INDUCING SOME SECONDARY METABOLITES FROM CALLUS CULTURES DERIVED FROM *Plantago psyllium* AND *Plantago major* EXPOSED TO COBALT STRESS

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ABSTRACT

This experiment was conducted to study the influence of cobalt concentrations on the production of seven flavonoid compounds in callus derived from *Plantago psyllium* L. and *Plantago major* L. Results showed that the best combination of 2,4-D and kinetin concentrations add to Muroshige and Skoog medium to obtain the highest fresh weight of 541.0 mg was 3.0 and 1.0 mg.L^{-1} respectively. *psyllium* stimulated callus produced the highest fresh weight of 365.7 mg. The addition of 75 ppm of cobalt resulted in a significantly lower fresh weight of *P. psyllium* callus (139.8 mg). The interaction between *Plantago* species and cobalt concentrations was significant. The callus inducted from *P. major* had significant increases of the scutallarein, apigenin, nepetin and luteolin compounds with 26.40, 22.64, 14.93 and 26.20 µg.100mg^{-1} dry weight, respectively. The production of the hispidulin compound was increased in *P. psyllium* at 29.40 µg.100mg^{-1} dry weight. Also, the addition of cobalt metal stimulated the production of flavonoids at 50 ppm cobalt producing the highest amounts of hispidulin and luteolin at 40.30 and 41.60 µg.100mg^{-1} dry weight, respectively. Meanwhile, 75 ppm cobalt treatment produced the highest amount of scutallarein, apigenin, nepetin and aucubin at 25.61, 23.25, 15.90 and 13.70 µg.100mg^{-1} dry weight, respectively. The callus inducted from *P. major* treated with 50 ppm of cobalt showed the highest production of scutallarein, apigenin and luteolin at 30.33, 32.26 and 51.90 µg.100mg^{-1} dry weight respectively. Baicalein reached 16.46 µg.100mg^{-1} dry weight, at 75 ppm of cobalt metal treatment in callus inducted from *P. psyllium*.

Keywords: plant tissue culture, medicinal plants, flavonoids, heavy metals.

إستحثاث بعض مركبات الأيض الثانوي المستمدة من كالس *Plantago psyllium* و *Plantago major* المُعرض لإجهاد الكوبلت

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المُستخلص

نُفذت تجربة بهدف دراسة تأثير التراكيز المختلفة من معدن الكوبلت في إنتاج سبعة مركبات فلافونيدية من كالس نباتي *Plantago psyllium* L. و *Plantago major* L.. أظهرت النتائج بأن اضافة التركيزين 3.0 و 1.0 ملغم.لتر$^{-1}$ من منظمي النمو 2,4-D والكاينتين الى وسط مورشايج وسكوج على الترتيب, قد أعطيا أفضل توليفة لإستحثاث الكالس فقد أعطت التوليفة أعلى وزن طري للكالس المُستحث بلغ 541.0 ملغم. أعطى المحفز من نبات *P. psyllium* أعلى وزن طري بلغ 365.7 ملغم. أدت إضافة الكوبلت بالتركيز 75 جزء بالمليون إلى إنخفاض معنوي للوزن الطري للكالس المستحث من *P. psyllium* (139.8 ملغم). كما أثر التداخل بين النوعين النباتيين وتراكيز الكوبلت معنوياً في الوزن الطري. الكالس المستحث من نبات *P. major* سبب في زيادة معنوية في انتاج مركبات scutallarein , nepetin , apigenin , luteolin بمقدار 26.40 , 22.64 , 14.93 , 26.20 مايكروغرام. 100 ملغم وزن جاف$^{-1}$ بالترتيب. كما ازداد انتاج نبات *P. psyllium* من مركب hispidulin وبمقدار 29.40 مايكروغرام. 100 ملغم وزن جاف$^{-1}$. أيضاً أدت إضافة معدن الكوبلت بالتركيز 50 جزء بالمليون إلى زيادة إنتاج مركبي hispidulin و luteolin بمقدار 40.30 , 41.60 مايكروغرام. 100 ملغم وزن جاف$^{-1}$بالترتيب. في ذات الوقت أدت المعاملة بمعدن الكوبلت وبالتركيز 75 جزء بالمليون إلى إنتاج عالي من مركبات scutallarein , apigenin , nepetin , aucubin وبمقدار 25.61 , 23.25 , 15.90 , 13.70 مايكروغرام. 100 ملغم وزن جاف$^{-1}$ بالترتيب. كما تبين بان الكالس المستحث من نبات *P. major* والمعامل بمعدن الكوبلت بالتركيز 50 جزء بالمليون قد حقق اعلى انتاج من مركبات scutallarein , apigenin , luteolin وبمقدار 30.33 , 32.26 , 51.90 مايكروغرام. 100 ملغم وزن جاف$^{-1}$ بالترتيب. أما مركب baicalein فقد وصل إنتاجه إلى 16.46 مايكروغرام. 100 ملغم وزن جاف$^{-1}$ عند معاملة الكالس المستحث من نبات *P. psyllium* بالتركيز 75 جزء بالمليون.

الكلمات المفتاحية: زراعة الأنسجة النباتية، نباتات طبية، الفلافونيد، المعادن الثقيلة.

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INTRODUCATION

The agricultural sector contributes to meeting the needs of other sectors. In particular, it provides raw materials for the pharmaceutical industry through the use of natural chemical compounds formed within plants. Many of which have been used in the past centuries to treat various diseases, some of them intractable. With the development of pharmaceutical sciences and the existence of modern methods of analysis. The need for these natural compounds to provide a safe treatment at a small cost and the difficulty of manufacturing some of them in the laboratory it is still necessary to obtain them from natural sources (7). The genus Plantago, belongs to the family Plantaginaceae, includes many important medicinal plants. There are more 275 Plantago species have been specified worldwide (20), includes many important medicinal plants. It has high active compounds for the therapy of many diseases such as antidiarrhoeal, anticonstipatium (18) antibacterial, antidiabetic, antinociceptive, antiviral (1), anti-inflammatory (4), antitussive (19) antioxidant (3), anticancer (10), antimicrobial, antitumor (12) anti-infective, antipyretic, anti-hemorrhagic, laxative, diuretic, hemostatic, and astringent (11). These therapeutic properties are due to their content of activity compounds including flavonoids, terpenoids, iridoid glycosides, alkaloids, terpenoids, phenolic compounds, vitamins, fatty acids and polysaccharides (1, 2, 15). Also, it interacts with the lithium, carbamazepine, iron supplements, warfarin, minerals and vitamine B12 supplements (7). Cultivation of many plant species in Iraq is difficult because of the harsh environmental conditions for their cultivation and growth. Biotechnology provides the solution for using plant tissue culture for the production of these compounds *in vitro* and even allows to control the metabolic pathways for these compounds by adding elicitors (14). Elicitor is the one of the stress types that improves the produce of secondary metabolites in the cells and plant tissue. Cobalt metal is classified into abiotic based on their nature. It has diverse effects on the pathways throught Impact on the cellular processes in the tissue system, Include gene expression, carbon partitioning, photosynthesis, lipid metabolism, carbohydrate metabolism, protein synthesis, osmotic homeostasis, and growth (8, 13). In this study, the callus from two species of *Plantago* genus was inducted by determining the appropriate combination of plant growth regulators and the production of some medicinal compounds from the callus was stimulated by treatment with varying concentrations of the cobalt heavy metal.

MATERIALS AND METHODS

Seeds germination

The process of seed germination was performed under sterile conditions inside the laboratory. The seeds were sterilized using 3.0% hypochlorite solution for 10 minutes, 70% ethanol solution for one minute and then washed with sterilized distilled water three times (16). Seeds were then incubated at 25 25±1 Cº and 1000 lux light intensity, for `6 hours per day.

Callus induction

The hypocotyl was used for callus induction after seed germination of the sterilized seeds by adding 2,4-D at the concentrations 1.0, 2.0 or 3.0 $mg.L^{-1}$ and Kinetin at 0.5 or 1.0 $mg.L^{-1}$ using completely randomized design with 10 replicates.

Callus initiation

The experiment was carried out under sterile conditions inside a biosafety cabinet. About 150 mg of callus was inoculated into 2.5 x 8.0 cm. tubes with 10 mL of MS medium and cobalt at 0, 25, 50 or 75 ppm. Culture were incubated under the conditions of same seed germination.

Extraction of flavonoids

Quantitative and qualitative estimation of flavonoids was done according to Bos (5). The induced *Plantago* calluses were stressed by different concentrations of cobalt metal. The calluses were dried at 40°C for 48 hours. Diathiapentene was used as extraction solution (9).

HPLC analysis

HPLC was used to estimate the quantity and quality of flavonoid compounds in the callus extracts. Aliquot of 25 µL of callus extracts was injected into a C-18 column with dimensions 50 x 4.6mm ID and the size of the particles 3 µm at 30°C. The flavonoid

compounds were estimated under the following conditions:

- Mobile phase was acetonitrile/ methanol/ deionized water (10:40:50, v/v).
-flow rate 0.8 ml.min^{-1}.
-Wavelength 280 nm.

The quantitative estimation of all flavonoids was calculated using the following equation:

Flavinoid compounds con. = $\frac{\text{Area of compound}}{\text{Area of standard}}$×Con. of Standard×No. of dilutions

- The concentration of standard compound was 25 mmol.m^{-1}.
- The number of dilutions was only one for the seven study compounds.

Statistical analysis

The statistical analysis of data was performed using the Discovery GenStat version 12.0 software. The value of the least significant difference was determined for comparisons among means at the probability %5 level.=

RESULTS AND DISCUSSION

Effect of *Plantago* species, concentrations of 2,4-D and kinetin, and their interaction on fresh weight of induced callus

Results in Table 1 show a significant effect for species, 2,4-D and kinetin concentrations on the fresh weight of the induced callus. *P. major* had the highest mean of fresh weight of 415.0 mg, by an increase of 6.14% compared to *P. psyllium*. Meanwhile, the growth regulators 2,4-D with kinetin at the concentration of 3.0×1.0 mg.L^{-1} produced the highest mean of fresh weight recorded 534.8 mg. The effect of the interaction between the two species of *Plantago* and 2,4-D with kinetin concentration was significant for *P. psyllium*, at 3.0×1.0 ml.L^{-1} which produced the highest mean of fresh weight of 541.0 mg. Meanwhile, the same of species produced the lowest weight of 211.2 mg when treated with 1.0×0.5 mg.L^{-1} of plant growth regulators.

Table 1. Influence of 2,4-D and kinetin interaction on *Plantago psyllium* L. and *Plantago major* L. callus fresh weight (mg) grown on MS media for 28 days

Concentrations of 2,4-D and Kin. (mg.L^{-1})	Species		Means
	P. psyllium	*P. major*	
1.0×0.5	211.2	216.4	213.8
1.0×1.0	420.2	403.0	411.6
2.0×0.5	326.1	430.4	378.2
2.0×1.0	406.4	478.9	442.7
3.0×0.5	441.5	432.6	437.0
3.0×1.0	541.0	528.6	534.8
Means	391.0	415.0	
L.S.D 0.05	Species = 23.27*	Con.=40.30**	Species ×Con.=56.99*

Effect of *Plantago* species, cobalt concentrations and their interaction on fresh weight of callus

Results show a variation in fresh weight of stimulated of callus (Figure 1). *P. psyllium* callus produced the highest fresh weight reached 365.7 mg, a significant increase by 7.56% compared to the *P. major* callus (Table 2). Increasing the concentrations of cobalt caused a significant decrease in the fresh weight. Cobalt at 75 ppm produced the lowest mean fresh weight (151.7) mg. The interaction between plant species and cobalt concentrations produced a significant effect on fresh weight. After treatment with 25 ppm cobalt, *P. psyllium* produced a significant increase in the fresh weight (541.0) mg. Meanwhile, *P. psyllium* callus stimulated by 75 ppm of cobalt produced the lowest fresh weight (139.8) mg. Plant species differe in their tolerance to the accumulation of heavy metals. The species *P. psyllium* was more tolerant than *P. major*. Therfore, it was able to produse a larger biomass. Both of species were affected by the increased concentration of cobalt expresied as callus fresh weight. As well as, these increase concentrations caused a significant decreases in the biomass of the plant tissue.

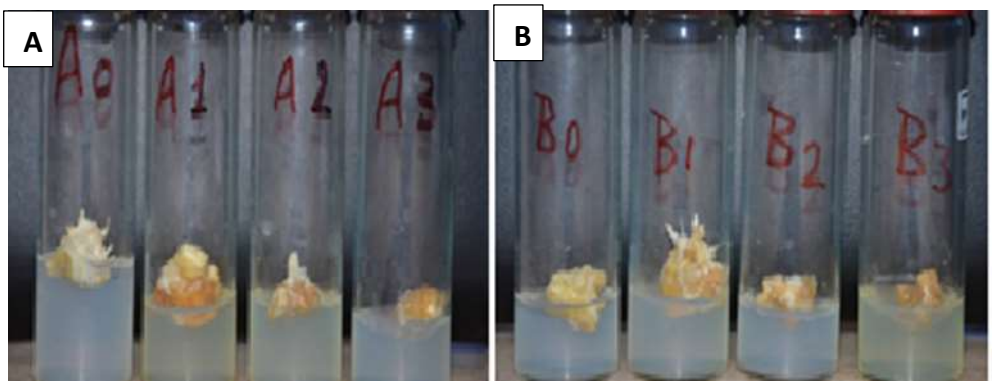

Figure 1. (A) Callus initiation from *Plantago psyllium* L. explants after adding some concentrations of cobalt metal to MS medium. (B) Callus initiation from *Plantago majar* L. explants after adding some concentrations of cobalt metal to MS medium

Table 2. Influence of *Plantago* species, cobalt concentrations, and their interaction on *Plantago psyllium* L. and *Plantago major* L. callus fresh weight (mg.) grown on MS media for 28 days

Concentration of Cobalt (ppm)	Species		Means
	P. psyllium L.	*P. major* L.	
0	497.6	503.0	500.3
25	541.0	438.7	489.8
50	284.3	254.6	269.5
75	139.8	163.6	151.7
Means	365.7	340.0	
L.S.D 0.05	Species.= 23.52*	Con.=33.26**	Species.×Con.= 47.04**

Effect of *Plantago* species, concentration of cobalt, and their interaction in the production of flavonoid

The effect of *Plantago* species was significant in the production of some flavonoid compounds (Table 3). *P. major* produced a significantly higher production of scutallarein, apigenin, nepetin and luteolin, which reached 26.40, 22.64, 14.93, and 26.20 µg.100 mg^{-1} dry weight, respectively. While *P. psyllium* showed a significantly higher production (%45.55) in hispidulin.

Table 3. Production of flavonoids compounds from *Plantago psyllium* L. and *Plantago major* L. *in vitro* after 28 days.

Species	Scutallarein	Apigenin	Nepetin	Aucubin	Baicalein	Hispidulin	Luteolin
P. psyllium	13.19	9.72	6,96	9.17	11.47	29.40	18.80
P. major	26.40	22.64	14.93	10.94	11.35	20.20	26.20
L.S.D 0.05	2.105**	3.771**	2.250**	N.S	N.S	7.580**	6.120**

The different concentrations of cobalt significantly increased the production of flavonoid compounds *in vitro* (Table 4). The addition of 75 ppm cobalt increased the production of scutallarein, apigenin, nepetin and aucubin, which reached 25.61, 23.25, 15.90 and 13.70 µg.100 mg^{-1} dry weight respectively, while the addition of cobalt at 50 ppm increased the production of hispidulin and luteolin which reached 40.20 and 41.60 µg.100 mg^{-1} dry weight, respectively.

Table 4. Production of flavonoids compounds from *Plantago psyllium* L. and *Plantago major* L. *in vitro* after stimulation by cobalt concentrations after 28 days.

Con. (ppm)	Scutallarein	Apigenin	Nepetin	Aucubin	Baicalein	Hispidulin	Luteolin
0	12.78	8.36	6.28	5.64	9.64	15.90	10.30
25	18.78	11.84	7.79	8.40	9.75	21.20	17.60
50	21.99	21.28	13.83	12.47	13.92	40.30	41.60
75	25.61	23.25	15.90	13.70	12.32	21.80	20.50
L.S.D 0.05	2.977**	5.333**	3.182**	4.505**	N.S	10.720**	8.650**

Table 5 shows a significant effect of the interaction between *Plantago* species and cobalt concentrations. The *P. major* callus stimulated by 50 ppm cobalt produced the highest production of scutallarein, apigenin and luteolin at 30.33, 32.26 and 51.90 µg.100 mg^{-1} dry weight, respectively. In addition, 75 ppm cobalt produced the highest producation of the baicalein compound by the *P. psyllium* callus at 16.46 µg.100 mg^{-1} dry weight. *P. psyllium* callus without the addition of cobalt produced the lowest production of scutallarein, apigenin, and baicalein at 6.41, 4.68, and 6.10 µg.100mg^{-1} dry weight respectively. However, the same cobalt concentration induced *P. major* to give the lowest production of luteolin at 9.40 µg.100 mg^{-1} dry weight. The stress caused by the cobalt caused an increase in the production and accumulation of secondary metabolites. There is a difference in the tolerance of plant species to different concentrations of cobalt metal. This is due to the different ability of these species in the disposal of heavy metals by various mechanisms. For example confining these compounds within the vacules, linking harmful ions with the compound glutathione and linking ions with amino acids and the weakness of these mechanisms reduce of biomass and stress obtain at the cellular part and then at the field of tissue (17).

Table 5. Production of flavonoids compounds from *Plantago psyllium* L. and *Plantago major* L. *in vitro* after stimulation by cobalt concentrations after 28 days

Con. (ppm)	Scutallarein	Apigenin	Nepetin	Aucubin	Baicalein	Hispidulin	Luteolin
	P. psyllium						
0	6.41	4.68	3.19	4.72	6.10	17.70	11.20
25	9.61	7.07	4.12	7.28	9.53	28.90	17.60
50	13.66	10.30	7.97	11.27	13.78	44.50	31.40
75	23.07	16.84	12.58	13.41	16.46	26.40	14.90
	P. major						
0	19.14	12.04	9.37	6.56	13.19	14.00	9.40
25	27.96	16.60	11.46	9.53	9.97	13.50	17.60
50	30.33	32.26	19.68	13.67	14.06	36.00	51.90
75	28.16	29.65	19.22	13.99	8.19	17.20	26.0
L.S.D 0.05	4.211**	7.543*	N.S	N.S	5.050**	N.S	12.24*

This study serves as an encouragement and knowledge for other researchers to continue working in production of natural compounds that are medically important to take advantage of its chemical components in the pharmaceutical industry. Stress is one method of inducing callus to stimulate the metabolic pathways responsible for the construction of secondary metabolic compounds. The use of cobalt is a feasible means that enable us to increase the production of these compounds.

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