Effects of some precursors on development of secondary products in tissues and media of embryogenic callus of date palm cv. Sewi

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ABSTRACT

This work was conducted to study the effect of pyruvic acid, squalene and cholesterol on the growth and development of metabolic products in the tissues and media of embryonic callus of date palm (Phoenix dactylifera L.) cv Sewi. Different concentrations (0.0, 0.01, 0.1, and 10.0 mg/l) from pyrovic acid, squalene, and cholesterol as precursors were added to the media. The obtained results showed clearly that morphogenesis characters responded differently to the different precursors used in this study. Squalene was the most suitable for stimulating and increasing embryos number specially with the concentration of 0.01 mg/l, while cholesterol had more stimulating effect on embryos fresh weight and volume followed by pyrovic acid in case of embryos volume. Moreover, pyrovic acid was the most suitable for steroids formation in embryos cells specially with the concentration of 0.1 mg/l followed by cholesterol (0.01 mg/l).

Key words: *Steroids, cholesterol, squalene, pyruvic acid, secondary products, embryogenesis, date palm.*

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) plant is widely distributed in Egypt, West Asia and North Africa and extensively planted in the Arab countries and to some extent, in Southern Europe. It is used for nutritive and therapeutic purposes. Their pollen grains are utilized as antisterility agent (Ateya, 1975). Plants and some animal products are used in folklore medicine for treatment of several diseases e.g. hypertension, cardiac diseases, kidney disjunction and diabetes, ... etc. However, nothing could be traced concerning drugs which are used in the treatment of sterility except date palm pollen grains, which are known by the Egyptians and Arabs to be nutritive and are used as antisterility agent. Cholesterol and coprostanol are the animal sterols, while B-sitosterol, campestrol, stigmasterol, ergosterol and brassicasterol are the principal plant sterols (Bailey, 1964). Cholesterol one of the typical animal sterols has recently been found to be rather widely distributed among plants. So far, cholesterol has been identified in the pollens of many plants including the date palm (Bennett *et al.*, 1996) and oil palm (Slover *et al.*, 1983).

Plants possess solar-powered biochemical factors, which manufacture what they need to survive (both primary and

secondary metabolites) from air, water, minerals, and energy from sunlight. Many species of higher plants biosynthesize and accumulate extractable organic substances in quantities sufficient to be economically useful as chemical feed-stocks or as raw materials for various scientific, technological, and commercial applications. Natural substances are employed, either directly or indirectly, by a large number of industries as the pharmaceuticals, cosmetics, foods, agrochemicals, chemurgic industries, industrial oils, flavors and fragrances, resins (e.g. rosin and tall oil), gums, natural rubber, waxes, saponins and other surfactants, pesticides e.g., insecticides and rodenticides), and many special products (Balandrin and Klocke, 1988 and Bosila *et al,* 2001).

The present investigation was planned to study the effect of some precursors on the growth and development of secondary metabolites synthesis (steroids) from date palm embryogenic callus of *(Phoenix dactylifera* L.) cv Sewi through tissue culture

MATERIALS AND METHODS

This work was conducted in The Central Laboratory of Date Palm Research and Development, Agricultural Research Center, Ministry of Agriculture, Egypt during the years 2001 and 2002 to study the effect of pyrovic acid, squalene, and cholesterol on the growth and development of metabolic products in the tissues and media of embryogenic callus of date palm cv. Sewi.

The optimum embryonic callus mass of date palm (*Phoenix dactylifera* L.) was used as explants. The embryogenic calli were divided into small pieces and cultured on MS solid medium supplemented with 10 NAA + 3 mg/l 2ip and treated with different concentrations of pyrovic acid, squalene and cholesterol (0.0, 0.01, 0.1, 1.0, 10.0 mg/l). Ten groups of jars each containing 25-ml medium for each treatment were arranged. The pH value was adjusted to 5.7 - 5.8 prior to autoclaving. The treatments were incubated in growth chambers at 27± 2°C in complete darkness. The following data were recorded on the embryonic callus tissues and their media after one month:

1. Volume of embryonic callus as follows
   +=2= small value. ++=3= moderate value. +++=4= high value.
2. Embryonic callus weight (g/embryo).
   Total steroids were calculated and determined by the spectrophotometer according to the methods described by Pharco (1993).
3. Separation and identification of steroids and sterols compounds in the *in vitro* culture of date palm were determined by gas liquid chromatography (G.L.C.)

The steroid composition of date palm *in vitro* culture treated with some precursors namely squalene, pyruvic acid and cholesterol were identified by gas liquid chromatography (G.L.C.) analysis. The adapted samples were chosen on the basis of their total steroids as well as those of high total steroids content in the media that were considered as promising treatments and subjected to G.L.C. analysis. It should be noticed that the obtained chromatograms represent not only the steroids but also all the compounds in the unsaponifiable fraction in the extracts of each treatment.

Identification and determination of steroids composition

The steroids and sterols were analyzed by Gas Liquid Chromatography (PYE UNICAM PRO – GC).

The chromatograms were fitted with a capillary column OV 17 (Methyl phenyl silicone) 1.5 m x 4mm.

Unsaponifiable materials separation was done by (GLC).

Temperature programming

Initial: 70°C, upper: 270°C, Rate: 10 C/ min., Injector: 250°C (N_2) carrier, Detector: 300°C and (H_2) Flame Ionization (FID).

Flow rate of gases

N_2: 30 ml/min, H_2: 33 ml/min, Air: 330 ml/min and Chart speed: 0.50 cm/min.

The following parameters were recorded

a) Steroid composition produced in embryonic callus tissues.

b) Steroids and sterols composition diffused to the embryonic callus medium.

The completely randomized design was used and data were subjected to analysis of variance. Separation of means among treatments was determind using L.S.D test at 5% probability level according to Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

1- Number of embryos

The obtained results show clearly that the highest number of embryos was obtained from explants grown on MS medium containing squalene, while the lowest number was recorded for cholesterol, regardless of precursor concentrations. In this concern, MS solid medium supplemented with 0.01 mg/l of squalene seems to be the most suitable medium with respect of forming the highest number of embryos/explant in this study (Table1).

Table (1): Effects of different precursor concentrations on the number of embryos / explant of date palm (Phoenix dactylifera L.) cv .Sewi.

Treatment	Pyrovic acid	Squalene	Cholesterol	Mean
Control	2.67	2.67	2.67	2.67
0.01 mg/l	2.00	6.67	2.00	3.56
0.1 mg/l	2.67	5.00	2.00	3.22
1.0 mg/l	3.00	4.67	2.00	3.22
10.0 mg/l	2.00	3.67	3.33	3.00
Mean	2.47	4.53	2.40	

L.S.D (A) = 0.49 L.S.D (B) = 0.38 L.S.D (A×B) = 0.85
A= precursors B= concentrations

2- Fresh weight of embryonic callus

Data in Table (2) clearly show that the highest weight of embryonic callus was recorded on MS basal medium supplemented with 0.10 mg/l of cholesterol followed by 1.0 mg/l of cholesterol.

On the other hand, the lowest amount of embryonic callus was formed by explants grown on MS basal medium supplemented with 10.0 mg/l of squalene, with significant differences between precursors, concentrations and their interactions.

Table (2): Effects of different precursor concentrations on the fresh weight of embryos / explant of date palm (Phoenix dactylifera L.) cv .Sewi.

Treatment mg/l	Pyrovic acid	Squalene	Cholesterol	Mean
Control	0.83	0.83	0.83	0.83
0.01	0.83	0.70	0.94	0.83
0.10	0.96	0.84	1.24	1.01
1.0	0.91	0.63	1.01	0.85
10.0	0.80	0.61	0.83	0.75
Mean	0.87	0.72	0.97	
L.S.D (A) = 0.11	L.S.D (B) = 0.08	L.S.D (A×B) = 0.18		
A= precursors	B= concentrations			

3- Volume of embryonic callus

Data presented in Table (3) indicated that the largest volumes were achieved with embryonic callus grown on medium contained 0.01 mg/l of pyrovic acid or/and 0.1mg/l cholesterol. Increasing pyrovic acid or cholesterol levels significantly decreased embryonic callus volume. While, the smallest volume of embryonic callus was observed with that grown on medium contained 1.0 mg/l of pyrovic acid or 0.1 mg/l squalene followed by 10.0 mg/l squalene which had fluctuating effects. Statistical analysis of variance showed that the variations in callus volume between precursors, concentrations and their interactions were of significant value.

Table (3): Effects of different precursors concentrations on volume of embryos / explant of date palm (Phoenix dactylifera L.) cv .Sewi.

Treatment mg/l	Pyrovic acid	Squalene	Cholesterol	Mean
Control	3.67	3.67	3.67	3.67
0.01	4.00	3.00	3.67	3.56
0.1	2.67	2.33	4.00	3.00
1.0	2.33	3.00	3.67	3.00
10.0	3.00	2.67	3.33	3.00
Mean	3.13	2.93	3.67	
L.S.D (A) = 0.35	L.S.D (B) = 0.27	L.S.D (A×B) = 0.61		
A= precursors	B= concentrations			

4- Steroids biosynthesis in callus tissues and media

It is clear from the data of Table (4) that steroid formation had responded differently to the different precursors (pyruvic acid, squalene and cholesterol) levels used in this study. Steroid formation was of positive correlation responses with increasing precursor levels from 0.0 mg/l to 0.1 mg/l in case of pyrovic acid and squalene or 1.0 mg/l for cholesterol, reaching its maximum value with 0.1 mg/l pyrovic acid which stimulated steroid formation by about 937.7% of that of the control, followed by 1.0 and 0.1 mg/l cholesterol, respectively.

The obtained results show also that MS medium supplemented with 0.1 mg/l squalene stimulated the process of steroid formation in callus tissues and increased it by about 482.2% of that of the control comparing with 284.4%

for 0.01 mg/l squalene, 446.6% for 1.0 mg/l squalene or 417.7% of that of the control for 10.0 mg/l squalene. The recorded data indicated that using 0.10 mg/l pyrovic acid on MS medium seemed to be the best precursor used in this study to stimulate steroid formation in callus tissues resulting in 937.7% of that of the control (Table5).

Table (4): Effects of different precursors concentrations on steroids formation (mg/g) of date palm (Phoenix dactylifera L.) cv .Sewi callus tissues.

Treatment mg/l	Pyrovic acid	Squalene	Cholesterol	Mean
Control	0.150	0.150	0.150	0.150
0.01	0.130	0.427	0.113	0.223
0.1	1.407	0.723	1.230	1.120
1.0	0.733	0.670	1.353	0.919
10.0	0.597	0.627	0.793	0.672
Mean	0.603	0.519	0.728	
L.S.D (A) = 0.135	L.S.D (B) =0.105	L.S.D (A×B) = 0.234		
A= precursors	B= concentrations			

Table (5): Effects of different precursors concentrations on steroids formation (% of control) of date palm (Phoenix dactylifera L.) cv . Sewi callus tissues.

Treatment mg/l	Pyrovic acid	Squalene	Cholesterol	Mean
Control	100	100	100	100
0.01	86.6	284.4	75.5	148.83
0.1	937.7	482.2	820.0	746.63
1.0	488.8	446.6	902.2	612.53
10.0	397.7	417.7	528.8	448.07
Mean	402.16	346.18	485.3	
L.S.D (A) = 27.57	L.S.D (B) = 21.35	L.S.D (A×B) = 47.7		
A= precursors	B= concentrations			

Data of Tables (6 and 7) clearly show that the used precursors inhibited, in most cases steroid biosynthesis processes in embryonic callus media as compared to control.

Table (6): Effects of different precursors concentrations on steroids formation (mg/g) of date palm (Phoenix dactylifera L.) cv. Sewi embryonic callus media.

Treatment mg/l	Pyrovic acid	Squalene	Cholesterol	Mean
Control	0.08	0.08	0.08	0.08
0.01	0.19	0.01	0.02	0.08
0.1	0.04	0.02	0.02	0.02
1.0	0.03	0.06	0.1	0.06
10.0	0.03	0.04	0.01	0.03
Mean	0.08	0.04	0.05	
L.S.D (A) 0.01	L.S.D (B) = 0.01	L.S.D (A×B) = 0.02		
A= precursors	B= concentration			

Table (7): Effects of different precursors concentrations on steroids formation (% of control) of date palm (Phoenix dactylifera L.) cv . Sewi embryonic callus media.

Treatment mg/l	Pyrovic acid	Squalene	Cholesterol	Mean
Control	100	100	100	100
0.01	254.4	17.5	26.3	99.4
0.1	55.26	30.7	3.07	38.89
1.0	44.7	74.5	135.9	86.03
10.0	42.5	48.2	17.5	36.07
Mean	99.37	54.18	62.08	

L.S.D (A) = 27.57 L.S.D (B) = 21.35 L.S.D (A×B) = 47.75
A= precursors B= concentrations

Table (8): Effects of some precursors on the steroid composition (%) in embryogenic callus tissues and medium of date palm (Phoenix dactylifera L.) Sewi cultivar.

Compound	RT	Tissues of Embryogenic callus				Medium of Embryogenic callus			
		Control	TE	PTE	TE	Control	SME	PME	CME
Cholesterol	15.08	1.14	2.30	0.17	0.05	-	-	-	0.68
Oestrone	19.8	-	2.78	88.23	-	-	-	-	-
Ethylene estradiol	21.3	2.92	-	-	-	-	14.56	0.123	73.23
Ethistrone	22.8	41.75	28.10	-	-	89.52	-	-	-
Ostriol	23.18	1.15	-	-	-	1.51	0.04	0.05	1.18
Stigmasterol	24.28	0.52	0.17	-	-	0.90	-	0.06	15.95
B-sitosterol	27.08	0.13	-	-	0.25	-	0.34	0.12	-

RT = Retintion Time.
STE = embryogenic callus tissues treated with 1.0 mg/l squalene.
PTE = embryogenic callus tissues treated with 0.01 mg/l pyravic acid.
CTE = embryogenic callus tissues treated with 1.0 mg/l cholesterol.
SME = embryogenic callus medium treated with 0.1 mg/l squalene .
PME = embryogenic callus medium treated with 0.1 mg/l pyrovic acid.
CME = embryogenic callus medium treated with 1.0 mg/l cholesterol.

Date presented in Table (8) show the biotransformation and biosynthesis of steroids in date palm embryogenic callus cultures under the effect of squalene, pyruvic acid and cholesterol precursors. Squalene at the rate of 1.0 mg/l stimulated the biosynthesis of the steroid formation by 2.30% cholesterol, 2.78% oestrone, 28.10% ethistrone and 0.17% stigmasterol. However, pyruvic acid treatment at the rate of 0.01 mg/l led to the formation of 0.17% cholesterol and 88.23% oestrone. Moreover, the identified compounds under the effect of cholesterol at the rate of 1.0 mg/l were cholesterol (0.05%) and B- sitosterol (0.25 %).

The data in Table (8) show that ethylene estradiol is the major steroid formed and diffused from the embryogenic callus to the growing medium regardless of the precursor used. In this concern, squalene 0.1 mg/l treatment formed in the medium 14.56% ethylene estradiol, 0.04% ostriol and 0.34% B-sitosterol. While pyruvic acid 0.1 mg/l treatment formed in the medium 0.123% ethylene estradiol, 0.05% ostriol, 0.06% stigma sterol and 0.12% B-sitosterol.

However, cholesterol 1.0 mg/l treatment had the highest effect on steroid formation in the embryonic callus medium resulting in 0.68 % cholesterol, 73.23 % ethylenestradiol , 1.18 % ostriol and 15.95 % B- sitosterol.

DISCUSSION

Tissues, which had differentiated roots, produced flavor intensities approaching that of fresh onion, although there were important qualitative differences particularly in respect of lachrymatory potency. Undifferentiated tissues, on the other hand, contained only very small amounts of flavor components owing to the absence of precursors rather than of the enzyme alliance (Freeman *et al.,* 1974). Precursor level in callus was only 2-10 % of that found in the intact plant. In undifferentiated callus, S-methyl-L-cysteine sulphoxide was present at a low concentration, while the major precursor of onion flavor, S-Trans-prop-l-enyl-L-cysteine was absent altogether (Selby *et al.,* 1979). But, in undifferentiated roots and shoots, this precursor was present again. Both crushed roots and shoots had the characteristic odor of onion, however, only crushed roots showed the lachrymatory effect (Turnbull *et al.,* 1981).

Addition of intermediates to the culture medium showed that the callus was capable of the final stages in the synthesis of S-Trans-prop-1-enyl-L-cysteine sulphoxide (Selby *et al.*, 1979). It has been frequently found that the increased production of onion aromas (monocotyledons) is chiefly attributed to the presence of roots in tissue cultures of *Allium cepa* (Freeman *et al.*, 1974; Fridborg 1971; Turnbull *et al.*, 1981).

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الملخص العربي

تأثيرات بعض البادئات على نمو وتكوين المنتجات الثانوية (الإستيرويدات) في الأنسجة والبيئة الغذائية للكالس الجنيني لنخيل البلح صنف السيوى باستخدام تقنيات زراعة الأنسجة

شريف فتحى الشرباصى
المعمل المركزى للأبحاث وتطوير نخيل البلح ــ مركز البحوث الزراعية
وزارة الزراعة ــ الجيزة ، ج . م . ع .

أجـرى هـذا البحـث في المعمل المركزى للأبحاث وتطوير نخيل البلح ــ مركز البحوث الزراعية بالجيزة ، لدراسة تأثير حمض البيروفـيك والأسـكوالين والكوليسترول على نمو وتكوين المنتجات الثانوية الناتجة من الأنسجة والبيئة الغذائية للكالس الجنيني لنخيل البلح صـنف سـيوى. حيـث اسـتخدمت التركـيزات المخـتلفة (صـفر ، ٠,٠١ ، ٠,١ ، ١ و ١٠ ملليجرام / لتر) من حمض البيروفيك والإسكوالين والكوليسترول كبادئات تضاف للبيئة الغذائية .

وتوضـح النـتائج المتحصل عليها أن الصفات المورفولوجية كانت مختلفة الاستجابة تبعا لاختلاف البادئ المستخدم في الدراسة . حيـث كان الإسكوالين أكثر تأثيراً بالنسبة لتكوين وزيادة عدد الأجنة خاصة عند تركيز ٠,٠١ ملليجرام/ لتر ، بينما كان الكوليسترول أكثر تأثـيراً واسـتجابة بالنسـبة للـوزن الطازج للأجنة ، لكن حمض البيروفيك كان أكثر تأثيرا على حجم الأجنة. بالإضافة لذلك فإن حمض البيروفـيك كان أكثرهم فاعلية على إنتاج الأستيرويدات في خلايا الأجنة خاصة عند معدل ٠,١ ملليجرام / لتر ويليه الكوليسترول بتركيز ٠,٠١ ملليجرام/ لتر .