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PIMPINELLA ANISUM IN VITRO PROPAGATION AND ITS TRANS-ANETHOLE OIL YIELD IN CALLUS INDUCED IN GROWING TIP WITH BIOTIC ELICITORS

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SUMMARY

The study investigated the stimulation of callus tissue from explants (growing apex, true leaves, and hypocotyl segments) of medicinal *Pimpinella anisum* plant using an L2 medium supplemented with different levels of growth regulators 2,4-D, $AgNO_3$, and biotic elicitors (*Aspergillus flavus*, *Mucor* spp., and yeast extract) to the secondary metabolite induction. Callus formation induced from the single node explants had culture on the L2 medium. The addition of yeast extract 750 mg L^{-1} to the medium was superior in the highest average fresh and dry weight of 435.79 and 0.616mg, respectively. The treatment at 300mg L^{-1} of *Mucor* spp. recorded the highest rate of fresh and dry weight of the callus tissue. When cultured on 500 mg L^{-1} of *Aspergillus flavus,* fresh and dry callus weights were 376.09 and 0.628mg, respectively. The different levels of biotic elicitors (*Aspergillus flavus*, *Mucor* spp., and yeast extract) stimulate the production of essential oil t-anethole from the callus tissue when added to the medium. The results also showed that the highest increase in the amount of vital oil t-anethole occurred when the medium included 500 mg L^{-1} of the yeast extract. It recorded 2.969 mg L^{-1} dry weight (DW). The MS medium supplied with 500 mg L^{-1} *Aspergillus flavus* provided a maximum value in the t-anethole essential oil, reaching 3.756 mg L^{-1} DW of the callus. Meanwhile, the 300 mg L^{-1} of *Mucor* spp. recorded the utmost amount of crucial oil t-anethole at 3.945 mg L^{-1} DW.

Keywords: *Pimpinella anisum* L., biotic elicitors, growth regulators, yeast extract, secondary metabolite, callus, in vitro

Key findings: The secondary metabolite compounds estimation ensued by quantitative and qualitative analysis using the High-Performance Liquid Chromatography (HPLC) device for extract samples separated from seedlings growing in vitro. For induction and multiplication of callus tissue, applying the seedling explants of the *Pimpinella anisum* plant occurred. The single-node explants had

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a high ability to induce callus, one of the best explants used to stimulate callus tissue, followed by hypocotyl segments, then dicotyledonous leaves. The addition of yeast extract led to a response affecting the average fresh and dry weight and the properties of the callus.

INTRODUCTION

The *Pimpinella anisum* plant has become one of the most valuable aromatic plants. It is an annual, local, indigenous herb that spreads in many regions of the Middle East, such as, Iran, Turkey, India, Egypt, and some other hot parts of the world. The *Pimpinella anisum* oil found in the seeds and the extract contains an anethole compound of about 85%, considered a bioactive compound. The seed oil also has other essential chemical compounds, such as, eugenol, methyl chavicol, estragole, and anisaldehyde (Bayram *et al.*, 2007). The addition of some stimuli to the cell system works to increase the biosynthesis of some cellular compounds.

Based on their nature, the stimuli can be biotic and abiotic (Park *et al.*, 2008; Subashini *et al*., 2105; Ewhayid *et al.*, 2022; Ibrahim, 2022). Newall *et al.* (1996) reported that the seeds and vegetative parts are the active portions of the plant, which may generally be in the form of extracted essential oil. Based on the previous on the importance of the plant medicinally and its containment of secondary metabolite compounds, it is imperative to enter the therapeutic industries; however, it has a low production compared with the actual requirements for these compounds. Some studies have indicated the possibility of increasing callus production of active substances, such as, alkaloids, saturated alkanes, flavonoids, and esters in plant cells when using abiotic stress factors causing stress to it (Karuppusamy, 2009; Hussain *et al.*, 2012).

Increasing the concentration of the sugar type added to the food medium beyond the specified amount constitutes stress in the tissue. In turn, it stimulates the production of some active substances, speeding up the withdrawal process and the absorption of alkaloids metabolizing products. In addition, their contribution to constructing (primary and intermediate) compounds involved in the structural pathways leads to the production of plants' specific compounds, with their estimation (Hussain *et al.*, 2012). Producing some effective secondary compounds is insufficient in most cases from its natural sources only and is unobtainable industrially, in addition to the difficulty in obtaining compounds with high purity. Therefore, tissue culture provided an industrial environment and a limited space for the quantitative and qualitative production of these compounds without being restricted to the growing season or providing large agricultural areas, as well as isolating and purifying them with high efficiency compared with extracting them from the whole plant (Zainab, 2002; Ibrahim *et al.*, 2022).

The elicitors served to enhance secondary metabolite production in plants. In addition to varied levels of 2,4-D, $AgNO_3$ will test as an influential precursor that may help induce callus tissue to produce bioactive compounds. The promising study probed to stimulate plant tissues to increase the production of secondary metabolite compounds by preparing the medium containing callus tissue with different concentrations of biotic elicitors (*Aspergillus flavus*, *Mucor* spp., and yeast extract). Likewise, the study sought to quantitatively and qualitatively detect the induced compounds by chromatographic analysis using the HPLC device for callus extracts separated from seedlings growing in vitro.

MATERIALS AND METHODS

The experiments progressed for the period from January 10, 2022 to January 30, 2023 in the following research centers:

• Ministry of Higher Education and Scientific Research, University of Tikrit, College of Agriculture, Department of Horticulture and

Landscape Engineering - to conduct tissue culture experiments related to adding biostimulants;

- Ministry of Science and Technology, Agricultural Research Department, Laboratory of the Biotechnology Research Center, Technology Department - to conduct tissue culture experiments related to the main study. The main objective was to induce callus and stimulate it to produce trans-anethole essential oil of the *Pimpinella anisum* plant outside the living body after exposing it to different levels of biotic elicitors (*Aspergillus flavus*, *Mucor* spp., and yeast extract).

Preparation of L2 nutrient medium (Phillips and Collins, 1979)

Preparing the L2 medium (Phillips and Collins, 1979) commenced in the laboratory by dissolving all its components in an appropriate volume of sterile distilled water, then adding sucrose at a concentration of 30 g L^{-1} and agar (7 g L^{-1}). Completing the final volume continued to one liter of distilled and sterilized water and adjusting the pH within 5.8–6.0. The medium sterilization used the Autoclave at a temperature of 121°C and a pressure of 1.04 kg cm^3 for 20 min. The medium serves to grow seeds and develop callus (Table 1).

Seed source and seedling production in vitro

Seeds of medicinal *Pimpinella anisum* L used in the study came from the agricultural offices in the Capital, Baghdad, Iraq. The seed sterilization proceeded in a laminar airflow table, followed by the seeds' immersion in glass beakers containing a solution of distilled water and a commercial minor sodium hypochlorite (NaOCl) solution. A concentration of 6% at the rate of 2 volumes of distilled and sterilized water: 2 volumes of sterile material for 15 min incurred washing three consecutive times with sterilized distilled water to remove traces of the sanitized material. Seeds' culturing on a medium (L2) of maximum strength of salts, with 10 replicates (culture tube) of five seeds/tube, calculated the percentages of contamination and germination in the seeds (Figure 1). The seeds sustained incubation at 25 °C ± 1 °C and a light of 1000 lux for 16 h per day. After 27 days of culture, excising the explant from the seedling after 30 days transpired to create callus tissue, which included the parts (the shoot apex, true leaves, and hypocotyl).

Induction and differentiation of callus tissue

The healthy and growing in vitro explant at one month's age became a source of explants to know the best part for producing callus tissue. Taking the choice parts created a wound in the growing apex and the true leaves, with the hypocotyl cut and cutting each chosen part to a length of 1.3 cm using a sterile scalpel and in a completely sterilized atmosphere. Then, the sterilized explants grown in sterile glass tubes contained (L2) medium supplemented with different concentrations of $AgNO_3$ (0, 2, and 4 mg L^{-1}) and 2,4-D (1, 2, and 4 mg L^{-1}), with the culture conducted at an average of 10: cut vegetative part: treatment. The process of

Table 1. Components of the L2 nutrient medium (Phillips and Collins, 1979) used for callus tissue formation.

No.	Quantity (mg)	Material
1	full power	Salts L2
2	0.1	Thiamine–Hcl
3	0.5	Pyridoxine-Hcl
4	0.5	Nicotinic acid
5	2.0	Glycine

Sucrose: 30 g L^{-1}, Myo-inositol: 100 mg L^{-1}, Agar-Agar: 8 g L^{-1}, pH: 5.7–5.8
: 1.5 mg L^{-1}, Biotin+ Ca- Pantothenate

Figure 1. Seedlings developed from the seeds of *Pimpinella anisum* L. on L2 medium (Phillips and Collins, 1979) with full strength of salts after 30 days of culture.

callus differentiation occurred, which included the transfer of induced callus from cuttings of explant (single nodes, true leaves, and hypocotyl, with a weight of 100 g/piece) on the surface of 20–30 ml of the medium intended for differentiation (an L2 medium containing interaction of $AgNO_3$ in concentrations of 0, 2, and 4 mg L^{-1} and 2,4-D in concentrations of 1, 2, and 4 mg L^{-1}). The samples' incubation in the incubator underwent controlled sterilization conditions.

Callus culture and maintenance

The new callus attained re-culturing from the plant cuttings on the nutrient medium L2 (Phillips and Collins, 1979) after 63 days from the start of culture. The callus tissue's transfer to a new nutrient medium involves the process of perpetuating the callus transfer to a fresh nutrient medium every 21 days to prevent the accumulation of accidental by-products that may have adverse effects on its growth and ensure the readiness of nutrients, as well as avert the accumulation of pigments. Depleting nutrients from the nutrient medium negatively affected the growth and differentiation of callus tissue (Verma *et al.*, 2016). The periods (21 and 63 days) of the age of the callus were the basis for determining its fresh weight and following up on its growth and differentiation.

The following characteristics bore scrutiny:

Callus induction traits - Included measurement of fresh and dry weight of induced callus from *Pimpinella anisum* L. seedlings.

Response to callus induction - The percentage of callus induction after complete formation calculated according to the equation by Ibrahim *et al.* (2020). The data recorded comprised the duration (days), the size of the callus (+: Very low, ++: Low, +++: High, ++++: Very High), and the nature of the callus.

Elicitor treatments

Biotic elicitors

The strains of *Aspergillus flavus* and *Mucor* spp. came from the National Chemical Laboratory (NLC), Bioanalyse, Turkey. The cultures' establishment on a potato dextrose agar (PDA) medium evolved. Adding the biotic elicitors had the concentrations of *Aspergillus flavus* (500, 750, and 1000 mg L^{-1}), *Mucor* spp. (300, 600, and 900 mg L^{-1}), and yeast extract (YE) (250, 500, and 750 mg L^{-1}) in the callus and cell culture media.

Preparation of biotic elicitors

The fungal cultures used for the elicitation are *Aspergillus flavus* and *Mucor* spp. obtained from the National Chemical Laboratory, Bioanalyse, Turkey. Yeast extract (YE) purchase came from the U.K. (Fisher Scientific). The YE elicitor preparation consisted of dissolving in distilled water to make an aqueous stock (10 mg L^{-1}) having a pH of 5.7. The fungi maintained on PDA slants gained transfer to 150 ml liquid PD broth medium in 500 ml Erlenmeyer flasks and received shaking at 100 ± 20 rpm on a rotary shaker at 35 °C for 29 days. The fungal suspension cultures continued to autoclave at 121 °C and 103 KPa for 21 min. The autoclaved cultures gained centrifuging and filtering through Whatman No. 2 filter papers to obtain the respective fungi's culture filtrate (CF). The cell residue washing used sterile double distilled water thrice, then drying at 60 °C for 24 ± 1 h in the oven and grinding in mortar and pestle. The obtained preparation sustained crushing as dried cell powder. The culture filtrate and dried cell powder remained stored at 4 °C–8 °C until further use.

Qualitative and quantitative evaluation of anethole compound using HPLC technique

Anethole standard solution preparation

A 120 microliter of anethole standard solution reached mixing to 92 ml sterilized distilled water and completing the volume to 100 ml by adding sterile distilled water to obtain solutions with a concentration of 1000 mg L^{-1} and from these stock solutions, potent concentrations prepared included 2, 8, and 100 mg L^{-1} (Wagner and Bladt, 1996).

HPLC technique

Using the HPLC technique in estimating the quality and quantity of a t-anethole compound was according to the method of Jurado *et al.* (2006). Then, the reading at 257 nm wavelength and determining retention time of *t*-anethole in samples followed the process of Ionkova *et al.* (1989).

RESULTS AND DISCUSSION

All the explants of the *Pimpinella anisum* plant showed a clear response in the process of developing and differentiating callus tissue in an L2 medium supplemented with different concentrations of growth regulators, including $AgNO_3$ in varying concentrations (0, 2, and 4 mg L^{-1}) and 2,4-D (1, 2, and 4 mg L^{-1}). The single nodes excelled in recording the highest response, which amounted to approximately 100%, with periods ranging between 3–7 days. Likewise, the reaction of the leaves reached about 100% on the same medium. On the hypocotyl cuttings' response to callus induction, it was better than the other explants. Percentages above for explants represent the average of the total percentages found in Tables 2, 3, and 4. The results appear in Tables 2, 3, and 4 on single node cuttings cultured on the L2 medium supplied with different interactions of $AgNO_3$ at 0, 2, and 4 mg L^{-1} and 2, 4-D at 1, 2, and 4 mg L^{-1}.

The callus tissue's induction rate reached 100% in all media supplied with 2.4 mg L^{-1} $AgNO_3$ + 2.4 mg L^{-1} 2,4-D. Meanwhile, the medium without $AgNO_3$ and amended with 1 mg L^{-1} 2,4-D was the most effective in responding to the formation of callus tissue. Data also showed that the callus formation of cotyledon on the L2 medium supplied with different combinations of $AgNO_3$ and 2, 4-D was significantly better (Table 1). The L2 medium with 0.0 mg L^{-1} $AgNO_3$ + 1 mg L^{-1} 2,4-D recorded the lowest response rate to the callus formation. The hypocotyl responded most to callus induction when cultured on the medium supplied with 4 mg L^{-1} $AgNO_3$ + 4 mg L^{-1} 2,4-D (Table 4). The results showed high callus stimulation from single nodes, leaves, and hypocotyl. The main reason for the response to the formation of callus tissue may be due to the conditions of the nutrient medium used and its containment of varying levels of growth regulators (Dixon, 1985; Byun and Pedersen, 1994). In general, this study's findings indicated a good response of *Pimpinella anisum* L. plants to an in vitro culture system, represented by the success of the explant selected for callus formation. The single nodes revealed the utmost response to

Table 2. Induction of callus tissue from single nodes of medicinal *Pimpinella anisum* plant *Pimpinella anisum* L in solid medium (L2) supplemented with varying levels of $AgNO_3$ and 2,4-D after six weeks of in vivo culture.

Growth regulators		The number of callus pieces produced	Induction percentage	Duration (days)
AgNO3	2,4-D			
0	1	4	40	3-9
0	2	5	50	6-10
0	4	7	70	3-5
2.0	1	9	90	3-7
2.0	2	10	100	3-5
2.0	4	10	100	3-4
4.0	1	7	70	5-7
4.0	2	10	100	4-6
4.0	4	100	100	3-7

• Number of cuttings planted in each treatment = 10.

Table 3. Induction of callus tissue from leaf cuttings of medicinal *Pimpinella anisum Pimpinella anisum* L in solid medium (L2) supplemented with varying levels of AgNO3 and 2,4-D after six weeks of in vivo culture.

Growth regulators		The number of callus pieces produced	Induction percentage	Duration (days)
AgNO3	2,4-D			
0	1	5	50	3- 5
0	2	7	70	8-10
0	4	5	50	10-12
2.0	1	2	20	5- 7
2.0	2	10	100	4-7
2.0	4	10	100	3-5
4.0	1	6	60	5-7
4.0	2	7	70	8-9
4.0	4	9	90	4-8

Number of cuttings planted in each treatment = 10.

Table 4. Induction of callus tissue from hypocotyl cuttings of *Pimpinella anisum* plant *Pimpinella anisum* L in solid medium (L2) supplemented with varying levels of $AgNO_3$ and 2,4-D after five weeks of in vitro culture.

Growth regulators		The number of callus pieces produced	Induction percentage	Duration (days)
AgNO3	2,4-D			
0	1	2	20	3-4
0	2	5	50	5-7
0	4	7	70	7-14
2.0	1	4	40	7-14
2.0	2	9	90	3-6
2.0	4	10	100	7-9
4.0	1	7	70	4-9
4.0	2	10	100	7-10
4.0	4	9	90	10-12

• Number of cuttings planted in each treatment = 10.

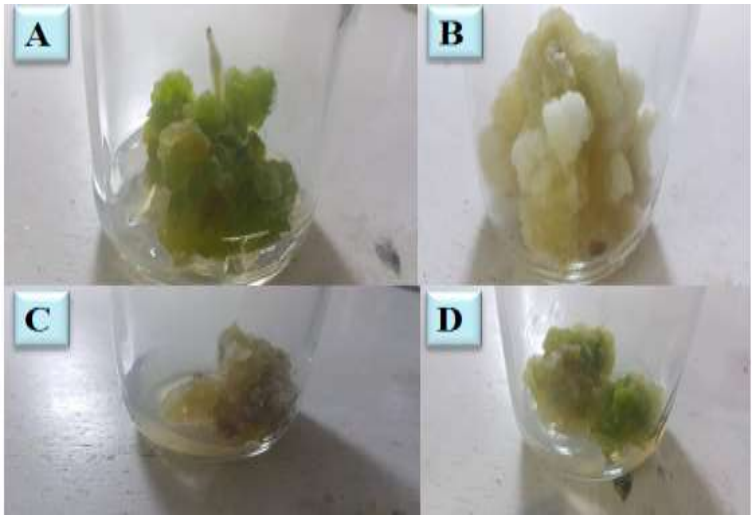

Figure 2. Effect of 2,4-D and ethylene antagonist $AgNO_3$ on dallus proliferation from leaves explants of *Pimpinella anisum* L cultured on L_2 media containing a. media L_2 + 4.0 mg 2,4-D + 2.0 mg $AgNO_3$; b. media L_2 + 4.0 mg 2,4-D + 2.0 mg $AgNO_3$; c. media L_2 + 4.0 mg 2,4-D + 2.0 mg $AgNO_3$; d. media L_2 + 4.0 mg 2,4-D + 2.0 mg $AgNO_3$ (Images were taken 35 days after the addition of 2,4-D and ethylene antagonist $AgNO_3$).

the callus tissue induction with a faster time than the rest of the explant, followed by the leaves and then the hypocotyl. The explant type has the potential energy of cells to form callus tissue (Bela and Shetty, 1999; Negrutui *et al.*, 1978; Hartmann *et al.*, 1990).

Effect of 2,4-D and ethylene antagonist $AgNO_3$

The callus formation induction of the medical *Pimpinella anisum* plant came from a single node in the L2 medium supported by the 2,4-D and $AgNO_3$ at 2 and 4 mg L^{-1}, respectively (Figure 2). All tested auxin concentrations (Table 5) effectively promoted callus formation by increasing fresh and dry weight, volume, and properties (callus texture). The highest mean of fresh and dry weight and volume of callus induced from the growing apex was successful at an overlap of 4 mg L^{-1} 2,4-D + 2 mg L^{-1} $AgNO_3$ (7.92 mg, 1.33 mg, and a ++++, respectively). It did not differ significantly from the rest of the overlap treatments. The L2 medium at 0.0 mg L^{-1} $AgNO_3$ + 1.0 mg L^{-1} 2,4-D recorded the lowest value in average fresh and dry weight and poor callus volume at 0.44 mg, 2.39 mg, and a +, respectively. It may be due to the toxic effects of metal ions on total cellular expression, and some previous research reports support this study's observation (Ishizaki *et al.*, 2000).

The callus induced from the bases of explants when cultured in all concentrations after less than a week of culture. At first, the fragment enlarged and began growing as an irregularly shaped mass of cut and scarred edges. However, it was apparent that the growth rate differed at varying concentrations of 2,4-D and $AgNO_3$. Callus tissue growth induction was rapid and vigorous in a medium enriched with 2,4-D and $AgNO_3$ interactions. Compared with the $AgNO_3$-free medium, it resulted in an etiolated, fragile, slow-formation callus produced in all concentrations free of the AgNO3 and a low concentration of 2,4-D. This weak callus growth may be due to complete or partial suppression of cellular totipotency by the hormone ethylene accumulated in the vessels of the cultures. These findings are consistent with those from Fei *et al.* (2000) and Sujana and Saivenkatesh (2017). It was also evident that the best stimulation of callus growth in the fresh and dry weight, texture brown, yellow, and brittle was at 4 mg L^{-1} 2,4-D + 2 mg L^{-1} $AgNO_3$ concentration.

Table 5. Effect of the interaction between the concentrations of 2,4-D and AgNO3 in the percentage of fresh weight (g) and dry weight (mg) in the induction of the first callus of single-node culture of *Pimpinella anisum* L medicinal plant after five weeks of culture on medium (L2).

Medium L2 mg L-1		Fresh weight	Dry weight	Intensity	Nature of callus
2,4-D	AgNO3	(g)	(mg)	formation	
1.0	0.0	2.39	0.44	+	Pale green, fragile
	0.2	3.55	0.87	++	Pale green, Fragile
	0.4	4.08	0.99	+++	Dark green, nodular
2.0	0.0	3.07	0.85	+++	Dense, robust hairy
	0.2	4.65	0.93	+++	light brown, hairy
	0.4	6.73	1.07	++++	Pale yellow, fragile
	0.0	4.93	0.95	+++	Dense hairy
4. 0	0.2	7.92	1.33	++++	Brown yellow, fragile
	0.4	5.88	1.09	++++	Yellow green, nodular
L.S.D. (0.05)		1.082 *	0.274 *		

Intensity of the callus: + Very low, ++ Low, +++ High, ++++ Very High

Table 6. Effect of different levels of biotic elicitors (yeast extract) on the average fresh and dry weight of callus of *Pimpinella anisum* L induced by the production of secondary metabolite compounds essential oil t-anethole after eight weeks of culture on medium (L2 + 4.0 mg 2.4 -D + 2.0 mg $AgNO_3$).

Nutrient medium biotic elicitors (mg/l)	Leaves Culture (media L_2 + 4.0 mg 2,4-D + 2.0 mg $AgNO_3$)			essential oil
Yeast extract	Fresh weight Mean (mg)	Dry weight Mean (mg)	Nature of callus	*t*-anethole
Without (Yeast extract)	213.62	0.188	light brown, hairy	0.830
250	296.45	0.219	Shiny cream, friable	1.600
500	322.75	0.415	dark green, hard	2.969
750	435.79	0.616	Cream, friable	2.056

LSD for Fresh weight 82.97; Dry weight = 0.2013; Essential oil t-anethole = 1.077.

The dilution of 2.0 mg L^{-1} 2,4-D + 4.0 mg L^{-1} $AgNO_3$ led to a light-yellow, friable callus formation. Also, this treatment combination caused a brown-yellow chalky-textured callus for some cultures. This color may refer to the active participation of 2,4-D in the polymerization of alkaloids, phenols, and other chemicals to produce brown callus (De, 1992). This brown callus appeared less frequent in the subculture. The 2.0 mg L^{-1} 2,4-D + 4.0 mg L^{-1} $AgNO_3$ treatment also produced light-green, fragile, and compact callus. This green color is attributable to chloroplast development (Sujana and Saivenkatesh, 2017). However, many previous scientific studies of this property have existed on the anti-aging effect of $AgNO_3$. They found that $AgNO_3$ inhibits the action of ethylene receptors or silver ions thought to compete for the ethylene ion-binding site (Beyer, 1976; McDaniel and Binder, 2012). Seldom, the accumulation of excess ethylene in culture tubes is auto-inhibitory to increase ethylene production (Yang and Hoffman, 1984). The $AgNO_3$ compound may provide a good level of hormones to the cells for unregulated cellular growth when it interacts with the endogenous hormones of excised explant and exogenous hormones added to the medium. The silver ions (Ag+) may inhibit ethylene competition by altering the ETR1 form (the ethylene receptor in cell walls) upon substitution of copper in the cofactor (Zobel and Roberts, 1978).

Effect of Biotic Elicitors (Yeast extract: YE) on the production of essential oil t-anethole

The yeast extract added to the nutrition medium positively influenced the fresh and dry weight of the callus (Table 6). The L2 medium

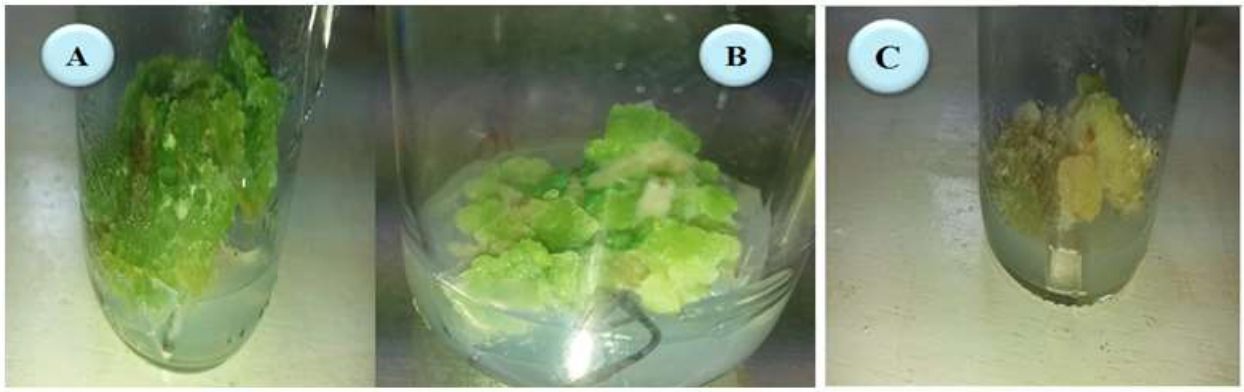

Figure 3. Effect of biotic elicitors (yeast extract *Mucor* sp. and *Aspergillus flavus*) on growth of callus cultures raised on cultured on L_2 media containing a. media L2 + 4.0 mg 2,4-D + 0.2 mg $AgNO_3$ + 500 mg/l Yeast extract; b. media L_2 + 4.0 mg 2,4-D + 0.4 mg $AgNO_3$ + 500 mg/l *Mucor* spp.; c. media L_2 + 4.0 mg 2,4-D + 0.2 mg $AgNO_3$ + 500 mg/l *Aspergillus flavus*.

supplied with 750 mg L^{-1} yeast extract recorded the highest callus fresh and dry weight, reaching 0.616 and 435.79 mg, respectively. Induced callus displayed a dark green, hard texture (Figure 3). It did not differ significantly from the two treatments (250 and 500 mg L^{-1}) of yeast extract with fresh and dry weights, reaching 296.45 and 0.219 and 322.75 and 0.415 mg, respectively. Fresh and dry weight was the most effective in producing and multiplying callus tissues, except for treatment without adding yeast extract. The fresh and dry weight was 0.188 and 213.62 mg. The induced callus was compact and friable (Figure 3). Likewise, increasing the concentration of yeast extract from the control treatment led to a gradual increase in the quantities of essential oil t-anethole in the callus.

The low concentrations of yeast extract 500 mg L^{-1} were the most effective in stimulating the callus to produce essential oil t-anethole, reaching 2.969 mg L^{-1} DW callus, which differed significantly from the rest of the concentrations. However, it considerably differed from the two treatments, 0 and 250 mg L^{-1}, which amounted to 0.830 and 1.600 mg L^{-1} DW of t-anethole, respectively. Kumar *et al.* (2013) found that the addition of various fungal extracts of *Penicillium notatum* and *Aspergillus niger* and some yeast extracts to the medium of callus formation of *Psoralea corylifolia* leaves increased the accumulation of bioactive compounds when added at different levels. Siddiqui *et al.* (2010) explained many scientific facts have occurred in secondary metabolism processes that may be affected by the presence of many fungi or their extracts. Azeez and Ibrahim (2013) showed an increased concentration of fungal elicitors and yeast extracts at 0.1, 0.25, 0.5, or 0.75 mg L^{-1} dilutions.

Wang *et al.* (2004) found that the treatment of cell cultures with vital effects of yeast extracts may increase the multiplication and growth of cultures of *Perilla frutescens* and boost the accumulation and production of active compounds anthocyanin and triterpenoids. Al-Mafargi (2010) and Almukhtar (2022) showed that the treatment of callus cultures with bioactive (fungi, bacteria, and yeast extract) caused an increase in the growth and development of callus tissue and raised the accumulation and production of essential oils of *Rosmarinus officinalis* L. leaves.

Effect of adding biotic elicitors (*Aspergillus flavus*) on essential oil t-anethole production

The L2 medium supplied with different concentrations of biotic stress (0.0, 500, 750, and 1000 mg L^{-1}) of *Aspergillus flavus* fungus

recorded the highest mean of fresh and dry weight of callus, amounting to 376.09 and 0.628 mg. Average fresh and dry weight gave a yellow-brown color and crumbly texture, respectively, at 500 mg L^{-1} of *Aspergillus flavus.* Its value differed from the control treatment. It recorded the lowest average fresh and dry weight, and the callus had a granular and brittle texture clustered around the edges of the leaf segments (Figure 3) (Table 7). It did not differ from the two treatments (750 and 1000 mg L^{-1}) of *Aspergillus flavus* statistically with the average fresh and dry weights, and the formation of callus was chalky (Table 7).

The biostimulants or fungi added to the media include many essential compounds that contribute to biosynthesis, such as, polysaccharides, glucans, chitin, and proteins (Siddique *et al.*, 2010). The fungus treatment recorded the lowest values for the fresh and dry weights of callus, reaching 0.620 and 0.066 mg. In vivo, it revealed a decrease in the fresh and dry weights and a boost in the accumulation of secondary metabolite compounds. The results appear in Table 7 on dried callus samples grown on a nutrient medium prepared with different concentrations of *Aspergillus flavus*. The averages of t-anethole varied depending on the concentrations of *Aspergillus flavus* added to the L2 medium. The dilutions of fungus added to the L2 medium significantly differed in the essential oil t-anethole concentrations compared with the control treatment.

The bioactive compound averages rose when adding 500 mg L^{-1} *Aspergillus flavus*. It reached 3.756 ppm DW callus of t-anethole. Most compound averages decreased in the control treatment, recording 0.830 mg L^{-1} DW callus versus the treatment of 1000 mg L^{-1} *Aspergillus flavus*. Notably, the best concentration of *Aspergillus flavus*, increasing the production of t-anethole in plant calluses, was at 500 mgL^{-1}, followed by the addition of 750 mg L^{-1} concentration. This study results agree with the findings of Ajungla (2007). DC recorded the highest value of total flavonoid, reaching 0.036 ± 0.010 mg/g after four days of exposure to the fungus. It aligns with a study that the addition of biotic elicitors, such as, fungi, bacteria, and yeasts, actively contributed to the accumulation of bioactive compounds in plant tissue and cell culture (Chodisetti *et al.*, 2013). Bais *et al.* (2002) indicated that adding different concentrations of *Aspergillus niger* in vitro to hairy root cultures of *Ocimum basilicum* significantly increased the buildup of rosmarinic acid. Similarly, Wibberley *et al*. (1994) and Zhou *et al.* (2007) obtained an accrual in the production of Salidroside compound from hairy roots of *Rhodiola sachalinensis* when adding concentrations of *Aspergillus niger*, *Ganoderma lucidum*, and *Coriolus versicolor* to the tissue culture medium.

Effect of adding biotic elicitors (*Mucor* spp.) on essential oil t-anethole production

The results showed that adding *Mucor* spp. to the nutrient medium gave the highest rate of fresh and dry weight of callus tissue when treated with 300 mg L^{-1} of *Mucor* spp. It excelled over the rest of *Mucor* spp. treatments, resulting in 341.03 and 0.713 mg fresh and dry weight, respectively (Table 8). The formed callus was yellow-green and friable (Figure 3). The control treatment caused a decrease in the average of the two weights (fresh and dry), as it recorded the lowest rate of 213.62 and 0.188 mg, respectively. The results in Table 8 show significant differences in the concentration of the studied secondary compounds when combining varied concentrations of *Mucor* spp. to the L2 medium. The dilution of 300 mg L^{-1} gave the maximum amount of essential oil t-anethole, amounting to 3.945 mg L^{-1} DW of callus. It did not differ significantly from the concentration of 600 mg L^{-1} of *Mucor* spp. at 2.788 mg L^{-1} DW of callus. It substantially contrasted with the concentration of 900 mg L^{-1} of *Mucor* spp. The control treatment recorded the minimum value of t-anethole. Hahn and Albersheim (1978) and Namdeo (2007) indicated that the addition of fungi or their extracts to suspension cultures of callus accumulated bioactive compounds, including calcifediol. The results showed that the *Pimpinella anisum* plant is a rich source of bioactive compounds.

Table 7. Effect of different levels of biotic elicitors (*Aspergillus flavus*) on the average fresh and dry weight of callus of *Pimpinella anisum* L. induced by the production of secondary metabolites essential oil t-anethole after eight weeks of cultivation on media (L2 + 4.0 mg 2.4 -D + 2.0 mg AgNO3).

Nutrient medium biotic elicitors	Culture (media L_2 + 4.0 mg 2,4-D + 2.0 mg AgNO3)			Essential oil
Aspergillus flavus (mg/l)	Fresh weight (mg)	Dry weight (mg)	Nature of callus	*t*-anethole
Without(*Aspergillus flavus)*	213.62	0.188	light brown, hairy	0.830
500	376.09	0.628	yellow brown , friable	3.756
750	289.23	0.376	Cream, friable	2.398
1000	271.08	0.314	green, yellow, fragile	1.920

LSD Fresh weight 68.867; Dry weight 0.252; Essential oil t-anethole 1.557.

Table 8. The effect of different levels of biotic elicitors (*Mucor* sp) on the average fresh and dry weights of callus of *Pimpinella anisum* L induced by the production of secondary metabolite compounds essential oil t-anethole after eight weeks of culture on medium (L2 + 4.0 mg 2.4 -D + 2.0 mg AgNO3) 300 mg/l.

Nutrient medium biotic elicitors (mg/l)	Culture (media L_2 + 4.0 mg 2,4-D + 2.0 mg AgNO3)			Essential oil
Mucor sp	Fresh weight Mean (mg)	Dry weight Mean (mg)	Nature of callus	*t*-anethole
Without (*Mucor* spp.)	213.62	0.188	light brown, hairy	0.830
300	341.03	0.713	yellow green, , friable	3.945
600	275.90	0.491	Pale green, friable	2.788
900	258.00	0.258	yellowish, little green, Fragile	1.462

LSD Fresh weight 89.302; Dry weight = 0.207; Essential oil t-anethole = 1.374.

CONCLUSIONS

The biotic elicitors usually affect in vitro callus proliferation. In *Pimpinella anisum*, the highest callus proliferation was on solid LS medium supplemented with growth regulators 2,4-D, $AgNO_3$, and biotic elicitors, supported by better fresh weight and dry weight of callus. It indicates *Pimpinella anisum* is a biotic elicitor's tolerant medicinal plant that may be lenient to a concentration above 500 mg L^{-1} of *Aspergillus flavus*, 300 mg L^{-1}, and 500 mg L^{-1} of yeast extract of the *Mucor* spp. in vitro culture. The biotic elicitors (*Aspergillus flavus*, *Mucor* spp., and yeast extract) induced the secondary metabolite essential oil t-anethole. Callus tissue induction came from single node explants when cultured on this medium.

REFERENCES

Ajungla L (2007). Tissue culture studies in *Datura metel* L. for production of secondary metabolites (Doctoral dissertation). Department of Botany, University of Pune, India.

Al-Mafargi KIR (2010). Study the effect of some biotic and abiotic factors on enhancement of essential oils and rosmarinic acid in Rosemary *Rosmarinus officinalis* L. vitro (M.Sc.). Al-Nahrain University, College of Science, Ministry of Higher Education, Iraq.

Almukhtar SA (2022). Plant growth regulators, licorice extract, and salts used in media for micropropagation of *Gardenia jasminoides*. *SABRAO J. Breed. Genet.* 54(5): 1149-1158. http://doi.org/10.54910/sabrao2022.54.5.16.

Azeez HA, Ibrahim KM (2013). Effect of biotic elicitors on secondary metabolite production in cell suspensions of *Hypericum triquetrifolium* Turra. *Bull. Univ. Agric. Sci. Vet. Med. Cluj-Napoca*. *Hort.* 70(1): 26-33. https://doi.org/10.15835/buasvmcn-hort:9264.

Bais HP, Walker TS, Schweizer HP, Vivanco JM (2002). Root specific elicitation and antimicrobial activity of rosmarinic acid in hairy root cultures of *Ocimum basilicum*. *Plant Physiol. Biochem.* 40(11): 983-995. https://doi.org/10.1016/S0981-9428(02) 01460-2.

Bayram I, Cetingul SI, Akkaya B, Uyarlar C (2007). Effect of aniseed (*Pimpinella anisum* L.) on egg production, quality, cholesterol levels, hatching results, and the antibody values in blood of laying quails (*Coturnix coturnix japonica*). *Arch. Zootech*. 10:73-77.

Bela J, Shetty K (1999). Somatic embryogenesis in anise (*Pimpinella anisum* L.): The effect of proline on embryogenic callus formation and ABA on advanced embryo development. *J. Food biochem*. 23(1), 17-32. https://doi.org/10.1111/j.1745-4514.1999.tb00002.x.

Beyer EM (1976). A potent inhibitor of ethylene action in plants. *Plant physiol*., 58(3), 268-271.

Byun SY, Pedersen H (1994). Two-phase airlift fermentor operation with elicitation for the enhanced production of enzophenanthridine alkaloids in cell suspensions of *Escherichia californica*. *Biotech. & Bioengin.* 44(1): 14-20. https://doi.org/10.1002/bit.260440104.

Chodisetti B, Rao K, Gandi S, Giri A (2013). Improved gymnemic acid production in the suspension cultures of *Gymnema sylvestre* through biotic elicitation. *Plant Biotechnol. Rep*. 7: 519-525. https://doi.org/ 10.1007/s11816-013-0290-3.

De KK (1992). Callus Culturing. In: An Introduction to Plant Tissue Culture. Now Central Book Agency, Calcutta, India, 167-168.

Dixon RA (1985). Plant Cell Culture. A practical approach. IRL, Oxford, UK.

Ewhayid BM, Ibrahim MA, Abdulzahra EM (2022). Effect of growth regulators, chitosan, and silver nanoparticles on callus induction and stimulating the Myricetin production in moringa (*Moringa oleifera* Lam.) Tree. *Texas J. Agric. Biol. Sci*., 10, 85-94.

Fei S, Read PE, Riordan TP (2000). Improvement of embryogenic callus induction and shoot regeneration of buffalograss by silver nitrate. *Plant Cell, Tissue and Organ Culture*, 60: 197-203. https://doi.org/ 10.1023/A:1006468324616.

Hahn MG, Albersheim P (1978). Host-pathogen interactions: XIV. Isolation and partial characterization of an elicitor from yeast extract. *Plant Physiol.* 62(1): 107-111. https://doi.org/10.1104/pp.62.1.107.

Hartmann HH, Kester DE, Davies FT (1990). Plant Propagation Principles and Practices. 5th Edition, Prentice Hall, Eaglewood Cliffs, 232-233.

Hussain MS, Fareed S, Ansari S, Rahman MA, Ahmad IZ, Saeed M (2012). Current approaches toward production of secondary plant metabolites. *J. Pharm. Biol. Allied Sci*. 4(1): 10-20.

Ibrahim M (2022). Role of Endogenous and Exogenous Hormones in Bioactive Compounds Production in Medicinal Plants via In Vitro Culture Technique. Plant Hormones-Recent Advances, New Perspectives and Applications. https://doi.org/10.5772/intechopen.102814.

Ibrahim MA, Al-Jabir HSS, Lafta AY (2022). Protocols of micropropagation of some medicinal plants. *Multidisci. Rev*., 5(3), 2022008-2022008. https://10.31893/multirev.2022008.

Ibrahim MA, Jerry AN, Khalil AI (2020). Salt tolerant cells selection of potato callus from culturing at different concentrations of sodium chloride by in vitro culture technique. *Plant Cell Biotech. Mol. Bio*., 21(37&38): 81-89.

Ionkova I, Witte L, Alfermann AW (1989). Production of alkaloids by transformed root cultures of *Datura innoxia*. *Planta Medica*, 55(02): 229-230. https://doi.org/10.1055/s-2006-961984.

Ishizaki T, Komai F, Msuda K, Megumi C (2000). Exogenous ethylene enhances formation of embryogenic callus and inhibits embryogenesis in cultures of explants of spinach roots. *J. Amer. Soc. Hort. Sci.*, 125(1): 21-24.

Jurado JM, Alcazar A, Pablos F, Martin MJ (2006). LC determination of anethole in aniseed drinks. *Chromatog.* 64: 223-226. https://doi.org/ 10.1365/s10337-006-0001-0.

Karuppusamy S (2009). A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ and cell cultures. *J Med Plants Res*, 3(13): 1222-1239.

Kumar A, Patil D, Rajamohanan PR, Ahmad A (2013). Isolation, purification and characterization of vinblastine and vincristine from endophytic fungus *Fusarium*

oxysporum isolated from *Catharanthus roseus*. *PloS one*, 8(9): e71805.https://doi.org/10.1371/journal.pone.0071805.

McDaniel BK, Binder BM (2012). Ethylene receptor 1 (ETR1) is sufficient and has the predominant role in mediating inhibition of ethylene responses by silver in *Arabidopsis thaliana*. *J. Bio. Chem.*, 287(31): 26094-26103. https://doi.org/10.1074/jbc.M112.383034.

Namdeo AG (2007). Plant cell elicitation for production of secondary metabolites: A review. *Pharm. Rev*, 1(1): 69-79.

Negrutui I, Jacobs M, Dorina CZ (1978). Some factors controlling in vitro morphogenesis of *Arabidopsis thaliana*. *Z. Pflanzen Physiol.* 86:113-124.

Newall CA, Anderson LA, Phillipson JD (1996). Herbal Medicines. A guide for health-care professionals. The Pharmaceutical Press. London, England.

Park SU, Uddin R, Xu H, Kim YK, Lee SY (2008). Biotechnological applications for rosmarinic acid production in plant. *Afri. J. Biotech*., 7(25): 4954-4965.

Phillips GC, Collins GB (1979). In vitro tissue culture of selected legumes and plant regeneration from callus cultures of red clover 1. *Crop Sci.*, 19(1): 59-64. https://doi.org/10.2135/cropsci1979.0011183X001900010014x.

Siddiqui ZH, Mujib A, Ahmad MM, Ali A (2010). Fungal Elicitors: A Potent Approach for Enhancing Secondary Metabolites in Cultured Cells. Fungal Biochemistry and Biotechnology Lap Lambert Academic Publishing AG & CO. KG, Saarbrücken, 88-104.

Subashini G, M. Paramathma and N. Manivannan (2015). Character association analysis for oil yield and yield components in BC4F1 population of interspecific cross (Jatropha curcas x Jatropha integerrima). *SABRAO J. Breed. Genet.* 47(4): 335-339.

Sujana P, Saivenkatesh K (2017). Effect of Silver Nitrate on Callus Cultures of Spilanthes Acmella Murr - An Endangered Toothache Medicinal Herb. Department of Botany, P.V.K.N. Govt College, Chittoor, A.P., India. 6(12): 1305-1315. https://doi.org/10.20959/wjpr201712-9791.

Verma SK, Das AK, Cingoz GS, Uslu E, Gurel E (2016). Influence of nutrient media on callus induction, somatic embryogenesis and plant regeneration in selected Turkish crocus species. *Biotech. Rep*., 10: 66-74. https://doi.org/10.1016/j.btre.2016.03.006.

Wagner H, Bladt S (1996). Plant Drug Analysis: A Thin Layer Chromatography Atlas. Springer Science & Business Media.

Wang JW, Xia ZH, Chu JH, Tan RX (2004). Simultaneous production of anthocyanin and triterpenoids in suspension cultures of *Perilla frutescens*. *Enz. Microbial. Tech.* 34(7): 651-656. https://doi.org/10.1016/j.enzmictec.2004.02.004.

Wibberley MS, Lenton JR, Neill SJ (1994). Sesquiterpenoid phytoalexins produced by hairy roots of *Nicotiana tabacum*. *Phytochem.* 37(2): 349-351. https://doi.org/10.1016/0031-9422(94)85059-3.

Yang SF, Hoffman NE (1984). Ethylene biosynthesis and its regulation in higher plants. *Ann. Rev. Plant physiol*., 35(1): 155-189.

Zainab JA (2002). The production of cardiac glycosides from the plant of zahr al kishtiban (*Digitalis purpure* l.) by using tissue culture technique. Ph.D. Thesis., University of Baghdad, Iraq.

Zhou X, Wu Y, Wang X, Liu B, Xu H (2007). Salidroside production by hairy roots of *Rhodiola sachalinensis* obtained after transformation with *Agrobacterium rhizogenes*. *Biol. Pharm. Bull.* 30(3): 439-442. https://doi.org/10.1248/bpb.30.439.

Zobel RW, Roberts LW (1978). Effects of low concentrations of ethylene on cell division and cytodifferentiation in lettuce pith expiants. *Can. J. Bot.*, 56(8): 987-990. https://doi.org/10.1139/b78-110.