), antibacterial (2), antihyperlipidemic and immune-system effects (20), anticancer and antimicrobial (11, 20), as well as digestive, diuretics, analgesics, anti-diarrheal and appetite stimulant (12, 19, 23). Plant tissue culture technology is one of the most modern and widely used methods of providing raw materials that can be used for therapeutic purposes or in the pharmaceutical industry. This is because this technology provides these materials throughout the year, thus overcoming all the problems and obstacles that can face traditional agriculture such as needing to cultivate at specific times dealing with adverse environmental conditions, and agricultural pests such as diseases, insects and weeds and costs of harvest and storage conditions. Another advantage of plant tissue cultures is the ability to provide raw materials with high purity with the possibility of controlling the paths of production of these compounds. This could be achieved through the use of some stressors that can stimulate the explant to produce a particular compound with attention to the type and concentration of the compounds used and the nature of the explants and their relation to the target compound to be produced (13). Several studies were revealed the possibility of increasing the secondary compounds produced by the callus tissue induced by different explant when exposing to some of the stressors. These compounds can then be isolated, purified and used as important material in different industries (6, 14). This study aimed to stimulate the production of some terpeniods compounds that are essential oils known for their biological effectiveness within the human body, by using several concentrations of PEG on hypocotyls and cotyledons of *Nigella sativa* L. plant.

MATERIALS AND METHODS

This study was carried out in tissue culture and biotechnology laboratory- Center of Desert Studies- University of Anbar. The *N. sativa* seeds were germinated to obtain sterilized seedlings for use as a source of the explant. Seeds were first washed under top water and then transferred to the biological safety cabinet for surface sterilization using 6.0% sodium hypochlorite with three drops of Tween 20 for 15 minutes (1). Then seeds were washed with sterile distilled water three times to remove excess sterilizing solution and then grown in Screw Vials containing hormone-free MS medium (18) supplemented with sucrose and agar by 30.0 and 7.0 $g.L^{-1}$ respectively. The cultured seeds were incubated at a temperature of 25 ± 1 ℃ and a daily illumination of 1000 Lux for 16 hours. To stimulate callus tissue for both hypocotyl and cotyledon. The resulting sprouts were grown under sterile conditions in vials containing 10mL of media containing 3.0$mg.L^{-1}$ 2.4-D and 1.0 $mg.L^{-1}$ Kin, with the same amount of sucrose and agar in the seed germination process (1). The tested PEG concentration were 0, 50, 100, 150 $g.L^{-1}$ About 100mg of callus tissue, which incubated in the previously mentioned conditions with 10 replicates for each concentration. The fresh and dry weights of the grown callus and the control treatments were calculated using a sensitive balance and seven replicates. The process of extraction was carried out in the laboratories of the Medical Research Department of the Ministry of Science and Technology, using three replicates according to Bos (7). For the extraction of terpeniods compounds, Callus that was induced from the two explants and stressed by PEG was dried at 40°C for 24 hours. Then 100 g. of dried material was ground and 2.0 ml of 96% pure ethyl alcohol was added. The samples were shaked for 24 hours. The solution was than filtered and placed in a beaker in the oven at 40°C for 24 hours to turn it into powder. The powder was then dissolved in 2.0 ml. of pure 96% ethyl alcohol. The active substances in the solution were estimated using HPLC (Shimadzu LC-10 ATVP). The HPLC was used to estimated the quantity and quality of monoterpeniods compounds in the tissues of

grown callus extracts, by injecting 25 µL into a C-18 column with dimensions (50 x 4.6mm ID) and the size of the particles 3µm and 30°C. The monoterpeniods compounds were estimated under the following conditions: methanol/ deionized water (90:10, v/v) mobile phase at a flow rate of 0.8ml.min^{-1}. The quantitative estimation of all monoterpeniod compounds was performed at the wavelength 280nm according the following equation:

$$\textit{terpeniods compound con.} = \textit{terpeniods compound con.} = \frac{\textit{Area of Compound}}{\textit{Area of Standard}} \times \textit{Con. of Standard} \times \textit{No. of dilutions}$$

The concentration of standard compound was 25 mmol.m^{-1}. The number of dilutions was only one for the seven study compounds. The Discovery Genestat version 12.0 software was used to perform statistical analysis of the data obtained using Completely Randomized Design (CRD) design as well calculated the least significant difference (LSD) at a significance level of 0.05.

RESULTS AND DISCUSSION

Fresh weight of the callus

Table 1 shows that there is a significant difference in the fresh weight of the induced hypocotyl and cotyledon, when treated with different concentrations of PEG. Table shows the superiority of hypocotyl by giving the highest fresh weight of 282.8 mg, a significant increase by 17.14% compared to the callus which was induced from cotyledons. The increase in the concentrations of PEG led to a significant decrease in the fresh weight of callus and control gave the highest value, which was 364.5 mg. The PEG concentration of 150 g.L^{-1} gave the lowest value of fresh weight 124.5 mg. The interaction between the two factors of the study revealed that there was a significant differences in fresh weight. The control of PEG with callus induced from hypocotyl gave a significant increase in the fresh weight (388.7 mg). while, the callus induced from cotyledons with a PEG concentration of 150 g.L^{-1} gave the lowest value at 114.0 mg. (Table 1)

Dry weight of the callus

Results in Table 2 indicate that there was a significant difference between the levels of the study factors and the interaction among them in the dry weight of the callus. The callus of the hypocotyl gave the highest value of 25.25 mg while, the callus induced from cotyledon gave a dry weight of 20.33 mg. Also, treatment with different PEG concentrations caused significant differences among them.

Table 1. Influence of explants, PEG concentrations and their interaction on *Nigella sativa* L. callus fresh weight (mg.) after four weeks on MS medium

Concentration (PEG) g.L^{-1}	Explants		Means
	Hypocotyls	Cotyledons	
0	388.7	340.4	364.5
50	353.0	283.8	318.4
100	254.6	227.2	240.9
150	135.0	114.0	124.5
Means	282.8	241.4	
L.S.D 0.05	Explants= 13.14**	PEG=18.59**	Explant×PEG= 26.28*

The control treatment gave the highest value of dry weight of 30.12 mg. An increase in PEG concentration caused a decrease in the dry weight of the callus produced from explant. Both 100 and 150 g.L^{-1} PEG treatment concentration differed significantly compared to control treatment 50 g.L^{-1} and gave 19.76 and 14.91 mg., respectively. There is a significant effect among the treatment interactions in the dry weight of the callus. However, the callus induced from the hypocotyl with the treatment of control PEG was 32.15 mg in dry weight. while, interaction of callus produced from the cotyledon with a 150 g.L^{-1} PEG gave the lowest value of dry weight 11.51 mg.

Table 2. Influence of explants, PEG concentrations and their interaction on *Nigella sativa* L. callus dry weight (mg.) after four weeks on MS medium

concentration (PEG) g.L^{-1}	Explants		Means
	Hypocotyls	Cotyledons	
0	32.15	28.08	30.12
50	29.34	23.43	26.38
100	21.22	18.31	19.76
150	18.30	11.51	14.91
Means	25.25	20.33	
L.S.D 0.05	Explants= 1.052**	PEG=1.487**	Explant×PEG= 2.103*

Production of terpeniods compounds

Results in Table 3 shows a significant differences in each of explants and the concentrations of PEG in the product quantity of thujone. Callus produced from cotyledon gave the highest value of thujone at 24.01 mg.100 mg^{-1} of dry weight and twice the quantity produced from the treatment of hypocotyl. In addition, the treatment of PEG resulted in changes in the level produced of this compounds. The PEG concentration of 150 $g.L^{-1}$ gave the highest dry weight of 23.29 $\mu g.100mg^{-1}$ and was superior compared to the other concentrations treatments (0, 50, 100) mg , which gave 11.61, 17.11 and 19.99 $\mu g.100mg.L^{-1}$ dry weight, respectively (Table 4). A significant difference was observed in the rate of compound production due to the interaction between the explants types and the PEG concentrations. The cotyledon callus with the PEG concentration of 100 $g.L^{-1}$ gave the highest amount of compound at 27.57 $\mu g.100mg^{-1}$ dry weight, while the hypocotyl callus tissue that was non-treated with PEG gave the lowest value of compound 5.83 $\mu g.100$ mg^{-1} dry weight (Table 5). Table 3 shows, the concentration of α-pinene compound was significantly different, with the highest value of 20.58 $\mu g.100$ mg^{-1} dry weight obtained from hypocotyl callus. The treatment of cotyledon callus gave the lowest value of 8.48 $\mu g.100mg^{-1}$ dry weight. PEG concentrations caused a significant change in the production of α-pinene compound, with the concentration of 150$g.L^{-1}$ giving the highest amount of 21.13 $\mu g.100mg^{-1}$ dry weight. However, the value was not significantly difference with that obtained from the PEG concentration of 100 $g.L^{-1}$. The increase was above the level of significance compared to amount of compound obtained from the control treatment and the 50 $g.L^{-1}$ treatment which gave 7.60 and 10.76 $\mu g.100$ mg^{-1} dry weight respectively (Table 4). The Interaction between the two factors resulted in a significant increase in the amount of this compound among the 150 studied treatments. The interaction treatment of the concentration of 100$g.L^{-1}$ cotyledon callus gave the highest mean of α-pinene of 29.33 $\mu g100.mg^{-1}$ dry weight and it was higher than most of the other study treatments. The control on hypocotyl callus gave 4.26 $\mu g.100$ mg^{-1} dry weight (Table 5). The results of Table 3 indicate that the production of camphene compound was affected by the two studied factors. The cotyledon callus gave a significant increase in its production of camphene compound at 13.58 $\mu g.100\mu g.mg^{-1}$ dry weight which is 31.77% higher compared to those produced by the hypocotyl callus. The rate production of this compound increased significantly by increasing the PEG treatment concentration. The PEG concentration of 150 $g.L^{-1}$ gave the highest amount of this compound at 14.45 $\mu g.100$ mg^{-1} dry weight. However, this was non-significantly different compared to the amount of compound obtained from the100$g.L^{-1}$ PEG treatment 12.57 $\mu g.100mg^{-1}$ dry weight,but significantly exceeded the control treatment and the 50$g.L^{-1}$ treatment 5.71 and 7.08 $\mu g.100mg^{-1}$ dry weight, respectively. Results shows non-significant effect of interaction in the production of the camphene compound (Table 5). Results shows in Table 3 non-significant differences between the explants types which were used as sources of callus in the production of mycene compound, while the different PEG concentration led to a significant difference in the production rate of the mycene compound. The PEG concentration of 150$g.L^{-1}$ gave 12.45 $\mu g.100mg^{-1}$ dry weight and it was significantly different compared to the control treatment and 50$g.L^{-1}$ PEG 5.13 and 7.64 $\mu g.100mg^{-1}$ dry weight, respectively (Table 4). The treatment interaction did not had a significant effect on the production of this compound (Table 5). As shown in Tables 3 and 4, there is non-significant difference in both of explants type and PEG concentrations in the production of limonene compound *in vitro*. However, the interaction between factors caused a significant difference in compound production (Table 5). The highest amount of this compound was 14.96 $\mu g.100mg^{-1}$ dry weight, which was obtained from hypocotyl and it significantly exceeded all of the studied treatments. Whereas the callus from the same explant that was non-treated with PEG gave the lowest production of limonene at 5.54 $\mu g.100$ mg^{-1} dry weight. Results Tables 3 and 4 shows that the level of production of borneol compound was

significantly different depending on the type of explant and PEG concentration. The hypocotyl callus was superior and gave the highest rate of this compound at 45.90% compared to the that produced by the cotyledon callus. An increase in the concentration of PEG gave a significant increase in the production of this compound, the concentration 100g.L^{-1} producing the most borneol at 36.60 µg.100 mg^{-1} dry weight. Which was significantly higher than all of the other studed factors. There is non-significant differences between the study factors in the amount of the production of borneol compounds (Table 5). As shows in Table 3, there was a significant effect of the explants used in the production of the carvacrol compound. The cotyledon callus was superior by producing 23.80 µg.100 mg^{-1} dry weight, compared to the hypocotyl callus. In addition, PEG treatments significantly increased carvacrol production, with 100g.L^{-1} PEG producing the highest value of 37.80 µg.100 mg^{-1} dry weight. This was significantly different compared to all of the other study treatments (0, 50, 150 g.L^{-1}) which gave 9.40, 16.00 and 18.60 µg.100mg^{-1} dry weight respectively (Table 4). Table 5 shows a significant effect of the interaction between the type of explant and PEG concentration. The PEG concentration of 100 mg.L^{-1} with callus indiucted from the cotyledon gave the highest amount of this compound at 47.20µg.100mg^{-1} dry weight, which was significantly different from all others concentration. The cotyledon callus without addition of PEG gave the lowest compound production of 8.50 µg.100 mg^{-1} dry weight. The differences between the explants types in fresh weight are caused by fundamental differences in the type and nature of the original tissue. As well as, there was a difference in their hormonal content, so the effect of stimulation was different between them (17). Results shows that polyethylene glycol (PEG) caused a significant increase in the production of terpenoids compounds and it may be the cause that the growing cells undergoing drouhgt stress may produce polyamines, which is the key to the formation of many secondary compounds (9). It increases the osmosis pressure of the cell, increases exchange of solubility in the cell and increases the synthesis of these compounds (22). The explant differed in their response to the concentration of PEG. For example, in Tables 3 and 4, found that the limonene compound was not significantly affected by explant types or addition of PEG. But, the interaction between them reached to the limits of the significance, thus indicating the explant has different responses to the PEG concentration (Table 5).

Table 3. *In Vitro* production of terpenoids compounds (µg.100 mg^{-1}dry weight) from callus induced from explants type of *Nigella sativa* after 30 days of culture

Explants	Thujone	α-pinene	Camphene	Mycene	Limonene	Borneol	Carvacrol
Hypocotyls	11.99	8.84	6.33	8.34	10.42	26.70	17.10
Cotyledons	24.01	20.58	13.58	9.94	10.32	18.30	23.80
L.S.D 0.05	1.909**	3.428**	2.045**	N.S	N.S	6.890**	5.560**

Table 4. *In Vitro* production of terpenoids compounds (µg.100 mg^{-1}dry weight) with different PEG concentrations of callus induction from *Nigella sativa* after 30 days of culture

Con. (PEG)	thujone	α-pinene	Camphene	Mycene	limonene	Borneol	Carvacrol
0	11.61	7.60	5.71	5.13	8.77	14.40	9.40
50	17.11	10.76	7.08	7.64	8.86	19.20	16.00
100	19.99	19.15	12.57	11.34	12.65	36.60	37.80
150	23.29	21.13	14.45	12.45	11.20	19.80	18.60
L.S.D 0.05	2.700**	4.849**	2.892**	4.098**	N.S	9.750	7.890**

Table 5. *In Vitro* production of terpenoids compounds (µg.100 mg^{-1}dry weight) with intraction between explants type and PEG concentrations of callus induction from *Nigella sativa* after 30 days of culture

Con. (PEG)	Thujone	α-pinene	Camphene	Mycene	limonene	borneol	Carvacrol
	Hypocotyls						
0	5.83	4.26	2.90	4.29	5.54	16.10	10.20
50	8.74	6.43	3.74	6.62	8.66	26.20	16.00
100	12.42	9.36	7.25	10.25	12.53	40.50	28.50
150	20.97	15.31	11.44	12.19	14.96	24.00	13.60
	Cotyledons						
0	17.40	10.95	8.52	5.96	11.99	12.70	8.50
50	25.48	15.09	10.42	8.66	9.06	12.20	16.00
100	27.57	29.33	17.89	12.42	12.78	32.80	47.20
150	25.60	26.96	17.47	12.72	7.45	15.60	23.70
L.S.D 0.05	3.819**	6.857*	N.S	N.S	4.591**	N.S	11.12*

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