The Study of *in Vitro* Regeneration and Growth Parameters in *Catharanthus roseus* L. under Application of Tryptophan

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Abstract

Periwinkle (*Catharanthus roseus* L.) is one of the most important medicinal plants that is commonly used for its anticancer alkaloids. In this investigation, the effects of different tryptophan concentrations (0, 150, 250 and 350 mg/l) on in vitro regeneration conditions were studied. The regeneration of different explants on different tryptophan-free media compositions showed that the best media for growing nodal segments explants was MS medium containing (BAP, 0.5 mg/l)+(NAA, 1 mg/l). However, the rooting media composition *was determined* as half MS medium supplemented with (IBA, 0.1 mg/l). After adding tryptophan into this optimal medium, the maximum shooting and rooting percentages were obtained with 250 and 350 mg/l tryptophan, respectively. Furthermore, the measurement of shoot and root length, dry and fresh weight of shoot, fresh weight of root, chlorophyll *a* and carotenoid content and also, soluble sugars content of shoot and root, revealed that the best medium was in the presence of 350 mg/l tryptophan, while the maximum amount of root dry weight and chlorophyll *b* were obtained by 250 mg/l tryptophan containing medium. Finally, the analysis of total proteins content of shoot and root showed a significant difference between all groups and higher content of proteins for shoots and roots was observed in 250 mg/l and 350 mg/l tryptophan, respectively.

Introduction

Periwinkle (*Catharanthus roseus* L.) G. Don) as a tropical plant is also well known as a famous medicinal plant due to its anticancer alkaloids. Vinblastine and Vincristine

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are two alkaloids which are used for their antitumor properties [1]. The interest carried by the investigators with this plant is due to the presence of these alkaloids and one with therapeutic significant activity such as Ajmalicine [2]. The great demand for these alkaloids and low naturally occurrence of the raw material of this plant, indicate the importance of studies on the enhancement of the periwinkle growth and development [3]. *In vitro* techniques are effective means that are patented by several scientists. Most of transformational methods which are applied to genetically modified plants require regeneration of whole plant from plants tissues and segments. Plant biotechnology offers an opportunity to grow cells, tissues, organs or entire plants under *in vitro* conditions and manipulate them to get the desired compounds [4]. Generally, in regeneration, shoots formed and grew out of the callus tissue that able to enhancement of producing significant amount of important metabolites with medicinal properties. Regeneration can be achieved through two ways, including organogenesis and somatic embryogenesis from different segments of the plants [3].

Tryptophan is one of the essential amino acids whose role is well known in plants. It has an indirect role on the growth via its influence on auxin synthesis which contributes in promoting plant growth and development [5]. On the other hand, tryptophan is a precursor for anticancer alkaloids producing in *C. roseus* [1]. Also, the role of tryptophan in the growth of several plants was reported by several investigators under *in vivo* conditions. Abou Dahab *et al*. [5] on *Philodendron erubescens,* Nahed *et al.* [6] on *Antirrhinum majus* and Attoa *et al.* [7] on *Iberis amara* reported that spraying of these plants by tryptophan increased the plant growth, but the evaluations of tryptophan functions under *in vitro* conditions have not been carried out yet. Because of the medicinal importance of *C. roseus*, the efforts for achievement of optimal conditions for regeneration of this plant were carried out in several investigations. Faheem *et al.* [8], found the better media for maximum shooting initiation and growth of periwinkle plants under *in vitro* conditions.

The aim of this study is to evaluate the role of several concentrations of tryptophan in induction and elongation of shoots and consequently growth of the whole plants under *in vitro* conditions.

Materials and methods

The explants used for this experiment were obtained from the seeds that had been grown under *in vitro* conditions. The seeds of periwinkle were surface sterilized using 70% EtOH for 30 sec and then were transferred to 1% hypochlorite (containing %5.25 chlorine) for 15 min. Then, they were washed by distilled water twice and were germinated aseptically on basal Murashige and Skoog (MS) medium for 5 weeks. Five old seedlings were used as plant materials.

Explants used for shooting induction were young leaf, petiole and nodal segments. These explants were placed in different shooting induction medium (MS) supplemented with various plant growth regulators including benzyl amino purine (BAP) in 0.5 and 0.25 mg/l, naphthalene acetic acid (NAA) in 1 and 0.5 mg/l and indole butyric acid (IBA) in 1.5 and 2 mg/l concentrations. The pH was adjusted to 5.6 prior to autoclave. The medium was supplemented with 30 g/l of sucrose and solidified by 0.8% agar. In order to identify the effect of tryptophan on growth of regenerated plants, various concentrations of this amino acid (0,150, 250 and 350 mg/l) were added to optimum medium. Watery solution of tryptophan solution was added separately to the autoclaved medium after filter sterilization. The experiments were carried out in four replications.

Root and shoot heights, fresh and dry weight of the shoot and root and the number of leaves/plant were determined as growth parameters. Total soluble sugar content in shoot and root systems were determined by Anthrone method described by Fales [9]. The total protein of shoots and roots were measured using the method of Bradford [10]. Also, photosynthetic pigments were extracted from the leaves in 80% acetone and the content of chlorophylls *a* and *b* and carotenoids were determined spectrophotometrically as described by Lichtenthaler and Wellburn (11).

Data were subjected to analysis of variance and one way ANOVA was applied to compare results between different groups using the Duncan and Tukey multiple range test ($P < 0.05$).

Results

In this study, we used half mature seedlings as a source of the explants. Germination of seeds completed within 7 days. Regeneration of periwinkle plants was carried out using different explants and media compositions. The shooting medium used in this experiment, was MS medium supplemented with different combinations of BAP, NAA and IBA. The young leaf segments cultured in different media compositions showed the optimal results in medium composed of MS + BAP (0.5 mg/l) + NAA (1mg/l), but this regeneration result was very lower than that of nodal segments (Tables1 and 2).

Also, nutrient medium (MS) supplemented with BAP (0.5 mg/l) + IBA (2 mg/l) did not show any regeneration results. Petiole explants did not show any regenerated sample and all of the explants were infected after 7 days of sowing among plant parts (Table3).

Table1. Effects of some plant growth regulators on *C.roseus* nodal segments regeneration

Concentration (mg/l)			Number of shoot/explants (mean ± SE)	Shooting (%)	Rooting (%)
BAP	NAA	IBA			
0.5	1		5.75 ± 0.625	100	10
0.5	0.5		3.00 ± 0.408	95	0
0.25	1		4.50 ± 0.645	83	0
0.5		1.5	2.70 ± 0.478	96	7
0.5		2	2.25 ± 0.750	79	0
0.5		1	1.70 ± 0.478	63	0

Table2. Effects of some plant growth regulators on *C.roseus* young leaf segments regeneration

Concentration (mg/l)			Number of shoot/explants (mean ± SE)	Shooting (%)	Rooting (%)
BAP	NAA	IBA			
0.50	1.0		0.50 ± 0.288	15	96
0.50	0.5		0.25 ± 0.250	2	73
0.25	1.0		1.00 ± 0.408	7	14
0.50		1.5	0.75 ± 0.478	5	63
0.50		2.0	0.00 ± 0.000	0	26
0.50		1.0	0.25 ± 0.250	2	47

Table3. Effects of different plant growth regulators on *C.roseus* petiols regeneration

Concentration (mg/l)			Number of shoot/explants (mean ± SE)	Shooting (%)	Rooting (%)
BAP	NAA	IBA			
0.50	1.0		0.00 ± 0.00	0	0
0.50	0.5		0.00 ± 0.00	0	0
0.25	1.0		0.00 ± 0.00	0	0
0.50		1.5	0.00 ± 0.00	0	0
0.50		2.0	0.00 ± 0.00	0	0
0.50		1.0	0.00 ± 0.00	0	0

During the time intervals between three experiments conducted on the explants, nodal segments regenerated on BAP (0.5 mg/l) + NAA (1 mg/l) showed emergence of shoots earlier than others (8 days) and after 7 weeks, the microshoots (3-4 cm in length) were transferred to rooting medium. Therefore, these explants and composition media were the best results for regeneration of *C. roseus* plants.

The composition of rooting media was half strength MS supplemented with four concentrations of IBA. The results showed that the best conditions for rooting induction were obtained in ½ MS medium containing 0.1 mg/l IBA (Table4). The initiation of roots was observed 15 days after transferring.

Table4. Effects of different concentrations of IBA on rooting of *C. roseus* explants

	Rooting (%)	
IBA concentration (mg/l)	MS medium	half MS medium
0.1	56	86
0.5	42	53
1.0	63	71
1.5	69	76

Tryptophan was added in three concentrations to these optimal shooting and rooting media. The analysis of regeneration properties in regenerated periwinkle plants after tryptophan addition showed that shooting percentage in media with 250 mg/l tryptophan

was higher than the others (Table5). Also, measurement of rooting percentage revealed that nutrient solution containing 350 mg/l tryptophan had the maximum rooting percentage (Table5). The evaluation of the number of shoots in media containing 350 mg/L tryptophan was higher than the others media (Table5).

Table5. Effect of tryptophan on *C. roseus* nodal segments regeneration

Tryptophan Concentration (mg/l)	Shooting (%)	Rooting (%)	Number of Shoot/Explants
0	87	83	7.75 ± 0.853
150	100	92	9.50 ± 1.040
250	95	95	8.75 ± 0.853
350	100	97	11.50 ± 1.322

The results of shoot and root length measurements indicated that the regenerated plants in media composition containing 350 mg/l tryptophan had higher shoots and roots length which were significant at 5% level (Table6). Also, the obtained results of shoots and roots fresh weights showed that increments in tryptophan concentrations have caused the enhancement of these factors, which were significant at %5 statistical level (Table6). By measurement of shoots and roots dry weights, we found out that the treatment of 250 and 350 mg/l tryptophan had the best results for shoots and roots, respectively (Table6).

Table6. Effect of tryptophan on growth parameters of regenerated *C.roseus* plants

Try. Con. (mg/l)	Shoot Length (cm)	Root Length (cm)	Shoot Fresh Weight (g)	Root Fresh Weight (g)	Shoot Dry Weight (g)	Root Dry Weight (g)
0	4.875 ± 0.286	1.050 ± 0.210	0.752 ± 0.099	0.385 ± 0.032	0.385 ± 0.065	0.013 ± 0.002
150	5.600 ± 0.460	1.075 ± 0.110	0.560 ± 0.055	0.415 ± 0.030	0.415 ± 0.061	0.022 ± 0.006
250	5.275 ± 0.535	1.337 ± 0.205	0.765 ± 0.055	0.537 ± 0.042	0.537 ± 0.085	0.079 ± 0.003
350	7.875 ± 0.642	2.925 ± 0.221	0.925 ± 0.079	0.755 ± 0.040	0.755 ± 0.080	0.023 ± 0.002

The evaluations of photosynthetic pigments demonstrated that plants grown under 350 mg/l tryptophan had the higher amounts of chlorophyll *a* and carotenoid that was significant at 5% level (Figures1 and 3). However, the chlorophyll *b* content showed the highest level due to 250 mg/l tryptophan treatment (Figure2).

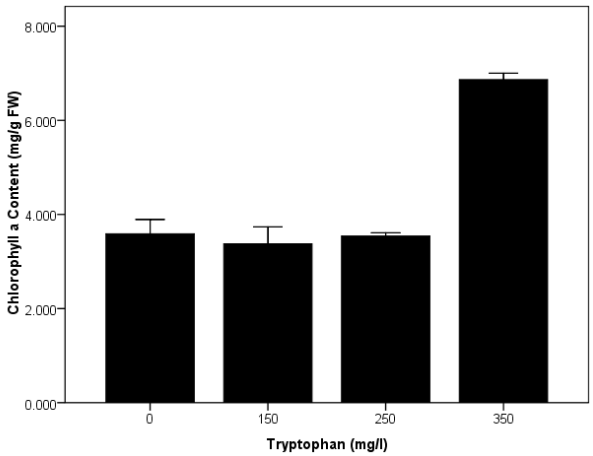

Figure1. Effect of tryptophan on chlorophyll *a* content of *C. roseus.* Results are shown as mean ± standard error (P < 0.05), obtained from four replicates

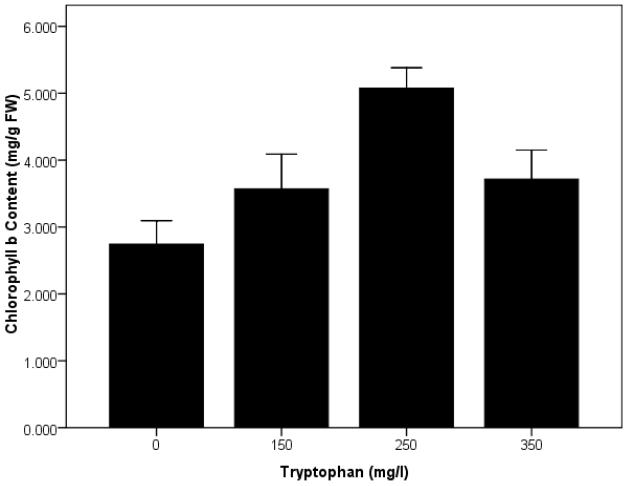

Figure2. Effect of tryptophan on chlorophyll *b* content of *C. roseus.* Results are shown as mean ± standard error (P < 0.05), obtained from four replicates

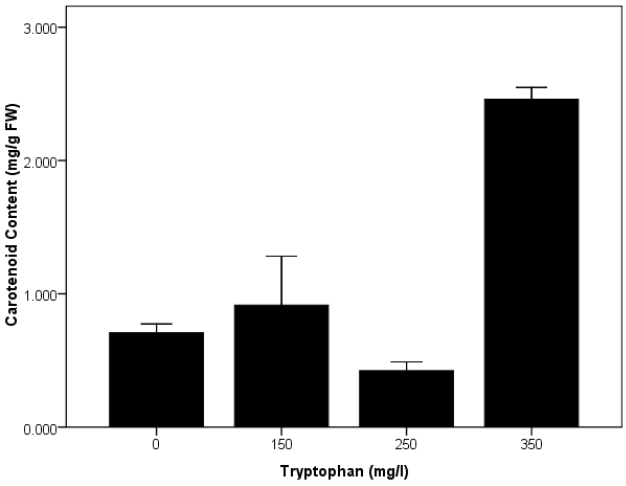

Figure3. Effect of tryptophan on carotenoid content of *C. roseus.* Results are shown as mean ± standard error (P < 0.05), obtained from four replicates

By measuring the total soluble sugars content in shoots and roots, we found that the amount of this factor was higher in samples regenerated under 350mg/l tryptophan containing culture than the other samples. It was significant at 5% level in all groups (Figures4 and 5).

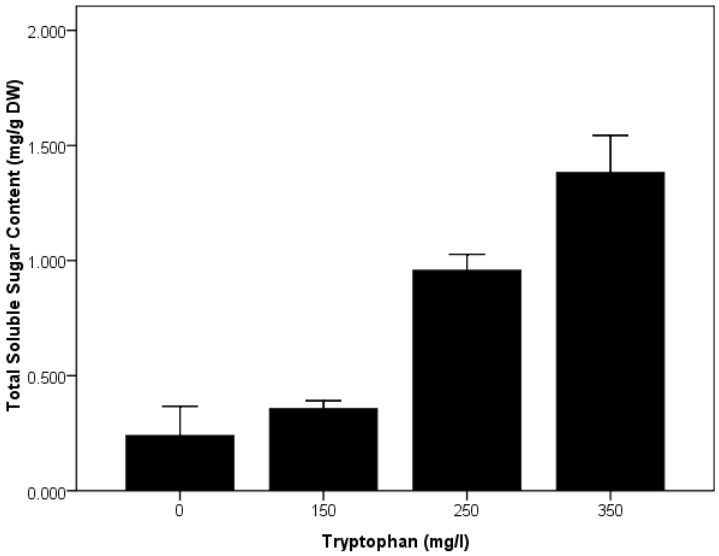

Figure4. Effect of tryptophan on total soluble sugars content of shoots in *C.roseus.* Results are shown as mean ± standard error (P < 0.05), obtained from four replicates

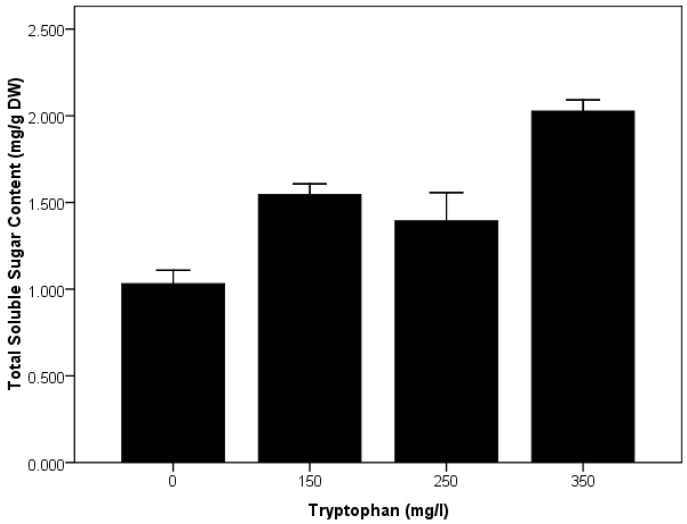

Figure5. Effect of tryptophan on total soluble sugars content of roots in *C.roseus.* Results are shown as mean ± standard error (P < 0.05), obtained from four replicates

Finally, the analysis of total proteins contents of shoots and roots showed significant differences between group samples and the 250 and 350 mg/l concentration of tryptophan in all treatments revealed the highest protein content in shoots and roots, respectively. (Figures6 and 7).

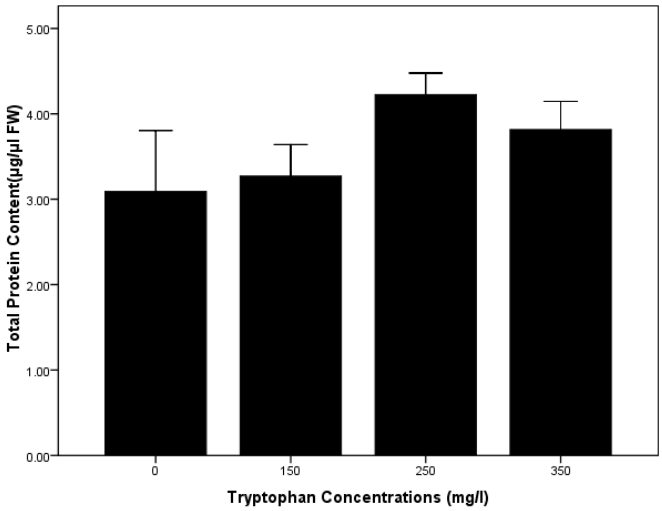

Figure6. Effect of tryptophan on total protein contents of shoots in *C. roseus.* Results are shown as mean ± standard error (P < 0.05), obtained from four replicates

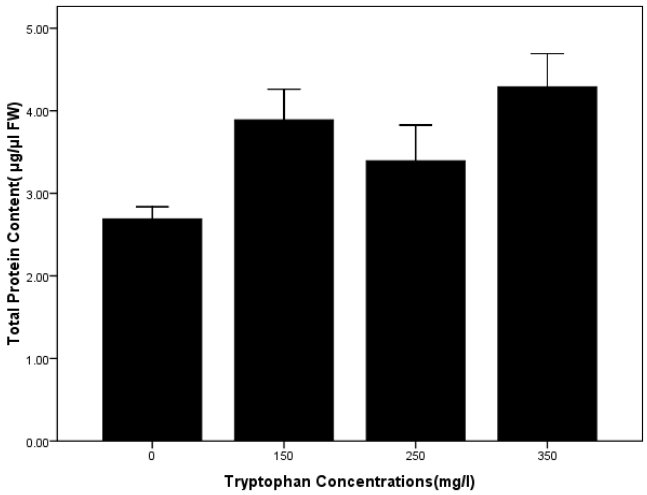

Figure7. Effect of tryptophan on total protein contents of roots in *C. roseus.* Results are shown as mean ± standard error (P < 0.05), obtained from four replicates

Discussion

Regeneration of periwinkle plants was carried out on different explants and media compositions. The results showed that the nodal segments in media containing MS supplemented with BAP (0.5 mg/l) + NAA (1 mg/l) showed the best results. This result was similar to Faheem *et al.* [8] findings on regeneration of *C. roseus*. The effect of auxin on number of shoots is due to the role of these phytohormones in apical dominance. On the other hand, increasing of NAA concentration compared to optimal

one (1mg/l) caused a decrease in number of shoots. It was demonstrated that NAA triggers cell elongation much more than cell division [12].

Rooting initiation was carried out on full and half strength MS medium supplemented with different concentrations of IBA. We found that the maximum rooting percentage may be achieved at half strength MS medium containing 0.1 mg/l IBA. This finding is similar to Faheem *et al.* [8] that reported the higher rooting percentages at MS medium with IBA (0.1 mg/l) in *C. roseus*. In the present study, we applied different concentrations of tryptophan using filtration after autoclave. The results revealed that the increment in tryptophan concentration increased the percentage of shooting and rooting. However, the results showed that tryptophan had much more effects on shooting than rooting and also, on all of growth parameters such as shoot and root length, shoot and root fresh and dry weight. These results may be due to the absorption of more amino acids by shoots prior to rooting process. Amino acids are commonly added to culture media as organic supplements and provide a source of nitrogen. The effect of this amino acid on shooting and rooting percentages and also on the number of shoots per explants may have resulted from the roles of amino acids in plant development. Shahsavari [13] used four levels of tryptophan and glutamine on the M5B5 medium and evaluated the role of these amino acids on the callus induction and regeneration of upland rice. He showed that the appropriate level of tryptophan caused a significant increase in regeneration frequency and callus induction. On the other hand, the positive effects of tryptophan on the growth of *C. roseus* could be due to its conversion into IAA [7]. According to Talaat *et al.* [14] study on *C.roseus* and Attoa *et al.* on *Iberis amara* [7], spraying of these plants with tryptophan may increase plant growth.

The measurement of photosynthetic pigments revealed that these parameters increased by increment of tryptophan concentrations. The effect of tryptophan on stimulating of photosynthetic pigments may be due to contribution of succinyl CoA and glycine in initiating of the pathway leading to chlorophyll formation. Hassanein *et al.*

[15] on *Foeniculum vulgare* and Milad *et al.* [16] on *Mentha viridis* reported that the foliar application of tryptophan on those plants led to an increase in these pigments content under *in vivo* conditions.

The analysis of total soluble sugar content in shoots and roots showed a gradual increase of this factor by tryptophan application in different concentrations. The role of tryptophan in increasing the total soluble sugar content is probably due to their role in the biosynthesis of chlorophyll pigments. Our findings are consistent with the results reported by the other investigators. Also foliar application of tryptophan under greenhouse conditions, Attoa *et al.* [7] on *Iberis amara* and Abdel Aziz *et al.* [17] on *Salvia farinacea* similar results were obtained.

Furthermore, in the study of total protein content, we found that the maximum amount of this factor was in 250 mg/l tryptophan compared with the control treatments. The role of tryptophan in protein contents have been previously reported by Talaat *et al.* [14] on *C. roseus* and Attoa *et al.* [7] on *Iberis amara* under *in vivo* conditions.

In conclusion, application of tryptophan by filtration into autoclaved culture medium may improve the regeneration and growth of *C. roseus* plants under *in vitro* conditions and also 350 mg/l tryptophan led to better results compared to other applied concentrations.

References

1. J. Aslam, S. Haque Khanand, Z. H. Siddiqui, "*Catharanthus roseus* (L.) G. Don. an important drug: its applications and production", Pharm Global, 4 (2010) 1-16.
2. M. M. Senoussi, J. Creche, M. Rideau, "Relation between hypoxia and alkaloid accumulation in *Catharanthus roseus* cell suspension", J Appl Sci Res, 3 (2007) 287-290.
3. A., Pietrosiuk, M. Futmanowa, B. Lata, "*Catharanthus roseus*; micropropagation and in vitro techniques", Phytochem Rev, 6 (2007) 459-473.
4. A. B. Ali Ahmed, A. S. Rao, M. V. Rao, "Somatic embryogenesis and plant regeneration from cell suspension culture of *Gymnema sylvestre",* KMITL Sci Tech J, 9 (2009) 18-25.
5. T. A. M. Abou Dahab, G. N. Abd El-Aziz, "Physiological effect of diphenylamine and tryptophan on the growth and chemical constituents of *Phylodendron erubescens* plants", World J Agri Sci, 2 (2006) 75-81.

6. A. A. Nahed, N. H. Mahgoub, A. A. M. Mazher,. "Physiological effect of phenylalanine and tryptophan on the growth and chemical constituents of *Antirrhinum majus* plants", Ozean J Appl Sci, 2 (2009) 399-407.
7. G. E. Attao, H. E. Wahba, A. A. Farahat, "Effect of some amino acids and sulphur fertilization on growth and chemical composition of *Iberis amara* L. plants, Egypt J Hort", 29 (2000) 17-37.
8. M. Faheem, S. Singh, B. S. Tanwer, M. Khan, A. Shahzad, "*In vitro* regeneration of multiplication shoots in *Catharanthus roseus*-an important medicinal plant", Adv Appl Sci Res, 2(2011) 208-213.
9. F. W. Fales, "The assimilation and degradation of carbohydrates by yeast cells", J Biol Chem, 193 (1951) 113-124.
10. M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding", Anal Biochem, 72 (1979) 248-254.
11. H. K. Lichtenthaler, A. R. Wellburn, "Determination of total carotenoids and chlorophylls a and b of leaf in different solvents", Biochem Soc Trans, 11 (1983) 591-592.
12. E. F. George, M. A. Hall, G. J. D. Klerk, "Plant propagation by tissue culture",Volume1. The Background, Springer, Dordrecht (2008).
13. E. Shahsavari, "Impact of tryptophan and glutamine on the tissue culture of upland rice", Plant Soil Environ, 57 (2011) 7-10.
14. I. M. Talaat, M. A. Bekheta, M. H. Mahgoub, "Physiological response of periwinkle plants (*Catharanthus roseus*) to tryptophan and puterscine", Intl J Agric Biol, 2(2005) 210-213.
15. R. A. M. Hassanein, "Effect of some amino acids, trace elements and irradiation on fennel (*Foeniculum vulgare* L.)", PhD Thesis, Faculty of Agriculture, Cairo University, Egypt (2003).
16. S. M. N. Milad, "Effect of tryptophan and methionine on the productivity of two *Mentha* species and American *Ocimum* plants", MSc, Faculty of Agriculture, Cairo University, Egypt (1998).
17. N. G. Abdel Aziz, L. K. Balbaa, "Influence of tyrosine and zinc on growth, flowering and chemical constituents of *Salvia farinacea* plants", J Appl Sci Res, 3 (2007) 1479-148.