Effect of Amino Acids on the Growth and Production of Steroids in Date Palm Using Tissue Culture Technique

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Abstract: The present investigation studied the effect of amino acids (Glutamine, Spermidine and Asparagine) with different concentration (50, 250.500 mg/l) used as precursors to produce secondary metabolites (steroids) and growth development during different stages (callus, embryoids and shooting) of date palm (Malakaby cv.). In Embryogenic callus stage, callus volume was the highest (4.00) when treated with any of the three amino acids, 50 mg/l of Glutamine or Asparagine showed no effect compared to the control giving the lowest callus volume (3.00). Total steroids in callus tissues clearly showed that using Glutamine 250 mg/l in medium gave the highest steroid content 0.662 mg/g and percentage (336% of control), while the lowest (0.111mg/g) was found with Asparagine (500mg/l) and 56.35% of control. Glutamine at 250 mg/l resulted in the highest weight of embryos (2.100 gm). As well as, 500 mg/l Spermidine seemed to be the best amino acid used in order to stimulate steroid biosynthesis resulting in 202.1% of control (0.782 mg/g). In shooting stage, according to the number of shoots, the highest number of shoots (2.33) was achieved with Glutamine and Spermidine at 500 mg/l. In shoot weight, the highest weight (7.267 gm) was achieved by using 500mg/l Glutamine , as to steroid biosynthesis in shooting stage, the best result obtained, were by using Glutamine at 500 mg/l which gave highest steroid biosynthesis (0.534mg/g), 206.0% of control.

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Keyword Glutamine, Spermidine, Asparagine

1. Introduction

Date palm *(phoenix dactylifera)* is a monocotyledonous dioecious plant. Dates are considered the main fruit in Arab nations such as Saudi Arabia, Iraq, and Egypt. Date palms are not only used as fruit producing trees, but are also used in landscape decoration. The major problem in date palm cultivation is the poor method of vegetative propagation. **(Badawy *et al*., 2005).** During ancient times, Egyptians recognized the growth of the date palm *(phoenix dactylifera)* and was recognized as being a fertility symbol **(Puchoaa and Ramburn, 2004).**

Cholesterol, Cholestanol and Coprostanol are the animal sterols, while, β-sitosterol, Compesterol, Stigmasterol, Ergosterol and Brassicasterol are the principle plant sterols **(Bailey, 1964)**. Cholesterol, one believed to be the typical animal sterol has recently been found to be rather widely distributed among plants. So far, cholesterol has been identified in the pollen of many plants including date palm **(Bennett et al., 1966)** and oil palm **(Slover et al., 1983).** Plant tissue cultures have long been regarded as a source of commercially important steroids, alkaloids and terpenes for pharmaceutical industry **(Bohm, 1980; Staba, 1980; Barz and Eills, 1981; Deus and Zenk, 1982). El-Sharabasy (2004)** indicated that the precursors have great effect in the biosynthesis of steroids in date palm callus and embryogenic callus cells.

The present investigation was planned to study the effect of some amino acids used as precursors to produce secondary metabolites (steroids and sterols), and its effect on plant development during different stages (callus, embryoids and shooting) of in vitro date palm cultivation (*Phoenix dactylifera* L.) Malakaby cultivar

2. Materials and Methods

This investigation was carried out in the laboratory of Plant Cell and Tissue Culture Department, Biotechnology Division, The Central Laboratory for Date Palm Research and Development, 2010.

Plant materials

To test the effect of Glutamine, Spermidine, and Asparagine as precursors on the growth, development

and secondary products synthesis (steroids) in embryogenic callus, somatic embryo and shooting stages of date palm.

In this experiment, embryogenic callus, somatic embryo and shooting stages of date palm (*Phoniex dactylefera L.)* were treated with 0, 50, 250 and 500 mg/l of Glutamine, Spermidine and Asparagine.

Tissue culture experiment

MS solid medium and 0.1 NAA were used for embryogenic callus and somatic embryo, while in shooting stage 0.05 mg/l BA + 0.1 NAA were used. Nine groups of jars having 25 ml medium for every treatment have been set. The pH value was regulated to 5.7- 5.8 before autoclaving. The cultures were then incubated at 27 ± 2°C temperature and 16 hours light/day photoperiod. The following data were analyzed after one month such as, Measurements on the callus tissues of embryogenic callus stage.(Volume of callus, Callus weight (gm),Total concentration of steroid hormones were calculated and verified by the spectrophotometer due to the methods illustrated by Pharco in 1993). , Measurements on the embryos tissues of embryoids stage (Number of embryos,Weight of embryo (gm),Total concentration of steroid hormones and Measurements taken on the shooting tissues of shooting stage (Shoot number, Weight of shoots and Total steroids)

Chemical analysis

Preparation of the unsaponfiable matter (U.S.M) according to Sharabasy (2001)

Quantitative Determination of Total Steroids in the unsaponifiable fraction by Spectrophotometer: The total steroids were determined in the unsaponifiable fraction by the reaction with Denigee reagent according to (Pharco 1993).

Statistical analysis

The experiments were carried out using completely randomized blocks design and with three replicates. The results were analyzed using analysis of variance and the means compared using L.S.D. at the 5% level, all obtained data were subjected analysis of variance completely randomized blocks according to **(Snedecor and Cochtan, 1980).**

3. Results

Effect of different amino acids as precursors on the embryogenic callus volume of embryogenic callus stage resulting from shoot tip of date palm (*Phoenix dactylifera L.*) Malakaby cultivar

Data shown in table 1 and figure 1 demonstrates the effect of the different precursor concentration on embryogenic callus value derived from shoot tip of date palm (Malakaby cultivar). These results illustrated that the greatest results (4.00) attained with embryogenic callus grown on medium contained 500 mg/l Glutamine, Spermidine or Asparagine. These three precursors with concentration 500mg/l showed the same best effect on callus volume, followed by medium treatment with 250 mg/l Spermidine (3.67). Using Glutamine and Asparagine with concentration 250mg/l showed the same effect on callus volume (3.33).

Alternatively, there was no observed change in the volume of callus when grown on medium contained 50 mg/l, which was shown in Asparagine and Glutamine as they were similar to the control (3.00), but in Spermidine there was a slight difference compared to the control (3.33).

Overall there was no examined decrease due to the addition of precursors with the concentration applied.

Table 1: Effect of different amino acids as precursors on the embryogenic callus volume of the embryogenic callus stage of date palm (*Phoenix dactylifera L.*) Malakaby cultivar.

Precursors (A)	(B) Concentration (mg/l)				Mean
	0.0	50	250	500	
Glutamine	3.00	3.00	3.33	4.00	3.33 A
Spermidine	3.00	3.33	3.67	4.00	3.50 A
Asparagine	3.00	3.00	3.33	4.00	3.33 A
Mean	3.00 B	3.11 B	3.44 B	4.00 A	

L.S.D at 0.05% A =0.7750 B = 0.8949 AB =1.550

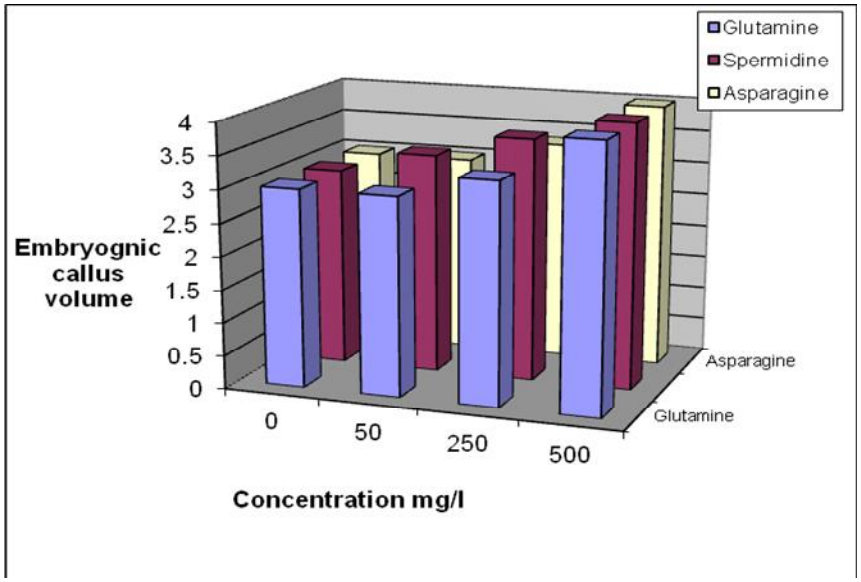

Figure 1: Effect of different amino acids as precursors on the embryogenic callus volume of the embryogenic callus stage of date palm (*Phoenix dactylifera L.*) Malakaby cultivar.

Effect of different amino acids as precursors on the production of total steroids as secondary metabolite in the embryogenic callus tissues of embryogenic callus stage of date palm (*Phoenix dactylifera L.*) Malakaby cultivar.

Data in Table 2 and Figure 2 shows the variations in steroid biosynthesis from the embryogenic callus tissues of date palm, Malakaby cultivar, under the effect of different amino acids (Glutamine, Spermidine, Asparagine) concentration used in this study. These results clearly showed that using Glutamine 250 mg/l in MS medium seemed as the most suitable amino acid to stimulate steroid biosynthesis 0.662 mg/g and the highest percentage (336% of control) followed by Asparagines 250m / l which gave steroid biosynthesis 0.261 mg/g and the percentage (132.5% of control) where Spermidine gave the lowest result in this stage. The data also showed that using high concentration of different amino acids specially 500 mg/l had negative effect on steroid biosynthesis which was 0.159, 0.135, and 0.111 with Glutamine, Spermidine, and Asparagine respectively.

Table 2: Effect of different amino acids as precursors on the production of total steroids as secondary product in the embryogenic callus tissues of embryogenic callus stage of date palm (*Phoenix dactylifera L.*) Malakaby cultivar.

Type of precursors	Concentration (mg/l)	Total steroids	
		Mg/g	% of control
Control	0.0	0.197	100
Glutamine	50 250 500	0.223 0.662 0.159	113.3 336.0 80.71
Spermidine	50 250 500	0.250 0.161 0.135	127.1 81.85 68.63
Asparagine	50 250 500	0.219 0.261 0.111	111.2 132.5 56.35

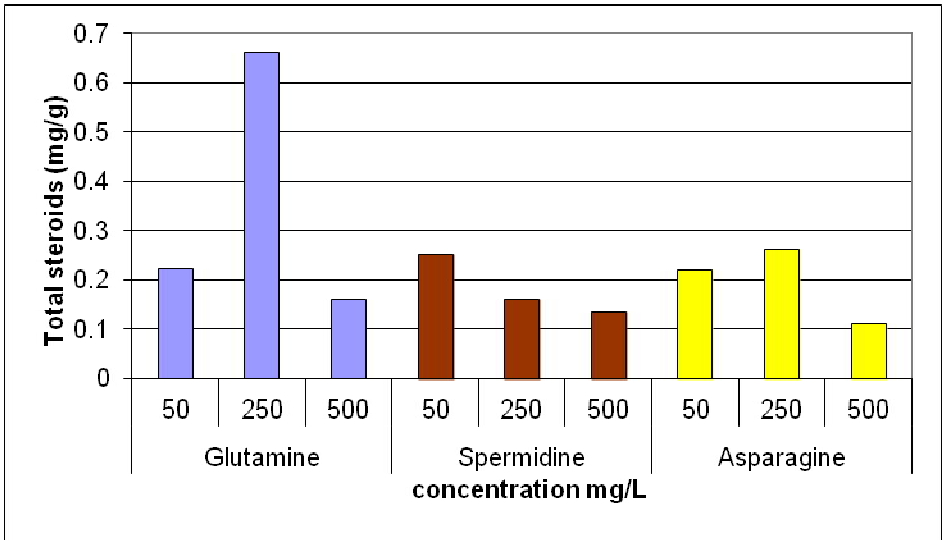

Figure 2: Effect of different amino acids as precursors on the production of total steroids as secondary product in the embryogenic callus tissues of embryogenic callus stage of date palm (*Phoenix dactylifera L.*) Malakaby cultivar.

Effect of different amino acids as precursors on the weight of embryos (gm) of somatic embryo stage of date palm (*Phoenix dactylifera L.*) Malakaby cultivar.

Data presented in Table 3 and Figure 3 illustrates the effect of Glutamine, Spermidine and Asparagine as precursors on embryos weight of date palm Malakaby cultivar. These results indicated that there were significant difference between different precursor's concentration and their interactions.

Statistical analysis of variance showed that MS medium supplemented with the low Asparagine concentration (50 mg/l) and the low Spermidine concentration (50 mg/l) had a significant stimulating effect to increase the weight of embryos derived from shoot tip. In Asparagine compared to the control, the weight of embryos increased from 0.400gm--1.67gm and in Spermidine, the weight of embryos compared to the control increased from 0.400gm--1.733gm. In contrast, the lowest increase in weight of embryos with concentration 50mg/l was observed in Glutamine (1.633 gm).

Glutamine added with concentration 250 mg/l to the medium showed the best increase in weight of embryos (2.100 gm), and then comes Spermidine (1.33 gm) and then Asparagine (1.267 gm).

In Glutamine, Spermidine and Asparagine with concentration 500 mg/l, there were no observed increase in weight (1.133 gm, 1.067 gm, and 1.30 gm) compared to the other concentration 50 and 250 mg/l, but actually a significant decrease.

In general, the highest weight of embryos (2.100 gm) in somatic embryo stage was achieved with Glutamine at 250 mg/l concentration while the lowest weight of embryos was obtained with Asparagine (1.067 gm).

Table 3: Effect of different amino acids as precursors on the weight of embryos (gm) of somatic embryo stage of date palm (Phoenix dactylifera L.) Malakaby cultivar.

Precursors (A)	(B) Concentration (mg/l)				Mean
	0.0	50	250	500	
Glutamine	0.400	1.633	2.100	1.133	1.317 A
Spermidine	0.400	1.733	1.33	1.067	1.333 A
Asparagine	0.400	1.867	1.267	1.300	1.208 A
Mean	0.400 C	1.744 A	1.567 AB	1.167 B	

L.S.D at 0.05% A = 0.3971 B = 0.4586 AB =0.7942

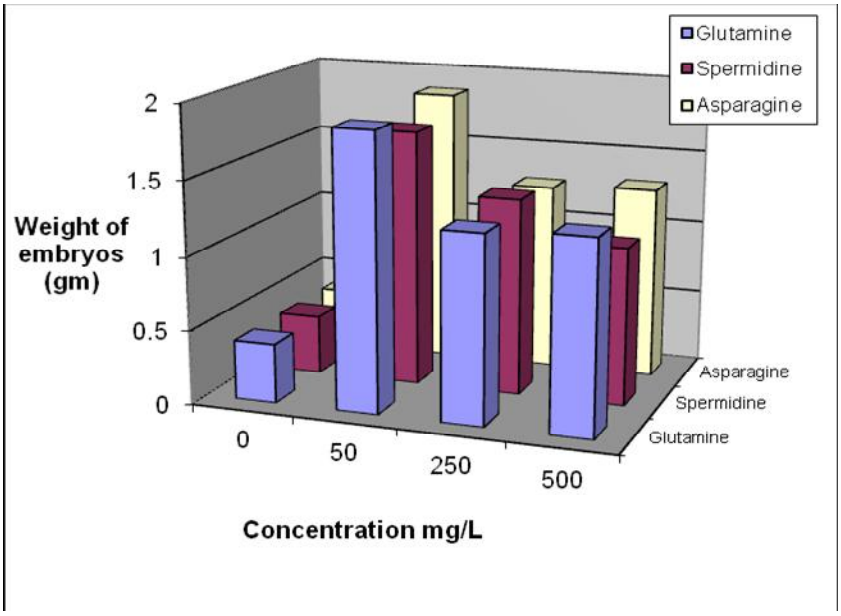

Figure 3: Effect of different amino acids as precursors on the weight of embryos (gm) of somatic embryo stage of date palm (*Phoenix dactylifera L.*) Malakaby cultivar.

Effect of different amino acids as precursors on the production of total steroids as secondary metabolites in the embryo tissues of somatic embryo stage of date palm.

Data in Table 4 and Figure 4 records that steroid biosynthesis responded differently to the different amino acids (Glutamine, Spermidine and Asparagine) concentration used in this study.

The obtained results showed that MS medium supplemented with Glutamine 500 mg/l stimulated the process of steroid biosynthesis and increased it by about 132.6 mg/g of control (0.513mg/g) comparing with 80.62% of control (0.496 mg/g) for 50 mg/l Glutamine , 128.2% of control (0.496 mg/g) for 250 mg/l Glutamine .

The recorded data indicate that using of 500 mg/l Spermidine seemed to be the best amino acid used in order to stimulate steroid biosynthesis resulting in 202.1% of control (0.782 mg/g) where of control (0.611 mg/g) for 250 mg/l and 83% of control (0.325 mg/g) for 50 mg/l. Whereas steroid biosynthesis showed negative correlation responses when Asparagine concentration reached 500mg/l which stimulate steroid biosynthesis by about 87.9% of control.

Table 4: Effect of different amino acids as precursors on the production of total steroids as secondary products in the embryo tissues of somatic embryo stage of date palm.

Type of precursors	Concentration (mg/l)	Total steroids	
		Mg/g	% of control
Control	0.0	0.387	100
Glutamine	50	0.312	80.62
	250	0.496	128.2
	500	0.513	132.6
Spermidine	50	0.325	83.0
	250	0.611	157.9
	500	0.782	202.1
Asparagine	50	0.270	69.8
	250	0.414	106.0
	500	0.340	87.9

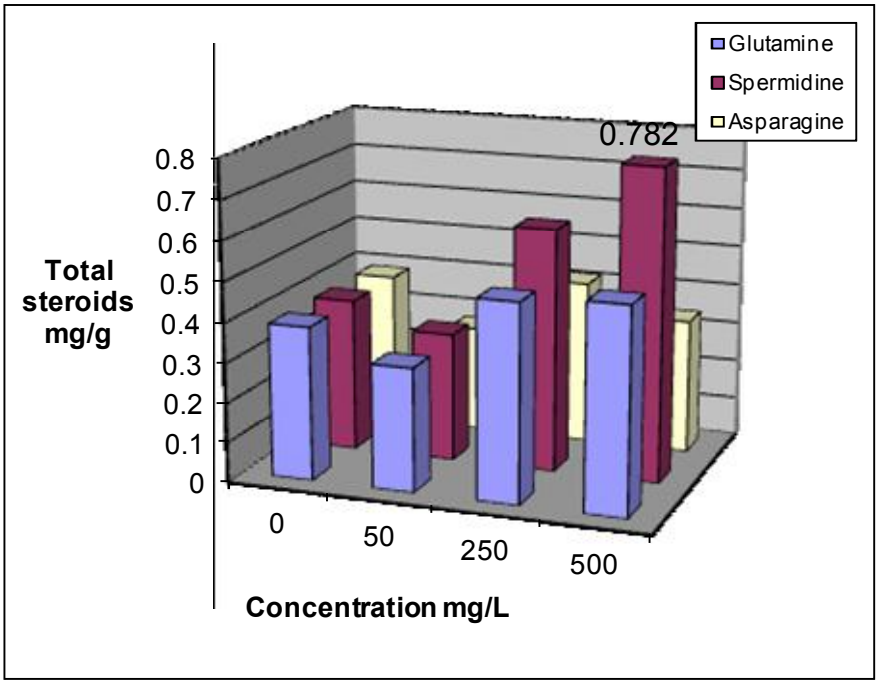

Figure 4: Effect of different amino acids as precursors on the production of total steroids as secondary products in the embryo tissues of somatic embryo stage of date palm.

Effect of different amino acids as precursors on the number of shoots during shooting stage of date palm (*Phoenix dactylifera L.*) Malakaby cultivar.

Data presented in Table 5 and Figure 5 illustrates the effect of Glutamine, Spermidine and Asparagine as precursors on number of shoot embryo of date palm Malakaby cv.From the presentation of data in Table (5) and Figure (5), it appears that shoot formation responded differently to the different precursors used in this study. As in Glutamine with concentration 50 mg/l added to the medium showed 2.00 shoot/embryo compared to Spermidine and Asparagine with the same concentration (50 mg/l) added did not show any increase in shoot/embryo value (1.333) compared with untreated explants. Giving Glutamine the highest value with this recorded concentration (50 mg/l).

Glutamine added with concentration 250 mg/l to the medium showed the best increase in shoot formation (2.33), and then comes Spermidine (1.667) and then Asparagine (1.373).

In general the highest number of shoots (2.33) was achieved with Glutamine and Spermidine at concentration 250 mg/l, while the lowest number of shoots (1.33) was obtained with Asparagine and Spermidine at concentration 50 mg/l. On the other hand, the best concentration achieved with 500 mg/l was (2.222). Glutamine and Spermidine showed the same number of shoot formation per embryo with concentration 500 mg/l, while Asparagine showed the lowest value (2.00).

Table 5. Effect of different amino acids as precursors on the number of shoots of shooting stage of date palm (*Phoenix dactylifera L.*) Malakaby cultivar.

Precursors (A)	**(B) Concentration (mg/l)**				**Mean**
	0.0	50	250	500	
Glutamine	1.333	2.00	2.33	2.333	2.00 A
Spermidine	1.333	1.333	1.667	2.333	1.667 A
Asparagine	1.333	1.333	1.373	2.00	1.50 A
Mean	1.333 B	1.556AB	1.778 AB	2.222A	

L.S.D at 0.05% A = 0.6821 B = 0.7876 AB =1.364

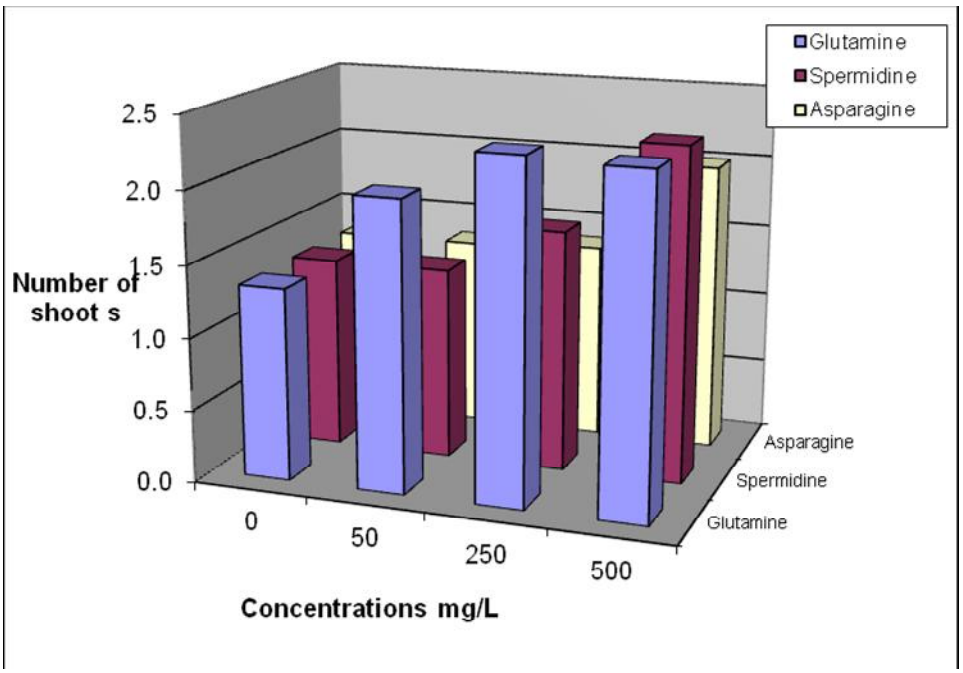

Figure 5: Effect of different amino acids as precursors on the number of shoots of shooting stage of date palm (*Phoenix dactylifera L.*) Malakaby cultivar.

3.1 Effect of different amino acids as precursors on shoot fresh weight (gm) of shooting stage of date palm (*Phoenix dactylifera L.*) Malakaby cultivar.

Data presented in Table 6 and Figure 6 clearly shows the effect of Glutamine, Spermidine and Asparagine precursors on shoot fresh weight (gm) of date palm (Malakaby cv.)

The highest weight of shoot was recorded for MS basal medium supplemented with 500 mg/l of Glutamine (7.267 gm), followed by 500mg/l of Spermidine (7.233 gm). On the other hand, the lowest weight of shoot formed was by explants grown on medium supplemented with 50 mg/l of Asparagine (4.133 gm), followed by control (4.00 gm).

Data of table (8) shows that there were a significant difference between different precursors and concentration.

Concerning the effect of concentration of each precursor added under test, it could be mentioned that increasing precursor level up to 500 mg/l increased shoot formation and weight.

Table 6: Effect of different amino acids that as precursors on shoot fresh weight (gm) of shooting stage of date palm (*Phoenix dactylifera L.*) Malakaby cultivar

Precursors (A)	**(B) Concentration (mg/l)**				**Mean**
	0.0	50	250	500	
Glutamine	4.00	6.133	7.00	7.267	6.100 A
Spermidine	4.00	4.167	5.167	7.233	5.142 A
Asparagine	4.00	4.133	4.167	5.967	4.567 A
Mean	4.00 B	4.811 AB	5.44 AB	6.822 A	

L.S.D at 0.05% A = 2.079 B = 2.400 AB =4.157

Effect of amino acid as precursors on the production of total steroids as secondary products in the shoot tissues of shooting stage of date palm.

Data of Table 7 and Figure 7 clearly shows that the different amino acids stimulate steroids biosynthesis processes in shoot tissues comparing with control in the most treatments.

Steroid biosynthesis positively correlated with increasing Glutamine concentration. The values recorded in this case were 0.157 mg/g (60.85% of control), 0.423 Mg/g (163.9 of control) and 0.534

Mg/g (206.0% of control) for 50, 250 and 500 mg/l Glutamine respectively. However, steroid biosynthesis in the shoot tissues was responding differently to Spermidine concentration, whereas it was decreased by increasing Spermidine level from 50mg/l to 500 mg/l which gave 0.393mg/g (152.3% of control), 0.273 Mg/g (105.8% of control) and 0.260 Mg/g (100.8% of control) respectively. Increasing Asparagine concentration from 50mg/l to 500 mg/l decreased steroid content in shoot tissues.

In general the best result, were in the usage of Glutamine at 500 mg/l which gave highest steroid biosynthesis content (0.534mg/g), 206.0% of control. The lowest (0.157 mg/g) was obtained with Glutamine 50 mg/l, 60.85% of control.

Table 7: Effect of amino acid as precursors on the production of total steroids as secondary products in the shoot tissues of shooting stage of date palm.

Type of precursors	Concentration (mg/l)	Total steroids	
		Mg/g	% of control
Control	0.0	0.258	100
Glutamine	50	0.157	60.85
	250	0.423	163.0
	500	0.534	206.0
Spermidine	50	0.393	152.3
	250	0.273	105.8
	500	0.260	100.8
Asparagine	50	0.390	151.2
	250	0.316	122.5
	500	0.285	110.5

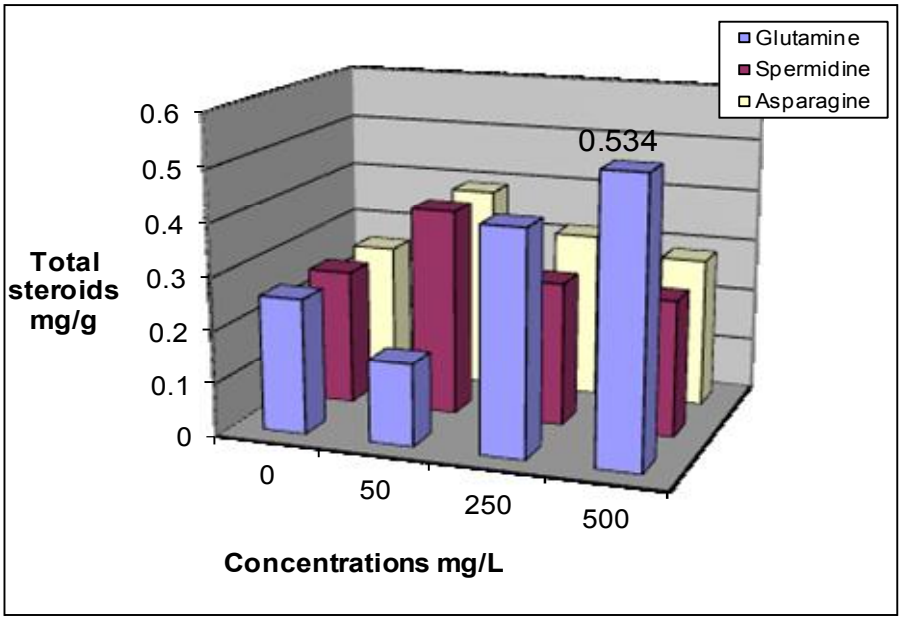

Figure 7: Effect of different precursors on the production of total steroids as secondary product in the shoot tissues of shooting stage of date palm (*Phoenix dactylifera L.*) Malakaby cultivar.

4. Discussion

Amino Acids are the essential components in the procedure of protein synthesis. A lot of studies have verified that amino acids can directly or indirectly control the physiological actions of the plant. Amino acids added to medium have been found to play a vital role on tissue culture techniques of certain species (Benson, 2000; Liu, 1993).

Amino acids have been applied in medium of numerous species as alfalfa, maize, sorghum, pineapple, rice and other monocots as a nitrogen supply in *in vitro* cultures to improve somatic

embryogenesis growth (Skokut et al., 1985; Claparols et al., 1993; Rao et al., 1995; Hamasaki et al., 2005; Grewel et al., 2006).

In Embryogenic callus stage there are a lot of causes that could lead to the increase or decrease in embryogenic callus volume and wasn't enclosed only in the amino acids that was added as precursors to examine growth rate of callus volume. For instance, Thomas and Rao (1985) found on oil palm that after several subcultures on MS medium with sequentially reduced 2.4-D the callus formed nodular callus masses which were maintained on a continuous source for the induction of embryogenesis.

Abou El-Nil (1989) reported various amino acids that resulted callus growth in plants and categorized them as follows: first Glutamine, second Asparagine, third Arginine, fourth Serine, fifth Glycine and sixth Alanine. Glutamine caused doubling callus growth compared to control.

El-Sharbasy in 2004 indicated that the highest value of callus volume in Sewi cultivars was achieved with callus grown on medium contained 0.01mg/l cholesterol, while lowest value was observed in case of adding 0.01mg/l of Squaline, and the highest callus weight was obtained when MS basal medium supplemented with 1 mg/l Squaline while the lowest was in 10 mg/l of Pyruvic acid.

Fermandes-Ferreira et al. (1991); Fermandes-Ferreira (1992) found that the sterols Compesterol, β-sitosterol, Avenasterol and the Triterpenal α-amyrin are the main constituents of the unsaponifiable fraction obtained from calli and suspended cells of *Euphorbia characias*. Squalene and trace amounts of hydrocarbons, namely nonacosane and hentriacontane, were also identified into the sunsaponifiable fractions obtained from these *in vitro* cultures.

El-Sharabasy 2004 indicated that the highest value of steroids diffused by the callus in the medium was Pyruvic acid in the concentration 10mg/l and recorded (609.1%) higher than control. While the lowest ones were those 0.01mg/l Pyruvic, 10mg/l Squalene and 0.01 mg/l Cholesterol.

El- Sharbasy 2004 indicated that the highest embryos weight was recorded with cholesterol treatment 0.1 mg/l and the lowest was that of pyruivic acid 10 mg/l.

Amino acids showed effects on the production of secondary metabolites, for instance, Walton in 1988 made experiments using transformed roots instead of cell suspensions, it showed that feeding Putrecine at 1-5 mM caused a 100% increase in the nicotine content of transformed roots of *Nicotiana rustica* compared to controls.

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