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Original Article

Effect of some elicitors on chemicals composition for *Nigella sativa* callus cultures

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ABSTRACT

Callus culture from leaf explants of *Nigella sativa* was used to study the effect of silver nitrate ($AgNO_3$) and salicylic acid (SA) at 0.5mM as elicitor compounds on the chemical composition of *N. sativa* for two and four days. Total fatty acids and fatty acid esters increased with $AgNO_3$ addition for two days represent 29.73% of total constituents, while essential oils constituents were enhanced by salicylic acid addition for two days represent 79.31% of total constituents. These elicitors (AgNO3 and SA) proved to be good elicitor compounds to increase secondary metabolites (fatty acids and essential oil) in *N. sativa* callus culture.

Key words: *Nigella sativa*, Sliver nitrate, Salicylic acid, Fatty acids, Essential oil, GC-MS analysis

INTRODUCTION

Plant volatile and nonvolatile secondary metabolites have wide applications in dietary regimens, food flavoring and preservation, folk medicine and fragrance industry. Application of plant materials as dietary regimens and preservatives is mainly due to their antioxidant, antimicrobial and other biological potentials. In this regard, a growing rate of research was conducted on many plant species in order to find new natural bioactive compounds in them.

Nigella sativa L. (Ranunculaceae) plant commonly known as Black seed or Black cumin a small elegant herb believed to be endogenous to the Mediterranean region but has been cultivated in other parts of the world including India and Pakistan [1]. Studies have revealed various therapeutic values of *N. sativa* such as anti-inflammatory, anti-analgesic, anti-stress, anticancer, antioxidant, antibacterial, antifungal, anti-parasitic and anti-asthmatic [2,3,4]. These activities have been predominantly attributed to the presence of active compounds in the fixed oils, volatile oils and different extracts studied [5]. The volatile oil of the seed (0.5–1.6%) is composed mainly of the monoterpenes p-cymene, γ-terpinene, α-pinene, β-pinene, α-thujene, carvacrol, thymol and thymoquinone [6,4]. Thymol, is one of the active compounds in *N. sativa* extract, plays an important role in the inhibition of cancer cells, has antibacterial activity against oral bacteria also, anti-inflammatory activity, fungicidal activity, carcinogenicity and can attach with the mutagenic substance, because thymol is one of the antioxidant phenolic compounds [7,8].

Therefore, *in vitro* produced cultures can be used an alternative for meeting out the demand of secondary metabolites within reasonable time and obtain them in large amount. Plant tissue culture refers to growing and multiplying of the cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment. Different plant tissue culture strategies have been extensively studied to improve the production of plant chemicals. Elicitation being one of them is done to enhance the production of secondary metabolites in plant cultures by using biotic/a biotic molecules. They are considered as signaling molecules as their incorporation in the cultures generates signal transduction cascade and leads to activation and expression of the related genes with the biosynthesis of metabolites and also stimulates plant's antioxidant defense system [9,10].

Shoot differentiation occurrence upon omission of coconut milk first studied, then the auxin from the medium or upon supplementation with indol acetic acid (2.0 mg/L) and coconut milk (15%) [11]. Production of Thymol from the leaf callus cultures of *N. sativa* under different hormonal effects has

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also been reported [7]. Some authors indicated the difficulty of inducing the synthesis of secondary products in suspension cultures of this species [12].

Therefore, the present study was proposed to investigate the effect of elicitor compounds (silver nitrate and salicylic acid) on chemicals composition of *N. sativa* callus culture.

MATERIALS AND METHODS

This study was carried out in Plant Biotechnology Department, Genetic Engineering and Biotechnology Division, National Research Centre, Egypt. Seeds of *Nigella sativa* were obtained from the Experimental Farm of Pharmacology, Faculty of Cairo University.

Sterilization and incubation conditions*:* Seeds were surface sterilized by washing thoroughly under tap water then rinsed with 70% ethanol for 30 seconds followed by washing with sterile water twice. They were then immersed in 50% sodium hypochlorite solution for 15min after which they were rinsed with sterile water four times and cultured on MS-medium [13] free hormones containing 3% sucrose and 0.7% agar. Seeds were transferred in a growth chamber at 26±1ºC under light conditions of 16 h photoperiod. After one-month seedlings were used as starting plant material for callus initiation.

Callus induction and maintenance: Stem, root and leaf segments were separated from the produced seedlings and cultured on solidified MS medium supplemented with different combinations of 2,4-dichlorophenoxy acetic acid (2,4-D) 1.0 and 2.0 mg/l and kinetin (kin) 0.5, 1.0, 1.5 and 2.0 mg/l. Cultures were kept in the culture room under temperature of 26±1ºC and light conditions of 16 h photoperiod. Initiated callus were observed after 4 weeks of cultivation then the percentage of callus induction was calculated based on the following equation.

Callus induction % =

$$\frac{\text{Number of calli}}{\text{Number of explants inoculated}} \times 100$$

Cell suspension cultures and elicitor compound addition: Cell suspension cultures were derived from friable callus of leaf explant and cultured in Erlenmeyer flasks (250 ml) containing 100 ml of liquid MS medium supplemented with 2,4-D (1.0 mg/l) and kinetin (1.5 mg/l). A biotic elicitors [Silver nitrate ($AgNO_3$) and Salicylic acid (SA)] at 0.5 mM were filter sterilized through a 0.45 µM bacteria-proof filter (Millipore) and added into sterile liquid media, callus control not contain elicitor compound. The cell suspensions were maintained at 110 rpm on a rotary shaker at 26±2C and 16 h photoperiod. The cells were harvested after two and four days of exposure for oil extraction.

Oil extraction from different N. sativa extracts: Extraction of *Nigella sativa* L. samples performed according to previous method [14]. The sample flask was charged with 5 g of seeds or dried calli then 30 mL of the mixture of acetone-dichloromethane (1:1 v:v) was added and put it in the boiling water bath under reflux for 3 h. The temperature of extracting solvent under reflux was about boiling point of solvent mixture nearly 80°C then the mixture of solvent separated and evaporated, the residues analyzed by GC-MS.

Gas chromatography-mass spectrometric analysis (GC-MS): The extracted oils were analyzed by gas chromatography-mass spectrometric (GC-MS). The GC-MS analysis was performed using a Thermo Scientific, Trace GC Ultra / ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30m, 0.251mm, 0.1 mm film thickness). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used, helium gas was used as the carrier gas at a constant flow rate of 1ml/min. The injector and MS transfer line temperature was set at 280 °C. The quantification of all the identified components was investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC-MS system.

RESULTS AND DISSCUSION (done)

The *N. sativa* extracts were coded as S (seeds), C (leaf callus control), Ag_2 (leaf callus treated with silver nitrate for two days), Ag_4 (leaf callus treated with silver nitrate for four days), SA_2 (leaf callus treated with salicylic acid for two days) and SA_4 (leaf callus treated with salicylic acid for four days).

Callus induction and maintenance: Leaf, stem and root segments were taken from 30th day *N. sativa* seedling and transferred on solidified MS medium supplemented with different concentrations of 2,4-D and kinetin. The percentage of callus induction of different explants was recorded as shown in table (1) and Fig (1). Data revealed that, leaf segments gave the best response of callus induction with green friable callus. The combination of 1.0 mg/l 2,4-D and 1.5 mg/l kin obtained the best results from three explants (leaf, stem and root). When 2,4-D concentration increased to 2.0 mg/l the

callus formation was decreased from three explants, moreover when the kinetin concentration increased with 2.0 mg/l 2,4-D the callus formation was inhibited from three explants. From these results, it can be summarized that the combination of 1.0 mg/l 2,4-D and 1.5 mg/l kin was most suitable for callus induction from three explants of *N. sativa* seedling, based on this result, the callus from three explants sub cultured on the best medium for three months. The callus derived from leaf explants gave the greatest amount of callus (Fig. 1), then callus derived from leaf explants treated with elicitor compounds (silver nitrate and salicylic acid) at 0.5 mM for two and four days. In this point the effect of different Kinetin (Kn) and Naphthalene acetic acid (NAA) concentrations on the callus induction of *Nigella sativa* seedling from different explants (leaf, epicotyls, hypocotyls and root) was studied [15].They revealed that MS media supplemented with 2.0 mg/l Kn and 1.0 mg/l NAA gave best callusing, also the epicotyls segment gave best and fast response with creamy white to greenish friable callus followed by leaf disc segment which gave compact, green callus but callusing from leaf explants was delayed when compared to epicotyls whereas, root and hypocotyls segments did not responded towards callusing. In our study, Kin was used at concentration 1.5 mg/l and the same concentration of auxin (1.0 mg/l 2, 4-D) to gain the highest percentages of callus induction from all explants examined.

***GC-MS analysis of different N. sativa extracts*:**

a. Fatty acids and Fatty acid esters constituents: For the mass production of leaf calli in comparing with others explants, the experiments for studying the effect of elicitation on the composition of callus oils were done.

Data tabulated in table (2) showed the GC-MS analysis of different *N. sativa* extracts which detected 21 fatty acids (FAs) and fatty acid esters (FAEs) among these different extracts, the highest level of total FAs and FAEs was obtained in the extract of leaf calli cultures exposed to 0.5 mM of $AgNO_3$ as elicitor for two days representing 29.73 % of total constituents followed by calli extract affected by 0.5 mM of SA as elicitor for two days representing 16.17 % of total constituents. Whereas the lowest FAs and FAEs levels were detected in the controlled calli extract (0%). All FAs were found in the ester form except three FAs are 17-Octadecynoic acid, Oleic acid and Icosapentaenoic acid.

Unsaturated FAs and FAEs was the most common in all extracts with the chain length (C9-C22) carbon atoms. Octadecadiynoic acid methyl ester (C18:2) was the most presence with various double bond substitutions. 2,5-octadecadiynoic acid methyl ester (C18:2) was found in high level in Ag_2 extract (20.92%) followed by hexadecatrienoic acid methyl ester (C16:1) in SA_2 extract (7.11% of all constituents). Three saturated F.A.Es were found including Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester and Stearic acid,3-octadecyloxy propyl ester in SA_4 extract and Methyl-9,9,10,10-D-4-octadecanoate in Ag_2 extract.

From these results it could be concluded that FAs and FAEs in the seeds extract differs from of those acids in the treated leaf callus cultures. Treating callus cultures of *N. sativa* with both $AgNO_3$ and SA as elicitor compounds increased total FAs and FAEs in comparison with untreated callus cultures and seeds extracts. Also, using of elicitor compounds ($AgNO_3$ and SA) for two days was better than using of these elicitors for four days.

Silver nitrate has been proven to be a good a biotic elicitor for accumulation of Spiroketal enol ether diacetylenes in Feverfew hairy root cultures [16], lettucenin A in lettuce [17] and sakuranetin in paddy leaves [18].

b. Essential oil constituents: The GC-MS analysis of different *N. sativa* extracts showed 40 different compounds for essential oil as shown in table (3).

The levels of essential oil ranging from 1.83% to 79.31% of total constituents, the largest amount of essential oil was found when the leaf callus extract was treated with 0.5 mM of SA as elicitor for two days (SA_2 extract) followed by using 0.5 mM of SA for four days (SA_4 extract) representing 79.31% and 21.07% respectively of total constituents. Whereas the lowest essential oil percentage (1.83% of total constituents) was found in the seeds extract (S). SA_2 extract showed the highest number and level of essential oils compared with the other treatments, it contained 15 different essential oils, 1,8-cineole was the highest percentage (39.56% of total constituents) followed by Ledene oxide (II) representing 15.40% of total constituents, while trans-Z-α-Bisabolene-Epoxide (0.26%) was the lowest percentage. The essential oil level in SA_4 extract (21.07%) was found as butyled hydroxyl toluene as the single essential oil constituent.

Whereas the seed extract (S extract) had the lowest essential oil level (1.83% of total constituents) because it contained a small amount of different essential oils constituents such as trans-Caryphylene (0.04% of total constituents) it was found in both Ag_2 and Ag_4 extracts with higher

quantities representing 1.79% and 1.10% respectively of total constituents. Also dotriacontane was found in C, Ag_2 and Ag_4 extracts with higher levels representing 5.96%, 1.10% and 0.67% respectively of total constituents. Some essential oil constituents such as trans-caryphyllene, caryophyllene oxide, butyled hydroxyl toluene and dotriacontane were most common in different extracts.

Thymol and thymoquinone (the major compounds of *N. sativa*) does not exist in different extracts may be for extraction method (boiling under reflux) was unsuitable for extracted them.

From pervious results it could be concluded that the using of elicitor compounds have a positive effect on essential oil constituent's formation. Calli treated by elicitors ($AgNO_3$ and SA) for two days was better than the same treatment for four days.

In this issue [19] cleared that the using of 0.5 mM silver nitrate was successful in eliciting thymol at the highest concentration within 48 hrs of exposure in *Thymus Vulgaris* plant as well as salicylic acid (SA) was effective in inducing secondary metabolites formation in plant cell culture or *in vivo* plants [20], they applied SA as elicitor compound at grown field lemon verbena (*Lippia citriodora*) plant and found that, SA application increased the content of some essential oils constituents including β-Pinene, 1,8-Cineol, ɣ-Terpinene, Terpinolene, cis limonene oxide, α-terpineol, neral, α-terpinyl acetate, geranyl acetate, ɣ-elemene, α-humulene, cubenol, spathulenol, globulol and epi-α-cadinol.

c. Other identified compounds: Data tabulated in table (4) show some compounds were detected in different extracts by GC- MS analysis, the highest one is diisooctyl phathalate (86.72%) found only in the control extract (C) followed by dioctyl phathalate found at high level in Ag_4 and SA_4 extracts (81.76% and 69.05% respectively). Also 2-pentanone-4-hydroxy-4-methyle found in the S and SA_4 extracts, it was higher in the S extract (8.25%). Other identified compounds were appeared at low levels in different extracts such as 24,25-dihydroxycholecalciferol, cholic acid, bis(2-ethylhexyl) phathalate, 1,25-dihydroxy vitamin D2 and ethyl iso allocholate.

CONCLUSION

The plan of the present work was to investigate the effect of some elicitor compounds on the chemical composition of *Nigella sativa* leaf callus culture in comparison with chemical composition with seeds extract and untreated calli. The study revealed that, using of silver nitrate and salicylic acid as elicitor compounds have a clear effect on the quantity and quality of chemical composition (fatty acids and volatile oils). These elicitor compounds enhanced total fatty acids and volatile oils constituents also, it led to the emergence of new compounds not found in both callus cultures control and seed extracts. Finally tissue culture techniques can be used to produce a new and different secondary metabolites from those found in the mother plant at higher ratio.

Table1. Effect of different combinations from 2,4-D and kinetin on callus induction from stem, root and leaf explants of *N. sativa*.

Concentration (mg/l)		% of callus induction		
		Stem	Root	Leaf
2,4-D	Kin			
1.0	0.1	85	83	87
1.0	0.5	83	55	85
1.0	1.0	80	77	84
1.0	1.5	100	90	100
2.0	0.1	66	66	70
2.0	0.5	0	0	0
2.0	1.0	0	0	0
2.0	2.0	0	0	0

Table 2. Percentage of fatty acids and fatty acid esters composition in different *Nigella sativa* extracts.

F.A. names	Molecular formula	Carbon chain of FA	% of FAs and FAEs in different samples					
			S	C	Ag_2	Ag_4	SA_2	SA_4
Ethyl-2,3-nonadienoate	$C_{11}H_{18}O_2$	C9:2	0.05					
Methyl-3,5-tetradecadiynoate	$C_{15}H_{22}O_2$	C14:2	0.08					
Hexadecatrienoic acid methyl ester	$C_{17}H_{28}O_2$	C16:1					7.11	
9,12-Hexadecadienoic acid, methyl ester	$C_{17}H_{30}O_2$	C16:2						0.22
7,10,13-Hexadecatrienoic acid, methyl ester	$C_{17}H_{28}O_2$	C16:3	0.10					
10-Heptadecan-8-ynoic acid methyl ester	$C_{18}H_{30}O_2$	C17:2			0.92		7.81	
17-Octadecynoic acid	$C_{18}H_{32}O_2$	C18:1					0.15	
Oleic acid	$C_{18}H_{34}O_2$	C18:1						0.09
Methyl-9,9,10,10-D-4-octadecanoate	$C_{19}H_{34}D_4O_2$	C18:0			4.11			
2,5-Octadecadiynoic acid methyl ester	$C_{19}H_{30}O_2$	C18:2			20.92		0.16	
6,8-Octadecadiynoic acid methyl ester	$C_{19}H_{30}O_2$	C18:2				0.79		
8,11-Octadecadiynoic acid, methyl ester	$C_{19}H_{30}O_2$	C18:2				0.23		
9,12-Octadecaneynoic acid methyl ester	$C_{19}H_{32}O_2$	C18:2			2.06			0.16
11,14-Octadecadiynoic acid methyl ester	$C_{19}H_{30}O_2$	C18:2			0.96			
Methyl 9,12-epithio 9,11-octadecanoate	$C_{19}H_{32}O_2S$	C20:5			0.76			
Icosapentaenoic acid	$C_{20}H_{30}O_2$	C20:5			0.76			
6,9,12,15-Docosatetraenoic acid methyl ester	$C_{23}H_{38}O_2$	C22:4					0.94	
Cis 7,10,13,16 docosatetraenoic acid methyl ester	$C_{23}H_{38}O_2$	C22:4				0.27		
Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	$C_{35}H_{68}O_5$	C16:0						0.35
Stearic acid,3-(octadecyloxy) propyl ester	$C_{39}H_{78}O_3$	C18:0						0.06
Oleic acid, 3-(octadecyloxy) propyl ester	$C_{39}H_{76}O_3$	C18:1				0.46		
Total (%) of total constituents			0.23		29.73	1.75	16.17	0.96

Table 3. Percentage of essential oils composition in different *Nigella sativa* extracts.

Essential oil name	% of essential oil in different samples					
	S	C	Ag_2	Ag_4	SA_2	SA_4
A-Elemene			1.13			
1,8-Cineole					39.56	
R-Limonen					0.48	
Cis-Ocimene				0.27	1.83	
Cis-4-Thujanol					0.66	
Trans-4-methoxy-Thujane	0.05					
Linalool formate	0.08					
Dihydro-Carveol					4.11	
Trans longipino-Carveol			0.72			
Isopino-Carveol					1.32	
Camphor		0.55				
Isomenthone				0.51		
A-Copaene			1.91	0.95		
A-Bourbonene				0.98		
Trans-Caryphyllene	0.04		1.97	1.10		
Aromandendrene					1.60	
Alloaromadendrene				0.35	1.89	
Trans-Z-α-Bisabolene-Epoxide					0.26	
A- Muurolene				0.29		
Junipene	0.51			0.17		
Benzyl benzoate		0.70				
Butyled hydroxyl toluene		3.88			6.94	21.07
D-Nerolidol	0.68					
Globulol					4.06	
Epiglobulol					0.27	
Caryophyllene oxide		0.33	1.28		0.27	
Ledene oxide (II)					15.40	
Calarene epoxide				4.21		
Isoaromadendrene					0.57	
Alloaromadendrene oxide (II)			4.14	0.26		
Cubedol				1.91		
Methyl jasmonate			1.28			
Hexa-hydrofarnesl		0.52				
Cedran-diol (8s,14)	0.04					
Cembrene	0.30					
Eicosane, 1-chloro	0.04					
Docosane	0.04					
Heptacosane			1.83			
Nonacosane			1.83			
Dotriacontane	0.05	5.96	1.10	0.67		
Total (% 0f total constituents)	1.83	11.94	17.19	11.67	79.31	21.07

Table4. Percentage of other identified compounds in different *Nigella sativa* extracts.

Compound name	% of compounds in different samples					
	S	C	Ag_2	Ag_4	SA_2	SA_4
2-Pentanone-4-hydroxy-4-methyl	8.25					4.00
24,25-Dihydroxycholecalciferol	0.17		1.48			
Cholic acid	0.10		0.78			
Bis(2-ethylhexyl) phathalate	0.29			0.16		
Dioctyl phathalate	6.98		11.43	81.76		69.05
Diisooctyl phathalate		86.72				
1,25-Dihydroxy vitamin D2			3.56	0.34		
Ethyl iso allocholate			2.44	0.14	0.24	0.31

Figure 1. *Nigella sativa* callus culture from root (R), stem (S) and leaf (L) explants after three months of sub culturing on MS-medium containing 1.0 mg/l 2,4-D and 1.5 mg/l kin.

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