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REGULAR ARTICLE

A new concept for production and scaling up of bioactive compounds from Egyptian date palm (Zaghlool) cultivar using bioreactor

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Abstract

A promising and successful protocol for enhancement and production of total phenolic and peroxides compounds from Egyptian date palm cultivar Zaghlool cells in stirred tank reactor was established. The influence of cell culture cultivation in combination with *Aspergillus niger* extract, and methyl-jasmonate elicitors incorporation in the feeding medium on cell growth patterns and production of active compounds was investigated. The maximum value of cell growth parameters and the highest content of bioactive compounds were obtained from elicitation of modified MS-medium with *Aspergillus niger* extract at 0.1% in combination with methyl-jasmonate (100 µM), as compared with other concentrations after 10 days of cultivation. The chemical analyses of phenolic and peroxidase compounds were spectrophotometrically performed.

Key words: Date palm, Elicitors, Phenolic and Peroxidase compounds, Bioreactor

Introduction

Date palm (*Phoenix dactylifera* L.) is a dioecious fruit tree native to the hot arid regions of the world, mainly grown in the Middle East and North Africa. Since ancient time this majestic plant has been recognized as the *tree of life* because of its integration into human settlement, wellbeing, and food security in hot and barren parts of the world, where only a few plant species can flourish (Al-Khayri, 2007). Date palm trees provide the most sustainable agro-ecosystems in harsh dry environments, providing raw materials for housing, furnishings, and many handcrafts. In addition the palm supplies nutritious delicious fruits that can be consumed fresh, dried, or processed, and are a source of sugars, minerals, and vitamins. Economically, date palm provides a major source of income for local farmers and associated industries in communities where it is grown. Biotechnology is a set of rapidly emerging and far-reaching new technologies with great promise in areas of sustainable food production, nutrition security, health care and environmental sustainability. The objective of biotechnology is to use its tools to help convert a country's diverse biological resources into useful products and processes that are accessible to its people for economic development and employment generation (Jain et al., 2011). Bioreactors have several advantages for the mass cultivation of plant cells: 1) better control for scale-up of cell suspension cultures under defined parameters for the production of bioactive compounds; 2) constant regulation of conditions at various stages of bioreactor operation; 3) easy and efficient handling of culture such as inoculation or harvest; 4) enhanced nutrient uptake by submerged culture conditions which stimulate the multiplication rate and a higher yield of bioactive compound and 5) large numbers of plantlets are easily produced and can be scaled-up (Fulzele and Heble, 1994; Othmani et al., 2011).

Phenolics are intermediates of phenylpropanoid metabolism (Cvikrová et al., 1996) and precursors of lignin (Lewis and Yamamoto, 1990) and phenylpropanoid phytoalexins (Kessmann et al., 1990). Their deposition in cell walls is an important defense-mechanism response to pathogen infection (Bolwell, et al., 1985). Plant cells cultivated *in vitro* synthesize phenolic compounds; however, in some cases changes in the quality and quantity of the substances are recommended (Zagoskina and Zamprometov, 1983). This is probably due to the specificity of tissue culture as an artificial biological system in which the basic function of phenols is to interfere with cell proliferation (Ozyigit et al., 2007). Longenbeck (1983) reported that a substitution pattern of phenols was affected

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according to the rate of IAA degradation. Also, he reported that phenols were found to react with hydrogen peroxide produced during IAA degradation, thereby protecting cellular constituents from their toxic effect.

Peroxidase is an enzyme known to play a very crucial role in scavenging free radicals within plant systems (Regalado et al., 2004) in addition to their involvement in various metabolic activities. Outside plant systems, this enzyme has several commercial applications, the major one being its use as an important component in chemical diagnostics and laboratory experiments (Ayala, 2000). A wide range of chemicals can be modified using peroxidase and hence it has various applications in waste water treatment to remove phenolics, and in the synthesis of various aromatic compounds. On the other hand**,** Booij et al. (1993) reported that the changes in soluble peroxidase correlated well with budding, and that the modification of peroxidase activities and expression of iso-peroxidase always preceded the morphological appearance of buds. Thus, evaluation and determination of peroxidase during subculture can lead to a better understanding of the physiological processes, as well as establish optimum conditions for the culture of date palm.

Elicitors are molecules that stimulate defense or stress–induced responses in plants (Radman et al., 2003). However, the broader definition of elicitors includes both substances of pathogenic origin and compounds released from plants by the action of pathogens (endogenous elicitors). Natural elicitors can be divided into two types: biotic and abiotic. The biotic elicitors have a biological origin, derived from the pathogen or from the plant itself, while abiotic elicitors are not of biological origin and are the result of physical factors and chemical compounds (Kumar and Shekhawat, 2009).

Zhao et al. (2000) reported that using combined biotic elicitor treatment of *Aspergillus niger* mycelium and tetramethyl ammonium bromide with *Catharanthus roseus*, cell cultures enhanced the accumulation of ajmalicine content, as compared with the control medium.

On other hand**,** Fritz et al. (2010) reported that jasmonic acid (JA) and methyl-jasmonate (MJ), are plant hormones involved in chemical and physiological defense responses. Moreover**,** Balbi and Devoto (2008) stated that JA and MJ are oxylipins (oxygenated fatty acids) that originate from linolenic acid released from chloroplast membranes by lipase enzymes and subsequently oxygenated by lipoxygenases (LOXs) to hydroperoxide derivatives. Elicitation or stress stimulus leads to a rapid release of α-linolenic acid from the lipid pool of the plant cell through which an intracellular signal cascade elicits secondary metabolite production important for plant defense (Memelink et al., 2001**)**. α-Linolenic acid is converted by a lipoxygenase, an allene oxide synthase (AOS) and an allene oxide cyclase (AOC) into the intermediate 12-oxo-phytodienoic acid. This compound is converted into JA through the action of a reeducate and three rounds of β-oxidation (Mueller, 1997; Menke et al*.,* 1999).

The main objective of this investigation focused on the effect of different concentrations among of *Aspergillus niger* (AN) and MJ as biotic elicitors on primordial leaf cell growth parameters; and to determine the accumulation rate of phenolic and peroxidase compounds in suspension cultures of Egyptian date palm (Zaghlool) cultivar using a bioreactor.

Material and Methods

Plant materials

Female date palm (*Phoenix dactylifera* L.) offshoots of Egyptian date palm cultivar (Zaghlool) were obtained from Rashed in North Egypt; the offshoots were separated during the fruiting stage from the mother plants. The measurements and parameters of the offshoots were 120-150 cm in height, 35 cm in diameter and 45-50 kg. in weight. These offshoots were used as mother plant materials for initiation of *in vitro* cultures.

Sterilization

Sterilization of the obtained shoot tip was carried out according the method described by Taha et al. (2010).

Nutrient media and callus production

Explants of sterilized primary basal leaves excised from the base of shoot tips were cultured on solidified Murashige and Skoog (1962) nutrient medium (MS) supplemented with 1.7 g/l phytagel and 30 g/l sucrose. The MS-nutrient medium was fortified by 170 mg/l NaH_2Po_4 $2H_2O$ + 200 mg/l H_2PO_4 + 1 mg/l thiamine HCI and 3 g/l activated charcoal and augmented with 3 mg/l 2,4-D + 3 mg/l 2iP and 5 mg/l BA. This is the best medium for callus production according to Taha et al. (2010). The pH of the culture medium was adjusted to 5.7 with 0.1 M NaOH or 0.1 M HCl before adding phytagel. The culture medium was dispensed into 150 ml jars, each containing 40 ml and autoclaved at 121°C ± 1 for 20 min. Cultures were incubated in darkness in a growth chamber at a constant temperature of 28°C ± 1 then incubated under light condition (2000 Lux) from cool white

fluorescent lamps, and subcultured every 6 weeks on fresh new medium. After three subcultures, white calli were initiated and observed.

Cell production

Calli from the previous experiment were retained and re-suspended in an agitated liquid MS medium containing 1 mg/l 2,4-D + 1 mg/l 2iP + 3mg/l NAA according to the best results obtained by Taha et al. (2010).

Establishment of bioreactor experiments

A 2-L turbine stirred tank bioreactor (STB) of the National Research Centre (NRC) was used with a working volume of 1.5-1.7 L (B. Braun, Biotech, International, Germany). The culture was aerated through a stainless steel sparger. The flow rate was set up according to the type of experiment and maintained at the normal level with a mass flow control system until the end of the culture period. Two six-bladed turbine impellers (D=45 mm) were used for mixing at a rotation speed of 120 rpm. The temperature was maintained at 26°C with a thermostatic outlet spongy sheet surrounding the vessel. Aeration was performed by filtered sterile air at the rate of 0.5 l/min. Dissolved oxygen concentrations were measured with a sterilizable oxygen electrode (Ingold). Dissolved oxygen concentration was monitored with a sterilizable pO2 electrode to maintain different levels of dissolved oxygen concentrations in the bioreactor broth, with the inlet air dosed by a mass flow controller connected with software and pO2 electrode. The bioreactor was inoculated with one part of suspension culture and five parts of medium, and the cell cultures were kept at 25°C. The MS-nutrient medium containing cell lines were introduced into a glass tank bioreactor under sterilized air condition. The parameters affecting either mass cell culture and/or enhancement of phenolic and peroxides compounds in lyophilized suspension culture of Zaghlool date palm cultivar were investigated, as follows:

- Effect of controlled pH medium at the degree of 5.7 using either 0.2 N NaOH or 0.2 N HCl with ADI 1030 Bio-controllers (Applikon) equipped with a sterilizable pH–electrode (Ingold) and peristaltic pumps for alkali and acid addition.
- Effect of uncontrolled of pH medium.
- Each experiment was done for 2 weeks after inoculation. At the end of 15 days of inoculation, the cells obtained were harvested and chemically analyzed for an accumulation of phenolic and peroxidease compounds. The percentage of these target compounds were recorded as the control treatment.
- Effect of two type of biotic elicitors at different concentrations on enhancement of cell growth parameters, phenolic and peroxidase compounds production.
- Effect of *Aspergillus niger* extract: An extract of the fungus, *A. niger* was obtained from The Department of Plant Pathology of the National Research Centre. Preparation of the fungus elicitor was carried out according to the method described by Taha (2002). In this experiment, 0, 0.1, 0.2 and 0.3% of suspended *A. niger* extract (P.C.V) were added to the culture media.
- Effect of methyl-jasmonate**.** In this experiment, 0, 50, 100 and 200 µM of methy-jasmonate were used.

Measurement of cell growth parameters

The fresh weight and dry weight (w/v) were determined using a sampling unit of suspension culture (2 ml) every 2 days for 2 weeks.

Total phenolics

Extraction and determination of total phenolics in different date palm cell lines were determined using Folin–Ciocalteau reagents (Singleton and Rossi, 1965). Date palm cell line extracts of (40 µl) or gallic acid standard were mixed with 1.8 ml of Folin–Ciocalteu reagent (prediluted 10-fold with distilled water) and maintained at room temperature for 5 min, then 1.2 ml of sodium bicarbonate (7.5%) was added to the mixture. After 1 hour at room temperature, the absorbance was measured at 765 nm. Results were expressed as ng gallic acid equivalents (GAE/g-dW) sample (Shui and Leong, 2006).

Peroxides

The peroxides activity in lyophilized cell cultures (cell and liquid medium) was monitored for total peroxides according to the method described by Wititsuwannakul et al. (1997).

Data analysis

All experiments were designed in a completely randomized design. The results obtained were statistically analyzed using standard error (SE) according to the method described by Snedecor and Cochran (1980).

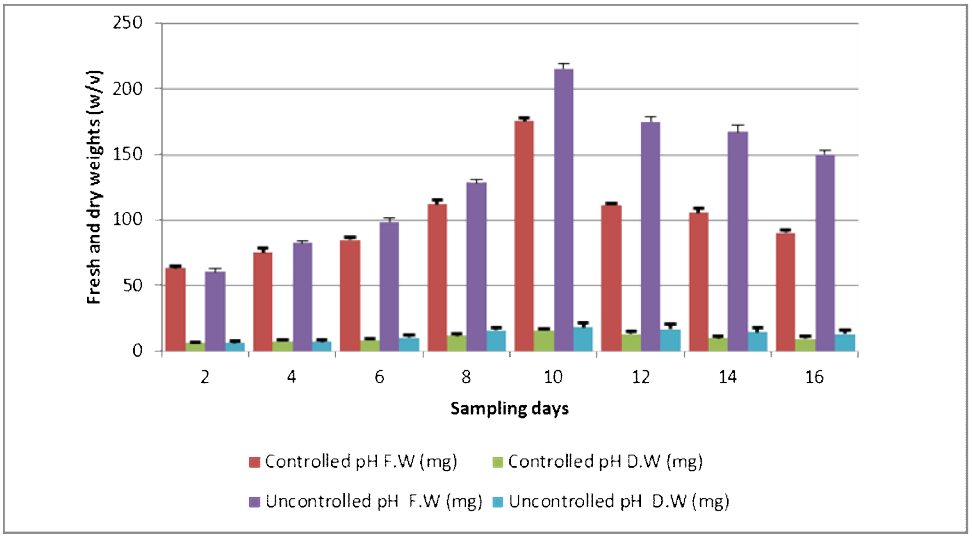

Figure 1. Effect of controlled and uncontrolled pH MS agitated liquid medium containing 1 mg/l 2,4-D + 1 mg/l 2iP + 3 mg/l NAA on cell growth parameters (fresh and dry weights) of Zaghlool date palm cultures, cultured in STB and incubated under 16/8 of daylight condition at 26±1ºC.

Results

Bioreactor experiments: Effect of different conditions on date palm cell growth parameters

1-Effect of controlled and uncontrolled pH medium

The effects of controlled and uncontrolled pH MS-liquid medium containing 1 mg/l 2-4-D, 1 mg/l 2iP and 3 mg/l NAA on Zaghlool cell growth parameters (fresh and dry weights) are presented in Figure 1. The scheduled time of measurement of different cell growth parameters i.e., fresh and dry weights was at intervals of 2 days for 16 days of culturing period. Culturing of date palm cells was carried out in 2-L turbine stirred tank bioreactor (STB) with a working volume of 1.5-1.7 liters. The highest values of fresh weight for controlled pH MS-medium 175.6 and 112.3 (w/v) were recorded in days 10 and 8, respectively. However those recorded 215.8 and 175.3 (w/v) in days 10 and 12, respectively, for uncontrolled pH MS-medium.

Regarding dry weights, the highest values recorded of 18.37 and 15.86 (w/v) were from uncontrolled and controlled pH of MS media, respectively.

Effect of *Aspergillus niger* extract

The effect of elicitation of uncontrolled MS medium with different concentrations of AN as a biotic elicitor on the optimization of different conditions affecting maximization of date palm cell growth parameters was investigated. Filtered and sterilized mycelium of AN as the biotic elector at different concentrations of 0, 0.1, 0.2, 0.3% was used. Sterilized filtrate of AN was used for enhancement of both cell growth parameters as well as the accumulation rate of total phenolic and peroxidase compounds in a suspension culture of Zaghlool date palm. Data presented in Table 1 clearly show that the highest value of fresh (245.4; 235.12; 223.17 and 197.8 w/v) and dry (24.12; 22.17; 20.15 and 18.2 w/v) weights were recorded with 0.1, 0.2, 0.0 and 0.3% of AN, respectively. The best results of fresh and dry weights were recorded at day 10 of cultivation, as compared with other schedule times. Furthermore, the optimum concentration of AN for achievement of different cell growth parameters was 0.1% compared with other concentrations.

Effect of methyl-jasmonate

MJ at different concentrations i.e., 0.50, 10 and 200 µM were used for scaling-up and production of mass cell cultures, as well as attainment of active compounds in suspension culture of Zaghlool date palm cultivar. Furthermore, MJ at different concentrations was incorporated with uncontrolled MS-medium containing 0.1% AN as shown in Table 2. The highest values of fresh (364.12; 331.45; 275.5; 249.75) and dry weights (39.28; 35.37; 31.05; 28.15) were recorded within fortified modified MS medium with 0, 50, 100 and 200 µM of MJ, for fresh and dry weights of cell cultures, respectively. In general these experiments clearly indicated that supplementation of uncontrolled MS liquid medium with 0.1% of AN and 100 µM of MJ enhanced mass cell production from Zaghlool

date palm cultivar using STB (Fig. 2) for 10 days, as compared with other concentrations and cultivation times.

Bioactive compounds determination

The total phenolic ng GAE/g dw and peroxidease (u/mg) compounds of lyophilized Zaghlool date palm cell cultures were determined in the previous experiments. Illustrated data in Figure 3 clearly show the effect of modified liquid MS-medium with either controlled or uncontrolled pH media or elicitation of culture medium with 0.1, 0.2 or 0.3% of AN or with 50, 100 or 200 µM of MJ, as biotic electors on attainment of total phenolic and peroxides compounds production in different cell lines of Zaghlool date palm cultivar. The highest values of total phenolic (25.35 ng GAE/g dw) and peroxides (5432 u/g) compounds were recorded with uncontrolled pH of MS-medium augmented with 0.1% of AN and 100 µM of MJ, compared to other supplementation and concentrations.

This study clearly indicates that combining different elicitors derived from the secondary metabolites process, especially total phenolic and peroxides compounds in date palm cell cultures, along with AN and MJ, play a critical role in elicitation of date palm cell cultures. Moreover, scaling-up and production of mass cell cultures and active compounds from date palm cell cultures using a serried tank bioreactor 2L was established.

Discussion

The scaling-up of mass cell cultures and maximization of bioactive products through bioreactors must be followed up. Bioreactors have two advantages over flasks for culturing plant cells. First, better control can be exerted on the system (i.e., pH and dissolved gas concentrations). Second, most bioreactors are scalable and hence better able to reproduce on a larger scale those conditions which were observed on a smaller scale to be the most desirable for culture performance. With the aim of implementing an industrial scale process, the behavior of cell culture in bioreactors has been receiving significant investigative attention (Zhong, 2001; Huang et al. 2002). However, understanding how to improve cell cultures through rational modification of the reactor environment remains a challenge.

One of the methods frequently used to increase the productivity of plant cell culture is use of so-called elicitors (Singh, 1996). Elicitors can be any type of compound that improves the production of phytoalexins (Muller, 1956; Kue, 1972). Phytoalexins are antibiotically active compounds, used by plants to resist microbial attacks (Darvill and Albersheim, 1984). Many secondary metabolites belong to the group of phytoalexins; therefore, if the correct elicitor can be found, it is possible to enhance the production of the desired secondary metabolite (Eilert, 1987). In addition, Wijnsma et al. (1985) reported that, the anthraquinons in *Cinchona ledgeriana* cell cultures were increased when the cells were treated with 0.5 mg/ml of AN as elicitor. JA activates stress response in cells in two ways: (1) JA produced at the wound site, serves as a mobile signal to activate responses in systemic tissues; (2) wound-induced production of a mobile signal other than JA that activates synthesis of the hormone in systemic tissues (Abraham and Howe, 2009). It can be concluded that setup of Zaghlool date palm cell culture in STB for 10 days in MS-liquid modified with uncontrolled pH medium achieved a maximum of different cell growth parameters compared them with controlled pH medium.

Table 1. Effect of elicitation of uncontrolled pH liquid MS-medium with different concentrations of Aspergillus niger extract on enhancement of fresh and dry weights of Zaghlool date palm suspension cultures. Cultures were carried out using serried tank bioreactor 2L for 2 weeks at 26 ºC under 16/8 daylight condition.

Days duration	MS medium* supplemented with different concentrations of Aspergillus niger (%).							
	0		0.1		0.2		0.3	
	FW	DW	FW	DW	FW	DW	FW	DW
0	61.84±3.15	8.66±1.52	95.38±3.25	9.16±1.69	64.25±2.95	8.74±1.53	59.4±2.63	8.42±1.42
5	100.5±5.22	10.82±1.79	125.63±5.93	12.63±2.48	119.6±4.87	17.3±2.23	107.6±3.52	11.2±2.15
10	223.17±7.5	20.15±2.24	245.43±8.25	24.12±3.54	235.12±7.96	22.17±2.56	197.8±5.18	18.2±2.36
15	185.3±6.13	17.42±2.15	215.37±7.61	21.7±3.15	205.14±6.85	19.63±3.15	191.6±6.45	15.87±2.05

*The pH of MS- medium was set up as uncontrolled. Each treatment was the average of 3 replicates ± Standard Error FW: Fresh weight (w/v); DW: Dry weight (w/v).

Table 2. Effect of incorporated of uncontrolled pH liquid MS-medium containing 0.1% of Aspergillus niger extract with different concentrations of methyl-jasmonate on enhancement of fresh and dry weight of Zaghlool date palm suspension cultures. Cultures was carried out using serried tank bioreactor 2L for 2 weeks at 26ºC.

Days duration	MS medium* supplemented with 0.1% of Aspergillus niger (%) and different concentrations of Methyl-Jasmonate (µM).							
	0		50		100		200	
	FW	DW	FW	DW	FW	DW	FW	DW
0	66.25±3.42	8.73±1.55	65.18±3.53	8.07±1.15	64.35±2.95	8.12±1.76	65.25±2.25	7.95±1.25
5	100.63±5.43	11.32±1.93	112.13±4.65	13.25±2.15	135.12±5.25	15.17±2.09	125.15±3.98	15.33±2.95
10	249.75±7.95	28.15±3.05	275.25±6.33	31.05±3.17	364.12±8.14	39.28±4.56	331.1±5.22	35.37±2.85
15	235.13±6.25	25.45±2.89	264.85±5.89	28.19±3.85	325.13±8.09	35.62±3.97	305.52±4.87	32.49±2.97

Each treatment was the average of 3 replicates ± Standard Error. *The pH of MS- medium was setup as uncontrolled. FW: Fresh weight (w/v); DW: Dry weight (w/v).

Relative to the results we obtained, Zabetakisa et al. (1999) mentioned that elicitation through the use of MJ increased the tropane alkaloid from *Datura stramonium* as compared with a fungal elicitor and oligoalacturonide. Also, Taha (2003) established an efficient protocol for enhancement of total alkaloids production from suspension cultures of *Atropa belladonna* using various concentrations of AN extract. He reported that the optimum augmentation of liquid MS-medium was 1 mg/l of NAA and BA and extract of AN at a concentration of 10% (~ 0.5 mg/ ml), gave the highest value for cell growth and total alkaloid accumulation in the different type of cell cultures after 10 days of cultivation.

In general, the results obtained may be due to enhancement, achievement and production of total phenolic and peroxides from cell cultures of Zaghlool date palm cultivar using advanced techniques of scaling up through bioreactors such as STB.

The highest values of total phenolic compound accumulation (25.37 ng GAE/g dw) of lyophilized date palm cell cultures was recorded with elicitation of uncontrolled MS medium with 0.1% of AN and 100 µM. The results obtained are in agreement with those of Taha et al. (2011) who reported that the maximum value of cell growth parameters and highest content of inulinase activity (0.395 u/ml) resulted from elicitation of augmented MS-medium with AN extract at the level of 0.2% in combination with MJ (150 µM), as compared with other concentrations after 2 weeks of cultivation. In addition the results obtained agree with those obtained by Del Río et al. (2003) who reported that the highest phenol levels were detected after 120 days in leaves, stems and roots (in that order) of *Olea europaea*. This is because leaves are the principal producers of phenolic compounds, the pathway of shikimic acid, which acts as the precursor of phenolic compounds, beginning in their photosynthetic cells. Several studies in different plant species have shown that various phenolic compounds are synthesized and accumulate in different leaf tissue (Del Río et al., 2000; Botía et al. 2001**)**. The present study revealed that the phenolic content increased quantitatively with the increase in age of suspension to week 3 of cultivation may be due to the hyperactivity of oxidative enzymes (Cochrane, 1994). An increase in production of phenolic compounds has been associated with a decrease in growth and a decline in protein synthesis.

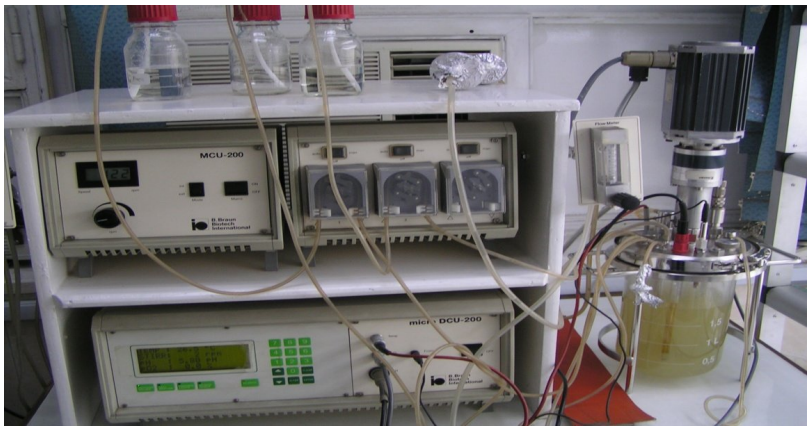

Figure 2. B-Braun Biotechnology International stirred tank bioreactor (2L).

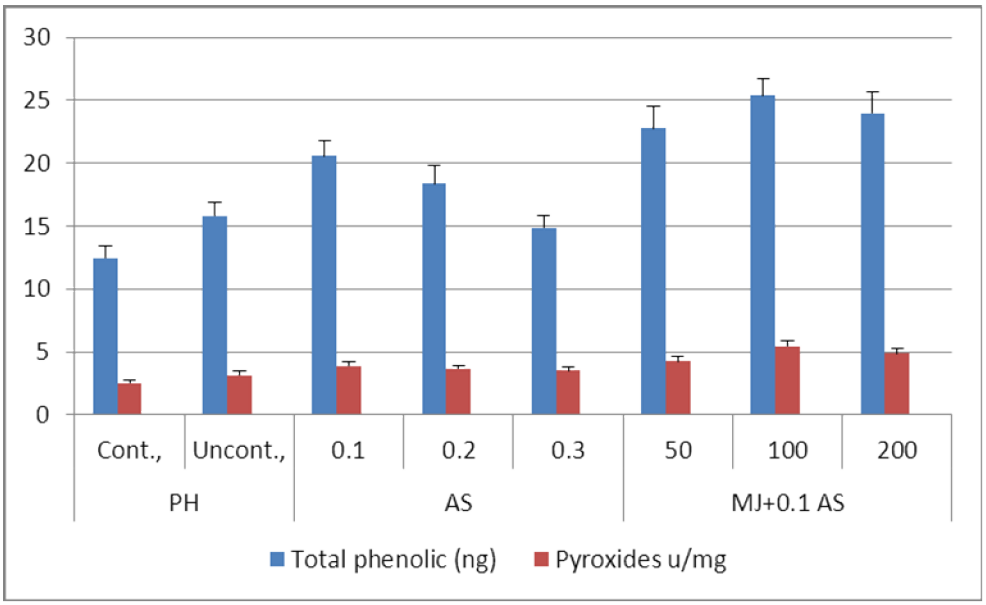

Figure 3. Effect of controlled and uncontrolled pH of MS-medium , or elicitation of uncontrolled pH liquid MS-medium with either Aspergillus niger extract (As %) or methyl-jasmonate (µM) incorporated with 0.1% of AS on accumulation of total phenolic (ng GAE/g dw) and peroxides (u/mg) of date palm lyophilized cell cultures. Cultures were carried out using STB 2L for 2 weeks at 26ºC and incubated under 16/8 of daylight condition.

Regarding, plant peroxidases, Obinger et al. (1996) reported that phenol oxidizing enzymes are widely used as markers in the plant kingdom, because of their high polymorphism. In agreement with our results, Azeqour et al. (2002) mentioned that date palm leaves contain highly active peroxidases. However since plant peroxidases are involved in many functions such as growth, vegetative development, resistance to biotic and abiotic stresses (Gonzalez-Verdejo et al., 2006; Mc Innis, 2006), the exact role of these enzymes is not yet understood in date palm. Plant cell and organ cultures grown *in vitro* usually exhibit changes in physiological and biochemical responses upon exposure to biotic and abiotic elicitors (Sircar and Mitra, 2008). Elicitation is a process of induced or enhanced synthesis of secondary metabolites by the plant cells to ensure their survival, persistence and competitiveness.

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