https://doi.org/10.31925/farmacia.2025.6.14 **ORIGINAL ARTICLE**

THE EFFECTS OF ELICITOR AND ABIOTIC STRESSOR MOLECULES ON PHENOLIC COMPOSITION OF *IN VITRO* CALLUS CULTURES OF ELECAMPANE (*INULA HELENIUM* L.)

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Abstract

This study focused on the effects of various stressors and elicitors on phenolic metabolism of *in vitro* callus cultures of Elecampane. 1 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) + 0,3 mg/L Kinetin (KN) formulation was detected as the most proper application for callus production. Quercetin was synthesized as the dominant phenolic compound of callus cultures. The highest values of total phenolics were obtained through the combination of 6-Benzylaminopurine + 1-Naphthaleneacetic acid (BAP+NAA) in the plant growth regulators (PGR) group (169 µg/g), ½ reduced Murashige and Skoog medium (MS) (108 µg/g) in the stressor group, and both abscisic acid (140 µg/g) and melatonin (138 µg/g) in the elicitor group. The application of various PGRs and elicitors enhanced the level of quercetin at pronounced levels (241-388 µg/g), but not in rutin (3-18 µg/g). Chlorogenic acid increased under silver nitrate (19 µg/g) and ¼ reduced MS stressors (17 µg/g) and melatonin (32 µg/g) and abscisic acid (24 µg/g) elicitors. These findings suggest that the combination of 2,4-D + KN, reduced MS, abscisic acid, and melatonin conditions stimulated the synthesis of phenolics through the biosynthetic pathway by increasing the stress resistance of Elecampane.

Rezumat

Au fost evaluate efectele *in vitro* a diverșilor factori de stres și elicitori asupra metabolismului fenolic al culturilor de calus de iarbă mare (*Inula helenium* L). Formularea 1 mg/L acid 2,4-diclorofenoxiacetic (2,4-D) + 0,3 mg/L kinetină (KN) a fost identificată ca fiind cea mai potrivită pentru obținerea de calus. Quercetina a fost principalul compus fenolic sintetizat al culturilor de calus. Cele mai ridicate valori ale fenolilor totali au fost obținute prin combinația 6-benzilaminopurină + acid naftalenacetic (BAP + NAA) în grupul regulatorilor de creștere (169 mg/g), mediul Murashige și Skoog (MS) la jumătate din concentrație (½ MS) în grupul factorilor de stres (108 mg/g), precum și acidul abscisic (140 mg/g) și melatonina (138 mg/g) în grupul elicitorilor. Aplicarea diverșilor regulatori de creștere și elicitori a crescut concentrația de quercetină sintetizată , dar nu și pe cea de rutină. Acidul clorogenic a crescut sub acțiunea nitratului de argint (19 mg/g) și ¼ a redus factorii de stres MS (17 mg/g) și elicitorii melatonină (32 mg/g) și acid abscisic (24 mg/g). Rezultatele sugerează că asocierea 2,4-D + KN, MS redus, acid abscisic și melatonină a stimulat sinteza fenolilor prin calea biosintetică, crescând rezistența la stres a *Inula helenium* L.

Keywords: *Inula helenium* L., elicitors, phenolic compounds, abiotic stress

Introduction

Medicinal plants and their products, including phyto-pharmaceuticals and nutraceuticals, are significant sources of pharmacologically important plant chemicals, as they possess a huge scale of metabolic profiles, have great opportunity for investigation and development of biotherapeutic agents [1-3]. Of these medicinal plants, *Inula* species from the *Asteraceae* family are common perennial herbs that have remarkable pharmacological activities and are of great scientific interest [4]. They contain more than 400 compounds, specifically sesquiterpene lactones, flavonoids, phenolic acids, and tannins, and thus have appropriate oral bioavailability, blood-brain barrier permeability, and low toxicity, which make them potential novel candidates for the pharmaceutical and cosmetical industries [5-6]. Among them, *Inula helenium*, which is commonly known as elecampane, horse-heal, elfdock, andız, andızotu, giyabavesir, tehlegezink, and perinuk, has been commonly used in folk medicine for centuries. It is naturally grown in several parts across the world, including Europe, Asia, Africa and North America. In folk medicine, it has been traditionally used to treat several ailments, including epilepsy, ulcers, hepatitis, and cancer [7-8].

Phenolics are significant plant chemicals that interact and adapt to the biotic environment and overcome stress conditions, and the construction of a defence mechanism. They play an integral role in helping plants to adapt and survive in harsh environments [9]. Production and accumulation of these phytochemicals are generally dependent on environmental conditions and abiotic and biotic stressors [10]. However, the production of these phytochemicals generally depends on seasonal and environmental factors, and they are generally located in certain tissues and organs which limits their potential availability in the plant matrix. Production of phenolic compounds through Plant Tissue Culture (PTC) is a rapid method which performed by callus growth, application of elicitors, abiotic stressors and precursors. PTC studies revealed the presence of a pronounced amount of phenolics, including chlorogenic and caffeic acids, in callus obtained from *Inula* species [11]. However, there is limited research on the production of secondary metabolites through plant tissue culture. Therefore, an optimized PTC protocol of *Inula helenium* is needed to enhance the production of its phytochemicals.

The principal aim of the present study was to evaluate the concentrations of pharmaceutically important phenolic compounds of *Inula helenium* under various stressors, including copper sulphate, copper chloride, silver nitrate, and reduced MS nutrient medium and elicitors including melatonin and abscisic acid of i*n vitro* germinated plant samples and callus samples obtained from leaf, stem, root, and cotyledon explants. Therefore, the present study was designed to (a) establish a standardized PTC protocol of *Inula helenium* L. by using plant growth regulators and (b) measure the effects of stressors and elicitors on the concentration of the total phenolic content and individual phenolic compounds of nature, pot, and callus samples by using proper spectrophotometric and chromatographic methods.

Materials and Methods

Plant materials and preliminary preparation of samples

Inula helenium L. seed samples were obtained from Zeytinburnu Curative Plants Garden, which was located in İstanbul, Türkiye on August 20th, 2021. Seed materials were transferred to the laboratory within a maximum of 1 hour and subsequently cleaned of contaminants with 70% aqueous ethanol for 2 minutes, 10% solution of 5.4% sodium hypochlorite for 75 minutes, and rinsed thrice with distilled water for 3 minutes, respectively. A total of 50% germination rate was obtained through the abovementioned process. They were then germinated in a flowerpot at laboratory conditions, and their aerial parts of plant samples were cut, lyophilized, labelled as “pot samples”, and stored at -20°C in plastic lock bags for analysis. The second part of the seed samples was cleaned of contaminants with 10% aqueous solution of 5.4% sodium hypochlorite for 10 min and rinsed thrice with distilled water. Then, seeds were cultured in small jars that contain MS medium (Murashige and Skoog), 3% sucrose and 0.6% agar to obtain aseptic seedlings as explant sources. The pH of all cultures was adjusted to 5.8 and incubated in a growth chamber at 25 ± 2°C, under a photoperiod of 16 h (photon flux density: 500 µmol-2sec-1). After germination, the identities of plant materials were done at Department of Pharmaceutical Botany, Van Yuzuncu Yil University, Van, Türkiye and a voucher specimen was stored at the Hakkari University Biodiversity Research Herbarium (Herbarium code: VPH-494; Collector code: GA-01).

Explant and callus production

Leaf, cotyledon, root, and shoot segments were aseptically excised from *in vitro* regenerated 4-week-old plantlets and used as explants to establish calli. A high number of contaminations were observed in explant samples and callus obtained from pot samples and hence they were not used for further studies. After application of several MS mediums supplemented with plant growth regulators, 1 mg/L 2,4-D + 0,3 mg/L KN dark culture conditions at 25°C was selected for callus production. Through these cultures, calli were taken, freeze-dried by using a lyophilizator (Teknosem TRS 2/2V, Gebze, Türkiye), tagged in locked bags, and subsequently stored at -20°C until analysis.

Shoot production

Apical segments, shoots, and leaves of aseptic seedlings were aseptically excised from 4-week-old plantlets and were planted in different nutrient media supplemented with different concentrations of various plant growth regulators (PGRs) for the initiation of the direct or indirect organogenesis process. MS, B5, SH media were used as nutrient media, and BA, Kinetin, 2,4-D and NAA were used as PGRs for shoot production. Explants in sterile petri dishes (90x15 mm) were incubated at 25 ± 2°C in a climatic (air-conditioned) room at 500 µmol-2sec-1 fluorescence illumination in a 16/8 light/dark photoperiod. Through these cultures, samples were harvested, lyophilized and tagged in locked bags and subsequently stored at -20°C until analysis.

Application of abiotic stressors and elicitors in vitro

The stressors (copper sulphate (25 µm), copper chloride (25 µm), silver nitrate (25 µM), and reduced MS nutrient medium and elicitors (melatonin (35 µM), and abscisic Acid (100 µM),) were applied to the calli and *in vitro* regenerated plants. Samples were harvested, lyophilized, and tagged in locked bags and subsequently stored at -20° C until analysis.

Extraction procedure

The plant materials obtained from pots, aseptic seedlings and calli were lyophilized under a vacuum at

-51°C. Subsequently, 100 mg of lyophilized materials were treated with 2 mL of acidified methanol (80% methanol and 1% HCl (v/v) in distilled water) and mixed well. Then, the mixed solutions were sonicated for 15 minutes, centrifuged (10 minutes, 10000 rpm, rotor FA-45-24-11, Eppendorf, Germany), and the pellets were re-extracted another two additional times. Aliquots (3 mL) of the combined supernatants were stored frozen (-20°C) until analysis.

Analysis of phenolic compounds

Phenolic compounds were identified by liquid chromatography-diode array-mass spectrometry (LC-DAD-MS/MS) and quantified by high-performance by high performance liquid chromatography-diode array detector (HPLC-DAD) as described previously [12].

Results and Discussion

Seed Germination, in vitro plant regeneration and callus production

Initially, seed samples of *Inula helenium* were incubated in sterile water for 12 hours and then planted properly in pots (Figure 1a). To obtain *in vitro* plants, the surfaces of the seed samples were cleaned of contaminants using 70% isopropyl alcohol for 2 minutes followed by using 10% NaOCl for 10 minutes and subsequently washed thrice with distilled water. Consequently, no contamination was observed. MS medium was used as an optimized media for *in vitro* plant regeneration (Figure 1b). Leaf, petiole, shoot tip, cotyledon, and root explants were cultured in MS media containing various concentrations and combinations of plant growth regulators including 2,4-D, IBA, BAP, NAA, and KN, followed by incubation for 1 month at 25°C, 16/8 photoperiod, or dark conditions (Table I and Figure 1c).

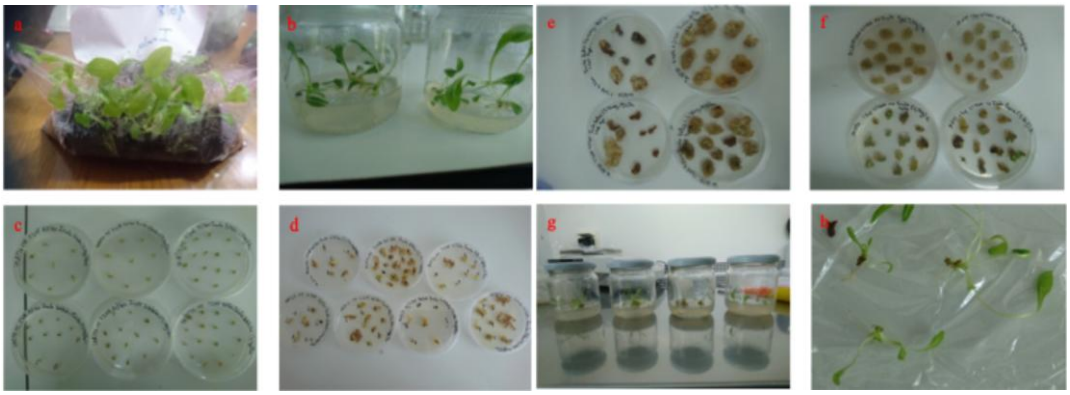

Figure 1.

In vitro plant regeneration and callus production: a) Pot plant sample, b) *in vitro* regenerated plant sample in MS media, c) explants cultured in MS media, d) callus samples regenerated in 1 mg/L 2,4-D + 0,3 mg/L KN at dark conditions, e-f) various callus formations under different plant growth regulators, g-h) *in vitro* shoot formation

Table I

Effect of plant growth regulators on explants

Plant Growth Regulator (mg/L)	Leaf	Petiole	Cotyledon	Shoot tip	Root
1 2,4-D	Callus	Callus	Callus	Callus	Callus
2 2,4-D	Callus	Callus	Callus	Callus	Callus
3 2,4-D	Callus	Callus	Callus	Callus	Callus
1 2,4-D + 0.1 KN	Callus	Callus	Callus	Callus	Callus
1 2,4-D + 0.3 KN	Callus	Callus	Callus	Callus	Callus
1,5 2,4-D + 0,3 KN	Callus	Callus	Callus	Callus	Callus
2 2,4-D + 0.3 KN	Callus	Callus	Callus	Callus	Callus
3 2,4-D + 0.3 KN	Callus	Callus	Callus	Callus	Callus
1 2,4-D + 0.6 KN	Callus	Callus	Callus	Callus	Callus
1 2,4-D + 1 KN	NA	NA	NA	NA	Callus
1 BAP	X	X	X	X	X
1 BAP + 1 IBA	X	X	X	Shoot	X
5 BAP + 2 2,4-D	Callus	Callus	Callus	Callus	Callus
1 BAP + 0,1 NAA	Callus	Callus	Callus	Shoot	Callus
2 NAA + 0,5 BAP	Callus	Callus	Callus	Callus	Callus
1 KN + 0,02 NAA	X	X	X	X	X
1 KN	NA	NA	NA	NA	Callus

NA: Not available, X: No formation of callus or shoot

Application of stressor and elicitor molecules on calli and in vitro regenerated plants

Calli used for stress and elicitor molecules application were selected from 1 mg/L 2,4-D + 0,3 mg/L KN media, which was incubated for 1 month for regeneration because of their high callus yield. As shown in Table II, 1/2 Reduced MS nutrient medium (2 weeks), 1/4 Reduced MS nutrient medium (2 weeks), 25 µM copper sulphate (1 week), 25 µM copper chloride (1 week), 25 µM silver nitrate (1 week) stressor and 100 µM abscisic acid (1 week), and 35 µM melatonin (1 week) elicitor molecules were applied to plant growth regulator-free *in vitro* regenerated plant samples at 25°C, 16/8 photoperiod.

The application of reduced MS nutrient medium stressors did not have a significant difference in viability, colour, and total weight increase compared to the control group (Table II and Figure 2). Copper sulphate and chloride applications also did not have significant difference in terms of colour and total weight increase but not viability compared to control group. However, application of silver nitrate had a remarkable difference in lower viability, different color (yellow), and decreased total weight increase compared to the control group (Table II and Figure 2). Concerning elicitor molecules, application of abscisic acid had a remarkable difference compared to the control group in terms of lower viability, different colour (deep brown), and decreased total weight increase, whereas melatonin application exhibited similar properties compared to the control group (Table II and Figure 2).

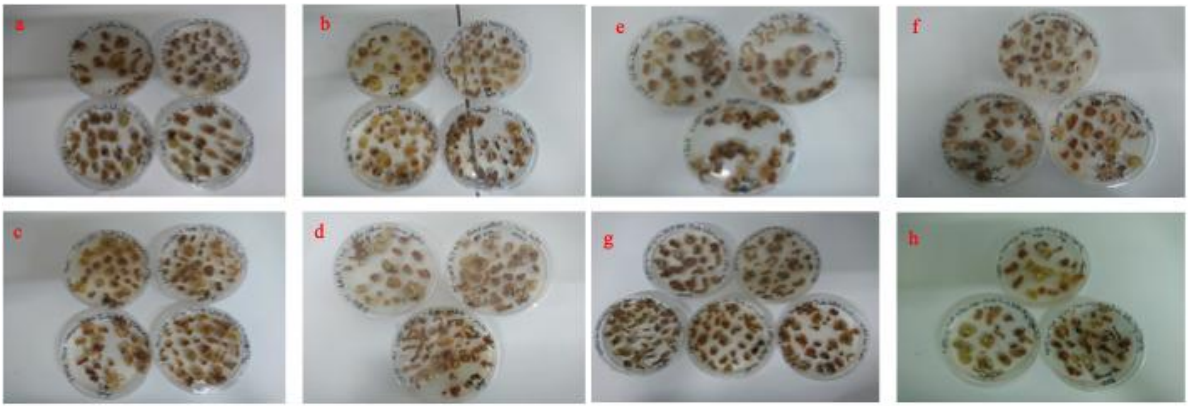

Figure 2.

Application of stressor and elicitor molecules on calli: a) Control, b) 1/2 reduced MS nutrient medium, c) 1/4 reduced MS nutrient medium, d) 25 µM copper sulfate, e) 25 µM copper chloride, f) 25 µM silver nitrate, g) 100 µM abscisic acid, h) 35 µM melatonin

Table II

Effect of stressors and elicitors on callus production

Application	Incubation time (week)	Callus		
		Viability	Colour	Total weight increase (%)
Control	2	High	White and brown	18.6
1/2 Reduced MS nutrient medium	2	High	White and brown	19.0
1/4 Reduced MS nutrient medium	2	High	White and brown	20.5
25 µM Copper sulfate	1	Medium	White and brown	18.6
25 µM Copper chloride	1	Medium	White and brown	20.0
25 µM Silver nitrate	1	Medium	Yellow	14.4
100 µM Abscisic acid	1	Low	Deep brown	6.7
35 µM Melatonin	1	High	White and brown	20.7

The effects of stressors and elicitors on *in vitro* regenerated plant development were summarized in Table III and shown in Figure 3. Like calli, reduced MS nutrient had similar viability, plant development, and average plant weight compared to the control group (Table III and Figure 3). The application of copper derivatives had similar effects on plant development, which were lower than the control group (Table III and Figure 3). Interestingly, silver nitrate and abscisic acid applications inhibited any plant development and resulted in lower viability and average weight compared to the control group, whereas melatonin caused relatively better effects (Table III and Figure 3).

Table III

Effect of stressors and elicitors on *in vitro* regenerated plant development

Application	Incubation time (week)	*In vitro* regenerated plant		
		Viability	Plant development	Average Plant Weight (g)
Control	2	High	Regular	0.090
1/2 Reduced MS nutrient medium	2	High	Regular	0.097
1/4 Reduced MS nutrient medium	2	High	Regular	0.088
25 µM Copper sulfate	1	High	Low	0.075
25 µM Copper chloride	1	High	Low	0.070
25 µM Silver nitrate	1	Low	-	0.038
100 µM Abscisic acid	1	Low	-	0.025
35 µM Melatonin	1	High	Low	0.067

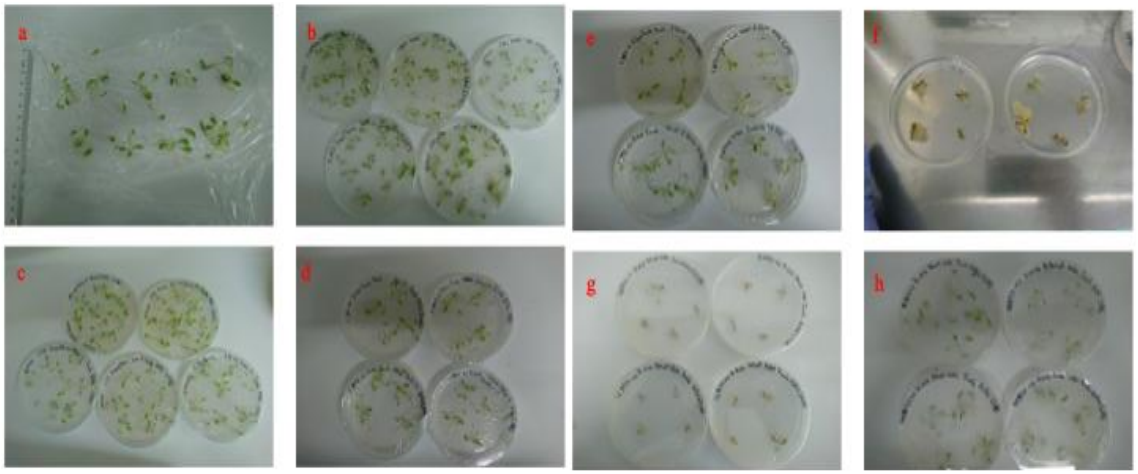

Figure 3.

Application of stressor and elicitor molecules on *in vitro* regenerated plant: a) Control, b) 1/2 reduced MS nutrient medium, c) 1/4 reduced MS nutrient medium, d) 25 µM copper sulphate, e) 25 µM copper chloride, f) 25 µM silver nitrate g) 100 µM abscisic acid, h) 35 µM melatonin

Effects of plant tissue culture applications on phenolic composition

Total phenolic content and individual phenolic compounds present in the sample matrix were presented in Table IV and Figure 4. A total of 4 major phenolic compounds were identified which were roughly composed of hydroxycinnamic acids and flavonols. Hydroxycinnamic acids were represented by chlorogenic and caffeic acids, whereas flavonols were represented by a common flavonol-quercetin and its rutinoside-rutin. Of all biomolecules, quercetin was found as the major key phenolic compound of callus cultures of *Inula helenium* (Table IV).

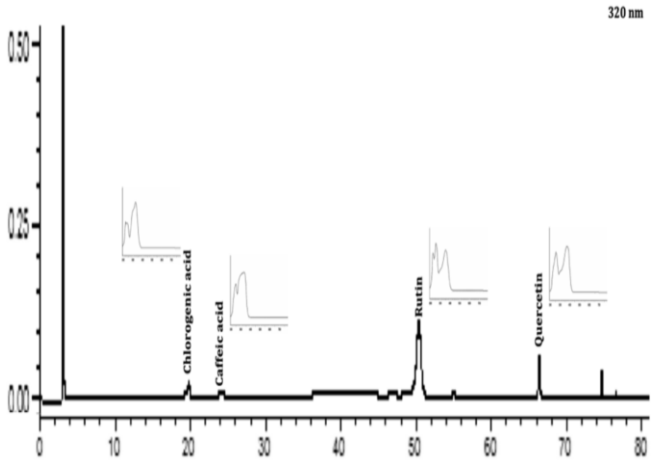

Figure 4.

Representative HPLC Profile of *Inula helenium* L. plant tissue culture samples

Table IV

Effects of plant tissue culture applications on phenolic composition (µg /g weight)

	Samples	Total phenolics [1]	Chlorogenic acid	Caffeic acid	Rutin	Quercetin
Control samples	Pot sample	86 ± 3	9 ± 0	8 ± 0	1 ± 0	1 ± 0
	In vitro regenerated samples without PGR	41 ± 3	1 ± 0	0.4 ± 0.0	1 ± 0	2 ± 0
	Organogenic sample (Stem)-MS 1 mg/L BAP + 0.1 mg/L NAA	130 ± 4	9 ± 0	1 ± 0	6 ± 1	33 ± 1
Callus samples	**+ Plant growth regulator**					
	MS 2 mg/L 2,4 D (Dark)	34 ± 2	4 ± 0	1 ± 0	10 ± 1	36 ± 1
	MS 1 mg/L 2,4 D + 0.3 mg/L KN (Dark)	115 ± 2	14 ± 0	5 ± 1	5 ± 1	321 ± 13
	MS 1 mg/L BAP + 0.1 mg/L NAA (16-8; Dark-Light)	169 ± 11	12 ± 0	3 ± 0	3 ± 0	256 ± 20
	MS 0.5 mg/L BAP + 2 mg/L NAA	118 ± 5	11 ± 0	2 ± 1	16 ± 1	241 ± 17
	+ Stressors applied					
	1/2 Reduced MS (1 mg/L 2,4 D + 0.3 mg/L KN)	108.1 ± 5	2 ± 0	1 ± 0	14 ± 1	88 ± 4
	1/4 Reduced MS (1 mg/L 2,4 D + 0.3 mg/L KN)	89 ± 5	17 ± 0	6 ± 1	4 ± 1	19 ± 1
	25 µM Silver nitrate MS (1 mg/L 2,4 D + 0.3 mg/L KN)	53 ± 3	19 ± 0	3 ± 1	30 ± 2	67 ± 8
	25 µM Copper sulphate MS (1 mg/L 2,4 D + 0.3 mg/L KN)	65 ± 2	9 ± 0	1 ± 0	11 ± 1	49 ± 3
	25 µM Copper chloride MS (1 mg/L 2,4 D + 0.3 mg/L KN)	82 ± 6	11 ± 1	3 ± 1	13 ± 1	66 ± 4
	+ Elicitors applied					
	100 µM Abscisic acid MS (1 mg/L 2,4 D + 0,3 mg/L KN)	140 ± 9	24 ± 0	5 ± 1	18 ± 1	312 ± 15
	35 µM Melatonin MS (1 mg/L 2,4 D + 0.3 mg/L KN)	138 ± 8	32 ± 0	2 ± 0	6 ± 1	388 ± 17
***In vitro* regenerated samples without PGR**	**+ Stressors applied**					
	1/2 Reduced MS	24 ± 1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	1 ± 0
	1/4 Reduced MS	21 ± 2	0.2 ± 0.0	0.1 ± 0.0	0.9 ± 0.0	11 ± 1
	25 µM Silver nitrate MS	15 ± 0	0.1 ± 0.0	0.04 ± 0.0	0.02 ± 0.0	1 ± 0
	25 µM Copper sulphate MS	17 ± 1	0.1 ± 0.0	0.1 ± 0.0	0.03 ± 0.0	3 ± 0
	25 µM Copper chloride MS	16 ± 2	0.1 ± 0.0	0.03 ± 0.0	0.03 ± 0.0.0.00	1 ± 0
	+ Elicitors applied					
	100 µM Abscisic acid MS	14 ± 0	0.1 ± 0.0	0.05 ± 0.0	0.01 ± 0.0	2 ± 0
	25 µM Melatonin MS	22 ± 2	0.1 ± 0.0	0.06 ± 0.0	0.01 ± 0.0	2 ± 0

[1] µg Gallic acid Eq/ g weight by HPLC; PGR: Plant growth regulator, MS: Murashige and Skoog medium, BAP: 6-Benzylaminopurine, NAA: 1-Naphthaleneacetic acid, 2,4-D: 2,4-Dichlorophenoxyacetic acid, KN: Kinetin

Among control samples, the organogenic sample treated with 1 mg/L BAP + 0,1 mg/L NAA had the highest amount of total phenolics and flavonols (quercetin and rutin), followed by the pot sample, and the *in vitro* regenerated sample without PGR. An organogenic sample treated with 1 mg/L BAP + 0.1 mg/L NAA was dominated by mainly flavonols (quercetin and rutin) and the pot sample was principally dominated by hydroxycinnamic acids (chlorogenic and caffeic acids). The lowest levels of total and individual phenolics were found in the *in vitro* regenerated sample without PGR (Table IV).

Callus samples treated with various combinations of PGR, including 2,4-D, KN, BAP, and NAA, had higher amounts of total and individual phenolics compared to control samples except for 2 mg/L 2,4-D in dark conditions. 1mg/L 2,4-D + 0,3 mg/L KN treated callus samples fulfill the optimum conditions for accumulation of total and individual phenolics, followed by 1 mg/L BAP + 0,1 mg/L NAA combination (Table IV). Therefore, stressor and elicitor molecules were applied 1mg/L 2,4-D + 0,3 mg/L KN-treated callus samples. Based on data obtained (Table IV), it could be concluded that elicitor compounds had superior phenolic compound-producing abilities, with both abscisic acid and melatonin exhibiting similar characteristics. With regards to stressors, the pattern of ½ reduced Ms nutrient medium > ¼ reduced MS nutrient medium > silver nitrate ≥ copper chloride > copper sulphate was obtained for phenolics production (Table IV).

The accumulation of chlorogenic and caffeic acids and rutin was only at trace levels among all *in vitro* regenerated samples without PGR. There was no significant effect of either stressors or elicitors applied. Solely, relatively lower amounts of total phenolics were obtained where the quercetin compound dominated as the major compound of the samples compared to control groups. Contrary to callus samples' chromatographic findings, stressors had superior stimulative effects than elicitors in terms of secondary metabolite synthesis and production (Table IV).

Various plant growth regulator combinations including 2 mg/L 2,4-D, 3 mg/L 2,4-D, 1,5 mg/L 2,4-D + 0,3 mg/L KN, 2 mg/L 2,4-D + 0,3 mg/L KN, 1 mg/L 2,4-D + 0,3 mg/L KN, 3 mg/L 2,4-D + 0,3 mg/L KN, 1 mg/L 2,4-D + 0,6 mg/L KN, 1 mg/L 2,4-D + 1 mg/L KN, 1 mg/L KN, 1 mg/L BAP + 1 mg/L IBA, 1 mg/L BAP + 0,1 mg/L NAA and 0,5 mg/L BAP + 2 mg/L NAA were examined for callus production in this study, which included some novel plant tissue culture applications of *Inula helenium*. The highest yield of callus production was obtained through 1 mg/L 2,4-D + 0,3 mg/L KN application which was coherent with previous reports [11] and could be explained through the callus stimulative effect of 2,4-D and the cellular division enhancing property of KN [13]. Gaspar and co-authors reported the negative effects of ethylene gas in calli which included inhibition of callus development and prevention of root formation [14]. The colours of callus samples became brownish after subculture process in this study, which could be associated with excessive synthesis of ethylene gas. Also, as shown in Table II, application of abscisic acid had a negative effect on callus color, which caused a conversion from white to deep brown. Abscisic acid could inhibit the effects of auxins, cause stomatal balance, defoliation, and senescence in plant physiology and inhibit callus development and organogenesis in plant tissue culture [14] which explains its negative effects on callus color, viability, and weight increase.

Phenolic compounds play crucial roles in biological functions of plant mechanisms such as UV absorbance, pollination, and signalling against abiotic stresses. The biosynthetic pathway of phenolics is generally linked to the activity of chalcone isomerase and ammonium lyase enzymes, stressors and elicitors [15]. The highest values of total phenolic concentration of callus samples were obtained through the combination of BAP+NAA in the plant growth regulators (PGR) group, ½ reduced MS (1 mg/L 2,4 D + 0,3 mg/L KN) in the stressor group, and both abscisic acid and melatonin in the elicitor group (Table IV). These findings are in accordance with Cingöz and Karakaş [16] who reported the pronounced effect of ½ MS stress on production of phenolics in calli of *Bellis perennis* and suggest that standardized laboratory conditions stimulated the accumulation of phenolic compounds of Elecampane. Also, Al Khayri [17], Riaz and co-authors [18] and Coşkun and co-authors [19] found the stimulative effects of different concentrations of melatonin on phenolic production in callus samples. It could be speculated that the combination of BAP+NAA, reduced MS, abscisic acid, and melatonin stimulated the synthesis of phenolics through the biosynthetic pathway by increasing the stress resistance of *Inula helenium*. The application of silver nitrate, copper sulphate, and copper chloride stressors decreased the levels of total phenolics in callus samples (Table IV), which could be associated with the probable toxic effects of abovementioned stressors on callus samples. The significant decrease of total phenolics in stressors and elicitors applied *in vitro* regenerated samples could be due to the lack of PGR in these samples.

Chlorogenic and caffeic acids are among the most common phenolic acids in the plant kingdom which are reported for their several food, medicinal, and industrial importance [20]. Our findings revealed that the amount of chlorogenic acid was increased under silver nitrate and ¼ reduced MS stressors and melatonin and abscisic acid elicitors. These results are coherent with previous studies [11, 16]. With regards to the caffeic acid compound, no significant change was detected. Caffeic acid involved in the synthesis of chlorogenic acid and lignin under biotic and abiotic stresses [18], which suggests its use as a precursor in chlorogenic acid synthesis in *Inula helenium*.

The flavonol compounds exhibited different responses to stressors and elicitors in this study. The application of various PGRs increased the level of quercetin at pronounced levels (8-10-fold), while slightly higher levels of rutin observed. Stressors had relatively lower effects on synthesis of quercetin but higher effects on rutin metabolism. On the other hand, elicitors have remarkable positive effects on quercetin synthesis, but not on rutin. The levels of both quercetin and rutin had no remarkable levels in *in vitro* regenerated samples without PGR (Table IV). These findings revealed the key role of 2,4-D and KN in flavonol synthesis of *Inula helenium*, which corroborates with the findings reported previously [18].

Conclusions

The application of various stressors and elicitors on phenolic metabolism of *in vitro* callus cultures of Elecampane revealed that MS nutrient medium supplemented with 1 mg/L 2,4-D + 0.3 mg/L KN + 35 µM melatonin stress in dark conditions might be a proper protocol to produce phenolic compounds-rich *Inula helenium* L. plant samples for scientific and industrial utilization.

Conflict of interest

The authors declare no conflict of interest.

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