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Improvement of Phenols Production by Amino Acids in Callus Cultures of *Verbascum thapsus* L.

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Abstract

A great mullein (*Verbascum thapsus* L.) was a famous species in Scrophulariaceae family. It was generally used as herbal medicine. Explants of *V. thapsus* (leaves and petioles) were cultured *in vitro* on Murashige and Skoog (MS) medium for shoot proliferation.Plantlet explants were cultured on MS medium supplemented with combination of Benzyl adenine (BA) and Naphthalene acetic acid (NAA) for callus induction. The best fresh and dry weight of callus formation was achieved using 0.5 mg/l BA. Quantitative analyses with High-performance liquid chromatography (HPLC) showed the content of phenols like Coumarin, Eugenol and Thymol were relatively low in leaves of mother plant, (10, 41, 310 ppm) respectively. The addition of different concentrations of amino acids as a precursor adding separately to the tissue culture medium led to raise the accumulation levels of phenolic compounds in callus tissue. Generally, the enhancement of accumulation depended on the type of amino acids and their concentration. The results showed 150 mg/l of Proline encouraged production of Comarin to 2752%, while 50 mg/l of Proline promoted accumulation of Eugenol to 290%. Whilst 150 mg/l of Tryptophan increased production of Thymol to 390%, in comparison with mother plant.

Keywords

***Verbascum thapsus*, Comarin, Eugenol, Thymol, Amino Acids**

1. Introduction

Verbascum is a genus of about 360 species of flowering plants in the Scrophulariaceae family. They are native

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to Europe and Asia, with the highest species diversity in the Mediterranean and Iranian Altoranih [1] [2]. The most important species is *Verbascum thapsus* L. Common names include mullein, common mullein, great mullein, wooly mullein, candlewick plant, velvet plant, blanket leaf, Aaron's rod, Jacob's staff, hedge taper, high taper, old man's flannel [3].

V. thapsus is rich plant in Phenolsso, it may be used wisely as an alternative medicine, it has been used as antiseptic, demulcent, narcotic, diuretic and has anti-microbial, anti-malarial, anti-oxidant and anti-inflammatory activities [4]-[7]. An aromatic, slightly bitter tea can be made by infusing dried leaves in boiling water [8]. The flowering stems can be dipped in wax and be used as torches and to make wicks for candle [9].

Plant phenols are secondary metabolites with diverse chemical nature and potential including: phenolic acids, flavonoids, tannins, coumarins, lignans and xanthones. Phenols are providing essential functions in the reproduction and the growth of the plants acting as defense mechanisms against pathogens, parasites, and predators [4] [10]. Plant tissue culture techniques are used as 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 [11].

Several endeavors have been recorded for enhanced synthesis of secondary metabolites *in vitro* cultures of different plant species, e.g. flavonoid productionin cultured tissue of *Hydrocotyle bonariensis* [12], Tropanealkaloids in *Hyoscyamus niger* [13], indole alkaloid in *Catharanthus roseus* [14], essential oil in *Origanum vulgare* L. and *Calendula officinalis* L. [15] [16].

Many researchers refer that metabolic engineering seems a promising approach to improve the cells production. So this study is conducted to experience the effect of amino acids as precursor feeding on the enhancement of phenolic compound accumulation *in vitro*.

2. Material and Methods

2.1. Source of Plant

Common mullein plants, *Verbascum thapsus* L. were collected at flowering stage from Al-Sulaimania mountains, Iraq on April 2013. *In vitro* plantlets were established according to Turker *et al*. [17]. Plantlet explants were used for induction of callus using (MS) medium [18] supplemented with different concentration of auxin 2,4-D (0, 0.5, 1 and 1.5 mg/l) and cytokinin BA (0, 0.5, 1, and 1.5 mg/l). Fresh and dry weights callus were measured after 6weeksof incubation at 25˚C ± 2˚C and 16 hrs light. The best callus production was selected after 6 weeks for further work. These callus were sub cultured each 4 weeks on fresh media supplemented with 0.5 mg/L of BA, and for two months continuously to maintain callus stock. An equal fresh weight of callus about (300 mg) was cultured on same medium which used in callus maintenance. Three concentrations 50, 100 and 150 mg/l of four different sources of amino acids: Proline, Glutamine, Tryptophan and Phenylalanine were used separately as precursor feeding for accumulation of phenolic compounds such as, Coumarin, Eugenol, and Thymol.

2.2. Extraction of Plant Materials

Leaves of mother plant and callus tissue were dried at 45˚C in oven for 48 hrs, and then kept at 4˚C. A 200 mg of dried plant material was taken for phenols extraction; the aliquots were shaken in 5 ml of methanol and incubated at room temperature for overnight. Plant material was centrifuged and filtered out using Whatman No.1, and 3 ml aliquots from each filtrate were filtered again using 0.22 µm syringe filters [19]. The existence and content of Coumarin, Eugenoland Thymol of callus tissue analyzed by HPLC. The leaves of mother plant were also analyzed for comparison.

2.3. Chromatographic (HPLC) Conditions

The RP-HPLC (Sykum-German) system with C18 reversed-phase column (250 × 4.6 mm) was used to detect of phenolic compound, Coumarin, Eugenol and Thymol. Acetonitrile and water in 75:25 (v/v) ratios was chosen as the mobile phase under a column temperature of 30˚C. The detection wavelength was set at 210 nm with a flow rate of 1.4 ml/min; the auto sampler injection volume was 20 µl. Quantitative method was analyzed by external standard. All standards were obtained from Sigma-Aldrich (USA).

2.4. Statistical Analysis

All experiments were carried out in 15 replicates. The experiment results were statistically analyzed by ANOVA with Two-way Analysis of Variance test using MINITAB11 statistical program. Experiments were carried out with completely randomized block design and the differences between groups were compared using LSD at $P \leq 0.05$.

3. Results and Discussion

3.1. Callus Production

Data recorded in **Table 1** showed the presence of significant effects of growth regulators used on callus production from plantlet explants. The best result of callus production was recorded with 0.5 mg/l of BA done highest value of mass callus production, (3672 and 218 mg) in fresh and dry weight respectively. The combination between BA and 2,4-D caused positive significant effect on fresh and dry weight of callus. However, supplementation of MS media with 2,4-D alone showed a little effect on callus production. Similar results were reported for callus induction from *Verbascum sinuatum* L. [20], they stated that a callus induction was induced on MS medium supplemented with different concentrations of BA and NAA, but no induction of callus was observed using NAA alone.

Callus inductions depended on kind and concentration of plant growth regulators, type of explant also played a considerable role in callus induction [21]. So the plantlet explant was selected in our study for initiation of callus according with [20] [22], they successfully, initiated the callus tissue of *V. speciosum* and *V. sinuatum* L.

3.2. Effect of Amino Acids on Mass Growth of Callus Culture

All treatments of amino acids (Proline, Glutamine, Tryptophan and Phenylalanine), gave different mass value of callus tissue. Generally, 150 mg/L of Glutamine added to MS medium done high response of fresh and dry weight reached to (3065 and 160 mg) respectively, (**Table 2**). Significantly, none of amino acids tested promoted production of callus. The results were in agreement with Urmantsva *et al*. [23], were found that of none of the amino acids tested enhanced biomass production in cell cultures of *Thalictrum minus*.

Table 1. Effect of 2,4-D and BA concentrations and their combinations on fresh and dry weight (mg) of callus induction on MS medium from plantlet explant of *V. thapsus*.

BA (mg/l)	2,4-D (mg/l)				Mean of BA
	0.0	0.5	1	1.5	
Callus fresh weight (mg)					
0.0	688	654	379	217	484
0.5	3672	2409	2669	2664	2854
1	3128	2514	2517	2250	2603
1.5	3020	2773	2855	3611	3065
Mean of 2,4-D	2624	2088	2105	2186	
LSD at $P \leq 0.05$	BA = 126		2,4-D = 126		Interaction = 252
Callus dry weight (mg)					
0.0	49	54	32	30	41
0.5	218	153	159	162	173
1	150	144	143	136	143
1.5	164	149	150	167	157
Mean of 2,4-D	145	126	122	123	
LSD at $P \leq 0.05$	BA = 6.5		2,4-D = 6.5		Interaction = 13.1

Table 2. Effect of different concentration of amino acids (mg/l) added to MS medium on callus fresh and dry weight of *V. thapsus* L. after 4 weeks of incubation (initial callus weight was 300 mg).

Concentration of amino acid (mg/l)	Amino acid			
	Proline	Glutamine	Tryptophan	Phenyl alanine
Callus fresh weight (mg)				
0.0	2548	2548	2548	2548
50	2758	2887	2857	2244
100	2193	2923	2951	1923
150	2112	3065	2709	2045
Mean	2402	2855	2766	2190
No statistically significant differences at $P \leq 0.05$				
Callus dry weight (mg)				
0.0	141	141	141	141
50	159	153	157	139
100	138	156	165	138
150	137	160	**158**	141
Mean	143	152	155	139
No statistically significant differences at $P \leq 0.05$				

The use of amino acids as an organic source of nitrogen was not usually desired in modern media, where a proper balance between $NO_3^-/NH4^+$ guaranteed the nitrogen requirement, *L. glutamine* was most commonly used as a nitrogen source in tissue culture [21].

3.3. Effect of Amino Acids on Accumulation of Phenolic Compound in Callus Culture of *V. thapsus*

The results in **Table 3** showed the low content of Coumarin (10 ppm) which achieved at mother plants, while *in vitro* callus tissues extract gave high significant values especially with amino acids treatments. It was noted that the addition of high concentration of Proline, Tryptophan and Phenylalanine (150 mg/l) led to produce high significant values of Coumarin (285.2, 88.2 and 146.8 ppm) respectively. Prolineat concentration of 150 mg/l successfully trigged the production of Coumarin (285.2 ppm), which was 2752% higher than mother plant. While the lowest concentration of Glutamine 50 mg/l gave the best production of Coumarin (113.2 ppm), 1032% more than mother plant.

Although Eugenol was scarcely detected (15.0 ppm) in callus culture of control treatment, (**Table 4**). This compound was highly produced when the callus was treated with low concentration of amino acids (50 and 100 mg/l). Treatment with Proline at 50 mg/l gave high percentage of increase; it was 290% more than mother plants, (**Figure 1**). On the other hand, similar values of Eugenol production (71.8, 68.0 and 74.9 ppm) were recorded in Glutamine treatments (50, 100 and 150 mg/l) respectively, with percentage of increasing ranged between (65% - 82%) more than mother plant.

The results illustrated in **Table 5** indicated that addition of Tryptophan and Phenylalanine as precursors led to increase the production of Thymol. The superiority in Thymol production was attained in medium contained 150 mg/l of Tryptophan, it was (1518.8 ppm), 390% higher compared with mother plants. Supplementation with both amino acids (Proline and Glutamine) as precursors caused reduction in Thymol concentrations to percentage of 30.97%, 95.66% and 38.60% respectively, compared with the control and mother plant.

Attempts to induce the yield of the secondary product by supplying precursors or abiotic elicitors are found to be effective in many cases. Amino acids have been used as organic nitrogen source in *in vitro* cultures of several

Table 3. Effect of amino acid added to MS medium on accumulation of Coumarin (ppm) in callus culture of *V. thapsus* after 4 weeks of incubation.

Concentration of amino acid (mg/l)		Coumarin (ppm)	Percentage (%) of Increase compared with mother plants (+)	Percentage (%) of decrease compared with mother plants (−)
Mother plants (leaves)		10		
Control (callus without amino acid)	0.0	90.2	+802	
	50	178.9	+1689	
Proline	100	121.8	+1118	
	150	285.2	+2752	
	50	113.2	+1032	
Glutamine	100	70.0	+600	
	150	41.1	+311	
	50	70.7	+607	
Tryptophan	100	70.4	+604	
	150	88.2	+782	
	50	45	+350	
Phenyl alanine	100	122.3	+1123	
	150	146.8	+1368	

Table 4. Effect of amino acid added to MS medium accumulation of Eugenol (ppm) in callus culture of *V. thapsus* after 4 weeks of incubation.

Concentration of amino acid (mg/l)		Eugenol (ppm)	Percentage (%) of Increase compared with mother plants (+)	Percentage (%) of decrease compared with mother plants (−)
Mother plants (leaves)		41.0		
Control (callus without amino acid)	0.0	15.0		−63
	50	160.3	+290	
Proline	100	57.8	+40	
	150	0.0		−100
	50	71.8	+75	
Glutamine	100	68.0	+65	
	150	74.9	+82	
	50	56.0	+36	
Tryptophan	100	51.0	+24	
	150	0.0		−100
	50	68.2	+66	
Phenyl alanine	100	57.6	+40	
	150	0.0		−100

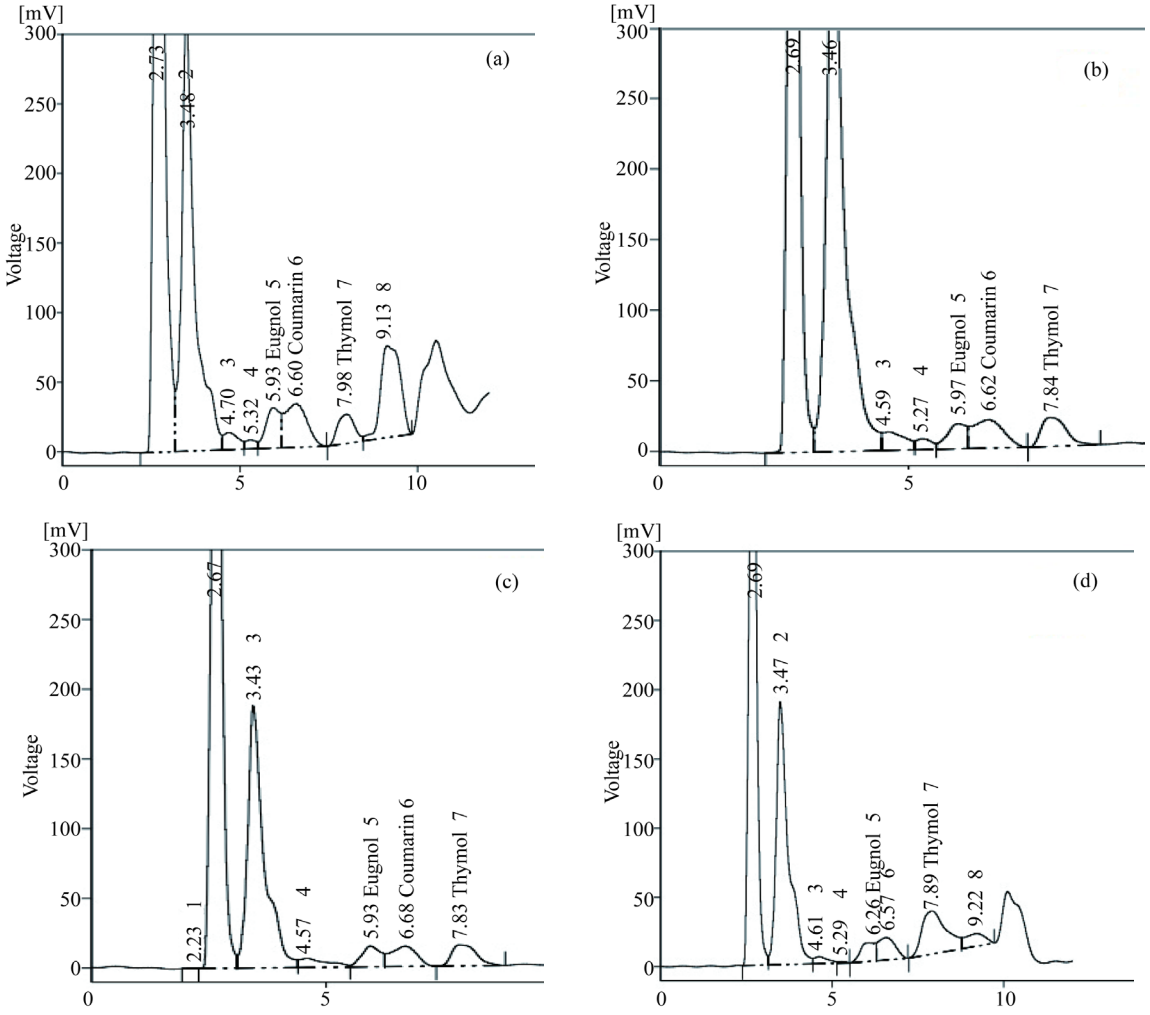

Figure 1. HPLC chromatograms for: (a) Proline (50 mg/l); (b) Glutamine (50 mg/l); (c) Tryptophan (50 mg/l); (d) Phenyl alanine (50 mg/l).

Table 5. Effect of amino acid added to MS medium on accumulation of Thymol (ppm) in callus culture of *V. thapsus* after 4 weeks of incubation.

Concentration of amino acid (mg/l)		Thymol (ppm)	Percentage (%) of Increase compared with mother plants (+)	Percentage (%) of decrease compared with mother plants (−)
Mother plants (leaves)		310.0		
Control (callus without amino acid)	0.0	519.4	+67	
	50	255.8		−17
Proline	100	290.0		−6
	150	224.4		−27
	50	272.8		−12
Glutamine	100	250.0		−19
	150	286.0		−7
	50	185.5		−40
Tryptophan	100	699.7	+125	
	150	1518.8	+390	
	50	153.6		−50
Phenyl alanine	100	296.2		−4
	150	763.7	+146	

species like sorghum [24], alfalfa [25], maize [26], rice [27] and other plants to enhance somatic embryogenesis and regeneration. The aromatic amino acids Phenylalanine and Tryptophan in plants are not only essential components for protein synthesis, but also serve as precursors for a wide range of secondary metabolites that are important for plant growth [28]. Hakkim *et al.* [29], found that the addition of Phenylalanine into agar medium improved in rosmarinic acid yield in *Ocimum sanctum* cell cultures. [30] suggests that Artimisinin production can be enhanced with the manipulation of medium by different amino acids in the callus cultured. Taha *et al*., [31] reported that highest value of mass cell cultures and indole alkaloids production in *Catharanthus roseus* were achieved with modified MS medium containing 300 mg/l of either L-glutamine for mass cell induction or L-typtophane for enhancement of total indole alkaloids. Also, Ahmed *et al*. [14], described that the indole alkaloid content of callus tissue of *Catharanthus roseus* was increased by amino acids supplementation. The effect of Proline on Thymol production in *Origanum vulgare* and on Hyoscyamine and Scopolamine in callus culture of *Hyoscyamus niger* has been studied by [13] [15], they were found Proline enhanced the secondary metabolites in callus tissue. Unsuccessful attempts to induce product yield may be due to our lack of knowledge concerning the timing of addition of such compounds, their uptake, and their compartmentation in relation to the enzymes involved in their utilization [32].

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References

[1] Zohary, M. (1974) Flora of Palestine. Israel Academy of Sciences and Humanities.

[2] Sharifnia, F. (2007) Notes on the Distribution and Taxonomy of *Verbascum* in Iran. *The Iranian Journal of Botany*, **13**, 30-32.

[3] Angier, B. and Foster, D.K. (2008) Field Guide to Medicinal Wild Plants. 2nd Edition, Stackpole Books, Mechanicsburg, 258.

[4] Tatli, I.I. and Akdemir, Z.S. (2004) Chemical Constituents of *Verbascum* L. Species. *FABAD J. Pharm. Sci.*, **31**, 85-96.

[5] Tatli, I.I. and Akdemir, Z.S. (2006) Cytotoxic Activity on Some *Verbascum* Species Growing in Turkey. *Hacettepe University Journal of the Faculty of Pharmacy*, **26**, 77-85.

[6] Tatli, I.I., Akdemir, Z.S., Yesilada, E. and Kueli, E. (2008) Anti-Inflammatory and Antinociceptive Potential of Major Phenolics from *Verbascum salviifolium* Bioss. *Zeitschrift für Naturforschung C*, **63**, 196-202.

[7] Turker, A.U. and Camper, N.D. (2002) Biological Activity of Common Mullein, a Medicinal Plant. *Journal of Ethnopharmacology*, **82**, 117-125. http://dx.doi.org/10.1016/S0378-8741(02)00186-1

[8] Facciola, S.C. (1990) A Source Book of Edible Plants. Kampong Publications, Vista, 677.

[9] R.H.S. (1988)The garden. *Royal Horticultural Society*, volume 113.

[10] Ghorpade, R.P., Chopra, A. and Nikam, T.D. (2011) Influence of Biotic and Abiotic Elicitors on Four Major Isomers of Boswellic Acid in Callus Culture of *Boswellia serrata* Roxb. *Plant Omics Journal*, **4**, 169-176.

[11] Alfen, N.K.V. (2014) Encyclopedia of Agriculture and Food Systems. 5-Volume Set, Elsevier/Academic Press, Amsterdam, 306.

[12] Masoumian, M., Arbakariya, A., Syahida, A. and Maziah, M. (2011) Effect of Precursors on Flavonoid Production by *Hydrocotyle bonariensis* Callus Tissues. *African Journal of Biotechnology*, **10**, 6021-6029.

[13] Aljibouri, A.M.J., Al-Samarraei, K.W., Abd, A.S., Mageed, D.M. and Ali, A.A. (2012) Alkaloids Production from Callus of *Hyoscyamusniger* L. *in Vitro*. *Journal of Life Sciences*, **6**, 874-882.

[14] Ahmed, F.A., Abdel-Fateh, O.M., Kobeasy, M.T. and Ahmed, O.K. (2000) Factors Affecting Growth and Indole Alkaloid Content of *Catharanthus calli* (*Catharanthus roseus* L.) Amino Acids, Casein Hydrolysate and Irradiation. *Arab Journal of Biotechnology*, **3**, 61-70.

[15] Al-Jibouri, A.M.J., Abd, A.S., Majeed, D.M. and Ismail, E.N. (2012) Influence of Abiotic Elicitors on Accumulation of Thymol in Callus Cultures of *Origanum vulgare* L. *Journal of Life Sciences*, **6**, 1094-1099.

[16] Al-Oubaidi, H.K.M. and Ameen, A.S.M. (2014) Increasing Secondary Metabolites of *Calendula officinalis* Using Salicylic acid *in Vitro*. *World Journal of Pharmacy and Pharmaceutical Sciences*, **13**, 1146-1155.

[17] Turker, A.U., Camper, N.D. and Gurel, E. (2001) *In Vitro* Culture of Common Mullein (*Verbascum thapsus* L.). *In Vi-*

tro Cellular and Developmental Biology-Plant, **37**, 40-43.

[18] Murashing, T. and Skoog, F. (1962) A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Culture. *Plant Physiology*, **15**, 473-497. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x

[19] Tamura, Y. and Nishibe, S. (2002) Changes in the Concentrations of Bioactive Compounds in Plantain Leaves. *Journal of Agricultural and Food Chemistry*, **50**, 2514-2518. http://dx.doi.org/10.1021/jf011490x

[20] Karamian, R. and Ghasemlou, F. (2014) Plant Regeneration via Organogenesis and Somatic Embryogenesis in *Verbascum sinuatum* L. *Acta Biologica Cracoviensia Series Botanica*, **56**, 97-103. http://dx.doi.org/10.2478/abcsb-2014-0010

[21] Pierik, R.L.M. (1997) *In Vitro* Culture of Higher Plants. Springer Science and Business Media B.V., Dordrecht.

[22] Karamian, R. and Ghasemlou, F. (2012-2013) Plant Regeneration via Somatic Embryogenesis and Organogenesis in V*erbascum speciosum* Schard. *Journal of Cell and Molecular Research*, **4**, 81-88.

[23] Urmantseva, V.V., Gaevskaya, O.A., Karyagina, T.B. and Bairamashvili, D.I. (2005) The Effect of Amino Acids as Components of Nutrient Medium on the Accumulation of Protoberberine Alkaloids in the Cell Culture of *Thalictrum minus*. *Russian Journal of Plant Physiology*, **52**, 388-391. http://dx.doi.org/10.1007/s11183-005-0058-x

[24] Asad, S., Arshad, M., Mansoor, S. and Zafar, Y. (2009) Effect of Various Amino Acids on Shoot Regeneration of Sugarcane (*Saccharum officinarum* L.). *African Journal of Biotechnology*, **8**, 1214-1218.

[25] Skokut, T.A., Manchester, J. and Schaefer, J. (1985) Regeneration in Alfalfa Tissue Culture. *Plant Physiology*, **79**, 579-583. http://dx.doi.org/10.1104/pp.79.3.579

[26] Claparols, I., Santos, M.A. and Torné, J.M. (1993) Influence of Some Exogenous Amino Acids on the Production of Maize Embryogenic Callus and on Endogenous Amino Acid Content. *Plant Cell, Tissue and Organ Culture*, **34**, 1-11. http://dx.doi.org/10.1007/BF00048457

[27] Grewal, D., Gill, R. and Gosal, S.S. (2006) Role of Cysteine in Enhancing Androgenesis and Regeneration of Indica Rice (*Oryza sativa* L.). *Plant Growth Regulation*, **49**, 43-47.

[28] Tzin, V. and Galili, G. (2010) The Biosynthetic Pathways for Shikimate and Aromatic Amino Acids in *Arabidopsis thaliana*. The Arabidopsis Book 8, e0132. http://dx.doi.org/10.1199/tab.0132

[29] Hakkim, F., Kalyani, S., Essa, M., Girij, S.A. and Song, H. (2011) Production of Rosmarinic in *Ocimum sanctum* Cell Cultures by the Influence of Sucrose, Phenylalanine, Yeast Extract, and Methyl Jasmonate. *International Journal of Biological and Medical Research*, **2**, 1070-1074.

[30] Zia, M., Mannan, A. and Chaudhary, M.F. (2007) Effect of Growth Regulators and Amino Acids on Artemisinin Production in the Callus of *Artemisia absinthium*. *Pakistan Journal of Botany*, **39**, 799-805.

[31] Taha, H.S., El-Bahr, M.K. and Seif-El-Nasr, M.M. (2009) *In Vitro* Studies on Egyptian *Catharanthus roseus* (L.) G. Don. IV: Manipulation of Some Amino Acids as Precursors for Enhanced of Indole Alkaloids Production in Suspension Cultures. *Australian Journal of Basic & Applied Sciences*, **3**, 3137-3144.

[32] Constabel, F. and Vasil, I.K. (1987) Cell Culture and Somatic Cell Genetics of Plants, Vol. 4: Cell Culture in Phytochemistry. Academic Press Inc., London.