J. of Plant Production, Vol. 1 (2): 319 - 332, 2010

ENHANCED SILYMARIN ACCUMULATION AS INFLUENCE OF MEDIUM COMPOSITION IN CELL SUSPENSION CULTURES OF *Silybum marianum* (L.) GAERTN.

Rabie, Kawthar A. E. ; Mona S. Abd El-Aal and H.H. Manaf
Agric. Botany Dept., Fac. of Agric., Ain Shams Univ., Shoubra El-Keima, Cairo, Egypt.

ABSTRACT

Silymarin production was examined in cell suspension cultures derived from hypocotyl of *Silybum marianum* (L.) Gaertn. MS medium supplemented with different concentrations of sucrose, glucose, KH_2PO_4, KH_2PO_4 + K_2SO_4, NH_4NO_3, $CaCl_2$, L-Phenylalanine, L-Tyrosine and yeast extract was used. Tyrosine 10 mg/l, 0.5 KH_2PO_4 + K_2SO_4 con., 0.75 NH_4NO_3 con., 0.75 KH_2PO_4 + K_2SO_4 con., glucose 30 g/l, 0.5 $CaCl_2.2H_2O$, tyrosine 20 mg/l and yeast extract 40 mg/l (3 days incubation period) showed the highest production of silymarin in callus cells to reach 79%, 57.5%, 46%, 24.2%, 21.2%, 19.7%, 9.7% and 8.6% over the control respectively. The same trend was obtained by HPLC (qualitative assay).
Keywords: *Silybum marianum* – Silymarin – Cell suspension cultures.

INTRODUCTION

The milk thistle Silybum marianum (L.) Gaertn is among the most ancient of all known herbal medicines. Various preparations of the plant, especially the fruits, have been used medicinally for over 2000 years, mainly for treatment of liver disorders (Morazzoni and Bombardelli, 1995). Silymarin is the pharmacological active principle of the fruit and is a constitutive natural compound which accumulates in the fruits of the milk thistle *Silybum marianum*, and is composed of an isomeric mixture of the flavonolignans silychristin, isosilychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B (Morazzoni and Bombardelli, 1995; Kim *et al.*, 2003; Lee and Liu, 2003). It has been shown that flavonolignans inhibit leucotriene production; this inhibition explains their anti-inflammatory and antifibrotic activity (Dehmlow *et al* 1996). Also, silymarin may be beneficial for reducing the chances for developing certain cancers (Katiyar *et al* 1997) and inhibit cholesterol biosynthesis (Krecman *et al* 1998).

There are several means for increasing the production of secondary metabolites by plant cell suspensioncultures. These are: 1- use of biotic or abiotic elicitors that could stimulate the metabolic pathways as in the intact plant; 2- addition of a precursor of the desired compound in the culture medium with a view to increasing its production or inducing changes in the flux of carbon to favour the expression of pathways leading to the compound(s) sought, i.e. alteration of secondary metabolism pathways of the control (Sasson 1991).

Tissue cultures derived from this species are able to produce silymarin, but to a lesser extent than that accumulated in the fruit (Alikaridis *et al* 2000). In the previous work with *S. marianum* cell cultures, growth and flavonolignan production were tested under different concentrations of the major components of the culture medium, only removal of calcium ions promoted silymarin accumulation (Cacho *et al* 1999). Elicitor treatment promotes secondary metabolite production (Sanchez-Sampedro *et al* 2005b).

The objectives of this work are to produce the natural medical products by means of plant cell suspension cultures under the optimum conditions, cell multiplication could be easily to yield their specific metabolites. Automated control of cell growth and rational regulation of metabolic processes would contribute to the reduction of labor cost and the improvement of productivity.

MATERIALS AND METHODS

Plant material

Achenes of *Silybum marianum* were obtained from National Research Center, Dokki, Egypt. Callus was formed from hypocotyl segments (5mm) as previously described (Manaf *et al.* 2009).

Cell suspension culture was established from half gram of callus, which initiated from hypocotyl on MS medium with growth regulators (BA 0.1 mg/l + NAA 1.0 mg/l) for 30 day and after three subcultures on the solid medium.

Media

Liquid MS basal medium supplemented with plant growth regulators (BA 0.1 mg/l and NAA 1.0 mg/l) was used. Different trals were made for increasing active ingredient (silymarin) from callus cells of silybum plant using suspension culture techniqe, these are:

1. Test different concentrations of the major components of the culture medium
    a. Sucrose 30 (control) 35, 40 and 45g/l.
    b. Glucose 30g /l.
    c. Half (0.085g/l) and three-quarter (0.1275g/l) concentration of potassium phosphate.
    d. Half (0.085g/l) and three-quarter (0.1275g/l) concentration of potassium phosphate supplemented with potassium sulfate for compensation potassium cation (0.05445g/l and 0.02723g/l respectively).
    e. Half (0.76228g/l) and three-quarter (1.78228g/l) concentration of amonium nitrate.
    f. Zero (0g/l), half (0.22g/l) and three-quarter (0.33g/l) concentration of calcium chloride.
2. Addition of a precursor compund in the culture medium
    a. L-Phenylalanine 10, 20, 30 and 40 mg/l.
    b. L-Tyrosine 10, 20, 30 and 40 mg/l.
3. Use of biotic elicitor

Yeast extract 40, 45, 50, 55 and 60 mg/l (done 3 days after transfer and incubated for 3 and 28 days).

Media were adjusted to pH 5.7, then dispensed into flasks (250 ml containing 50 ml of the testing media. Culture media were autoclaved at 121°C and 1.1 kg/cm^2 for 20 min.

Culture conditions

Callus were incubated under light condition for 16h/day photoperiod at intensity of 6000 Lux from white fluorescent lamps at 25ºC $\pm$ 1 in orbital platform shaker. The experiment was conducted in plant tissue culture laboratory, Agric. Bot. Dept., Fac. Of Agric., Ain Shams Univ. Callus growth was measured as fresh and dry weights after 28 days from culturing the callus and the growth index of the cultures was determined as:

$$\frac{\text{Final FW} - \text{initial FW}}{\text{Initial FW}}$$

In addition, silymarin (the main active ingredient) content in callus cells was determined. The experiment was conducted in a complete randomized design with three replicates. The obtained results were subjected to statistical analysis of variance according to method described by Snedecor and Cochran (1980). The statistical analysis of data was done by (SAS 1996) computer program, and means were compared by LSD method**.**

Flavonolignan extraction

The culture medium was separated from the biomass by filtration and flavonolignans were extracted three times with two volumes of ethylacetate. The combined extracts were dried at 40°C and re-suspended in 1ml methanol. The cells yield was freeze-dried; 0.1 g of the sample was extracted with methanol at 80°C for 4 h. The extracts were dried and re-dissolved in 1ml of methanol (Sánchez-Sampedro *et al*., 2005a).

Determination by UV spectrophotometer

The corresponding absorbance was measured on spectrophotometer (Shimadzu UV-160) at 288 nm.

HPLC assay of silymarin

The HPLC column was a Hewlett Packard (hp 1100) ODS reversed phase 4.6 ×200 mm. The mobile phase was a mixture of 34 volumes of methanol and 66 volumes of acetic acid: water (5:55, v/v) at 1ml min−1 and detection at 288 nm.

RESULTS AND DISCUSION

1. Effect of culture medium composition

Data illustrated in table (1) showed the effect of culture medium composition on callus growth and silymarin content in callus cells. There are significant differences in callus fresh weight and growth index between the control and different treatments. The change in calcium amount of MS medium almost gave the same effect on callus fresh weight and growth index. The treatment which has 0.5 phosphate concentration from KH_2PO_4 + K_2SO_4 achieved the highest callus frresh and dry weights followed by half strength of the total nitrogen in MS medium. Meanwhile, the highest concentration of sucrose (45g/l) recorded the lowest callus fresh weight and growth index. The sucrose (35g/l) treatment and all calcium chlorid treatments showed nearly the same effect on callus dry weight. On the other hand, the lowest callus dry weight was recorded by the treatment which contains 30g/l glucose.

Regarding silmarin content, it is obvious that different concentrations of sucrose (30, 35 and 40g/l) have the same effect on silymarin content except the highest concentration (45g/l) which achived the lowest level of significance in silymarin content (nearly half the control). The result showed that the treatments which have 0.5 phosphate concentration from KH_2PO_4 + K_2SO_4**,** 0.75 nitrogen concentration, 0.75 phosphate concentration from KH_2PO_4 + K_2SO_4, glucose 30g/l and CaCl2 half concentration recorded the highest silymarin content to reach 57.5%, 46%, 24.2%, 21.2% and 19.6% respectively over the control. On the other hand, the lowest level of silymarin (about half the control) was detected by using 0.75 phosphate concentration from KH_2PO_4 or sucrose 45g/l without any significant difference between the two treatments.

Table (1): Effect of different concentrations of the major components of culture medium on callus growth(fresh and dry weights (g), as well as growth index) and silymarin content (µg/g) d.w. cells.

Treatments	Conc. g/l	F. w. (g)	D. w. (g)	Growth index	Silymarin µg/g
Sucrose (Control)	30	4.66 g	0.314 d	8.32 g	25.68 d
Glucose	0	2.98 l	0.160 i	4.95 l	31.13 b
Sucrose	35	3.15 k	0.264 gh	5.30 k	25.62 d
	40	3.88 h	0.294 ef	6.76 h	25.56 d
	45	2.80 m	0.342 c	4.60 m	13.74 g
KH_2PO_4	0.1275	3.46 j	0.260 h	5.93 j	12.46 g
	0.085	3.79 i	0.280 fg	6.57 i	21.33 e
KH_2PO_4 + K_2SO_4	0.1275 + 0.2723	5.40 d	0.356 c	9.80 d	31.89 b
	0.085 + 0.05445	7.26 a	0.488 a	13.53 a	40.46 a
NH_4NO_3	1.7823	4.99 f	0.310 de	8.98 f	37.50 a
	0.7623	6.54 b	0.280 b	12.08 b	26.94 cd
$CaCl_2.2H_2O$	0.33	5.18 e	0.268 gh	9.36 e	18.04 ef
	0.22	5.17 e	0.264 gh	9.33 e	30.73 bc
	0.00	5.81 c	0.266 gh	10.63 c	14.68 fg
	LSD(5%)	0.0674	0.0185	0.0337	4.0252

-Values are means of 3 replicates.

In this respect, Mizukami *et al.* (1991) found that the anthocyanin content in callus cultures of roselle (*Hibiscus sabdariffa* L.) was the highest with 12-30 mM nitrogen and decreased significantly with increasing nitogen or phosphate levels while the cell growth increased, the highest cell growth was obtained when 80 mM potassium nitrate was added to the medium as sole nitrogen source. Therefore, the normal phosphate concentration in LS medium was found to be super optimal for the anthocyanin formation and suboptimal for the cell growth in *Hibiscus sabdariffa*. They reported that anthocyanin was little affected by changes of $CaCl_2$ concentration except that it was slightly inhibited by extremely high concentration (10 times higher than the normal level) of $CaCl_2$. Also the optimal carbon source was sucrose in 3% concentration for both growth and anthocyanin production callus cultures of roselle (*Hibiscus sabdariffa* L.).

Also Hippolyte *et al.* (1992) reported that an increment of sucrose concentration (5%) increased rosmarinic acid production by cell suspension cultures of sage *(Salvia officinalis* L.) and can be increased 10-fold to attain 6.4 g/l under optimal conditions. Indrayanto *et al*. (1993) found that increasing Ca ions concentration from 1.5 to 6 mM can slightly increase the phytosteroids content in callus cultures of Agave amaniensis but further increases up to 12mM tend to decrease it. Depletion of Ca ion in media increased the formation of hecogenin (108 times). The addition of 2 and 3 mM of ethylene glycol tetraacetic acid (EGTA) increased significantly the hecogenin content (4 and 2 times) and the addition of verapamil reduced the hecogenin content. Zhong *et al*. (1994) showed that the cell cultures of *Perilla frutescens*, growing on LS medium, released more anthocyanin with 40 to 50 g sucrose/l compared with the control of 30 g sucrose/l of medium. Ilieva and Pavlov (1997) showed that 7% sucrose in the nutrient medium ensured a steady growth of *Lavandula vera* MM cell suspension culture and increased the yield of rosmarinic acid. Cacho *et al.* (1999) reported that the removal of calcium ions from silybum marianum medium promoted flavonolignan accumulation, although under this condition, growth was significantly reduced. Ilieva and Pavlov (1999) found that the cultivation of *Lavandula vera* in cell suspension with 0.09 g ammonium ions/l (1/4 of standard medium) ensured intensive growth (16 g dry biomass/l) and enhanced biosynthesis of (rosmarinic acid) RA (15 mg/g dry biomass). Cultivation in a medium with 1/2-fold concentration of nitrate ions led to accumulation of 11 mg RA/g dry biomass which was twice as much as in the standard LS medium. Kim *et al*. (1999) found that sucrose among tested carbon sources increased the camptothecin production in suspension cultures of *Camptotheca acuminata*. Nakao *et al.* (1999) reported that the elevated levels of calcium chloride in the culture medium played an important role in activating the accumulation of flavanols and induced an increase in flavanol contents of the Polygonum hydropiper cells. Xu *et al.* (1999) reported that sucrose has positive effects on salidroside production from *Rhodiola sachalinensis*; the highest content was in medium containing 40 g/l sucrose. Sudha and Ravishankar (2002) studied the production of capsaicin by cell suspension cultures of *Capsicum frutescens* mediated through the calcium channel. Administration of the calcium ionophore A23187 resulted in a 1.43-fold enhancement of the total capsaicin production in the cell suspension cultures. Treatments wherein the calcium channel modulators verapamil and chlorpromazine were administered resulted in lower growth and capsaicin production, suggesting that calcium is involved in the signal transduction of capsaicin pathway in the suspension culture. Vanisree *et al.* (2004), mentioned that 6% sucrose was found to be optimum for the growth of *Dioscorea doryophora* cell suspension culture, Although cells cultured in a 3% sucrose medium produced more diosgenin. They also reported that glucose was to be a better carbon source than sucrose and fructose for increasing the production of imperator in *Angelica dahurica* cell suspension culture.

While Kim *et al*. (2005) investigated that saponin production in the root culture of *Panax ginseng* with various concentrations of nitrogen were ranged from 127.6-1148 mg/l. The optimal concentration of nitrogen on root growth and saponin production were 382.7 mg/l, also found the optimal concentrations of phosphate on root growth and saponin production were 40 mg/l. They noticed that the optimal concentration of sucrose on root growth and saponin production in the root culture was 30 g/l. Sánchez-Sampedro *et al.* (2005a) treated the cell cultures of Silybum marianum (L.) Gaertn with calcium ionophore A23187. The specific Ca^{2+}chelator, EGTA, enhanced the silymarin content in cells by 200% and its secretion by 3–4 times. The inorganic ion La+3, as well as the calcium channel inhibitor verapamil, also stimulated production. Several reagents known to block intracellular calcium movement, such as ruthenium red, thapsigargin and TMB-8 appreciably increased silymarin accumulation. These results suggest that inhibition of external and internal calcium fluxes plays a significant role in flavonolignan metabolism of S. marianum cell cultures. Sujanya *et al.* (2008) studied the effect of reducing phosphate concentrations from ¾ (0.937 mM), ½ (0.625 mM), ¼ (0.3125 mM) to complete reduction of potassium dihydrogen phosphate on biomass content and azadirachtin production in cell suspensions of *Azadirachta indica*. Total phosphate reduction raised intracellular azadirachtin production. They also showed the effect of variations in total nitrogen availability in the medium in terms of different ratios of nitrate: ammonium. that the ratio 4:1 revealed a profound effect, leading to a 1.5-fold increase in the total extra cellular azadirachtin production (5.59 mg/l) in cell suspensions of *Azadirachta indica* over the standard MS medium. Also they reported that reduction in sucrose (15mg/l) in the medium exhibited a reduction in biomass and absence of azadirachtin.

2. Effect of addation a precursor compound in the culture medium

The results for the effect of addition a precursor compound in the culture medium on callus growth and silymarin content in callus cells were presented in table (2).

Table (2): The fresh and dry weights (g), growth index and silymarin content (µg/g) d.w. cells under treatment by various concentration of phenylalanine and tyrosine as precursor.

Treatments	Conc. mg/l	F. w. (g)	D. w. (g)	Growth index	Silymarin µg/g
control	0.0	4.66 e	0.314 d	8.32 f	25.68 bc
Phenylalanine	10	8.32 a	0.496 a	15.63 a	9.65 e
	20	5.89 b	0.315 d	10.78 b	19.22 cd
	30	5.56 c	0.342 b	10.12 c	13.76 de
	40	4.95 d	0.282 e	8.91 d	8.52 e
Tyrosine	10	4.93 d	0.326 c	8.86 e	46.00 a
	20	2.50 f	0.154 f	4.00 g	28.17 b
	30	1.01 g	0.126 g	1.02 h	22.66 bc
	40	0.51 h	0.078 h	0.02 I	25.04 bc
	LSD(5%)	0.0328	0.0033	0.0292	6.4575

-Values are means of 3 replicates.

It is obvious that all phenylalanine concentrations and tyrosine 10 mg/l increased significantly callus fresh weight and growth index over the control and the reverse was true for the rest of tyrosine concentrations. Meanwhile, phenyalanine treatments with 10 & 30 mg/l and tyrosine 10 mg/l led to significant increament in callus dry weight as compared to the control. On the other hand, phenyalanine treatment at 20 mg/l gave the same effect as the control on the callus dry weight, while the rest of treatments decreased significantly callus dry weight than the control. Tyrosine treatments at 10 and 20 mg/l acheived signicant increase in silymarin content than the control. Meanwhile, there are no significant differences in silymarin content between tyrosine treatments at 30 & 40 mg/l and the control. On the other wise, all phenyalanine treatments decreased significantly silymarin content than the control.

In this respect Dhandapaani *et al.* (1977) reported that incubation of callus tissue of *Trigonella foenugraecum* with L-phenylalanine led to the incorporation of the labeled amino acid into P- caumaric acid, caffeic acid and ferulic acid. El Bahr and Shang (1990) found that phenylalanine increased the total alkaloid content on *Datura sp*. Sicha *et al.* (1991) reported that phenylalanine increased the production of cinnamic and caffeic acid up to 2.5 mmol in the callus culture of *Echinacea purpurea* and *Echinacea pallida* plants derived from seeds. Fett-Neto *et al.* (1994) found that feeding phenylalanine to the callus cultures of *Taxus cuspidata* increased the taxol yield and suggested that this is due to the involvement of these amino acids as a precursor for the N-benzoylphenylalanine side chain of taxol. They reported that the greatest increases in taxol accumulation are observed in the presence of various concentrations of phenylalanine (1mM for callus; 0.05, 0.1 and 0.2 mM for their suspensions). Ju and Byun (1994) found that tyrosine feeding without any treatment resulted in no significant increase of alkaloid production in suspension cultures of *Eschscholtzia californica*. Shushma *et al.* (1994) reported the effect of phenylalanine on the biosynthesis of bergapton in callus of *Ammi majus* seedling. They found that coumarin production significantly increased for 20 days when 100, 1000 or 10000 mg/l L-phenylalanine was added to the medium. Refaat and Naguib (1998) found that amino acid application improved the yield and oil quality of *Mentha piperita*. L-tyrosine at 25 ppm produced oil yields higher than the control and increased the total carbohydrates percentage in the leaves. Pavlov and Ilieva (1999) reported that adding phenylalanine to *Lavandula vera* MM cell suspension culture (0.1- 0.5 g/l) enhanced accumulation of caffeic acid in parallel with rosmarinic acid. When 0.3 g/l phenylalanine was added, the yield of rosmarinic and caffeic acids reached 87 mg/l and 60 mg/l respectively, compared with 68 mg/l and 4 mg/l in controls (without phenylalanine). Manaf (2004) found that the addition of 20.0 mg/l tyrosine to cell suspension culture of *Echinacea purpurea* showed increasing in both of total caffeic acid derivatives and carbohydrates content. On the other hand, the addition of 10.0 mg/l tyrosine showed increasing in alkamides content. Vanisree *et al.* (2004) reported that supplementation of an amino acid precursor such as tyrosine to the *Corydalis yanhusuo* culture medium may further improve the production of corydaline and tetrahydropalmatine.

3. Effect of addation biotic elicitor (yeast extract) in the culture medium

The result for the effect of addition yeast extract after three and twenty eight days incubation period in the culture medium on callus growth and silymarin content in callus cells were presented in table (3).

Table (3): The fresh and dry weights (g), growth index and silymarin content (µg/g) d.w. cells under treatment by various concentration of yeast extract after 3 and 28 day incubation period.

Treatment	Conc. mg/l	F. w. (g)	D. w. (g)	Growth index	Silymarin µg/g
3 days incubation period	0.0	0.56 d	0.024 d	0.12 e	22.25 b
	40	0.74 a	0.028 c	0.48 a	24.16 a
	45	0.71 ab	0.030 bc	0.42 b	22.29 b
	50	0.68 bc	0.032 b	0.36 c	20.87 c
	55	0.65 c	0.036 a	0.30 d	15.67 d
	60	0.54 d	0.020 e	0.10 f	15.27 d
	LSD	0.036	0.004	0.018	0.490
28 days incubation period	0.0	4.66 f	0.314 c	8.32 f	25.68 a
	40	11.79 a	0.760 a	22.58 a	10.15 c
	45	11.20 b	0.740 a	21.04 b	13.29 b
	50	10.66 c	0.730 a	20.31 c	11.70 bc
	55	10.35 d	0.580 b	19.69 d	10.31 c
	60	9.51 e	0.590 b	18.02 e	9.58 c
	LSD(5%)	0.148	0.032	0.018	2.486

-Values are means of 3 replicates.

Three days incubation period:

The callus fresh weight and growth index increased significantly than the control with decreasing yeast extract concentration from 55 to 40 mg/l. The reverse was true for callus dry weight. On the other hand, the concentration of 60 mg/l led to decrease callus growth than the control. Regarding silymarin content, the treatment with 40 mg/l recorded the highest silymarin content. Meantime, the concentrations of 50, 55 and 60 mg/l reduced significantly silymarin content than the control while, silymarin content was equal to the control by 45 mg/l application.

Twenty eight days incubation period:

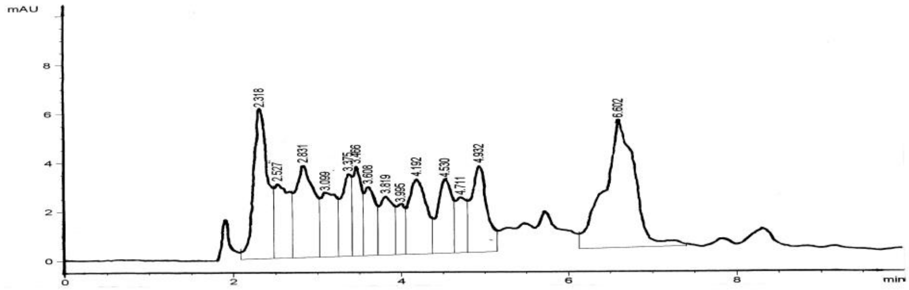
Significant increment was achieved in callus growth than the control with decreasing yeast extract concentration. On the other hand, there is no significant difference in callus dry weight between 40, 45 and 50 mg/l concentrations. As well, the same effect was detected in callus dry weight by 55 and 60 mg/l concentrations.

As for silymarin content, all yeast extract concentrations after 28 days incubation period led to significant decrement in silymarin content than the control reached about half or less as compared to the contorl. On the other hand, there is no significant difference in silymarin content between 40, 55 and 60 mg/l concentrations.

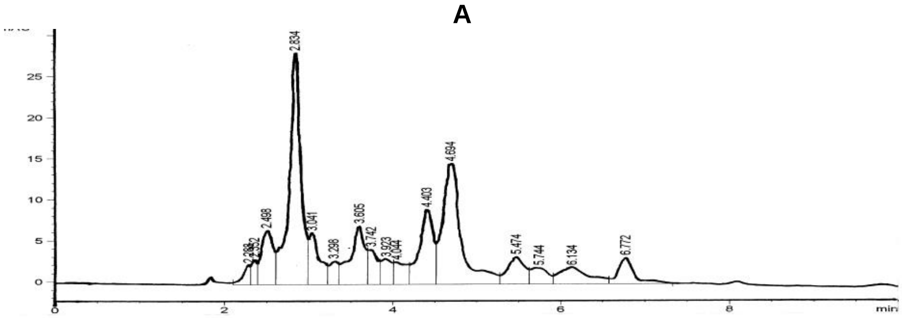
In this respect, Kim *et al* (2001) found that the addition of yeast extract preparation at 50 *µ*g ml^{-1} to *Agastache rugosa* cell suspension elevated the rosmarinic acid content up to 5.7-fold of that found in nonelicited suspension cells. Lu *et al* (2001) found that yeast extract significantly improved saponin production. The highest additive level of the seven ginsenosides tested was 2.07% (dry weight basis), which was 28-fold higher than that in the control. The optimum time to add either elicitor was found to be on the day of inoculation. The addition of either elicitor did not show as significant an influence on cell growth as on saponin production from *Panax ginseng* cell suspensions. Sánchez-Sampedro *et al* (2005b) reported the effect of yeast extract on the production of silymarin in *Silybum marianum* cultures. Only yeast extract stimulated production in both cells and the culture medium at the non-toxic concentrations. A slight increase was observed in the silymarin content in the biomass, in no case higher than 50%, and three-fold higher levels of products accumulated in the medium. Shams-Ardakani *et al* (2005) studied the possibility of enhancment accumulation of podophyllotoxin (PTOX) in cell suspension of *Linum album* by yeast extract elicitors for 24 or 48 hr in MS medium. Yeast extract (0.8 mg/ml) had a little effect after 48 hours. Krajewska-Patan *et al* (2007) found that the elicitation did not result in significant increase of the content of active compounds for callus cultures from *Salvia milthiorrhiza*. The increase of the tanshinones content in trace amount was noticed in callus cultured on solid medium. The decrease of rosmarinic acid content in elicited tissues was noticed on solid medium. Baldi and Dixit (2008) reported that the addition of yeast extract elicitor has slightly enhanced artemisinin levels by cell cultures of *Artemisia annua*. Cho *et al* (2008) found that benzophenanthridine alkaloid accumulation induced to mg/l (2.5 times) by yeast extract 0.2 g/l in *Eschscholtzia californica* suspension cultures and sanguinarine (5.5 times).

It is obvious from the prior results that tyrosine 10 mg/l, KH_2PO_4 0.085 g/l + K_2SO_4 0.5445 g/l, NH_4NO_3 0.7623 g/l, KH_2PO_4 0.1275 g/l + K_2SO_4 0.2723 g/l, glucose 30 g/l, $CaCl_2.2H_2O$ 0.22 g/l, tyrosine 20 mg/l and yeast extract 40 mg/l (3 days incubation period) showed the highest production of silymarin in callus cells to reach 79%, 57.5%, 46%, 24.2%, 21.2%, 19.7%, 9.7% and 8.6% over the control respectively.

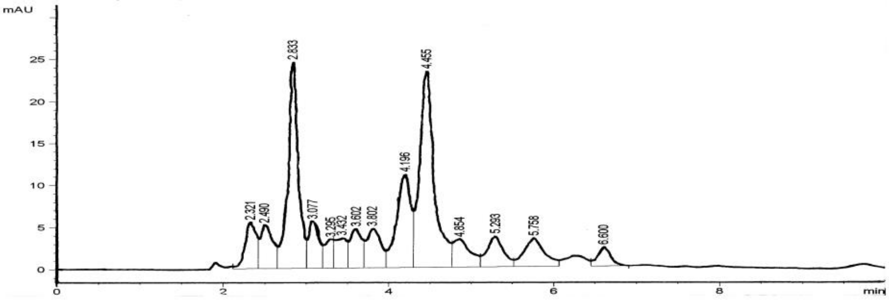
The same trend was obtained by HPLC (qualitative assay). For example the treatment 10 mg/l tyrosine (which has 1102.67 from total peak area) achieved the highest silymarin content which recorded 79% over the control (which has 531.16 from total peak area) followed by KH_2PO_4 0.085 g/l + K_2SO_4 0.5445 g/l, NH_4NO_3 0.7623 g/l & $CaCl_2.2H_2O$ 0.22g/l (which have 1074.17, 978.23 and 787.23 from total peak area respectively) (Fig. 1).


mAU
8
6
4
2
0
2.318
2.527
2.831
3.099
3.375
3.466
3.606
3.819
3.995
4.192
4.530
4.711
4.932
6.602
0
2
4
6
8
min

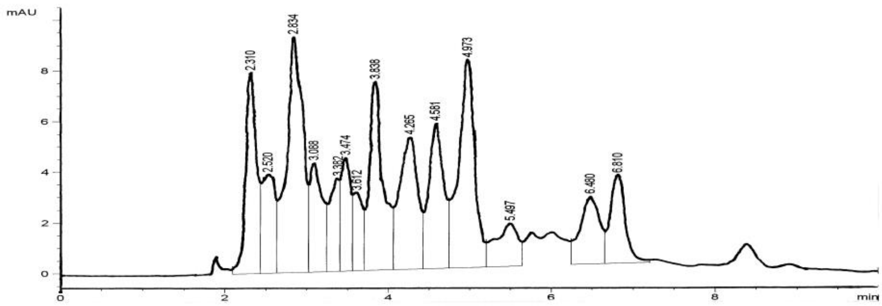
A


25
20
15
10
5
0
2.834
2.498
3.041
3.298
3.605
3.742
3.923
4.044
4.403
4.694
5.474
5.744
6.134
6.772
0
2
4
6
8
min

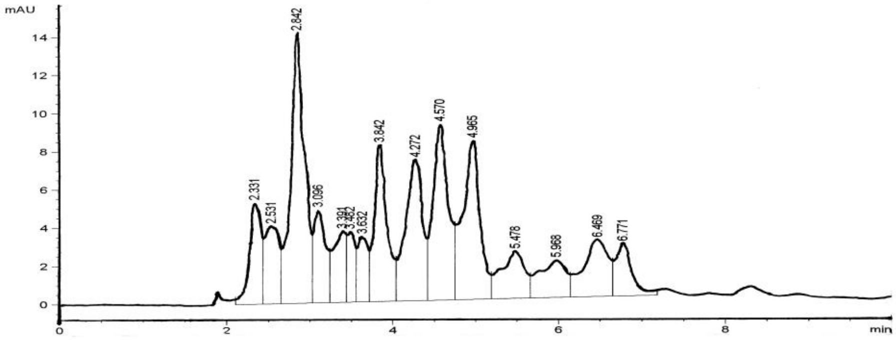
B


mAU
25
20
15
10
5
0
2.321
2.490
2.833
3.077
3.295
3.432
3.602
3.802
4.196
4.455
4.854
5.293
5.758
6.600
0
2
4
6
8
min

C


mAU
8
6
4
2
0
2.310
2.520
2.834
3.088
3.382
3.474
3.612
3.838
4.265
4.581
4.973
5.497
6.480
6.810
0
2
4
6
8
min

D



E

Fig.(1): HPLC analysis of silymarin in sample of cells treated by
A: Control B: Tyrosine10 mg/l C: 0.5 KH_2PO_4 + K_2SO_4
D: 0.75 NH_4NO_3 E: 0.5 $CaCl_2.2H_2O$

REFERENCES

Alikaridis, F.; D. Papadakis; K. Pantelia and T. Kephalas (2000). Flavonolignan production from *Silybum marianum* transformed and untransformed root cultures. Fitoterapia. 71: 379-384.

Baldi, A. and V.K. Dixit (2008). Enhanced artemisinin production by cell cultures of *Artemisia annua.* Current Trends in Biotech. and Pharmacy, 2(2): 341-348.

Cacho, M.; M. Morán; P. Corchete and J. Fernández-Tárrago (1999). Influence of medium composition on the accumulation of flavonolignans in cultured cells of *Silybum marianum* (L.) Gaertn. Plant Sci. 144: 63-68.

Cho, H.Y.; S.Y. Son; H.S. Rhee; S.Y.H. Yoon; C.W.T. Lee-Parsons and J.M. Park (2008). Synergistic effects of sequential treatment with methyl jasmonate, salicylic acid and yeast extract on benzophenanthridine alkaloid accumulation and protein expression in *Eschscholtzia californica* suspension cultures. J. Biotech. 135: 117-122.

Dehmlow, C.; J. Erhard and H. Groot (1996). Inhibition of kupffer cell functions as an explanation for the hepatoprotective properties of silibinin. Hepatology. 23: 749-754.

Dhandapani, M.; A. Antony and P.V.S. Rao (1977). Biosynthesis of phenolic compounds in hypocotyl callus cultures of fenugreek (*Trigonella foenumgreacum L.*). Ind. J. Exper. Bio. 15(3):204-207.

El-Bahr, C.S. and F.Y. Shang (1990). Ethylene, the gaseous plant hormone. Biosciences. 34(4): 217.

Fett-Neto, A.G.; S.J. Melanson; S.A. Nicholson; J.J. Pennington and F. Dicosmo (1994). Improved taxol yield by aromatic carboxylic acid and amino acid feeding to cell cultures of *Taxus cuspidate*. Biotech. and Bioeng. 44(8): 967-971.

Hippolyte, I.; B. Marin; J.C. Baccou and R. Jonard (1992) Growth and rosmarinic acid production in cell suspension cultures of *Salvia offcinalis* L. Plant Cell Reports, 11: 109-112.

Ilieva, M. and A. Pavlov (1997). Rosmarinic acid production by *Lavandula vera* MM cell-suspension culture. Appl Microbiol. Biotech. 47: 683-688.

Ilieva, M. and A. Pavlov (1999). Rosmarinic acid production by *Lavandula vera* MM cell-suspension culture: nitrogen effect. World J. Microbiol. and Biotech. 15: 711-714.

Indrayanto, G.; L. Rahayu; A. Rahman and P.E. Noeraeni (1993). Effect of calcium, strontium, and magnesium ions on the formation of phytosteroids in callus cultures of *Agave amaniensis*. Planta Med. 59: 97-98.

Ju, Y.W. and S.Y. Byun (1994). Precursor feeding experiments with elicitation in suspension cultures of *Eschscholtzia californica*. Plant Tissue Culture. 11(2): 112-115

Katiyar, S.K.; N.J. Korman; H. Mukthar and R. Agrawal (1997). Protective effects of silymarin against photocarcinogenesis in a mouse skin model. J. Natl. Cancer Inst. 89: 556–566.

Kim, H.K.; S.R. Oh; H.K. Lee and H. Huh (2001) Benzothiadiazole enhances the elicitation of rosmarinic acid production in a suspension culture of *Agastache rugosa* O. Kuntze. Biotech. 23: 55-60.

Kim, J.H.; E.J. Chang and H. Oh (2005). Saponin production in submerged adventitious root culture of *Panax ginseng* as affected by culture conditions and elicitors. Asia Pacific J. Molecular Biol. and Biotech. 13(2): 87-91.

Kim, N.C.; T.N. Graf; C.M. Sparacino; M.C. Wani and M.E. Wall (2003). Complete isolation and characterization of silybins and isosilybins from milk thistle (*Silybum marianum*). Org. Biomol. Chem. 1, 1684–1689.

Kim, S.H.; B.K. Hur and S.Y. Byun (1999). Effect of sugar concentration on camptothecin production in cell suspension cultures of *Camptotheca acuminate*. Biotech. Bioprocess Eng. 4: 277-280.

Krajewska –Patan, A.; M. Dreger; M. Górska–Paukszta; A. Mścisz; S. Mielcarek; M. Baraniak; W. Buchwald; R. Marecik; W. Grajek and P.M. Mrozikiewicz (2007). *Salvia milthiorrhiza* Bunge *in vitro* cultivation in callus cultures. Herba Polonica. 53(4): 88-96.

Krecman, V.; N. Skottova; D. Walterova; J. Ulrichova and V. Simanek (1998). Silymarin inhibits the development of diet-induced hypercholesterolemia in rats. Planta Medica, 64(2): 138-142.

Lee, D.Y.W. and Y.Z.J. Liu (2003). Molecular structure and stereochemistry of silybin A, silybin B, isosilybin A, and isosilybin B, isolated from *Silybum marianum* (milk thistle). Nat. Prod. 66, 1171–1174.

Lu, M.B.; H.L. Wong and W.L. Teng (2001). Effects of elicitation on the production of saponin in cell culture of *Panax ginseng*. Plant Cell Reports, 20: 674-677.

Manaf, H.H.A. (2004). Botanical Studies of *Echinacea Purpurea* in Relation to Production of Active Ingredients. Ph.D. Thesis. Faculty of Agriculture, Ain Shams University.

Manaf, H.H.; Kawthar A.E. Rabie and Mona S. Abd El-Aal (2009). *In vito* callus formation and plant regeneration of *Silybum marianum* (L.) Gaertn. Annals of Agric. Sci., 54(2): 283-289.

Mizukami, H.; M. Nakamura; K. Tomita; K.Higuchi and H. Ohashi (1991). Effects of macronutrients on anthocyanin production in roselle (*Hibiscus sabdariffa* L.) callus cultures. Plant Tissue Culture, 8(1): 14-20.

Morazzoni, P. and E. Bombardelli (1995). *Silybum marianum* (*Carduus marianus*). Fitoterapia. 66: 3-42.

Murashige, T. and F.A. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Nakao, M.; K. Ono and S. Takio (1999). The effect of calcium on flavanol production in cell suspension cultures of *Polygonum hydropiper*. Plant Cell Reports, 18: 759–763.

Pavlov, A. and M. Ilieva (1999). The influence of phenylalanine on accumulation of rosmarinic acid by *Lavandula vera* MM cell culture. World J. Microbio. and Biotech. 15: 397-399.

Refaat, A.M. and N.Y. Naguib (1998). Peppermint yield and oil quality as affected by application of some amino acids. Bull. Fac. Agric., Cairo Univ. 49(1): 89-98.

Sánchez-Sampedro, M.A.; J. Fernández-Tárrago and P. Corchete (2005a). Enhanced Silymarin accumulation is related to calcium deprivation in cell suspension cultures of *Silybum marianum* (L.) Gaertn. J. Plant Physiol. 162: 1177-1182.

Sánchez-Sampedro, M.A.; J. Fernández-Tárrago and P. Corchete (2005b). Yeast extract and methyl jasmonate-induced silymarin production in cell cultures of *Silybum marianum* (L.) Gaertn. J. Biotech. 119: 60–69.

SAS (1996). Statistical analysis system, SAS User's Guide: Statistics. SAS Institute Inc., Editor, Cary, NC.

Sasson, A. (1991). Production of useful biochemicals by higher-plant cell cultures: biotechnological and economic aspects. *Options Méditerranéennes* - Série Séminaires - no 14 - 1991: 59-74.

Shams-Ardakani, M.; S. Hemmati and A. Mohagheghzadeh (2005). Effect of elicitors on the enhancement of podophyllotoxin biosynthesis in suspension cultures of *Linum album.* DARU 13(2): 56-60.

Shushma, K.; A. Ashok and S. Carewal (1994). Growth and alkaloidal production in suspension cultures of *Hyoscyamus muticus* as influenced by various cultural parameters. J. Med. Plant Res. 47: 11-16.

Sicha, J.; H. Becker; J. Hbik; T. Siatka and I. Hrones (1991). Callus cultures of the genus Echinacea. II. Effect of phenylalanine on the growth of cultures and the production of cinnamic acid. Pharmazie. 46 (5): 363-364.

Snedecor, G.W. and W.G. Cochran (1980). Statistical Analysis Methods. 6th Ed. Iowa State Univ. Press. Ames., Iowa, USA.

Sudha, G. and G. A. Ravishankar (2002). Influence of calcium channel modulators in capsaicin production by cell suspension cultures of *Capsicum frutescens* Mill. Current Sci. 83 (4): 480-484.

Sujanya, S.; B.P. Devi and I. Sai (2008). *In vitro* production of azadirachtin from cell suspension cultures of *Azadirachta indica*. J. of Biosci. 33(1): 113- 120.

Vanisree, M.; C. Lee; S. Lo; S.M. Nalawade; C.Y. Lin and H. Tsay (2004). Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. Bot. Bull. of Acad. Sinica, 45: 1-22.

Xu, J.F.; P.Q. Ying; A.M. Han and Z.G. Su (1999). Enhanced salidroside production in liquid-cultivated compact callus aggregates of *Rhodiola sachalinensis* manipulation of plant growth regulators and sucrose. Plant Cell, Tissue and Organ Culture. 55: 53-58.

Zhong, J.J.; G.R. Xu and T. Yoshida (1994). Effects of initial sucrose concentration on excretion of anthocyanin pigments in suspended cultures of *Perilla frutescens* cells. World J. Microbio. and Biotech. 10(5): 590-592.

تأثير تركيب البيئة على حث تراكم السيلمارين فى معلقات خلايا الحرشف البرى

كوثر على إمام ربيع ، منى شعبان عبد العال و حسام حسن مناف
قسم النبات الزراعى - كلية الزراعة – جامعة عين شمس

تم إختبار السيلمارين فى مزارع المعلقات الخلوية الناتجة من السويقة الجنينية السفلى لنبات الحرشف البرى. وقد أستخدمت بيئة MS المضاف اليها عدة تركيزات من السكروز والجلوكوز وفوسفات البوتاسيوم وفوسفات البوتاسيوم + كبريتات البوتاسيوم ونترات الأمونيوم وكلوريد الكالسيوم والفينيل الانين والتيروزين ومستخلص الخميرة. وقد سجل التيروزين ١٠ملجم/لتر و وفوسفات البوتاسيوم ٠,٠٨٥ جم/لتر+ كبريتات البوتاسيوم ٠,٥٤٤٥ جم/لتر ونترات الامونيوم ٠,٧٦٢٣ جم/لتر وفوسفات البوتاسيوم ٠,١٢٧٥ جم/لتر+ كبريتات البوتاسيوم ٠,٢٧٢٣ جم/لتر والجلوكوز٣٠ جم/لتر وكلوريد الكالسيوم ٠,٢٢ جم/لتر والتيروزين ٢٠ملجم/لتر ومستخلص الخميرة ٤٠ ملجم/لتر (ثلاثة ايام فترة تحضين) أعلى أنتاج للسيلمارين فى خلايا الكالس وصل الى ٧٩% , ٥٧,٥ %, ٤٦%, ٢٤,٢%, ٢١,٢% , ١٩,٧%, ٩,٧%, ٨,٦% على التوالى مقارنة بالكنترول. وتحقق ذلك نوعياً بإختبار HPLC.

قام بتحكيم البحث

أ.د / محب طه صقر	**كلية الزراعة – جامعة المنصورة**
أ.د / محمد عبد الرسول محمد	**كلية الزراعة – جامعة عين شمس**