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SOME FACTORS AFFECTING *IN VITRO* SECONDARY METABOLITES PRODUCTION FROM *VERBESINA ENCELIOIDES* CALLUS CULTURES

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ABSTRACT

The present work was conducted in order to investigate the effect of explant type (leaf and stem internode) and auxin type (2,4-Dichlorophenoxyacetic acid, 2,4-D and Naphthalene Acetic Acid ,NAA) at different concentrations (0.00, 0.25, 0.50, 1.00 and 2.00 mg/l) as well as different concentrations (0, 5, 10, 20 and 40 mg/l) of L-phenylalanine(Phe) or tryptophan (Trp) on *Verbesina encelioides* callus cultures growth and production of secondary metabolites. Obtained results demonstrated that using leaf explant and supplementation MS medium with 0.5 mg/l NAA could enhanced and recorded the ultimate values of callus fresh weight, antioxidant activity (%), total flavonoids, total phenolic compounds contents and yields of *Verbesina encelioides* callus. Also, when the highest concentration (40 mg/l) of either L-phenylalanine or tryptophan was combined with 0.5 mg/l NAA, the maximum values of the abovementioned parameters were obtained. L-phenylalanine proved to be more effective than tryptophan in this regard.

Key Words: *Verbesina encelioides*, callus cultures, antioxidant activity, total flavonoids, total phenolic compounds, auxin, tryptophan, and phenylalanine.

INTRODUCTION

In vitro production of secondary metabolites in cell or tissue cultures has many advantages such as; year-round availability of cultures, only specific cells organs can be grown *in vitro*, secretory products can be easily purified, production of a single compound can be enhanced through the use of chemical elicitors, biotransformation or by transgene expression.The production of recombinant biopharmaceuticals such as mammalian hormones, enzymes, vaccines, and monoclonal antibodies, high consumption requires large industrial production, and secure from a natural hazardous condition **(Iffat, 2019)**. Moreover, depending on the plant species, traditional agricultural methods often require months to years to obtain a crop **(Kieran *et al*., 1997)**.

Verbesina is a genus of the sunflower family (Asteraceae) which contains over 60 species and is one of the largest families in the world**.** *Verbesina encelioides* (American dog weed, butter daisy, crown-beard, golden crown-beard or South African daisy) is native to the United States and Mexico and naturalized elsewhere. Seeds (achenes) are greyish brown, flattened, and broadly winged along margins. Long periods of seed dormancy and high germination rates are reported **(Karnawat *et al*., 2010).**

Verbesina produces a range of eudesmane sesquiterpenes with cinnamate or a derived ester group, of which, a- and b-verbesinolcoumarates were the first reported examples. Several species are also source of elemanolides, diterpenes, flavonoids and biological active guanidines as galegine, the toxic principle of *V. encelioides* Benth. **(Amaro-Luis *et al*., 2002).**

The plant is used traditionally to treat stomach diseases, heamorrhoides. The roots are used for retention of water, bladder inflammation and also as a blood purifier **(Bhattacharjee, 1998)**. Leaves are used as a poultice to relieve sore legs to treat rheumatism and the juice is used as a laxative **(Parrota, 2001)**. Later, **Jain *et al*., 1988 , 2007 and 2008 a & b** found that it also possessed antimicrobial, antiviral, antitumour, hypoglycemic and anti-implantation efficacies. The major phytoconstituents from the different parts are terpenoids, flavonoids, aromatic compounds .. etc. **(Bohlmann *et al.*, 1980)**.

However, there are some efforts **(Karnawat *et al*., 2010, Jain *et al*., 2010 and Karnawat *et al.*, 2011)** have been done to propagate *Verbesina encelioides* via *in vitro* culture techniques, up till now there is no published paper concerning *in vitro* production of secondary metabolites through tissue culture techniques from *Verbesina encelioides*.

Therefore, this study was designed to investigate the effect of auxin (2,4-D and NAA) and two precursors (L-phenylalanine and tryptophan) on *in vitro Verbesina encelioides* callus cultures growth and production of secondary metabolites.

Auxin is an essential plant hormone that controls nearly every aspect of a plant's life, from embryo development to organ senescence **(Brumos *et al*., 2014).** In *in vitro* cultures both the quality and quantity of auxin initially present in media administered during culture development have a marked effect on secondary metabolites production **(Iffat, 2019).**

Wink (2010) suggested that phenylalanine, tyrosine and tryptophan are the primary metabolites which serve as precursors for many natural (secondary) products such as flavonoids, phenolic acids, coumarins, alkaloids, glucosinolates and cyanogenic glycosides. Another interpretation for the stimulatory effect of tryptophan on flavonoids and phenolic compounds synthesis may be the indirect effect of tryptophan on production of these compounds through stimulation of auxin synthesis (especially IAA). It was repeatedly demonstrated that auxin stimulated *in vitro* accumulation of phenolic compounds **(Constabel and Vasil, 1987)**.

The main objective of this study was to investigate the effect of explant type (leaf and stem internode) and auxin type (2,4-D and NAA) at different concentrations (0.00, 0.25, 0.50, 1.00 and 2.00 mg/l) as well as different concentrations (0, 5, 10, 20 and 40 mg/l) of L-phenylalanine or tryptophan on *Verbesina encelioides* callus cultures growth and production of secondary metabolites.

MATERIALS AND METHODS

This study was carried out in Plant Tissue Culture Laboratory of Horticulture Department, Faculty of Agriculture, Zagazig University, throughout the period of 2015- 2020.

Seeds of *Verbesina encelioides* Benth. were obtained from the Experimental Farm of Faculty of Agriculture, Zagazig University. Seeds were kept under running tap water with soap for 2 h. Surface sterilization was achieved by immersion the seeds in fungicide (Rhizolex, 2 g l^{-1} for 30 min), $HgCl_2$ (0.1% for 10 min), then in ethyl alcohol 70% for 30 sec and finally in sodium hypochlorite solution (1% for 20 min). The seeds were thoroughly rinsed three times with sterile distilled water after each previous step. Seeds were cultured in jars (60 × 120 mm) containing basal medium with 30 g/l sucrose, free from growth regulators and solidified with 0.8% agar (HiMedia) (**Murashige and Skoog ,1962)**. Medium pH was adjusted at 5.8 before autoclaving. The culture jars were autoclaved at 1.3 kg/cm^2 for 20 min. The seeds were incubated for germination for six weeks at 25±2 ºC under 16 h photoperiod with 2000 Lux light intensity obtained from fluorescent tubes.

Initiation of undifferentiated callus was carried out by using leaf, and internode stem explants (0.5 cm long) obtained from germinated seeds. Explants were inoculated on Murashige and Skoog media (M&S) medium containing different concentrations (0.0, 0.25, 0.5, 1.0 or 2.0 mg/l) of 2,4-D or NAA in order to induction of calls. Each treatment was

consisted of 15 jars (60 × 120 mm) and each one contained about 50 ml of medium.

Since 0.5 mg/l NAA proved to be the best treatment for callus induction and secondary metabolites production from the above-mentioned experiment, this concentration of NAA was tested either alone (as control) or combined with different concentrations of L-phenylalanine (0, 5, 10, 20 and 40 mg/l) or tryptophan (0, 5, 10, 20 and 40 mg/l). Leaf explant was used in this experiment since it proved to be more promising than stem explant in this regard.

All above mentioned experiment cultures were incubated for 8 weeks at 25±2 °C under 16 h. photoperiod with 2000 Lux light intensity obtained from fluorescent tubes.

For all above mentioned experiments, after 8 weeks fresh weight of callus (g), antioxidant activity (%), content of total flavonoids (mg QE/g extract) and content of total phenolic compounds (mg gallic acid/g extract) were determined. Antioxidant activity (%) assay was carried out as described by **Ratty *et al.* (1988)**. Content of total flavonoids was determined according to the method described by **Ordon *et al*. (2006)**. While content of total phenolic compounds (TPC) was determined according to **Bray and Thorpe (1954)**. Also, total flavonoids yield (mg QE) and TPC yield (mg gallic acid) were estimated by multiplying content of total flavonoids (mg QE/g extract) and content of total phenolic compounds (mg gallic acid/g extract) by callus fresh weight (g), respectively.

The statistical layout of all experiments was simple completely randomized design. All collected data were analyzed with analysis of variance (ANOVA) procedure using the MSTAT-C Statistical Software Package **(Michigan State University, 1983)**. Differences between means were compared by using Duncan multiple range test **(Gomez and Gomez. 1984).**

RESULTS AND DISCUSSION

Effect of explant type and auxin type and concentration on callus growth and secondary metabolites production:

From data presented in **Table 1**, it was observed that, supplementation the medium with 0.5 mg/l NAA was the most effective treatment for increasing callus fresh weight since it produced the maximum mean value (2.614 g) of this parameter. Also, using leaf explant gave higher mean callus fresh weight (2.417 g) compared with stem explant (0.984 g). The ultimate callus fresh weight (3.725 g) was

detected when leaf explant was cultured on MS medium contained 0.5 mg/l NAA.

Antioxidant activity followed the similar trend of callus fresh weight since the highest mean value (45.47 %) of this character was recorded with 0.5 mg/l NAA treatment. Also, the highest mean value (44.86 %) of antioxidant activity was recorded with leaf explant compared with stem explant which recorded 15.89 %. The maximum antioxidant activity was detected as leaf explant was cultured on MS medium fortified with 0.5 mg/l NAA.

Table 1. Effect of auxin concentration and explant source on callus fresh weight (g) and antioxidant activity (%) of *Verbesina encelioides*.

Auxin conc. (mg/l)	Callus fresh weight (g)			Antioxidant activity (%)		
	Leaf	Stem	Mean	Leaf	Stem	Mean
0.00	0.000 i	0.000 i	0.000 I	0.00 i	0.00 i	0.00 I
0.25 2,4-D	2.244 e	0.953 e	1.599 E	41.46 e	14.55 e	28.00 E
0.50 2,4-D	2.005 f	0.780 f	1.393 F	37.14 f	12.58 f	24.86 F
1.00 2,4-D	1.749 g	0.627 g	1.188 G	33.52 g	10.44 g	21.98 G
2.00 2,4-D	1.546 h	0.421 h	0.983 H	28.91 h	9.62 h	19.26 H
0.25 NAA	2.965 b	1.318 b	2.141 B	56.56 b	20.74 b	38.65 B
0.50 NAA	3.725 a	1.503 a	2.614 A	66.05 a	24.90 a	45.47 A
1.00 NAA	2.677 c	1.222 c	1.949 C	50.12 c	18.22 c	34.17 C
2.00 NAA	2.432 d	1.050 d	1.741 D	45.09 d	16.09 d	30.59 D
Mean	2.417 A	0.984 B		44.86 A	15.89 B	

Means having the same letter (s) within the same column are not significantly different according to Duncan's multiple range test at 5% level of probability.

Total flavonoids content test referred that NAA surpassed 2,4-D concerning total flavonoids content of callus especially when used at 0.5 mg/l as this concentration produced the maximum mean value (5.791 mg QE/g extract) of this parameter **(Table 2)**. Higher and lower concentrations of NAA were less effective than this concentration in this regard. Leaf explant was more pronounced than stem explant in this connection since the former one produced more than threefold of total flavonoids content compared with the later one. The greatest total flavonoids content (8.722 mg QE/g extract) was determined in callus produced from leaf explant inoculated on MS medium supplemented with 0.5 mg/l NAA.

Total flavonoids yield calculation proved the efficiency of using leaf as explant source and 0.5 mg/l NAA as auxin treatment for elevating flavonoids production since this treatment resulted in the maximum total flavonoids yield (32.492 mg QE).

Table 2. Effect of auxin concentration and explant source on total flavonoids content (mg QE/g FW) and yield (mg QE/g) of *Verbesina encelioides*

Auxin conc. (mg/l)	Total flavonoids content (mg QE/g FW)			Total flavonoids yield (mg QE)		
	Leaf	Stem	Mean	Leaf	Stem	Mean
0.00	0.00 h	0.000 i	0.000 I	0.000 h	0.000 i	0.000 I
0.25 2,4-D	4.829 e	1.437 e	3.133 E	10.840 e	1.371 e	6.105 E
0.50 2,4-D	4.145 f	1.249 f	2.697 F	8.314 f	0.975 f	4.644 F
1.00 2,4-D	3.714 g	1.046 g	2.380 G	6.498 g	0.656 g	3.577 g
2.00 2,4-D	3.601 g	0.863 h	2.232 H	5.559 g	0.363 h	2.961 H
0.25 NAA	6.874 b	2.344 b	4.609 B	20.389 b	3.091 b	11.740 B
0.50 NAA	8.722 a	2.860 a	5.791 A	32.492 a	4.298 a	18.395 A
1.00 NAA	5.769 c	1.957 c	3.863 C	15.447 c	2.392 c	8.919 C
2.00 NAA	5.425 d	1.713 d	3.569 D	13.197 d	1.799 d	7.498 D
Mean	5.385 A	1.684 B		14.092 A	1.868 B	

Means having the same letter (s) within the same column are not significantly different according to Duncan's multiple range test at 5% level of probability.

Data in **Table 3** indicate that all NAA concentrations surpassed their equivalents of 2,4-D concerning total phenolic compounds content. Again 0.5 mg/l NAA proved to be the most efficient treatment in this regard since it produced the maximum mean value (27.025 mg gallic acid/g extract) of this parameter compared with all other treatments. Leaf explant callus produced about four times more of total phenolic compounds content compared with stem explant callus. It is worthy to be note that callus obtained from leaf explant cultured on MS medium provided with 0.5 mg/l NAA gained the maximum total phenolic compounds content (41.557 mg gallic acid/g extract) compared with all other applied treatments.

The effectualness of the above-mentioned treatment (0.5 mg/l NAA) on raising total phenolic compounds production was proved by estimation the yield of these compounds, especially when it was combined with using leaf explant as callus source. This combined treatment achieved the highest significant yield of total phenolic compounds (80.240 mg gallic acid) compared with all applied treatments.

The present results proved that supplementation the medium with auxin was essential for callus induction. This observation was earlier demonstrated by many investigators since auxin was known to play an important role in callus induction and proliferation **(Nic-Can and Loyola-Vargas, 2016)**. This might be attributed to auxin as it promotes the biosynthesis of ethylene by increasing the activity of 1-aminocylopropane-1-carboxylic acid (ACC) syntheses **(Kende, 1989)**.

The above-mentioned results indicated that NAA was more efficient than 2,4-D in callus induction and growth. This result was in the same line with those obtained by **Irvani *et al*. (2010), Mousavi *et al*. (2012), Jayaraman *et al.* (2014) and Kumar *et al*. (2014)**. Moreover, depressive effect of high concentration of auxin (especially 2,4-D) on callus induction which discovered in the current study was previously stated by many researchers such as **Malik *et al*. (2003), Irvani *et al*. (2010) and Verma *et al*. (2012)**. The inhibitory effect of high concentrations of 2, 4-D on callus induction may be due to its effect as the herbicide **(Wernicke and Mikovits, 1984)**.

The results of the present work clearly demonstrate that NAA was more effective than 2,4-D concerning flavonoids and phenolic compounds production from callus as well as improving anti-oxidant activity of obtained callus. This results are supported by the findings of **Kákoniová *et al*. (2009), Ong *et al*. (2011) and Kumar *et al*. (2014)**. Also, **Wernicke and Mikovits (1984)** referred that high concentrations of 2, 4-D were able to inhibit callusing of basal segments of wheat and this may be due to its herbicidal effect.

It is worth to mention that it could be observed that there was a positive correlation between high total phenol content & total flavonoid content and antioxidant activities in the above-mentioned data. This correlation was previously stated in callus cultures of *Canscora decussata* and they attributed that to the fact that both phenol and flavonoid content contributed in all the antioxidant assays tested **(Kousalya and Bai, 2016).**

Table 3. Effect of auxin concentration and explant source on TPC* content (mg gallic acid/g FW) and yield (mg gallic acid) of *Verbesina encelioides*

Auxin conc. (mg/l)	TPC content (mg gallic acid/g FW)			TPC yield (mg gallic acid)		
	Leaf	Stem	Mean	Leaf	Stem	Mean
0.00	0.000 i	0.000 i	0.000 I	0.000 i	0.000 i	0.000 I
0.25 2,4-D	24.515 e	5.584 e	15.050 E	55.024 e	5.318 e	30.172 E
0.50 2,4-D	19.824 f	4.229 f	12.026 F	39.731 f	3.300 f	21.515 F
1.00 2,4-D	17.526 g	3.581 g	10.554 G	30.650 g	2.246 g	16.448 G
2.00 2,4-D	15.083 h	2.655 h	8.868 H	23.325 h	1.124 h	12.225 H
0.25 NAA	34.440 b	10.317 b	22.379 B	102.060 b	13.599 b	57.828 B
0.50 NAA	41.557 a	12.493 a	27.025 A	154.820 a	18.780 a	86.800 A
1.00 NAA	29.987 c	7.556 c	18.772 C	80.240 c	9.233 c	44.737 C
2.00 NAA	27.597 d	6.584 d	17.091 D	67.134 d	6.915 d	37.025 D
Mean	26.316 A	6.625 B		69.123 A	7.564 B	

Means having the same letter (s) within the same column are not significantly different according to Duncan's multiple range test at 5% level of probability.
***Total phenolic compounds**

Effect of phenylalanine concentration combined with 0.5 mg/l NAA on callus growth and secondary metabolites production:

Supplementation the medium with low concentration (5 mg/l) of phenylalanine did not significantly affect callus fresh weight **(Table 4)**. However, increasing of phenylalanine concentration to be 10 or 20 mg/l significantly enhanced this parameter without significant difference between both concentrations. The heaviest callus (4.671 g) were estimated when medium was fortified with the highest concentration of phenylalanine (40 mg/l).

Antioxidant activity was significantly elevated as phenylalanine concentration increased. It reached its ultimate value (90.17 %) as medium was augmented with the highest concentration (40 mg/l) of phenylalanine.

There was a liner relationship between total flavonoids content as well as total flavonoids yield and phenylalanine concentration **(Table 4)**. The maximum total flavonoids content (43.69 mg QE/g) and total flavonoids yield (204.08 mg QE) were detected when medium was enriched with the ultimate concentration of phenylalanine (40 mg/l). This treatment gave about 5 fold and 6.5 fold more total flavonoids content and total flavonoids yield as compared with control treatment, respectively.

Similar trend of total flavonoids content and total flavonoids yield was observed with total phenolic compounds content and yield **(Table 4)**. The superiority of using the highest concentration (40 mg/l) of phenylalanine for improving total phenolic compounds content and yield compared with all other tested concentrations was proved in the current study.

The greatest values of total phenolic compounds content (72.60 mg gallic acid/g) and yield (339.14 mg gallic acid) were estimated as maximum concentration (40 mg/l) of phenylalanine were used. These parameters were increased by about 174.8 and 229 % compared to control treatment, respectively.

The enhancing effect of supplementation the medium with phenylalanine on callus growth was early demonstrated by **Shinde *et al.* (2009)** on *Hydrocotyle bonariensis* and **Mathur and Goswami (2014)** on *Maytenus emarginata* and proved by our findings.

The present results clear that augmentation the medium, especially at high concentration, with phenylalanine resulted in stimulation of flavonoids and phenolic compounds accumulation in callus. This observation is previously supported by the findings of many researchers such as **Bemani *et al.* (2013)** on *Corylus avellana*, **Mathur and Goswami (2014)** on *Maytenus emarginata*, **Arafa *et al.* (2015)** on carrot, **Mobin *et al.* (2015)** on *Echinacea purpurea*, **Al-Duraid *et al.* (2019)** on fenugreek and **Demirci *et al.* (2020)** on *Echinacea purpurea*. This may be due to that phenylalanine is basic substance of the phenylpropanoide path leading to the formation of phenolic compounds, flavonoids, tannins, coumarone and anthocyanin. The amino acids phenylalanine and tyrosine derived from the shikimic acid pathway are the most

common origin of polyphenols **(Vogt, 2010 and Al-Mohammad & Al-Taey, 2019).** Moreover, phenylalanine is the substrate of phenylalanine ammonia-lyase which catalyses the reductive de-amination of phenylalanine into trans-cinnamic acid. This is the first step in the biosynthesis pathway of plant phenolic compounds **(Kubota *et al*., 2001)**.

The enhancing effect of phenylalanine on antioxidant activity was demonstrated in this study and previously proved by **Bemani *et al*. (2013)** on *Corylus avellana*, **Arafa *et al*. (2015)** on carrot and **Al- Duraid *et al*. (2019)** on fenugreek. This increase in antioxidant activity was correlated with increasing in total flavonoids and phenolic compound contents. This because phenolic compounds, especially flavonoids, show various types of biological activity especially its antioxidant property **(Smoleń and Sady 2009)**.

Table 4. Effect of different phenylalanine (Phe) concentrations combined with 0.5 mg/l NAA on callus growth and secondary metabolites production from *Verbesina encelioides* leaf explant

Phe conc. (mg/l)	Callus fresh weight (g)	Antioxidant activity (%)	Total flavonoids content (mg QE/g extract)	Total flavonoids yield (mg QE)	TPC* content (mg gallic acid/g extract)	TPC yield (mg gallic acid)
0.0	3.565 c	66.03 e	8.76 e	31.23 e	41.53 e	148.06 e
5.0	3.634 c	70.62 d	13.39 d	48.67 d	50.48 d	183.45 d
10.0	3.975 b	78.10 c	22.15 c	88.07 c	56.36 c	224.05 c
20.0	4.005 b	84.46 b	27.04 b	108.29 b	58.00 b	232.32 b
40.0	4.671 a	90.17 a	43.69 a	204.08 a	72.60 a	339.14 a

Means having the same letter (s) within the same column are not significantly different according to Duncan's multiple range test at 5% level of probability.
***Total phenolic compounds**

Effect of tryptophan concentration combined with 0.5 mg/l NAA on callus growth and secondary metabolites production:

Data in **Table 5** clear that low and medium concentrations (5 and 10 mg/l) of tryptophan had no significant effect on callus fresh weight, while higher concentrations (20 and 40 mg/l) significantly enhanced callus fresh weight. The heaviest callus (4.062 g) was gained from the highest concentration (40 mg/l) of glutamine.

Antioxidant activity was gradually enhanced as tryptophan concentration increased **(Table 5)**. The maximum tryptophan concentration (40 mg/l) surpassed all other investigated concentrations in this regard and achieved 82.42 % antioxidant activity.

Data tabulated in **Table 5** show that there was a linear relationship between tryptophan concentration and total flavonoids content or yield. The ultimate total flavonoids content (18.26 mg QE/g) and yield (74.17 mg QE) were resulted from the highest concentration (40 mg/l) of glutamine.

As shown in **Table 5,** there was a positive correlation between both total phenolic compounds content as well as yield and tryptophan concentration. The highest concentrations of tryptophan exceeded all other tested concentration and recorded the maximum total phenolic compounds content (63.65 mg gallic acid/g) and yield (258.58 mg gallic acid).

The above mentioned result demonstrated that addition of tryptophan enhanced callus formation. This result was earlier indicated by **Siriwardana and Nabors (1983)** on rice. This may be attributed to the fact that tryptophan is a precursor of IAA in plants, and exogenous applications have been demonstrated to increase IAA synthesis in plant tissues **(Kefeli, 1978)**.

According to the present results, it is clear that tryptophan had a significant effect on improving the production of flavonoids and phenolic compounds and consequently antioxidant activity. These results are in accordance with those discovered either *in vivo* by **Mona and Sadak (2007)** on canola, **El-Bassiouny and Abdel-Monem (2016)** on sunflower and **Bakry *et al.* (2016)** on *Chenopodium quinoa* or *in vitro* by **Al-Jibouri (2016)** on *Verbascum thapsus*. In this connection, **Wink (2010)** suggested that phenylalanine, tyrosine and tryptophan are the primary metabolites which serve as precursors for many natural (secondary) products such as flavonoids, phenolic acids, coumarins, alkaloids, glucosinolates and cyanogenic glycosides. Another interpretation for the stimulatory effect of tryptophan on flavonoids and phenolic compounds synthesis may be the indirect effect of tryptophan on production of these compounds through stimulation of auxin synthesis (especially IAA). It was repeatedly demonstrated that auxin stimulated *in vitro* accumulation of phenolic compounds **(Constabel and Vasil, 1987).**

Table 5. Effect of different tryptophan (Trp) concentrations combined with 0.5 mg/l NAA on callus growth and secondary metabolites production from *Verbesina encelioides* leaf explant

Trp conc. (mg/l)	Callus fresh weight (g)	Antioxidant activity (%)	Total flavonoids content (mg QE/g extract)	Total flavonoids yield (mg QE)	TPC* content (mg gallic acid/g extract)	TPC yield (mg gallic acid)
0.0	3.565 c	66.03 e	8.76 e	31.23 e	41.53 e	148.06 e
5.0	3.528 c	69.67 d	10.38 d	36.63 d	49.70 d	175.36 d
10.0	3.589 c	74.41 c	11.16 c	40.07 c	50.78 c	182.26 c
20.0	3.841 b	78.42 b	14.51 b	55.75 b	56.34 b	216.41 b
40.0	4.062 a	82.42 a	18.26 a	74.17 a	63.65 a	258.58 a

Means having the same letter (s) within the same column are not significantly different according to Duncan's multiple range test at 5% level of probability.
***Total phenolic compounds**

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بعض العوامل المؤثرة على الإنتاج المعملي لنواتج التمثيل الغذائي الثانوية من مزارع الكلس لنبات الفربيزينا

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أجري هذا العمل بهدف دراسة تأثير نوع المنفصل النباتي (ورقة أو قطعة ساقية) و نوع الأكسين (نفثالين حمض الخليك أو 4.2 داي كلوروفينوكسي حمض الخليك) بتركيزات مختلفة (صفر، 0.25، 0.50، 1.0 و 2.0 مللجم/لتر) بالإضافة الى إستخدام تركيزات مختلفة (صفر، 5، 10، 20 و 40 مللجم/لتر) من الفينيل الانين أو التربتوفان على نمو الكلس و إنتاج نواتج التمثيل الغذائي الثانوية من مزارع كلس نبات الفربيزينا. و النتائج المتحصل عليها أثبتت أن إستخدام الورقة كمنفصل نباتي مع زراعتها على بيئة موراشيج و سكوج المزودة بتركيز نصف مللجم/لتر نفثالين حمض الخليك قد أدت لحدوث زيادة و تحقيق أقصى القيم من كل من وزن الكلس الطازج و النسبة المئوية للنشاط المضاد للأكسدة و محتوى الكلس من المركبات الفلافونيدية و المركبات الفينولية الكلية و كذلك محصول الكلس من كل منهما. كما أن إضافة أعلى تركيز من الفينيل الانين أو التربتوفان مشتركاً مع نصف مللجم/لتر نفثالين حمض الخليك قد أدى للحصول على أعلى القيم للصفات السابق ذكرها. و قد أثبت الفينيل الانين أنه أكثر فاعلية من التربتوفان في هذا الصدد.