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INFLUENCE OF THE EXTRACT OF BIOTIC ELICITOR *ASPERGILLUS NIGER* ON THE PRODUCTION OF FURANOCOUMARINS IN CALLUS CULTURES OF *RUTA GRAVEOLENS* L.

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Abstract

The study was performed in the laboratory of plant cell and tissue culture, Department of Biology, College of Education for Pure Sciences, University of Diyala. The aim of this study was to investigate the influence of the extract of biotic elicitor *Aspergillus niger* at different concentrations (0.0, 1.0, 1.5, 2.0 ml L^{-1}) on the production of furanocoumarins in callus cultures of *Ruta graveolens* L., after the cultures were growing for 30 days on Murashige and Skoog (1962) (MS) medium supplemented with 2.0 mg L^{-1} 2, 4-Dichlorophenoxy acetic acid (2,4-D) combined with 0.5 mg L^{-1} (Kin), the effect of the extract of *A.niger* on the production of Psoralen, Xanthotoxin and Bergapten was studied. Using Fast Liquid Chromatographic Column (FLCC) Technique, data showed the presence of the above three mentioned furanocoumarins in callus culture when compared with the standard samples. Psoralen, Xanthotoxin and Bergapten recorded their highest concentrations when the extract of the fungus *A.niger* was added to the growth medium at the concentration of 2.0 ml L^{-1}, which reached 2.387 mg g^{-1}, 0.537 mg g^{-1} and 6.606 mg g^{-1} respectively compared with the controls which were 0.913 mg g^{-1}, 0.244 mg g^{-1} and 3.983 mg g^{-1} respectively. The results indicate that plant tissue culture is constant and continuous source for the plant secondary metabolites such as Psoralen, Xanthotoxin and Bergapten and can serve as a suitable alternative for field plants.

Key words : *Ruta graveolens* L., Furanocoumarins, Biotic elicitor, Callus cultures.

Introduction

Ruta graveolens L. is perennial medical plant belongs to the family Rutaceae (Al- Kateb, 2000), widely distributed and has a long history in traditional medicine, as it had been used in medicine for more than 1500 year ago (Bowen *et al.,* 1988). Poutaroud *et al.,* (2000) mentioned that the plant has a strong smell and produce different types of secondary metabolites, that give it the medical and pharmaceutical importance, like essential oils, alkaloids, flavonoids and furanocoumarins (Al- Mahdawe, 2018a). It was also reported that it contains carotenoids, chlorophylls and a number of compounds that have antimicrobial activities such as acridone (Wessner *et al.*, 1999).

Other studies also mentioned that the whole plant and plants cell and tissue cultures produce a lot of secondary metabolites which have medical, pharmaceutical and physiological importance (Karuppusamy, 2009; Al-Mahdawe *et al.,* (2018) a, b,; Bruni *et al.,* 2019; Ahmad *et al.,* 2020).

Plant cell and tissue cultures are considered the fastest typical source, for the in vitro production of continuous large quantities of pharmaceutical compounds, away from the constraints of the environmental conditions without its interference with other compounds, as might happen during its isolation from the whole plant (Karuppusamy, 2009).

Biosynthesis and accumulation of the secondary metabolites occur in the plants exposed to different biotic and abiotic stresses, these compounds allow the plants to stay alive under harsh conditions in their environment. Depending on this principle, some strategies were developed and used to encourage the in vitro production of secondary metabolites, these strategies include treatment with microbial, physical and chemical agents

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known as elicitors (Yue, 2016). It became known now that the use fungi is considered to be one of the best biotic elicitors. Because it stimulate the plant's cells to produce the secondary metabolites such as flavonoids and phenols as in the case of using the fungus *A. niger* as a biotic elicitor (Shanker and Shanker, 2016; Ibrahim *et al.*, 2019). It also plays an important role in the field of biotechnology to produce chemical substances, enzymes and medical drugs (Baker and Bennett, 2008).

Furanocoumarins are a class of phenolic compounds belongs to the secondary metabolites which include 1500 compounds of coumarins (Bougrguad *et al.*, 2006), these compounds are basically found in four families of higher plants namely *Apiacea*, *Rutaceae*, *Moraceae* and *Fabaceae* (Ojala *et al.*, 2000). In general these compounds are more abundant in the flowering plants, mature seeds and fruits (Poutaraud *et al.*, 2000), produced in the plants as a defense mechanism against predators like insects, mammals and fungi. Furanocoumarins have physiological effects, the most important one of these is their interference with the liver enzyme cytochrome P450 which is present in the intestine, as a result, their vital advantages such as anti-inflammatory, antioxidant and anticancer properties were reported by different studies (Diwan and Malpathak, 2009).

The aim of this study is to extract, identify and estimate the above three furanocoumarins from callus cultures of *Ruta graveolens* L.

Materials and Methods

Ruta graveolens L. Seedling were obtained from Baladroze arboretum, Baladroze Agricultural Department which belongs to the Diyala Agricultural Directorate, Diyala, Iraq., and transferred to the green house at the Department of Biology, College of Education for Pure Science, University of Diyala. The plants were monitored and were classified by IBN-AL-HAITHAM herbarium, University of Baghdad.

The stem pieces were cut off from *Ruta graveolens*, washed in a running tap water for 20 minutes prior to its sterilization with sodium hypochlorate (NaOCl) (the percentage of active chloride is 6%), using 1.0 volume of sterilizing material: 9.0 volumes of sterile distilled water for 20 minutes with shaking, then the plant pieces were washed with sterile distilled water three times for five minutes each time, to remove any traces of sterilizing substance. Sterilized stem pieces were cut into 1.0 cm pieces and the sterilized plant materials were transferred on 20 ml of Murashige and Skoog (1962) (MS) solid medium supplemented with plant growth regulators in a 100 ml conical flasks and the samples were kept in growth room at 25 $\pm$ 2°C for a consecutive photoperiod (16 h light/ 8 h dark), (Al-Mahdawe, 2013). MS medium was used in the experiments for callus induction and maintenance supplemented with a suitable growth regulators. MS medium was prepared by dissolving the salts of its constituents in a suitable volume of distilled water, then 7.0 g of agar was dissolved in 500 ml distilled water and added to the first solution and then completed to the final volume of one liter by distilled water and the PH of the medium was 5.7-5.8, then the medium was divided in glass vials each one containing 20 ml and then sterilized by autoclave.

The sterilized stem pieces were used as explants for the callus induction as described by Nadir (2018). The changes that occurred in the plants part was monitored until it is completely converted to callus and the wet weight of the callus was measured after 30 days.

The fungus *A.niger* was isolated from the air according to the method described by Collee *et al.*, (1996). Then the fungus was identified as *A.niger* by method described by Ellis *et al.*, (2007). Using the method mentioned by Vakil and Mendhulkar (2013), the fungus was grown and its extract was prepared.

To study the effect of *A.niger* extract on the callus culture growth and their contents of furanocoumarins, pieces of induced callus from stem explants weighing one (1.0) gram was transferred to glass vials each containing 20 ml of MS medium enriched with 2.0 mg L^{-1} 2,4-Dichloro phenoxy Acetic Acid (2,4-D) combined with 0.5 mg L^{-1} Kinetin (Kin) to which 1.0, 1.5 or 2.0 ml L^{-1} of *A.niger* extract was added, and kept in the growth room for 30 days. After that the wet weight of each callus samples was measured and the callus texture and color was studied. Then its contents of furanocoumarins were determined.

Extraction, isolation and estimation of furanocoumarins from callus samples were performed according to the method described by Manjula and Sushma (2016). The separation and estimation occurred by using Fast Liquid Chromatographic Column (FLCC) instrument Shimaduz 10AV- LC equipped with binary delivery pump model LC-10A Shimaduz, the eluted peaks were monitored by UV-Vis 10A-SPD spectrophotometer. 20 µL of the sample was injected into the FLCC system according to the optimum conditions (Table 1). The concentration for each constituent was calculated by the comparison of the peak area of authentic standard with that of the samples under the same optimum conditions. The concentration of the samples were calculated using the following equation:

$$(X)\text{Concentration} = \frac{\text{Area under the curve of the sample}}{\text{Area under the curve of the standard}} \times \text{concentration of the standard} \times \text{dilution factor}$$

Results and Discussion

The results table 2 showed that the stems grown on MS medium supplemented with the growth regulators were superior in their ability for callus induction compared with the control treatment. MS medium enriched with 1.5, 2.0 mg L^{-1} 2,4-D combined with 0.5 mg L^{-1} Kin stimulated callus induction and achieved highest value (100%) compared with the control treatment whose induction was (0%), the induction rate decreased to 50% in 0.5 and 1.0 mg L^{-1} 2,4-D treatments. The results also indicate that the highest average callus wet weight was recorded at 1.5 and 2.0 mg L^{-1} 2,4-D which reached 1.785 g and 2.584 g respectively. Which was superior to the treatments at the concentration of 0.5, 1.0 mg L^{-1} 2,4-D that were 0.116 and 0.178 g $piece^{-1}$. The explants of the control treatment characterised by their swelling, while the shape of the stem pieces grown on MS medium fortified with 2.0 mg L^{-1} 2,4-D became dumbbell shape, the callus tissue formed in the cutting regions, and was characterized by its friable texture and its light green colour. The explants lost their entire shape and changed to a piece of callus after 30 days of growing on 0.5, 1.0, 1.5 mg L^{-1} 2,4-D. The callus of these concentrations had a solid texture and light green colour.

Table 1: The optimum conditions of identification of standard furanocoumarins from the selected samples.

Mobile phase (V:V)	**0.1% phosphoric acid: Methanol: acetonitrile (10: 45: 45)**
Flow rate of the mobile phase	1ml/ minutes
Volume of the injected sample	25 microliter
Separation temperature	30 ˚C
Type of the detector	UV-Vis 10A-SPD Spectrophotometer at wave length of 280 nm.
The reference	Manjula and Sushma, 2016.

Table 2: Percentage of callus induction, average wet weight and the effect on the callus size of callus derived from sterilized stem of *Ruta graveolens* L., on MS medium supplemented with different concentration of 2,4-D + Kin.

Concentration growth regulators (mg L^{-1})		**Induction %**	**Average of wet weight (g $piece^{-1}$)**	**Callus size**
2,4-D	Kin			
0.0	0.5	0	0.065 D	- - -
0.5		50	0.116 C	+ +
1.0		50	0.178 C	+ +
1.5		100	1.785 B	+ + +
2.0		100	2.584 A	+ + +

Number of repeats: 10 Pieces/ treatment.

The averages that have similar letters do not have significant statistical differences.

Oxygen plays an important role in the process of callus induction through increasing cells activity in building the basic substances for growth. In addition to the effect that results due to the hormonal combination between cytokinins and auxins (Hedden and Stephen, 2006). Auxins act on expansion of the cellular wall and on increasing nucleic acids metabolism especially RNAs, that play essential role in proteins biosynthesis, which are important for the cell division and reproduction. Auge (1984) pointed out that 2,4-D is one of highly active auxins and gives high callus initiation response. This response helps cell division and elongation.

Cytokinins affect protein regulation and carbohydrate metabolism, consequently stimulate cells division (Mahesh, 2008). In addition to the important role that played by cytokinins for increasing callus wet weight via their important effects on increasing cell division. Cytokinins also act on increasing the division of meristemic and parenchymal cells that lost their differentiation and converting them to meristemic cells. Which lead to increasing the size of different tissues of plant organs whether they are still attached to their mother plant or separated and grown in sterilized nutritional media (Al-Shahat, 2003; Delloloio, 2007). Goodwin (1985) showed the necessity of the addition of auxins and cytokinins to culture medium for callus induction. Cytokinins in the presence of oxygen act as a key for the initiation of the cellular division, the adenine which form part of the cytokinin molecule, could be the ideal part which lead to the optimum balance.

The results table 3 revealed that there are no significant differences between weights of the callus grown on MS medium, treated with *A.niger* extract, in terms of weights of callus measured after 30 days of culture. The highest average wet weight of callus was 8.642 g when the cultures were treated with the fungus extract at the concentration of 2.0 ml L^{-1}, which did not differ significantly from the control (6.774 g).

Al-Khazragi (2012) mentioned that if there are no significant differences between the callus weights after 30 days of culture, in spite of the presence of fungus extract at different concentration as elicitor. Could be explained by the probability that the effect of the fungus is exclusively for stimulating the plant defense processes

Table 3: The effect of different concentrations of *A.niger* extract on the wet weight of the callus induced from the stem of the plant *Ruta graveolens* L.

Callus wet weight (g)	Concentration of the fungus (ml L^{-1})
6.774 a	0.0
7.040 a	1.0
7.314 a	1.5
8.642 a	2.0

The values with the same letter do not differ significantly according to Duncan's test at the probability level of 0.05.

and production of the secondary metabolites, without affecting cell weight.

Identification of furanocoumarins-Psoralen, Xanthotoxin and Bergapten-using FLCC instrument (Table 4) showed that furanocoumarins were present in callus cultures grown on MS medium enriched with 2.0 mg L^{-1} 2,4-D combined with 0.5 mg L^{-1} Kin, for 30 days, indicated by the retention times of the standards (Fig. 1). The results indicated that the addition of *A.niger* extract, has a strong effect on increasing the concentration of Psoralen, Xanthotoxin and Bergapten in the callus cultures, grown on MS medium supplemented with 2.0 mg L^{-1} 2,4-D combined with 0.5 mg L^{-1} Kin, to which *A.niger* extract was added at the concentration of 1.0, 1.5 and 2.0 ml L^{-1}. There is a direct increment of psoralen concentration with the increasing of *A.niger* extract concentration. The concentration of 1.0 ml L^{-1} extract, recorded a value of 0.929 mg g^{-1} psoralen (Fig. 3). This value increased to became 2.378 mg g^{-1} at the concentration of 1.5 ml L^{-1} extract (Fig. 4), and reached the highest value of 2.387 mg g^{-1} psoralen at the concentration of the 2.0 ml L^{-1} extract (Fig. 5), compared with the control treatment which was 0.507 mg g^{-1} (Fig. 2). Xanthotoxin concentration increased directly with the extract concentration, xanthotoxin concentrations were 0.070, 0.537 and 0.862 mg g^{-1} at 1.0, 1.5 and 2.0 ml L^{-1} extract respectively (Fig. 4), compared with the control treatment which was 0.244 mg g^{-1}. Bergapten concentrations were 3.678, 5.468 and 6.606 mg/g^{-1} at the fungus extract concentration of 1.0, 1.5 and 2.0 ml L^{-1} respectively, compared with the control treatment which 3.983 mg g^{-1}.

The effect of the addition of *A.niger* extract on increasing the production and accumulation of furanocoumarins in callus culture (Table 4). Could be explained according to the fact that plant cells give their highest production when they are under stressful conditions or at study state or clumped together. There is a number of important factors that affect callus production and accumulation of secondary metabolites, such as, the elicitor concentration, the length of exposure to the elicitor, the formation of nutrients, the age and the culture status (Al-Sumaidai, 2017). Bohlman *et al*., (1995) also reported that the addition of the extract of the fungus *Rhodotorula rubrum*, to the cell cultures of *Ruta graveolens*, stimulated a fast accumulation of furanocoumarins, due to the induction of the enzyme Phenylalanine ammonia lyase (PAL). Which is one of the enzymes involved in furanocoumarins biosynthesis pathway. Ibrahim *et al*.,(2019), mentioned that the addition of *A.niger* extract at a concentration of 2.0 ml L^{-1} to callus culture of the plant *Calendula officinalis* L., produced the highest concentration of salicylic acid 1.147 mg g^{-1} in comparison of the control treatment which reached 0.428 mg g^{-1}. Manjula and Mythili (2012) also reported that the addition of *A.niger* extract at a concentration of 2.0 ml L^{-1} to callus cultures of the plant *Marsilea quadrifolia*, resulted in an increment in the growth of the plant and the concentration of carbohydrate and protein as primary metabolites, which reflected later on increment of the

Table 4: Retention times of the isolated furanocoumarins, Psoralen, Xanthotoxin and Bergapten and their percentages in callus cultures from the stems of *Ruta graveolens* L. grown for 30 days on MS medium supplemented with 2.0 mg L^{-1} 2,4-D combined with 0.5 mg L^{-1} Kin, to which different concentrations of *A.niger* extract were added.

The source of the furanocoumarins	Dilution factor			Retention time (minute) for furanocoumarins			Area under the curve of the furanocoumarins			Presence of furanocoumarins (mg g^{-1})		
	P	X	B	P	X	B	P	X	B	P	X	B
The standard furanocoumarins	-	-	-	2.30	3.15	5.00	178214	178169	182083	100	100	100
Callus control	100	100	100	2.31	3.16	5.01	65143	17421	290105	0.913	0.244	3.983
Callus which 1.0 ml L^{-1} *A.niger* extract was added	100	100	100	2.31	3.14	5.02	66237	5000	267943	0.929	0.070	3.678
Callus which 1.5 ml L^{-1} *A.niger* extract was added	100	100	100	2.32	3.16	5.01	169588	58695	398270	2.378	0.862	5.468
Callus which 2.0 ml L^{-1} *A.niger* extract was added	100	100	100	2.33	3.16	5.01	170224	38324	481184	2.387	0.537	6.606

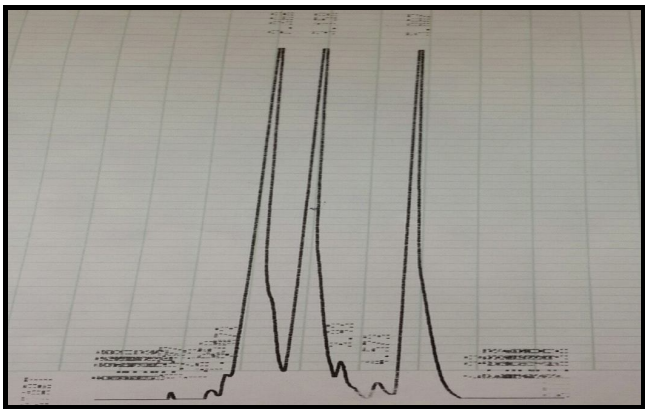

Fig. 1: Standards curve for furanocoumarins using FLCC.

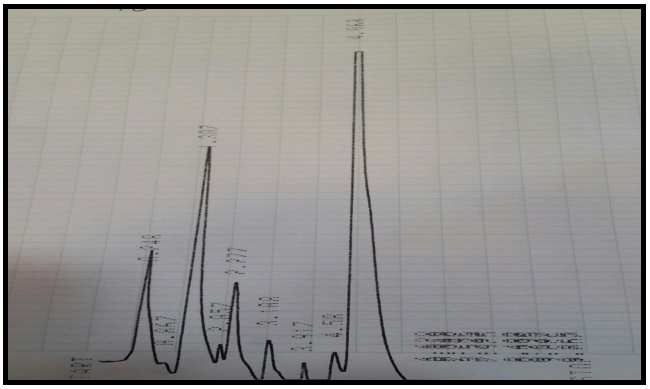

Fig. 2: Profile of furanocoumarins isolated from stem callus of *Ruta graveolens* L. after 30 days of growth, using FLCC.

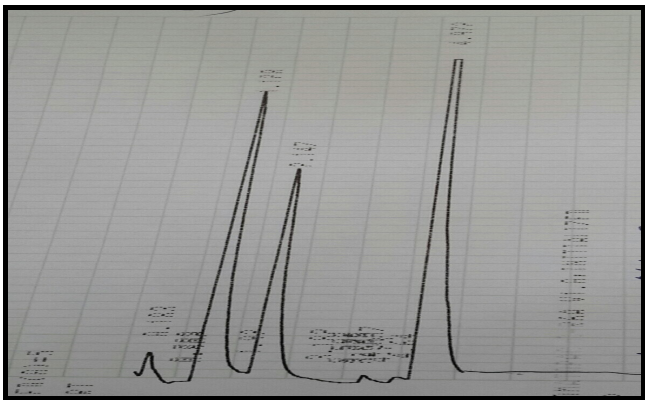

Fig. 3: Profile of furanocoumarins isolated from stem callus of *Ruta graveolens* L., to which extract of the fungus *A.niger* was added at a concentration of 1.0 ml L^{-1} for 30 days, using FLCC.

accumulation of the secondary metabolites, such as, the phenolic substances and flavonoid in the roots and the branches.

It is concluded that the addition of *Aspergillus niger* extract at the concentration of 2.0 ml L^{-1}, gives the highest concentration of furanocoumarins (psoralen, xanthotoxin and bergapten) in the callus cultures, of *Ruta graveolens*

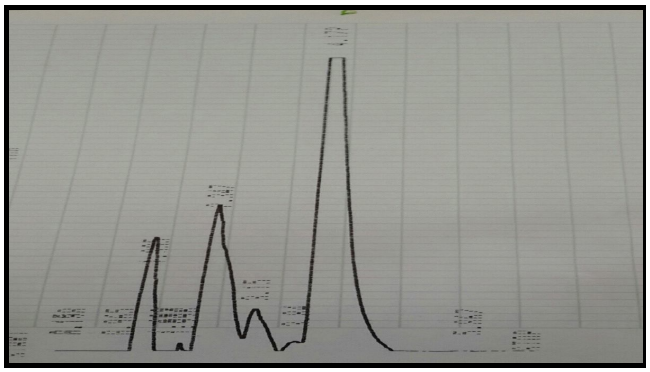

Fig. 4: Profile of furanocoumarins isolated from stem callus of *Ruta graveolens* L., to which extract of the fungus *A.niger* was added at a concentration of 1.5 ml L^{-1} for 30 days, using FLCC.

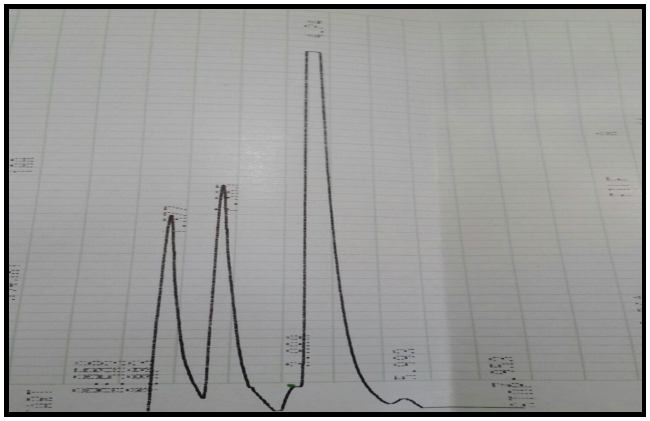

Fig. 5: Profile of furanocoumarins isolated from stem callus of *Ruta graveolens* L., to which extract of the fungus *A.niger* was added at a concentration of 2.0 ml L^{-1} for 30 days, using FLCC.

L. after 30 days of growth.

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