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Effect of *Spirulina Platensis* Extract on Growth, Phenolic Compounds and Antioxidant Activities of *Sisymbrium Irio* Callus and Cell Suspension Cultures

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Abstract: Several trials for induction of Sisymbrium irio L. callus were carried out on Murashige and Skoog (MS) medium supplemented with different concentrations of auxins and kinetin. Maintenance of calli on MS medium treated with Spirulina aqueous extract (SAE) as a source of vitamins and phytohormones was achieved. Growth and growth parameters of calli revealed that an obvious extension in the exponential phase with variable enhancement of the growth rate by 1 to 4 times more than irrespective control. Doubling time (t_d) and the relative growth rate (RGR) of callus were also estimated at different treatments. Antioxidant capacities of calli grown on MS medium supplemented with different SAE were enhanced up to 12 times more than control calli. The previous results were coincidentally with an obvious accumulation of both flavonoids and total phenolic compounds in cultures treated with SAE. The complementary between the previous results manifested the longevity and viability of calli on modified MS medium. Antioxidant activity also confirmed the different results. Successful suspension cultures were established using MS medium without vitamins and phytohormones and supplied with 18% SAE. Growth parameters, total flavonoids, phenolic compounds and antioxidant activity were highly increased compared with the untreated cultures.

Key words: *Sisymbriu Irio, Spirulina Platensis ,phenolic compounds , antioxidant activities, callus cultures, NAA, Kinetin,murashige and skoog.*

INTRODUCTION

Plants are major source of natural products, many of which can be used as pharmaceuticals, agrochemicals, flavor and fragrance ingredients, food additives, and pesticides (Mulabagal and Tsay, 2004). Since many of these products (secondary metabolites) are obtained by direct extraction from plants grown in natural habitat, several factors can alter their yield. *In vitro* cell cultures have been developed as promising alternative for agricultural processes in producing valuable phytochemicals (Nath and Buragohain, 2005; Langhansova *et al*., 2005; Srivastava and Srivastava, 2007). The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products.

The seeds of *Sisymbrium irio* L. are expectorant and restorative and are used externally as stimulating poultice (Kirtikar & Basu, 1984). The leaves exhibited antibacterial action against both Gram-positive, Gram-negative organisms and fungi. (Al-Gendy, 2008). Moreover, the extract of the seeds exhibits antipyretic, analgesic and antibacterial effects (Vohora *et al*., 1980).

Spirulina platensis, a blue-green alga is gaining worldwide popularity as a food supplement (Belay, 1997), being one of the most nutritious, concentrated foods known to man. It is gaining worldwide popularity as a food supplement. It has been shown to be an excellent source of proteins (Cohen, 1997; Colla *et al*., 2007), polyunsaturated fatty acids (Sajilata, 2008), pigments (Rangel-Yagui *et al*., 2004; Madhyastha and Vatsala, 2007), vitamins and phenolics (Colla *et al*., 2007; Ogbonda *et al*., 2007). Moreover, the antioxidant properties of *S. platensis* and its extracts have attracted the attention of researchers (Estrada, 2001).

In this regards our aims are to exchange the external plant growth hormones and the vitamins in plant tissue culture media by natural algal extract of *Spirulina platensis*.

Plant Material:

Fresh plant of *Sisymbrium irio* L. (London rocket), Brassicaceae, was collected during the flowering stage in April 2005 from Western Desert near Alexandria-North Coast Road, Egypt. The plant was kindly identified by Prof. Dr. H.A.Hussein, Botany Dept., Faculty of Science, Zagazig University. Voucher samples were kept

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Preparation of Tissue Culture Media:

Murashige & Skoog medium (Murashige and Skoog, 1962) was supplemented with different combinations of auxin; 2,4-dichlorophenoxy acetic acid (2,4-D) or α-naphthalene acetic acid (NAA) at 0.5, 1 or 2 mg/l and kinetin (kn) at 0.1, 0.5 or 1 mg/l. The pH of the medium was adjusted to 5.8 ± 0.1. For preparation of solid medium, 8g/l Bacto-agar was added. The medium was dispersed in 100 ml screw capped glass jars; 30 ml per each and autoclaved at 121ºC for 20 min.

Surface Sterilization of the Seeds:

Seeds were surface sterilized by immersion in 70 % (v/v) ethanol for 0.5-1min, followed by soaking in 30 % (v/v) aqueous H_2O_2 solution containing 1 % (v/v) Tween 80 for 3-7 min with frequent shaking. Seeds were washed 3 times with sterilized distilled water, distributed on the surface of a sterile 0.8 % (w/v) Bacto-agar without medium under aseptic conditions and incubated at 25 ± 2 ºC in the dark.

Induction and Maintenance of Callus Cultures:

0.5-1 cm long pieces of hypocotyls or cotyledonary leaves were placed on the surface of sterilized MS medium containing different combinations of the phytohormones as mentioned above. All cultures were incubated at 25 ± 2 ºC in the dark. Sufficient calli were transferred to fresh medium every 4 weeks.

Cultivation Condition and Algal Mass Production:

Erlenmeyer flasks 2 L, containing 1.8 L of nutritive medium of Zarrouk (Zarrouk, 1966) were used. The bioreactor inoculated by *Spirulina platensis* with initial biomass concentration of 0.16 g/l. Aeration was conducted using air pump according to Costa *et al*., (2002). The bioreactor was incubated at 30 ˚C under illumination with 40 watt day light type fluorescent lamps (2500 lux) with 12h light /dark photoperiod according to Vonshak *et al*., (1982) for 21 days. At the end of incubation periods, the algal cells were aseptically separated and washed 3 times with distilled water to remove the salts from the algal surface then dried at 50 ˚C. The dried tissue was powdered and stored in plastic container till used.

Extraction and Identification of Both Vitamins and Phytohormones of S. Platensis

Vitamins:

Ten grams of tissue fresh weight were homogenized with methanol for extraction of water soluble vitamins while acetone–chloroform (30:70 v/v) was used for extraction of fat soluble vitamins (Qian & Sheng, 1998). The mixtures were shaken on a vortex mixer for 5 min, centrifuged at 4000 rpm for 5 min and filtered through a Millipore filter (45 µm). The filtrates were evaporated under nitrogen and the residues were re-dissolved in 1ml water for water soluble vitamins and in 1 ml butanol for fat soluble vitamins. Analysis of vitamins was performed using HPLC (table 1).

Table 1: Conditions of HPLC analysis of vitamins and phytohormones of *S. platensis*

Condition	Vitamins	Phytohormones	Jasmonic acid& MEJA
Model	HP 1050		
Column	C18 (150mmx4.6mmx5µm)		RP-18 (250mmx4mmx5µm)
Mobile phase	-Acetic acid: water (6:94v/v) for water soluble vitamins. -Methanol: water (98:2v/v) for fat soluble vitamins (in 0.1 M acetic acid)	-30 % methanol with 0.1 M H_3PO_4) for GA_3 -55 % methanol for ABA -35 % methanol (in 1 % acetic acid) for IAA and -40 % methanol (adjusted to pH 3.5 with 0.1 M H_3PO_4) for cytokinin	-55 % methanol (in 0.1% acetic acid) (adjusted to pH 3.0
Detector	UV detector		
Detector wave length	-220nm (fat soluble vit.) -254nm (water soluble vit.)	-208nm (GA_3) -265nm (ABA) -280nm (IAA) -245nm (Kn)	-254nm
Flow rate	1ml/min		
Injection volume	10µl		

Phytohormones:

Ten grams of fresh tissue per sample were homogenized with 70 % methanol and stirred overnight at 4 ˚C. The extract was filtered and evaporated under vacuum to give an aqueous residue. The aqueous phase was adjusted to pH 8.5 with a 0.1 M phosphate buffer and partitioned three times with ethyl acetate (basic fraction). The aqueous phase was adjusted to pH 2.5 with 1N HC1 and partitioned three times with ethyl acetate. The basic and acidic ethyl acetate fractions were passed through anhydrous sodium sulphate and evaporated under vacuum. The dried residues containing hormones were dissolved in methanol and stored in vials at 4˚°C. The acidic ethyl acetate-soluble fraction was used for determination of acidic hormones such as indole acetic acid (IAA), abscisic acid (ABA), gibrellin (GA_3) and jasmonic acid (JA), whereas the basic fraction was used for determination of cytokinin (e.g. benzyl adenine BA) and methyl jasmonate (MeJA) (Shindy and Smith, 1975; Chen, 1990). HPLC analysis was carried out according to Guinn and Brummett, (1990); Baydar and Ulger, (1998) and Wasternack and Parthier, (1997).

HPLC Analysis:

Conditions of HPLC analysis is mentioned in table 1. Peak identification was performed by comparing the retention times with pure standards (Sigma-Aldrich, Deisenhofer-Germany) and the concentrations were calculated from integrated areas of sample and the corresponding standards.

Preparation of Spirulina aquous extract (SAE):

Air dried *Spirulina platensis* cells (5 g) were powdered under cooling conditions in sterile dist. water. The slurry was filtered through 50 mesh polyester rope and centrifuged at 5000 rpm for 10min. The clear supernatant was taken and made up to 100 ml then, sterilized through Millipore membrane 0.25µ.This sterilized *Spirulina* aqueous extract (SAE) represents the stock sources for vitamins and phytohormones. It was added to the following treatments at 2 % - 10 % (v/v) for the treatment (B) and 2 % - 20 % (v/v) for the third and fourth treatments (C & D) under sterile condition as follow:

A-medium: MS medium supplemented with 1 mg/l 2,4-D and 0.5 mg/l Kn (control).
B-medium: MS medium prepared with different concentrations of SAE instead of vitamins and supplemented with 1 mg/l 2,4-D and 0.5 mg/l Kn.
C-medium: MS medium prepared with different concentrations of SAE instead of phytohormones.
D-medium: MS medium prepared with different concentrations of SAE instead of vitamins and phytohormones.

Establishment of Cell Suspension Culture:

Establishment of cell suspension cultures was carried out using friable callus at stationary phase obtained from each of solid D-medium supplemented with 18% (v/v) SAE and A medium without SAE. Each fraction was transferred to 50ml liquid medium of the same composition in 250 ml Erlenmeyer flasks. Cultures were kept on an orbital shaker at 110 rpm in the dark at 25 ± 2 ºC and subcultures were performed every two weeks.

Growth Parameters in Callus and Cell Suspension Cultures:

Dry weight (CDW) (oven dried calli at 60 º C) and growth index (GI) were carried out according to (Dobberstein and Staba, 1969). Growth of established callus cultures was determined as a relative growth rate (RGR) after 28 days subculture period (Singer, 1986).

$$GI = \frac{final\,CDW - initial\,CDW}{initial\,CDW}$$

$$RGR = \frac{3(W_1^{1/3} - W_0^{1/3})}{(t_1 - t_0)}$$

Where **W_2= final CDW (at t_1), W_0= initial CDW (at t_0), t_1-t_0 = 28 days.**

$$t_d = \frac{t \log 2}{\log \frac{w_1}{w_0}}$$

Where t_d = D.W. doubling time, t=unit time(28 days), W_1=CDW at time t,W_0=CDW at time zero:

The growth curves of the calli grown on the previous treatments were carried out according to the method adopted by Dobberstein and Staba (1969). Growth of the cell lines was recorded along the incubation period. Equal amounts of calli were transferred to fresh media and the samples were collected to measure the fresh weight (CFW) and dry weight (CDW) every 3 days. Growth curve was carried out for suspension growing on medium D supplemented with 18% v/v SAE**.** Packed cell volume (PCV) was also measured for the suspension. The mean values of 5 readings were plotted against time to get growth curves (Fig. 1-4)

Antioxidant Activity:

The antioxidant activity was estimated following DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging described by Sandoval *et al*., (2002) with some modifications. Fresh calli or suspension cells (1 g) were homogenized with 5 ml methanol, centrifuged at 10,000 rpm for 10 min and the supernatant was completed to 10 ml. Methanol extract of the calli (0.5 ml) and 0.5 ml methanol solution of DPPH (1000µM) were mixed. The decrease in absorbance at 515 nm was determined after 1 hr. The antioxidant activity of the extract against the DPPH radicals was expressed as follow:

% inhibition = $[(A_{initial} - A_{final})/A_{initial}] \times 100$, where $A_{initial}$ was the absorbance at time 0 min, and A_{final} was the absorbance of the sample at time 1 hr. All samples were analyzed 3 times.

Determination of Total Phenolic Content:

Total phenolic compounds were colorimetrically estimated using Folin-Denis reagent according to Strycharz and Shetty, (2002) with some modification. Methanol extract of the calli or suspension cells (prepared as described above) was mixed with 1 ml of 95 % ethanol, 5 ml of distilled water and 0.5 ml of 50 % (v/v) Folin-Denis reagent. The extract was replaced by 1 ml methanol for the blank. After 5 min, 1 ml of 5 % (w/v) Na_2Co_3 was mixed with samples and tubes were incubated in the dark for 10 min. Absorbance was measured at 725 nm. The total phenolic content was given based on the standard curve which was prepared using standard gallic acid at 0-20mg/ml.

Quantitative Determination of Total Flavonoids of Callus and Suspension Cultures:

Ethanol extracts (0.5 ml) were mixed with 1.5 ml of 95 % ethanol (v/v), followed by 0.1 ml of 10 % aluminum chloride (w/v), 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. Aluminium chloride was substituted by the same volume of distilled water in blank. The flavonoid content was calculated using a standard calibration curve of rutin solution and expressed as milligrams of rutin equivalent per gram dry weight of sample. The calibration curve of rutin was done by using standard rutin solutions (0-10 µg/ml) in 80 % ethanol (v/v) and treated as extracts. (Hung and Morita, 2008; Kosalec *et al*., 2004).

Biochemical Analysis of Callus and Cell Suspension Cultures:

Activities of Catalase, Polyphenol Oxidase and Peroxidase:

Extraction: (Kar & Mishra, 1976)

Callus fresh weight (0.1 g) was homogenized in cold phosphate buffer (0.05 M at pH 6.5). The homogenate was centrifuged at 10,000 rpm for 10 min. The supernatant was made up to 10 ml and used as enzyme source. The protein content in the enzyme sources was estimated according to Lowry *et al*., (1951) as follow:

Reagents:

(1) Alkaline sodium carbonate solution: (2%w/v in 0.1M Na OH)
(2) Copper sulphate-sodium potassium tartarate solution: 5g $CuSO_4.5H_2O$ dissolved in potassium sodium tartarate (10 g/l).
(3) Alkaline solution was prepared by mixing 50 ml of reagent No.1 with 1 ml of reagent No.2.
(4) Folin-phenol reagent: Commercial Folin reagent diluted with an equal volume of water just before use.

Five ml of alkaline solution were added to 1 ml of the extract. After 10 min, 0.5 ml of diluted Folin reagent was added, left to stand 20 min, then the optical density was determined at 750 nm. A standard curve of bovine albumin solution in concentrations of 20-200 mg/ml was plotted.

Assay of Catalase Activity (CAT) (Turkan et al., 2005):

The assay mixture (5 ml) comprised 300 µM of phosphate buffer (pH 6.8), 100 µM of hydrogen peroxide and 1 ml of the enzyme extract. After incubation at 25ºC for 5 min, the reaction was stopped by the addition of 10 ml H_2SO_4 (2% v/v). The residual H_2O_2 was titrated against 0.01 N $KMnO_4$ until a faint purple color persisted for at least 15 seconds. A control was run at the same time, in which the enzyme activity was stopped at zero time. Catalase activity was expressed as µ mol H_2O_2 destroyed/ mg protein/ min.

Assay of Polyphenol Oxidase Activity (PPO) (Beyer and Fridovich, 1987):

The assay mixture (5 ml) containing 125 µM of phosphate buffer (pH 6.8), 100 µM of pyrogallol and 1 ml of crude enzyme extract was prepared. After incubation at 25 ºC for 5 min, the reaction was stopped by the addition of 1ml H_2SO_4 (10% v/v). The color intensity was measured at 430 nm and the enzyme activity was expressed as the change in the optical density/ mg protein/ ml

Assay of Peroxidase Activity (POX) (Racusen and Foote, 1965):

The assay mixture (5 ml) contained 300 µM of phosphate buffer (pH 6.8), 50 µM catechol, 50 µM H_2O_2 and 1 ml of crude enzyme extract. After incubation at 25 ºC for 5 min, the reaction was stopped by the addition of 1 ml 10% H_2SO_4 (v/v). The color intensity was read at 430 nm and the enzyme activity was expressed as the change in the optical density/ mg protein/ min.

RESULTS AND DISCUSSION

Induction of Sisymbrium Irio Callus:

For induction of *Sisymbrium irio* callus, different explants were cultured on MS medium supplemented with auxin (2,4-D or NAA) in combination with Kn at different concentrations table (2) & plate(1). The results revealed that, the seed and hypocotyl explants were more responsive than the cotyledonary leaves. This may suggest that levels of endogenous phytohormones or their sensitivity might vary between organs. Explants usually require auxins and cytokinins in their culture medium (Gurel *et al*., 2001; Perez-Frances, *et al*., 1995; Nowak and Miczyński, 2002; Gang *et al*., 2003). Although growth regulators may help to induce direct regeneration, cells in some plants appear to pass towards the formation of adventitious roots so the ratios between auxins and cytokinin should be estimated (Stickens *et al*., 1996). In a similar manner 2,4-D is the most effective synthetic auxin for promoting callus with inhibiting shoot formation (Ali and Hasnain, 2007). Moreover, NAA in combination with Kn gave compact callus with hairy root formation on the surface of the culture as small white lumps. For the previous results all cultures which contained NAA were discontinued

Maintenance of Sisymbrium Irio Callus:

Results in table (3) & plate (2) revealed that the calli produced from seeds retained high proliferation rate for 2 subcultures. Afterwards, in the 3rd and 4th subcultures the callus grew slower then turned brown and didn't survive in subsequent subcultures irrespective to the phytohormones concentration. Such retardation may be related to the oxidation of phenolic compounds (Chen and Wang, 1995; Ji *et al*., 1998). In the same line Arnaldos *et al*., (2001) and Laukkanen *et al*., (1999) found that, oxidized phenolic substances generally induced a suppressive effect *in vitro* proliferation due to their inhibitory effects on some essential enzyme activity. Also results in table (3) indicate that the optimal combination of phytohormones for maintenance and growth of hypocotyls derived callus was 1 mg/l 2,4-D with 0.5 mg/l Kn. However, 2 mg/l 2, 4-D plus 0.5 mg/l Kn provided the suitable condition for callus initiation. These results may be attributed to the supra optimum concentration of auxin that retard the growth (Meijer *et al*., 1999). In a similar situation, Stickens *et al*., (1996) indicated that auxin, cytokinin ratios represent important signals in the formation of cell phenotype. Also Skoog and Miller, (1957) investigated that the regulation of organogenesis *in vitro* related to auxins and cytokinin in culture media.

Growth of Callus Cultures under the Effect of Spirulina Aqueous Extract (Sae) as a Source of Vitamins and Phytohormones:

Data represented in Figs (1 and 2) & plate (3) revealed that the growth yields of calli treated with SAE as source of vitamins (Fig. 1) or hormones (Fig 2) were higher than that of the control culture (untreated culture) where most of the previous concentration of SAE used exerted a maximum promotive effect on the growth as well as the corresponding parameters (RGR=0.037) with least doubling time (t_d=11.8) in MS with 8% SAE as vitamin sources and both parameters reached (RGR =0.034 with t_d=12.65) when the medium

supplemented with 20% SAE as phytohormones (table 4 and 5) & plate (4). Moreover, the omission of both vitamins and phytohormones from MS medium and its compensation with different concentrations of SAE affected the obtained growth parameters table (6), fig. (3) &plate(5). The previous results were in concomitant with Amin and Omar, (2002) who found that cyanobacterial exudates of *Nostoc piscinale* and *Anabaena fertillissima* as biotic natural source of vitamins and phytohormones led to maximum fresh and dry weight of callus tissue as the medium under investigation prepared without the biotic source. The stimulatory responses of *S. irio* calli may be related to that *S. platensis* itself is a super supplement source for vitamins and phytohormones. The previous results may be explained on the basis that SAE has accumulative effect because of its multivitamins which included β-carotene, Inositol as the most dominant fraction of vitamins, whereas, riboflavine, thiamine, vitamin E and ascorbic acid are more or less indicated with lower concentrations table(7) & plate(6). Moreover, HPLC profile revealed the presence of several phytohormones including indol acetic acid and cytotokinin besides gibbrilic, abscisic and jasmonic acids table (7)&plate(6). The ratio between IAA and BA was 8 to 1. This ratio is the detrimental factor that maintains the callus and it plays a role in the promotive pattern of it (Stickens *et al*., 1996; Liu *et al*., 1997).

Table 2: Induction of *Sisymbrium irio* callus on MS medium supplemented with different concentrations of 2,4-D and Kn in the dark.

Phytohormones (mg/l)		Explant	Date of callus appearance	Color	Consistency	Growth
2,4-D	Kn2					
2.0	1.0	Hypocotyl	5th week	Pale yellow	Heterogeneous	***
		Seed	5th week	Dark yellow	Heterogeneous	***
2.0	0.5	Hypocotyl	6th week	Pale yellow	Friable	***
		Seed	6th week	Dark yellow	Friable	***
2.0	0.1	Hypocotyl	6th week	Pale yellow	Friable	**
		Seed	6th week	Dark yellow	Friable	**
1.0	1	Hypocotyl	8th week	Pale yellow	Heterogeneous	**
		Seed	8th week	Dark yellow	Heterogeneous	**
1.0	0.5	Hypocotyl	8th week	Pale yellow	Friable	**
		Seed	8th week	Dark yellow	Friable	**
1.0	0.1	Hypocotyl	9th week	Pale yellow	Friable	0
		Seed	9th week	Dark yellow	Friable	0

All data are the main of 10-replicates. *: Mild growth, **: Moderate growth, ***: High growth

Table 3: Growth parameters of *Sisymbrium irio* callus grown for 28 days on MS medium supplemented with different concentrations of phytohormones.

Phytohormones (mg/l)		CFW (g/30ml)	CDW (g/30ml)	Growth index	RGR($gg^{-1}d^{-1}$)	t_d (Day)
2,4-D	Kn					
1	0.1	5.85±0.25	0.41±0.02	3.09±0.15	0.030	13.76
1	0.5	6.78±0.26	0.49±0.02	3.47±0.20	0.033	12.99
1	1	5.80±0.17	0.42±0.01	2.50±0.07	0.027	15.49
2	0.1	5.82±0.11	0.36±0.01	2.28±0.05	0.025	16.37
2	0.5	5.60±0.26	0.38±0.02	2.46±0.25	0.026	15.66
2	1	5.30±0.29	0.40±0.02	2.32±0.15	0.026	16.12

All data are the mean of 5 replicates ± SD

Table 4: Growth parameters of *Sisymbrium irio* callus grown for 28 days on MS medium without vitamins and supplemented with different concentrations of SAE (medium B)

SAE Conc (v/v)	CFW(g/30ml)	CDW(g/30ml)	Growth index	RGR($gg^{-1}d^{-1}$)	t_d (day)
0%	1.24±0.17	0.09±0.02	------	-----	-----
2%	4.27±0.53	0.30±0.02	1.72±0.17	0.020	19.34
4%	5.65±0.17	0.39±0.02	2.54±0.17	0.027	15.33
6%	7.05±0.11	0.50±0.02	3.17±0.15	0.032	13.60
8%	7.72±0.33	0.57±0.03	4.17±0.26	0.037	11.80

All data are the mean of 5 replicates ± SD

Table 5: Growth parameters of *Sisymbrium irio* callus grown for 28 days on MS medium without phytohormones and supplemented with different concentrations of SAE (medium C)

SAE Conc (v/v)	CFW (g/30ml)	CDW (g/30ml)	Growth index	RGR ($gg^{-1}d^{-1}$)	t_d (day)
0%	-----	-----	----	-----	----
12%	5.04±0.16	0.31±0.01	1.82±0.14	0.021	18.73
14%	5.45±0.40	0.38±0.03	2.45±0.24	0.026	15.66
16%	6.10±0.16	0.42±0.01	2.83±0.13	0.029	14.49
18%	6.95±0.19	0.51±0.01	3.63±0.13	0.034	12.65
20%	6.84±0.25	0.50±0.02	3.54±0.15	0.034	12.82

All data are the mean of 5 replicates ± SD

Table 6: Growth parameters of *Sisymbrium irio* callus grown for 28 days on MS medium without both vitamins and phytohormones and supplemented with different concentrations of SAE (medium D)

SAE Conc (v/v)	CFW (g/30ml)	CDW (g/30ml)	Growth index	RGR ($gg^{-1}d^{-1}$)	t_d (day)
0%	----	----	----	----	----
12%	5.09±0.32	0.34±0.02	2.09±0.23	0.023	17.20
14%	5.75±0.36	0.41±0.03	2.74±0.27	0.028	14.75
16%	6.63±0.39	0.47±0.03	3.29±0.31	0.032	13.36
18%	7.59±0.28	0.56±0.02	4.08±0.27	0.037	11.93
20%	7.50±0.28	0.56±0.02	3.66±0.20	0.035	12.60

All data are the mean of 5 replicates ± SD

Table 7: HPLC analysis for vitamins and phytohormones of SAE

Vitamins	Concentration (mg/100 g dry weight)	Phytohormones	Concentration (µg / 10 g dry weight)
Biotin	----	IAA	536
Ascorbic acid	1.79	GA	80
Thiamin (B1)	4.14	BA	60
Riboflavin (B2)	3.45	ABA	1.0
Pantothenic acid (B5)	0.06	JA	0.0126
Pyridoxin (B6)	0.12	MeJA	0.002
Folic acid	0.10		
Inositol	63.51		
Vitamin E	5.00		
β-Carotene	122.54		

Table 8: Growth parameters of *Sisymbrium irio* cell suspension cultures grown for 15 days on MS medium without both vitamins and phytohormones and supplemented with different concentrations of SAE

Medium	SAE Conc (v/v)	CFW (g/100ml)	CDW (g/100ml)	PCV (%)	Growth index	RGR ($gg^{-1}d^{-1}$)	t_d (day)
A	0%	32.72±3.3	1.60±0.16	42.92±4.6	4.98±0.84	0.049	12.11
D	18%	46.15±3.8	2.5±0.41	66.4±10.8	8.89±1.63	0.065	9.07

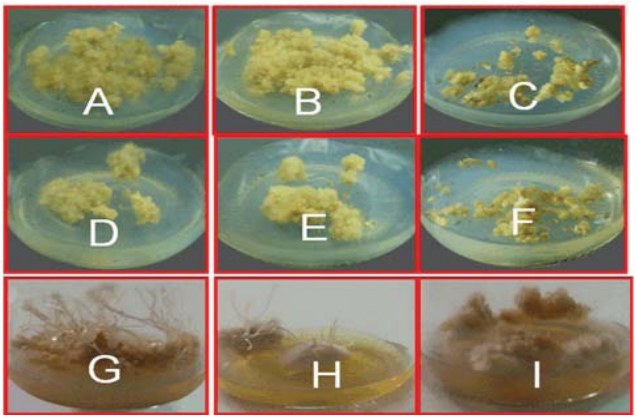

Plate1: Induction of Sisymbrium irio callus on MS medium supplemented with (A) 2 mg/l 2,4-D + 1 mg/l Kn, (B) 2 mg/l 2,4-D + 0.5 mg/l Kn, (C) 2 mg/l 2,4-D + 0.1 mg/l Kn, (D) 1 mg/l 2,4-D + 1 mg/l Kn, (E) 1 mg/l 2,4-D + 0.5 mg/l Kn, (F) 1 mg/l 2,4-D + 0.1 mg/l Kn, (G) 2 mg/l NAA+ 1 mg/l Kn, (H) 2 mg/l NAA+ 1 mg/l Kn, (I) 1 mg/l NAA+ 1 mg/l Kn.

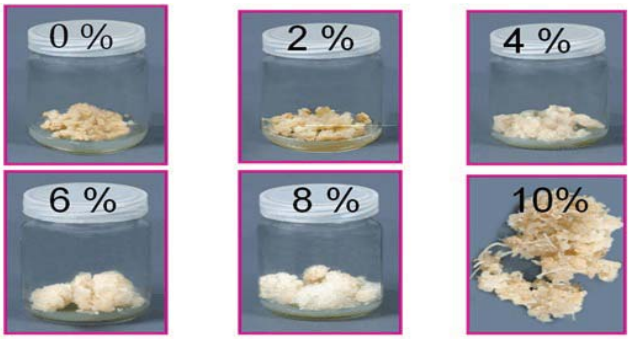

Plate 2: Growth of Sisymbrium irio calli grown for 28 days on complete MS medium supplemented with different concentrations (up to 10 %) of SAE.

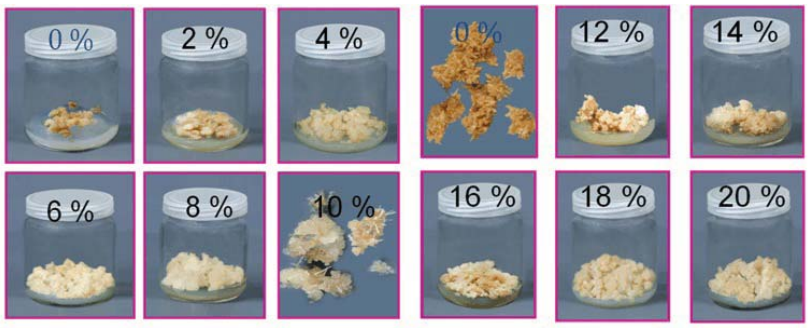

Plate. 3: Growth of Sisymbrium irio calli grown for 28 days on MS medium without vitamins and supplemented with different concentrations (up to 10 %) of SAE.

Plate. 4: Growth parameters of Sisymbrium irio calli grown for 28 days on MS medium without phytohormones and supplemented with different concentrations (up to 20 %) of SAE.

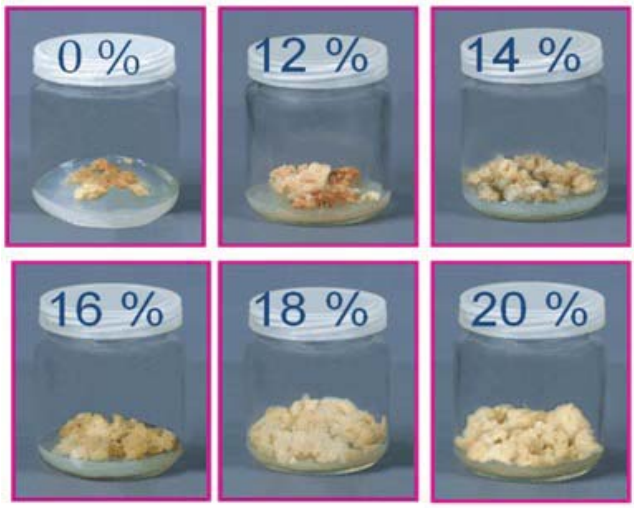

Plate. 5: Growth of Sisymbrium irio calli grown for 28 days on MS medium without both vitamins and phytohormones and supplemented with different concentrations (up to 20 %) of SAE.

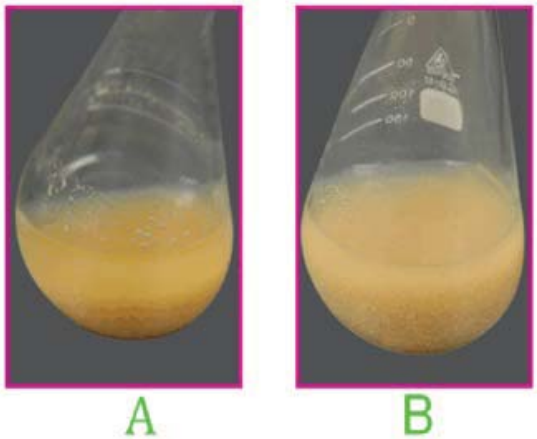

Plate. 6: Growth of Sisymbrium irio cell suspension cultures grown for 15 days in (A) medium A without SAE and (B) medium D supplemented with 18 % SAE.

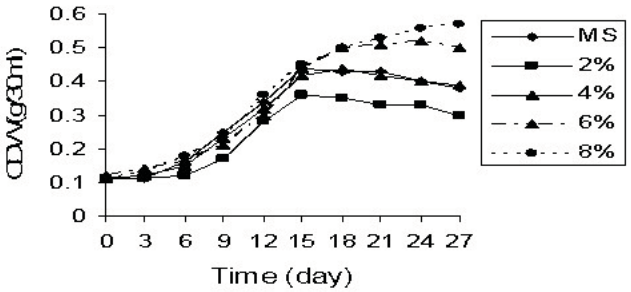

Fig. 1: Growth curves of Sisymbrium irio callus grown for 28 days on MS medium without vitamins and supplemented with different concentrations of SAE.

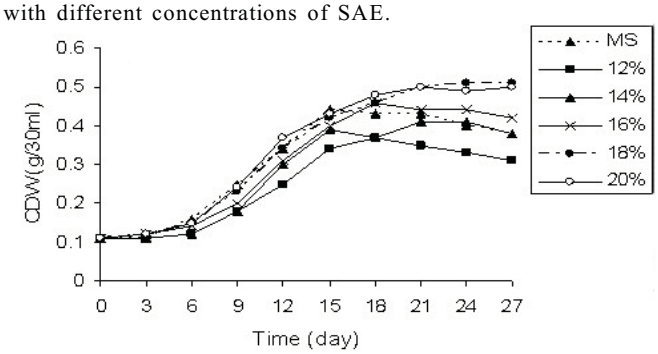

Fig. 2: Growth curves of Sisymbrium irio callus grown for 28 days on MS medium without phytohormones and supplemented with different concentrations of SAE

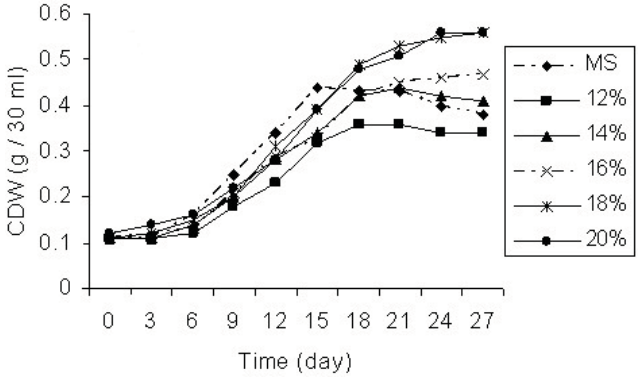

Fig. 3: Growth curves of Sisymbrium irio callus grown for 28 days on MS medium without both vitamins and phytohormones and supplemented with different concentrations of SAE

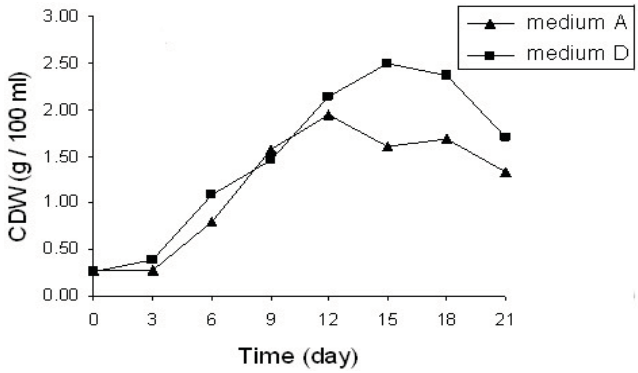

Fig. 4: Growth curves of Sisymbrium irio suspension grown for 21 days on MS medium free of vitamins and phytohormones and supplemented with 18% v/v SAE

Establishment of Cell Suspension Culture:

Data presented in table (8) revealed that, similarly as with callus cultures, the growth rate of cell suspension culture grown in medium D supplemented with 18 % SAE was higher than that in medium A (control) where RGR =0.065 $gg^{-1}d^{-1}$ and RGR= 0.049 $gg^{-1}d^{-1}$ respectively. However, the cells in suspension cultures grew with higher rates than in callus cultures. The time courses of growth represented in Fig. (4) revealed that an extension in the exponential phase to 18 days for cell grown in medium D supplemented with 18 % SAE meanwhile it extended to only 9 days for medium A. The previous extensions was coincident with the maximum biomass production in medium D supplemented with 18 % SAE, rather than the corresponding biomass production in the other medium A. The promotive effects in cell suspension culture grown on medium D supplemented with SAE may be related to the composition of SAE since it comprises phytohormones (Adam, 1999; Karthikeyan, 2006), vitamins (Misra and Kaushik, 1989) and micronutrients (Vonshak *et al*., 1982). The inorganic micronutrients significantly promote the callus growth in Rose leaves (Kintzios *et al*., 2000). Moreover, inositol could be essential during cell division and enlargement. Additionally, myo inisitol phosphate esters are important sources. Not only the presence of *Spirulina* in the medium is the causal agent for promotion of the growth but it may be related to the mechanical agitation which prevents the aggregation of the cells into mass as well it promotes adequate gaseous exchange between the culture medium and culture air (Street, 1973). Moreover, Yeoman and Aitchison, (1973) indicated that movement of the tissue as a result of agitation facilitate the polarization of the tissues due to gravity and elimination the nutrients gradients within the medium and gently wash the tissue with the nutrients.

Antioxidant Activity, Total Phenolic and Flavonoid Contents:

The results in table (9 & 10) demonstrate that, the antioxidant capacity of SAE-treated cultures was higher than that of the control cultures (untreated cultures). The extracts of calli grown on media B supplemented with 8 % SAE showed the best scavenging capacity toward DPPH (669 %) followed by the extracts of calli grown on media C and D supplemented with 18 % SAE (339 % and 353 % respectively). On the other hand, the extract of callus grown on medium D without SAE exhibited the least DPPH scavenging capacity (26 %). By comparing the results presented in table 9 and 10, it can be concluded that, the antioxidant capacity of cell suspension cultures is 3 and 5 folds higher than that of callus cultures grown on medium A and D respectively. The enhancement of the antioxidant activities by SAE may be related to that SAE is an elicitor that elicited production of some antioxidant metabolites in these cultures. In this regard, phenolic and flavonoid contents were elevated to more 15 times than the corresponding controls as the MS supplemented with 8% and 18% SAE. By comparing the results presented in Table (9) with those in table (10), it can be concluded that, the cell suspension cultures showed higher phenolic and flavonoids contents as compared with the callus cultures grown on the same media composition.

These results manifested the correlation between the efficiency of antioxidant activities and the accumulation of phenolic compounds. Data were coincident with Aljadi and Kamaruddin, (2004) and Li *et al*., (2008). Moreover, there are many other secondary metabolites, which have antioxidant activities in *Sisymbrium irio* such as glucosinolates, isothiocyanates, ascorbic acid and carotenoids **(**Guil, *et al*., 1997; Al-Gendy, 2008). Other phytochemicals may be also increased by addition of SAE to the culture medium. In consistent with our results, the relationship between phenolic content and antioxidant activity has been also evaluated **(**Sun and Ho, 2005; Guo *et al*., 2008) where a positive correlation between phenolic content and antioxidant activities was found.

Antioxidant Enzymes:

Beside antioxidant metabolites, other important compositions of antioxidant system are antioxidant enzymes for calli and cell suspension treated with SAE, where the activities of different enzymes were sharply decreased especially in calli treated with SAE (table 11 and 12). The activities of CAT, POX and PPO in cell suspension cultures were significantly lower than those of callus cultures grown on the same medium composition.

The previous reductions of antioxidant enzymes may be attributed to scavenging reactive oxygen species by SAE. In accordance to our results, several studies demonstrated that *S. platensis* or its extracts could have antioxidant and radical scavenging properties (Miranda et al., 1998; Mendes *et al*., 2003; Subhashini *et al*., 2004). It is generally assumed that the POX and PPO are the two major enzymes responsible for oxidation of phenolic compounds (Sheen and Calvert, 1969; George and Sherrington, 1984). Consequently, the decline in POX and PPO activities in SAE-treated cultures may be the reason for delaying senescence and browning of the calli in these cultures. Benson (2000) evoked that tissue culture manipulations cause major metabolic and developmental changes, some of which may predispose *in vitro* cultures to increase free radical formation, resulting in cellular dysfunction and recalcitrant cultures. The formation of free radicals may result in an increased activity of antioxidant enzymes such as peroxidase and catalase (Salin and Bridges, 1981; Thompson, 1987), which act to overcome the effect of reactive oxygen species (Benson, 2000).

Table 9: Effect of different concentrations of *Spirulina* aqueous extract (SAE) on the contents of total phenolic compounds, total flavonoids and antioxidant activities of *Sisymbrium irio* callus

Medium	SAE Conc(v/v)	Total flavonoids (mg / gm DW)	Total phenolic (mg / gm DW)	% Scavenging activity
Complete MS (A)	0%	1.2±0.15	0.79±0.21	49.22 ± 2.96
MS without vitamins (B)	0%	0.19±0.08	0.11±0.09	42.42 ± 9.60
	8%	3.06±0.06	1.94±0.37	668.51±58.68
MS without phytohormones (C)	0%	0.63±0.05	0.33±0.16	30.60 ± 2.63
	18%	2.20±0.06	1.18±0.05	338.61±0.06
MS without vitamins and phytohormones (D)	0%	0.13±0.03	0.11±0.09	25.73 ± 0.63
	18%	2.05±0.06	1.37±0.09	352.77±10.22

All data are the mean of 5 replicates ± SD

Table 10: Effect of *Spirulina* aqueous extract (SAE) on the contents of total phenolics, total flavonoids and antioxidant activities of *Sisymbrium irio* suspension cells

Medium	SAE Conc (v/v)	Total flavonoids (mg / gm DW)	Total phenolics (mg / gm DW)	% Scavenging activity
Complete MS (A)	0%	1.81±0.22	1.07±0.08	132.79±2.49
MS without vitamins and phytohormones (D)	18%	4.60±0.24	3.2535±0.59	1058.00±135.58

All data are the mean of 5 replicates ± SD

Table 11: Effect of different concentrations of *Spirulina* aqueous extract (SAE) on the activities of antioxidant enzymes extracted from *Sisymbrium irio* callus

Medium	SAE Conc (v/v)	Catalase (µmol H_2O_2 /mg protein/min)	P.P.O. (change O.D./mg protein/min)	Peroxidase (chang O.D./mg protein/min)
Complete MS (A)	0%	5.28±0.41	0.053±0.00	0.25±0.01
MS without vitamins (B)	0%	11.29±0.21	0.076±0.00	0.42±0.01
	8%	1.18±0.10	0.015±0.00	0.10±0.01
MS without phytohormones (C)	0%	13.65±0.24	0.072±0.01	0.50±0.01
	18%	0.66±0.00	0.012±0.00	0.11±0.00
MS without both vitamins and phytohormones (D)	0%	13.33±0.13	0.078±0.00	0.53±0.01
	18%	0.36±0.00	0.016±0.00	0.09±0.00

All data are the mean of 5 replicates ± SDO. D.: Optical density

Table 12: Effect of different concentrations of *Spirulina* aqueous extract (SAE) on the activities of antioxidant enzymes extracted from *Sisymbrium irio* suspension cells

Medium	SAE Conc (v/v)	Catalase (µmol H_2O_2 /mg protein /min)	P.P.O. (change O.D. /mg protein/min)	Peroxidase (change O.D./mg protein/min)
Complete MS (A)	0%	4.08±0.12	0.024±0.00	0.20±0.01
MS without both vitamins and phytohormones (D)	18%	0.28±0.04	0.008±0.00	0.05±0.00

All data are the mean of 5 replicates ± SD

Conclusion:

Different callus and suspension cultures of *S. irio* were successfully established under different conditions using MS medium. SAE could substitute phytohormones and vitamins with resultant increase of growth parameters as well as total phenolic, antioxidant activity and flavonoid production. Antioxidant capacity was increased up to 12 times more than the untreated cultures. Total phenolic and flavonoid contents increased to 2.5 times comparing with the untreated cultures. Suspension cultures showed higher antioxidant activity, total flavonoid and phenolic contents compared with the callus cultures. Antioxidant enzymes; CAT, PPO and POX were highly reduced in callus and suspension cultures upon addition of SAE. These results may suggest the use of *S. platensis* as a cheaper substitute for the used phytohormones and vitamins to promote the growth and production of secondary metabolites *in vitro*. To our knowledge, this is the first report for the use of *S. platensis* as growth promoter and productivity enhancer for establishment of *in vitro* cell cultures of *S. irio*.

REFERENCES

Adam, M.S., 1999. The promotive effect of the cyanobacterium *Nostoc muscorm* on the growth of some crop plants. Acta Microbiologica. Polonica, 48(2): 163-171.

Al-Gendy, A.A., 2008. Phytochemical and biological screening of glucosinolates and volatile constituents of different Brassicaceae plants growing in Egypt. Bull. Fac. Pharm. Cairo Univ., 46(2): 235-244.

Ali, B. and S. Hasnain, 2007. Efficacy of bacterial auxin on *in vitro* growth of *Brassica oleracea* L. World J. Microbiol. Biotechnol., 23(6): 779-784.

Aljadi, A.M. and M.Y. Kamaruddin, 2004. Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys, Food Chem., 85(4): 513-518.

Amin, G.H. and Omar, H.H. (2002): Cyanobacterial exudates as a natural biotic source for tissue cultures media. Egypt J. Biotechnol., 11: 190-205.

Arnaldos, T.L., R. Munoz, M.A. Ferrer and A.A. Calderon, 2001. Changes in phenol content during strawberry (*Fragaria x ananasa*, cv. Chandler) callus culture. Physiol. Plant., 113: 315-322.

Baydar, H. and S. Ulger, 1998. Correlations between changes in the amount of endogenous phytohormones and flowering in the safflower (*Carthamus tinctorius* L.). Tr. J. of Biology, 22: 421-425.

Belay, A., 1997. Mass culture of *Spirulina* (Arthrospira) outdoors. The Earthrise Farms experience. In: Vonshak, A., ed. *Spirulina platensis (Arthrospira):* Physiology, cell-biology and biotechnology. Taylor and Francis. London. pp: 131-158.

Benson, E.E., 2000 In vitro plant recalcitrance, do free radicals have a role in plant tissue culture recalcitrance?. *In Vitro* Cell Dev. Biol.- Plant, 36(3): 163-170.

Beyer, W.F. and I. Fridovich, 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in condition. Anal. Biochem., 161(2): 559–566.

Chen, W.S., 1990. Endogenous growth substances in xylem and shoot tip diffusate of lychee in relation to flowering. Hort. Science, 25(3): 314-315.

Chen, X.F. and K.F. Wang, 1995. The changes of enzymatic browning factors during the development of peaches. Acta Horticulturae Sinica, 22(3): 230–234.

Cohen, Z., 1997. The chemical of *Spirulina*. In: Vonshak, A. (ed.), *Spirulina platensis* (Arthrospira), Physiology, Cell-Biology and Biotechnology. Taylor and Francis, London., pp: 175-204.

Colla, L.M., C.O. Reinehr, C. Reichert and J.A.V. Costa, 2007. Production of biomass and nutraceutical compounds by *Spirulina platensis* under different temperature and nitrogen regimes. Bioresour. Technol. 98 (7): 1489–1493.

Costa, J.A.V., L.M. Colla, P.D. Filho, K. Kabke and A. Weber, 2002. Modelling of *Spirulina platensis* growth in fresh water using response surface methodology. World Journal of Microbiology and Biotechnology, 18(7): 603-607.

Dobberstein, R.H. and E.J. Staba, 1969. Ipomoea, -Violacea-D Rivea-Corymbosa-D and Argyreia-Nervosa-D tissue cultures: influence of various chemical factors on indole alkaloid production and growth. Lloydia., 32 (2): 141-147.

Estrada, J.E., P. Bescós and Villar Del A.M. Fresno, 2001. Antioxidant activity of different fractions of *Spirulina platensis* protean extract. Il Farmaco., 56: 497–500.

Gang, Y.Y., G.S. Du, D.J. Shi, M.Z. Wang, X.D. Li and Z.L. Hua, 2003. Establishment of *in vitro* regeneration system of the *Atrichum mosses*. Acta Bot. Sin., 45(12): 1475-1480.

George, E.F. and P.D. Sherrington, 1984. Plant Propagation by Tissue Culture. Handbook and Directory of Commercial Laboratories. Exergetics Ltd, Edington, UK., pp: 334-340.

Guil, J.L., I. Rodríguez-García and E. Torija, 1997. Nutritional and toxic factors in selected wild edible plants. Plant Foods Hum. Nutr., 51(2): 99-107.

Guinn, G. and D.L. Brummett, 1990. Solid-phase extraction of cytokinins from aqueous solutions with C_{18} cartridges and their use in a rapid purification procedure. Plant Growth Regulation, 9(4): 305-314.

Guo, D.J., H.L. Cheng, S.W. Chan and P.H. Yu, 2008. Antioxidative activities and the total phenolic contents of tonic Chinese medicinal herbs. Inflammopharmacology, 16(5): 201-207.

Gűrel, S., E. Gűrel and Z. Kaya, 2001. Callus development and indirect shoot regeneration from seedling explants of sugar beet (*Beta vulgaris* L.) cultured *in vitro*. Turkish J. Bot., 25: 25-33.

Hung, P.V. and N. Morita, 2008. Distribution of phenolic compounds in the graded flours milled from whole buckwheat grains and their antioxidant capacities. Food chemistry, 109(2): 325-331.

Ji, H., H.X. Zhang and H.B. Ge, 1998. The relationship between browning ratio in PPO and phenols of pear explants. Journal of Sichuan Agricultural University, 16(3): 310–313.

Kar, M. and D. Mishra, 1976. Catalase, peroxidase and polyphyenol oxidase activities during Rice leaf Senescence. Plant Physiol., 57: 315-319.

Karthikeyan, N., 2006. Characterization of cyanobacteria from the rhizosphere of wheat. M.Sc dissertation, Division of Microbiology, Post Graduate School, Indian Agricultural Research Institute, New Delhi.

Kirtikar, K.R. and B.D. Basu, 1984. Indian Medicinal Plants, vol. III, 2nd ed. Periodical Expert Book Agency, Delhi., 2190-2210.

Kintzios, S., J.B. Drossopoulos and C. Lymperopoulos, 2000. Effect of vitamins and inorganic micronutrients on callus growth and somatic embryogenesis from young mature leaves of rose. J. Plant Nutr., 23(10): 1407-1420.

Kosalec, I., M. Bakmaz, S. Pepeljnjak and S. Vladimir-Knežević, 2004. Quantitative analysis of the flavonoids in raw propolis from northern Croatia. Acat pharma., 54: 65-72.

Langhansova, L., P. Marsik and T. Vanek, 2005. Production of saponins from *Panax ginseng* suspension and adventitious root cultures. Biol. Plant., 49(3): 463-465.

Laukkanen, H., H. Häggman, S. Kontunen-Soppela and A. Hohtola, 1999. Tissue browning of *in vitro* cultures of Scots pine: Role of peroxidase and polyphenol oxidase. Physiol. Plant, 106(3): 337-343.

Li, H.B., C.C. Wong, K.W. Cheng and F. Chen, 2008. Antioxidant properties *in vitro* and total phenolic contents in methanol extracts from medicinal plants. LWT-Food Science and Technology, 41(3): 385-390.

Liu, Z.H., W.C. Wang and S.Y. Yan, 1997. Effects of hormone treatment on callus formation and endogenous indole-acetic acid and polyamine contents of soybean hypocotyl cultivated *in vitro*. Bot. Bull. Acad. Sin., 38: 171-176.

Lobban, C.S., D.J. Chapman and B.P. Kremer, 1988. Experimental phycology. A laboratory manual. Cambridge. Univ. Press. Cambridge, 20-21: 27-34.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275.

Madhyastha, H.K. and T.M. Vatsala, 2007. Pigment production in *Spirulina fussiformis* in different photophysical conditions. Biomol. Eng., 24(3): 301–305.

Meijer, E.A., De S.C. Vries and A.P. Mordhorst, 1999. Co-culture with *Daucus carota* somatic embryos reveals high 2,4-D uptake and release rates of *Arabidopsis thaliana* cultured cells. Plant Cell Reports, 18: 656-663.

Mendes, R.L., B.P. Nobre, M.T. Cardoso, A.P. Pereira and A.F. Palavra, 2003. Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae. Inorg. Chim. Acta, 356: 328-334.

Miranda, M.S., R.G. Cintra, S.B. Barros and J. Mancini-Fiho, 1998. Antioxidant activity of the microalga *Spirulina maxima*. Brazilian Journal of Medical Biology Research, 31(8): 1075-1079.

Misra, S. and B.D. Kaushik, 1989. Growth promoting substances of cyanobacteria. I. Vitamins and their influence on rice plant, Proc. Indian Nat. Sci. Acad., Part B Biol. Sci., 55(4): 295-300.

Mulabagal, V. and H.S. Tsay, 2004. Plant cell cultures an alternative and efficient source for the production of biologically important secondary metabolites. International Journal of Applied Sciences and Engineering, 2: 29-48.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15(3): 473–497.

Nath, S. and A.K. Buragohain, 2005. Establishment of callus and cell suspension cultures of *Centella asiatica*. Biol. Plant., 49: 411-413.

Nowak, B. and K. Miczyński, 2002. The course and efficiency of organogenesis on leaf explants of Plum ′Wegierka Zwykla (*Prunus Domestica* L.) induced by cytokinins. Elec. J. Polish Agric. Univ. Biotechnol., 5: 120-124.

Ogbonda, K.H., R.E. Aminigo and G.O. Abu, 2007. Influence of temperature and pH on biomass production and protein biosynthesis in a putative *Spirulina sp*. Bioresour. Technol., 98: 2207–2211.

Pérez-Francés, J.F., A.J. Exposito and J.A. Rodriguez, 1995. Effect of different factors on *in vitro* multiplication of *Leucadendron* 'Safari Sunset' (Proteaceae). Acta Hort., 387: 115-120.

Qian, H. and M. Sheng, 1998. Simultaneous determination of fat-soluble vitamins A, D and E and pro-vitamin D_2 in animal feeds by one-step extraction and high-performance liquid chromatography analysis. J. Chromatogr. A., 825(2): 127-133.

Racusen, D. and M. Foote, 1965. Protein synthesis in dark-grown bean leaves. Can. J. Bot., 43: 817-824.

Rangel-Yagui, C.O., E.D.G. Danesi, J.C.M. Carvalho and S. Sato, 2004. Chlorophyll production from *Spirulina platensis*: cultivation with urea addition by fed-batch process. Bioresour. Technol., 92(2): 133–141.

Sajilata, M.G., R.S. Singhal and M.Y. Kamat, 2008. Fractionation of lipids and purification of γ-linolenic acid (GLA) from *Spirulina platensis*. Food Chem., 109(3): 580–586.

Salin, M.L. and S.M. Bridges, 1981. Chemiluminescence in wounded root tissue. Plant Physiol., 67: 43-46.

Sandoval, M., N.N. Okuhama, F.M. Angeles and V.V.Melchor, *et al.,* 2002. Antioxidant activity of the cruciferous vegetable Maca (*Lepidium meyenii*). Food Chem., 79(2): 207–213.

Sheen, S.J. and J. Calvert, 1969. Studies on polyphenol content activities and isoenzymes of polyphenol oxidase, and peroxidase during air-curing in three tobacco types. Plant Physiol., 44(2): 199-204.

Shindy, W. and O.E. Smith, 1975. Identification of plant hormones from cotton ovules. Plant Physiol., 55(3): 550-554

Singer, S.R., 1986. Analyzing growth in cell cultures. I. Calculating growth rates. Can. J. Bot., 64: 233-237.

Skoog, F. and C.O. Miller, 1957. Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Sym. Soc. Exp. Biol., 11: 118-131.

Srivastava, S. and A.K. Srivastava, 2007. Hairy root culture for mass-production of high-value secondary metabolites. Critical Reviews in Biotechnology, 27(1): 29-43.

Stickens, D., W. Tao and J.P. Verbelen, 1996. A single cell model system to study hormone signal transduction. Plant Growth Regul., 18(1-2): 149-154.

Street, H.E., 1973. Cell (suspension) cultures techniques. In plant tissue and cell culture (Street, H.E. ed), Blackwell scientific publications, Oxford London Edinburgh Melbourne

Strycharz, S. and K. Shetty, 2002. Response of oregano (*Origanum vulgare* L.) clonal lines to Pseudomonas sp. Z strain and ploydye R-478 and implications for hyperhydricity prevention in tissue culture. Process Biochem., 38: 343–350.

Subhashini, J., S.V.K. Mahipal, M.C. Reddy, M.M. Reddy, A. Rachamallu and P. Reddanna, 2004. Molecular mechanisms in C-Phycocyanin induced apoptosis in human chronic myeloid leukemia cell line-K562. Biochem. Pharmacol. 68: 453-462.

Sun, T. and C.T. Ho, 2005. Antioxidant activities of buckwheat extracts, Food Chemistry, 90(4): 743–749.

Thompson, J.E., R.L. Legge and R.F. Barber, 1987. The role of free radicals in senescence and wounding. New Phytol., 105(3): 317-344.

Turkan, I., M. Bor, F. Ozdemir and H. Koca, 2005. Differential responses of lipid peroxidation and antioxidants in the leaves of drought tolerant *P. acutifolius* Gray and drought-sensitive *P. vulgaris* L. subjected to polyethylene glycol mediated water stress. Plant Sci., 168(1): 223–231.

Vohora, S.B., S.A.H. Naqvi and I. Kumar, 1980. Antipyretic, analgesic and antimicrobial studies on *Sisymbrium irio*. Planta Medica, 38: 255-259.

Vonshak, A., A. Abeliovich, S. Boussiba, S. Arad and A. Rich mond, 1982. Production of *Spirulina* biomass: effects of environmental factors and population density, Biomass, 2: 175-185.

Wasternack, C. and B. Parthier, 1997. Jasmonate – signaled plant gene expression. Trends Plant Sci., 2: 302-307.

Yeoman, M.M. and P.A. Aitchisan, 1973. Growth patterns in tissue (callus) cultures. In: Plant tissue and cell culture (Street, H.E.; ed), Blackwell scientific publications, Oxford London Edinburgh Melbourne

Zarrouk, C., 1966. Contribution à l′étude d′une cyanophycée influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de *Spirulina maxima* (Setch. et Gardner) Geitler (Ph. D. thèse). Univ. de Paris.