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The effect of biotic and abiotic elicitors on Dianthalexin production from the callus of *Dianthus caryophyllus*

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Abstract : Biotic (*Fusarium oxysporum*) and abiotic (salicylic acid) as an elicitors were examined on the induction of phytoalexin (Dianthalexin) from carnation (*Dianthus caryophyllus*) callus from leaf. MS medium with BA in concentrations (0.0, 0.5,1.0, 1.5, 2.0 or 2.5) mg/l and 2,4-D (0.0, 1.0, 2.0, 3.0, 4.0 or 5.0) mg/l which use for callus induction, fungal solution in (0.0, 2.0 or 4.0) ml/l and (0.0, 1.0 or 2.0) mg/l of salicylic acid were added to medium. Alcoholic extraction of callus tissues was analyzed by high-performance liquid chromatography (HPLC). The results showed that the dianthalexin highest level (58.29) µg/ml on MS medium with 1.0 mg/L BA and 2,4-D and fungal elicitor was used at 1.0ml/l in three days period followed salicylic acid (58.19) µg/ml after six days incubation period.
Keywords : Phytoalexin, elicitors, *Fusarium oxysporum*, callus induction.

Introduction:

Carnation (*Dianthus caryophyllus*L.), family Caryophyllaceae is one of the ornamental plants, it is grown in many countries on the world and believed it's to be the native of Mediterranean region(1).It is one of the most economically important cut flowers which due to the beauty of its colors and distinctive smell. It has pharmacological and aromatic properties which can use for the medical purposes. There are many therapeutic benefits of carnation oil for example anti-inflammatory, active as a muscle relaxant, tonic for strengthening the stomach and for dental pain (2,3).

Carnation plant has alkaloids, saponins, phenolic compounds, volatile oil. Some of these secondary metabolites are plant defense compound like phytoalexin.

Phytoalexins are low molecular weight antimicrobial compounds both synthesized by and accumulated in plants as a response to biotic like fungus or becteria and abiotic like salts or metals. Inducible secondary metabolites possessing antimicrobial activity toward phytopathogens (3,4). Structurally diverse antimicrobial secondary metabolites are produced by plants in response to infection by microorganisms.(5)Phytoalexins accumulate at infection sites and they inhibit the growth of fungi and bacteria in vitro therefore, it is logical to consider them as possible plant defense compounds against diseases (6).

Dianthalexin an alkaloidal phytoalexin isolated from elicited tissue of *Dianthus caryophyllus*. It is a key structural fragment in a range of biologically active compounds. Work by medicinal chemist had led to a number of drugs. Dianthalexin have cytotoxic activity, herbicidal properties, act as novel active substances for cardiovascular system and have relaxing effect on smooth muscle (6,7).

Plant tissue culture is one of the technique can provide the better alternative for the large-scale production of secondary metabolites by manipulating the conditions of environment and medium, selecting high

yielding cell clones, precursor feeding, elicitation and hairy root culture.(2,8).The aims of this study was focused on investigate the effect of biotic and abiotic elicitors to increase the amount of phytoalexin (dianthalexin) in tissue culture.

Materials and methods

Seed culture

Carnation seeds washed by water than surface sterilized with 70% ethanol for 30 second, followed by submerged in 4% sodium hydrochloride (Clorox) for 10 min with continuous shaking to increase the efficiency of sterilization, and then rinsed with sterilized distilled water three-time for (5) minutes at each time to remove the remains of Clorox and planted in universal vials containing MS medium (9) free of growth regulators and a rate one seed in each tube. Culture subjected to photoperiod 16/8 hours (light/dark) in a growth chamber. The temperature was set at 25ºC. Germination was measured after 14 days.

Callus induction

Seedling obtained was cut into 0.5 cm long under aseptic condition and culturing on MS medium supplemented with BA(0.0, 0.5, 0.1, 1.5, 2.0 or 2.5) mg/l and 2,4-D (0.0, 1.0, 2.0, 3.0, 4.0 or 5.0) mg/l

l.**Preparation of abiotic elicitors**

From the best previously step of callus induction, salicylic acid (SA)as abiotic elicitor chosen for culturing in MS medium, it was adding in concentrations (0.0, 1.0 or 2.0) mg/l.

Preparation of biotic elicitors

The fungus *Fusarium oxysporum* was used as biotic elicitor which obtained from microbiology laboratory of Biology Department in Collage of Science for Women.

The fungal strains were cultured on potato dextrose agar (PDA). The cultures were incubated for (5-7) days in 25±2,ºC, then the cultures autoclaved at121ºC for 15 min. The solution obtained was stored at 4^{O}C for future use (10).

Callus culture with elicitors

One gram of callus was cultured on MS media with biotic elicitors at concentrations 0.0, 2.0, or 4.0 ml/l, and with abiotic elicitors at concentrations 0.0, 1.0, or 2.0 mg/l, then incubation at 25±2 ºC for 16/8 (light/ dark)in 3 and 6 days. Five gram of callus powder was extracted with 50 ml (70%) methanol for six hours. The filtrate was evaporated at room temperature, the extract then stored in a refrigerator at 4 ºC for future use.

HPLC analysis

Quantitative and quantitative estimation of dianthalexin in crude extract was performed by using HPLC analysis. The main compounds were separated on fast liquid chromatography(FLC) column beneath the optional conditions:

-Column: phenomenex C-18, 3µm size of particle (50×2.0mm I.D).
-Mobile phase: 0.5% phosphoric acid: methanol, 23:70, v:v
-Detection: under UV at 275 nm.
-Injection volume: 20µl
-Flow rate: 1.4 ml/min.

These concentrations were calculated as follow:

Concentration of compound (µg/g)=$\frac{peak\ area\ of\ compound}{dx peak\ area\ of\ standard}$ × concentration of standard× dilution factor (11)

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters. Least significant difference –LSD test was used to significant compared between means in this study.(12)

Results and discussions

Callus induction was investigated using MS medium supplemented with different concentrations of 2,4-D and BA. Leaf explants were induced callus 100% in medium containing1.0 mg/l 2,4-D and 0.5 or 1.0 mg/l BA after 15 days of culture as showed in table (1).

Table (1) Effect of BA and 2, 4-D on callus induction (%) from leaf explants the response (%) of leaf explants to callus induction after 30 days culture on MS medium.

2,4-D	BA						LSD value
	0.0	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**	
0.0	0.00	80.00	80.00	80.00	70.00	70.00	6.977 *
1.0	70.00	100	90.00	90.00	70.00	80.00	7.561 *
2.0	70.00	90.00	100	80.00	80.00	80.00	7.432 *
3.0	80.00	70.00	70.00	80.00	90.00	70.00	6.936 *
4.0	60.00	80.00	80.00	70.00	80.00	80.00	6.844 *
5.0	60.00	70.00	70.00	80.00	90.00	90.00	7.028 *
LSD value	7.502	7.822 *	8.094 *	7.641 *	7.149 *	7.035 *	---
* (P<0.05).							

Properties of callus were friable, granular, well proliferated and greenish yellow to creamy. The success of the introduction callus from explan due to their quick response (after 5 days) use suitable of type regulators and its concentrations for the appearance of callus.Perhaps due to certain percentage of auxins, cytokinins and different balance between the two influences in the callus growth or because of the high concentrations of BA and lack of compatibility with the internal content of plant hormones explants which used, causing delay or stop the occurrence divisions (2,8).

The effect of *F. oxysporum* and salicylic acid elicitors on stimulation dianthalexin in callus as showed in table(2) and figure 1,2,3. The best results of dianthalexin production when callus was treating with 4.0 ml/l after 3 days (68.05µg/ml) followed by SA at concentration 1.0 mg/l (58.29 µg/ml) compared with control (untreated) which gave (28.15 µg/ml)

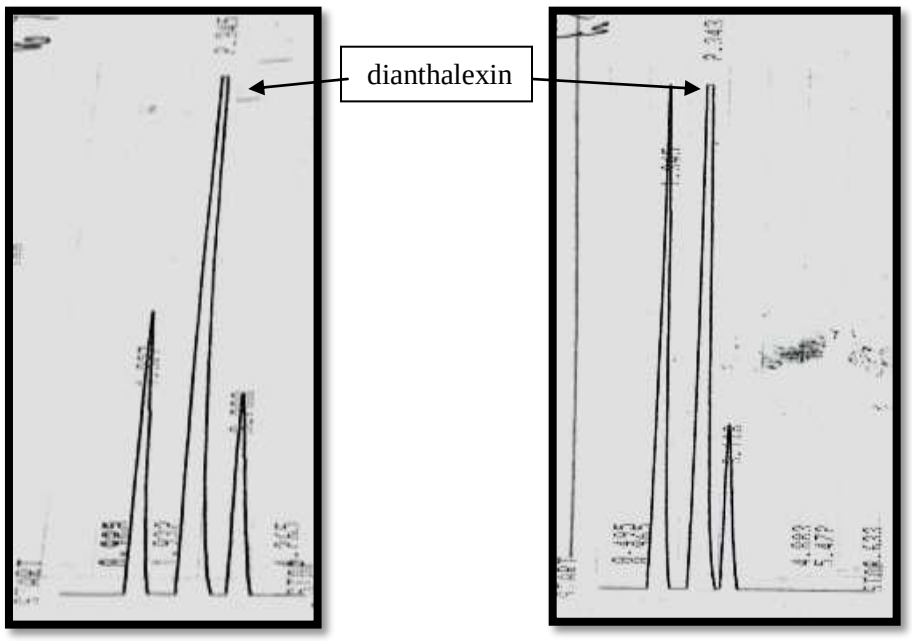
Table (2) Effect of *Fusaruimoxysporum*and salicylic acid on Dianthalexin quantity (µg/ml) estimation of carnation callusafterand6days

Untreated callus(gm)	elicitor	3 days		6 days	
		Con. 1*	**Con. 2****	**Con.1**	**Con. 2**
28.15	*F.oxysporum* (ml/l)	58.19	68.05	38.70	32.51
	Salicylic acid (mg/l)	47.55	29.80	58.29	37.01

Dianthalexin was increased when the plant under stress conditions, so used biotic and abiotic elicitors to stimulate Dianthalexin in *Dianthus caryophyllus* ,*F.oxysporum* enhanced accumulation of Dianthalexin in callus tissue.

(*) con1: 2.0 ml/l F. oxysporum, 1.0 mg/l SA

(**) con2: 4.0 ml/l F. oxysporum, 2.0 mg/l SA



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Figure(1,2) HPLC analysis of callus treated with 4.0 ml/l *F. oxysporum* and callus treated with 1.0 mg/l salicylic acid

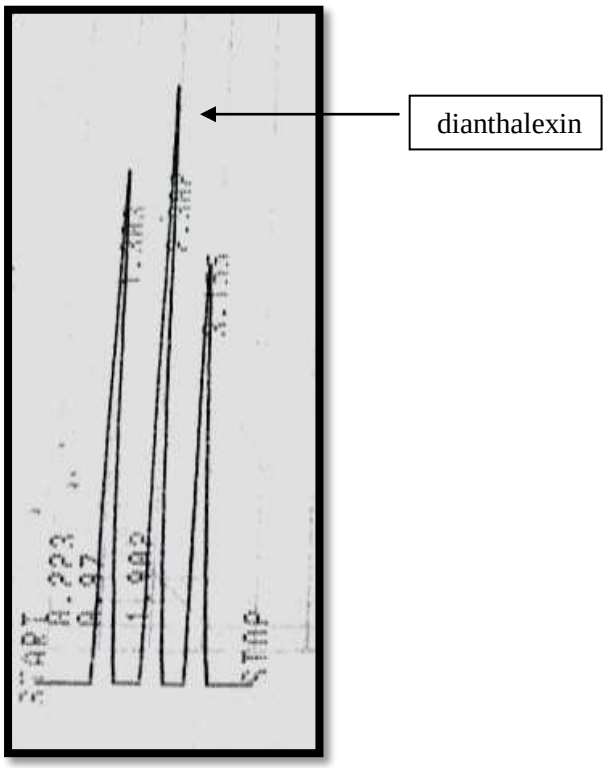

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Figure (3) HPLC analysis for untreated callus

When plant cell culture is exposure to any type of the elicitors a rapid arrangement of biochemical reaction occur like elicitor binding to plasma membrane receptor it means modify in protein phosphorylation pattern, protein kinase activation, mitogen-activated protein kinase and G protein activation (13), then quickchanges in ion fluxes upon the membrane. The ion of C^{+2} stream to the cytoplasm from extra cellular environment and induction of K^{+}andCl^{-} efflux. Thismechanism due to reduce in membrane polarization. (14,15,16)

Inbiotic elicitors state: following detection of microorganism by identification elicitor molecules released through plant pathogen interaction, generally indicative transduction pathways lead to production active oxygen species (AOS), phytpalexin biosynthesis, strengthening of plant cell associated with phenyl propanoid compounds, callus sedimentation , defense enzymes are synthesis , and led to accumulation of pathogenesis-related (PR) proteins (17).AOS cause hypersensitive response (HR) in plants (18).The hypersensitive response is making a trigger by the plant after it recognizes a pathogen, the pathogen identification usually occurs when avirulence (Avr) gene product, which secrete and bind with the product of plant resistense (R) gene . When both the R gene and the corresponding Avr gene are found, recognition occurs, lead to active resistance of the plant and the avirulence of the pathogen. Binding these two partners led to active a single transduction which cause activation plant defense responses (phytoalexin)(19,20).

This result in agreement with (3), who reported similar results for enhancing Dianthalexin in suspension culture of *Dianthus caryophyllus* treated with fungal elicitors.The accumulation of dianthalexin was followed in the cell which treated with elicitor preparation 3 days after inoculation; dianthalexin synthesis began and increases to a maximum about 24 hours after treatment. So, the concentration in the cells decrease gradually and 6 days after treatment only small amounts were detectable (4,21).

The addition of SA promotes accumulation of dianthalexin, the highest concentration of dianthalexin detected at 1.0 mg/l SA which gave 58.19 µg/ml. The responses of culture to elicitation associated with fact that SA is one of the key endogenous signals involve in activation plant defense response and its ability to produce pathogenesis related proteins in a plant, even in the absence of pathogenic organism (22,23).

SA was using as elicitor for production alkaloids, flavonoids, terpenes, phenolic compounds and phytoalexin, Phytoalexin in tissue is decrease when increase SA. Concentration in medid after 96 hours. (24,25)

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