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IN VITRO INFLUENCES OF SUGAR NUTRITION AND LIGHT CONDITION ON ACCUMULATION OF SOME PHYTOCHEMICALS IN *VERBASCUM THAPSUS* L. CULTURE

Ashwaq S. Abed[1], Duha M. Majeed[1]*, Mohammed A. Mohammed[2] and Abedaljasim M. Jasim Al-Jibouri

[1]Biotechnology Research Center, Al-Nahrain University, Baghdad 10072, Iraq.
[2]College of Agriculture, University of Anbar, Iraq.

Abstract

Verbasceae plants are known for the high diversity of their secondary metabolites. Most of them were used as traditional and healthful effects, therefore the aim of this project focused on enhancing the accumulation of some phytochemical compounds in callus tissue of common mullein (*Verbascum thapsus* L.) that cultivated under abiotic elicitors (internal and external stimuli), their included; sugar nutrition (six types of sugars as a carbon source were added separately to the medium with two concentrations 30 and 60 g/l), likewise exposure to different light conditions. Changes in three phytochemicals concentrations (coumarin, eugenol and thymol) were determined using HPLC analysis.

Compared to the control (plant mother), the callus tissues showed a motivating increase in coumarin and eugenol content, while decreasing in thymol concentrations. Likewise, the incubation at blue and red light condition, also the exposure to the ultraviolet radiation (UV-C) highly enhanced production of coumarin and eugenol compounds. While the most *in vitro* treatments inhibited the production of thymol compared to mother plants. The data indicate that sugars and light stresses may be trigger the assembly and accumulation of some phytochemical compounds in cultures of common mullein (*in vitro*).

Key words: *Verbascum thapsus*, Comarin, Eugenol, Thymol, Amino Acids, Sucrose, UVlight.

Introduction

The genus of *Verbascum thapsus* L. belongs to the Verbasceae (Scrophulariaceae) family, known as mullein, common or great mullein, grows best on stony ground in wasteland, roadsides, forest clearings and pastures (Parker, 2003). *Verbascum* species are rich in active compounds, are used for several years as flavoring medication. Most components of plant have medicine and demulcent properties, are used as anodyne, antiseptic, spasmolytic, emollient, nervine, vulnerary, analgesic, antihistaminic, anticancer, anti-oxidant, antiviral, biological process, antimycotic agent and cardio depressant (Tatli and Akdemir, 2006; Huang *et al*., 2009). Additionally, they are used for traditionally uses like hair coloring and candle wicks (Riaza, 2013). Several researchers instructed that medicinal impact can be due to their content of pharmaceutical compounds like saccharide, mucilage, saponins, alkaloids and lots of phenolic compounds (Turker and Gurel, 2005).

Phytochemicals play necessary roles in acclimating the plant to its surrounding environment (Swain, 2013), these include; flavonoids, phenolic acids, coumarins, eugenol, thymol, tannins, stilbenes, curcuminoids, quinones, lignans and others (Bileflimi, 2004; Huang *et al.*, 2009). *In vitro* biotechnological methods offer promising ways for enhancement the accumulation of plant secondary metabolites, plant tissue culture techniques are used an alternative method for production and accumulation of secondary metabolites in situations when plant material is rare or difficult to acquire and when chemical synthesis of their metabolites is low or not possible (Ajungla *et al.*, 2009).

There are different aspects of tissue culture that used for the accumulation of secondary metabolites under *in vitro* conditions, (Ahmad *et al.*, 2013) reviewed these aspects; optimization of cultural conditions, elicitation, precursor feeding, influence of growth regulators and permeabilization. Elicitors are classified as biotic or abiotic stress, internal or external stimuli for yielding the increased

***Author for correspondence :** E-mail: duham.majeed@brc.nahrainuniv.edu.iq, duha_majeed@yahoo.com

levels of plant products; for example, UV radiation was effective stimuli on phenolic compounds in broad bean seedlings (Younis *et al.,* 2010). Carbohydrates can even be *used* as a *carbon* supply to manage the assembly of of secondary metabolites in *Fusarium avenaceum* (Sørensen and Giese, 2013). Salicylic acid as a chemical stress increased the synthesis of secondary metabolites in root cultures of *Datura metel* (Ajungla *et al.,* 2009). Also, it's been found that content of thymol was increased in the callus cultures of *Origanum vulgare* by using proline as amino acid supply (Al-Jibouri *et al.,* 2012a). Hyoscyamine and scopolamine enhanced in *Hyoscyamus niger* cultures by employing abiotic elicitors; sucrose, NaCl, proline and BA were added separately to MS medium (Al-Jibouri *et al.,* 2012b). Another successful example was Chavan (Chavan *et al.*, 2011) who enhanced the accumulation of α-tocopherol and pigment productions in cell cultures of *Carthamus tinctorius* utilizing abiotic elicitors, NaCl and $MgSO_4$. Light conditions have been mentioned to provide a significant influence on phytochemicals of *broccoli sprouts* (Perez-Balibrea *et al.,* 2008). Furthermore, in a variety of plant species, the synthesis of flavonoid compounds was induced by UV radiation as an adaptive response, because aromatic secondary metabolites engage in protect plant DNA from the damaging effects of UV radiations (Li *et al.*, 1993; Dixon and Paiva, 1995). Therefore, the accumulation of plant active compounds may be stirred by precursors, elicitors or environmental stresses. The target of this study was elevating the accumulation of some important phenolic compounds (coumarins, eugenol and thymol) in callus tissue of *Verbascum thapsus* L. using different kinds and levels of sugars or light conditions as internal and external stimuli (abiotic elicitors).

Material and methods

Plant materials

This study was applied at Biotechnology Research Center, Al-Nahrain University, Baghdad, Iraq. Healthy *in vitro* plantlets of *Verbascum thapsus* L. were used as source of explant for callus induction, these plantlets were achieved according with (Al-Jibouri *et al.,* 2016), (MS) medium (Murashing and Skoog, 1962), was supplemented with 0.5 mg/l of BA for initiation of callus tissue, after 6 weeks of incubation at 25±2°C with dark condition, the successfully induced callus was re-cultured each four weeks in fresh medium for two months continuously to maintain callus stock.

Abiotic elicitation: Equal weights of fresh callus (about 320 mg) were cultivated on the same medium that employed in maintenance of callus. Two kinds of abiotic elicitors have been added to the medium separately, including;

• Sugars nutrition: 2 concentrations (30 and 60 mg/l) of 3 different sources of sugars; 3 monosaccharides (Fructose, aldohexose and Mannose), one disaccharides (Sucrose) and 2 sugar alcohol (Mannitol and Sorbitol) were used separately as carbon source-internal stimuli on secondary metabolites production. All cultures were incubated at 25±2°C with dark condition.

• Light condition: Four sources of light were used as external stimuli on synthesis of secondary metabolites compared with dark period; fluorescent, blue and Red lights were executed under 16 h. photoperiod per day, finally the irradiation exposure to UV-C light (254 nm) was for 10, 15 and 20 minutes, then these treatments were incubated under white light period for 16 h/day.

Fresh and dry weights of callus were measured after one month of incubation. Three phytochemicals compounds were analyzed for qualitative and quantitative analysis.

Samples extraction and chromatography

Extraction of some phytochemicals compounds from the callus and dried mother plant were performed as described by (Tamura and Nishibe, 2002), 200 mg of each sample was soaked overnight with 5 ml of methanol at room temperature. After centrifugation, Whatman filter papers with 0.22 µm syringe filters were used for filtration the supernatant to make it ready to be used chromatographic analysis.

Each extract was analyzed with High-performance liquid chromatography (HPLC), (Sykum-German) system; Separation was performed on C-18 column (250 × 4.6 mm) at 30°C. Acetonitrile and water in 75:25 (v/v) was set as the mobile phase. The elution mode was in an isocratic program with a flow rate of 1.4 $mL \cdot min^{-1}$; injection volume (20µl); UV detection was at (210 nm). Quantitative method was performed by external calibration. All standards (coumarin, eugenol and thymol) were obtained from Sigma-Aldrich (USA).

Statistical analysis

In vitro experiments were applied in 20 replicates. All data relating to experiments were analyzed by ANOVA (single factor) using MINITAB 11 statistical program. The variations among means of groups were compared using LSD test at 0.05 level of significance.

Results

Effect of sugars and Light conditions on in vitro growth factors

In our results, growth rate parameters differed significantly (Table 1) in response to the various sugars applied. Sucrose and Glucose gave highest growth activities of callus; they were recorded at (60 mg/l) of sugars, (4349.0 and 3149.5 mg), respectively for fresh

Table 1: Influences of sugars on *in vitro* growth factors of *Verbascum thapsus* L. (fresh and dry weight) of callus (mg) grown on MS medium for 30 days. Initial weight was 320 mg.

Sugars nutrition (g/l)		Fresh weight (mg)	Dry weight (mg)
Sucrose	30	2321.6	140.6
	60	4349.0	301.8
Glucose	30	2936.4	149.6
	60	3149.5	176.5
Fructose	30	1016.4	64.2
	60	1314.0	84.6
Mannose	30	660.3	69.1
	60	469.0	61.0
Mannitol	30	335.0	33.1
	60	338.0	35.0
Sorbitol	30	272.8	24.0
	60	235.3	23.4
LSD $_{0.05}$		453.09	22.08

weight and (301.8, 176.5 mg), respectively for dry weight). Whereas, the lowest biomass production occurred with mannose and sugar alcohols (Mannitol and Sorbitol) treatments, they significantly inhibited callus growth parameters.

Light conditions, shows greatest growth development at white light and UV-C irradiation treatments. The highest values for fresh and dry weights were registered at UV-C light at 20 min. (4227.1 and 216.1, respectively), whereas the lowest weights were monitored at dark condition (2321.6 and 140.6, respectively), (Table 2).

Generally, different Light conditions caused statistical promoting in growth parameters of callus compared with blue light and dark condition.

Effect of sugars and Light conditions on accumulation of phytochemical compounds (coumarin, eugenol and thymol)

HPLC analyzed contents of coumarin, eugenol and thymol in leaves of mother plant (*Verbascum thapsus* L.); they were at (10, 41 and 310 ppm) respectively. The results in table 3, illustrated that addition of various kinds of sugars to the medium culture separately as internal stimuli enhanced the assembly of phenolic compounds in callus tissue; (30 g/l) of sucrose or glucose led to raise values of coumarin to (90.2 and 42.4 ppm) respectively, which were (+802 and +324) higher than mother plant. Whilst the other sugar treatments did not give any sensible results. Furthermore, the most levels of sugars were led to high production of eugenol compared to mother plant, While these treatments decreased thymol biosynthesis in callus culture, except (60g/l) of sucrose raised thymol content to (1260 ppm), to reach (+306) higher than mother plant, (Fig. 1).

Table 2: Influences of light conditions on *in vitro* growth factors of *Verbascum thapsus* L. (fresh and dry weight of callus (mg)) grown on MS medium for 30 days, Initial weight was 320 mg.

Light conditions	Fresh weight (mg)	Dry weight (mg)
Dark	2321.6	140.6
White light	3946.8	216.1
Blue light	2634	167.6
Red light	3410.7	191.9
UV-C light (10 min)	3228.3	194
UV-C light (15 min)	3398.6	209
UV-C light (20 min)	4227.1	216.1
LSD $_{0.05}$	520.4	22.7

Table 4, demonstrated the effect of different lights

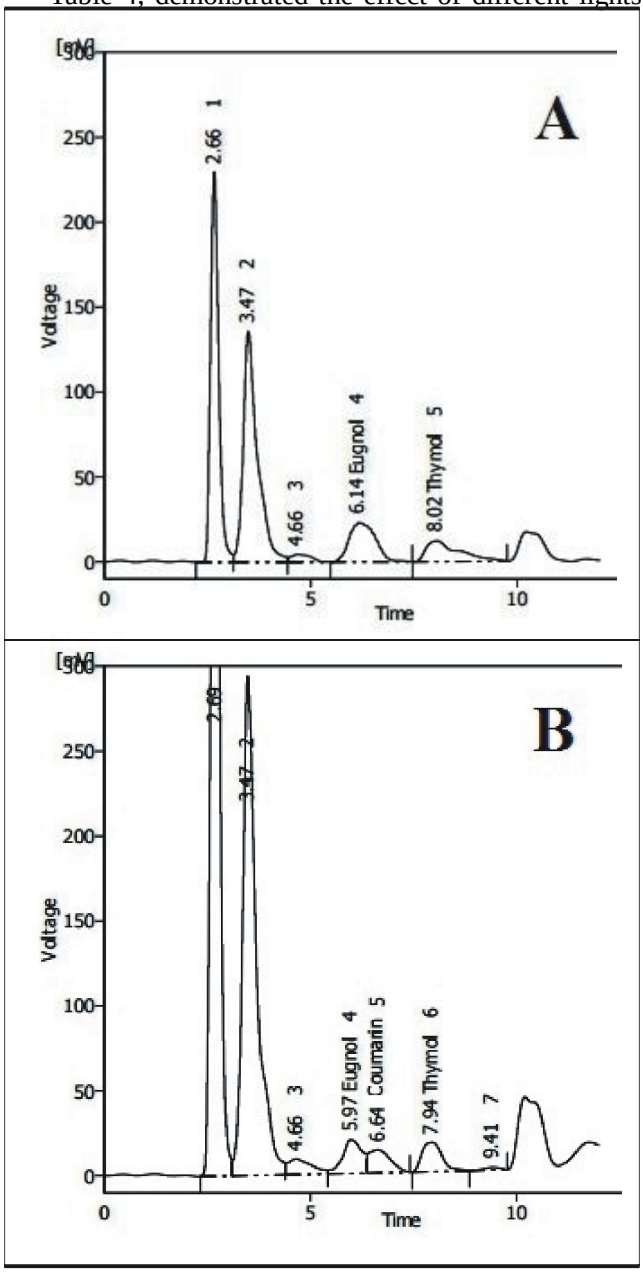

Fig. 1: HPLC chromatograms for: (A) Glucose (60 g/l); (B) UV-C light (20 min).

Table 4: Effect of light conditions on accumulation of coumarin, eugenol and thymol in callus culture of *Verbascum thapsu* L. after 4 weeks of incubation.

Light Conditions	Coumarin		Eugenol		Thymol	
	ppm	% of control	ppm	% of control	ppm	% of control
Control (*mother plant*)	10		41.0		310	
Dark	90.2	802	104.6	155	79.1	-74
White light	52.7	427	11	-73	Trace	Trace
Blue light	Trace	Trace	352.5	760	142.3	-117
Red light	127.1	1171	128.1	212	179.4	-42
UV-C light (10 min)	Trace	Trace	112.3	174	211.4	-32
UV-C light (15 min)	61.8	518	118.8	190	199.6	-35
UV-C light (20 min)	103.8	938	67.5	64	304	-2

on phenolic compounds production. Most *in vitro* treatment (with or absent of lights) enhanced the production of coumarin and eugenol compared with mother plant, maximum production of coumarin was observed at red light (127.1ppm), it was (+1171) higher than mother plant. At the same time, blue light successfully promoted the accumulation of eugenol in the callus tissue, reached to (352.5 ppm), (+760) more than mother plant. Generally, exposure to UV-C for 20 min positively affected the growth of callus tissue and accumulation of coumarin more than other time treatments of UV light, While the shorter times of exposure (10 and 15 min) led to enhancing production of eugenol more than (20 min). Fig. 1, appeared HPLC analysis of two different treatments, glucose at (60 g/l) and UV-C light at (20 min).

Discussion

The above results suggest that different carbohydrate sources can be used as abiotic factor which tend plants to stimulate the production of secondary metabolites. Sugars are a soluble carbohydrate, used as a vital carbon source, play important role in primary metabolic pathways such as glycolysis and photosynthesis, also as osmotic stress agent (Seigler, 2012). Structurally, the cell wall of plants is mostly made up of various sugars such as cellulose, glucose, mannose etc. (Alberts *et al.*, 2002). Furthermore, the accumulation of phenols can be enhanced under conditions where plants have excess carbon more than level which can be needed for plant growth (Ilvessalo and Tuomi, 1989). For instance, sucrose and mannitol have been used for highly production of phenolic compounds and other metabolites in *broccoli sprouts* (Guo *et al.*, 2011).

Most Plant species can produce ultraviolet absorbing substances such as anthocyanins, terpenoids and phenolic compounds that protect from UV damage and can also scavenge free-radicals, coumarins structure can be activated by UV radiation, causing phototoxic reactions (Mulder-Krieger *et al.*, 1988; Matsuki, 1996). Recent proofs by biochemists demonstrated that UV and blue light activate the phytochrome, this reaction enhances the activity of most enzymes involved in synthesis of phenols (Swain, 2013). Quantity analysis test showed thymol to be the main phenolic components which decreased in amount under most stress conditions, similar results have been notified by (Mulder-Krieger *et al.*, 1988) whose reported the amount of flavonoids and essential oils in the peel explants were relatively high as compared to those accumulated in the callus culture. But other reports described that *in vitro* cultures of *Carum copticum* and *Hypericum perforatum* succeeded to produce different flavonoids.

It has been demonstrated that biosynthesis and accumulation of metabolites depending on the related genes expression which are transcribed to functional enzymes, (Treutter, 2010; Hamzah and Hasso, 2019). The exposure to elicitors induces many signaling molecules such as jasmonic acid, salicylic acid or ethylene that lead to stimulate the expression of genes which involving in defense responses (Matsuura *et al.*, 2012).

Table 3: Effect of Sugars nutrition (g/l) added to MS medium on accumulation of coumarin, eugenol and thymol in callus culture of *Verbascum thapsus* L. after 4 weeks of incubation.

Sugars nutrition (g/l)		Coumarin		Eugenol		Thymol	
		ppm	% of control	ppm	% of control	ppm	% of control
Control (*mother plant*)		10		41.0		310	
Sucrose	30	90.2	+802	104.6	+155.1	79.1	-74
	60	-	-	151.6	+269.7	1260	+306
Glucose	30	42.4	+324	-	-	-	-
	60	-	-	220.4	+437.5	248.6	-20
Fructose	30	-	-	244.2	+495.6	137.3	-55
	60	-	-	171.9	+319.2	18.0	-94
Mannose	30	-	-	142.3	+247	196.3	-36
	60	-	-	106.1	+158.7	190.0	-38

Conclusion

Sugar nutrition, UV or light conditions are powerful tools to improve plant secondary metabolism pathways, particularly the phenolic content. The current results support using the above elicitors treatments, to provide large-

scale strategies for supporting yields of secondary compounds in tissue culture of *Verbascum thapsus*. However, these effects vary depending on the type of elicitor, duration and their concentrations, but they could play a substantial role in biosynthetic pathways to increase production of commercially phytochemical compounds.

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