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ARTICLE

# A new approach for achievement of inulin accumulation in suspension cultures of Jerusalem artichoke (*Helianthus tuberosus*) using biotic elicitors

**H.S. Taha [a,*], A.M. Abd El-Kawy [b,1], M. Abd El-Kareem Fathalla [c]**

[a] *Plant Biotechnology Department, National Research Centre, Dokki, Cairo, Egypt*
[b] *Plant Department, Science Collage for Girls, Al-Azhr University, Cairo, Egypt*
[c] *Plant Resources Department, City for Scientific Research and Technology Applications, Borg El-Arab, Alexandria, Egypt*

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*Aspergillus niger*;
Methyl-Jasmonate;
Inulin accumulation

**Abstract** A promising protocol for achievement the accumulation rate of inulin compound in a suspension culture of Jerusalem artichoke (*Helianthus tuberosus*) was established. The effect of incorporated of cell cultures in combining with two type of biotic elicitors *Aspergillus niger* extract and Methyl-Jasmonate incorporation feeding medium on leaf cell growth patterns and production of inulin was investigated. The maximum value of cell growth parameters and highest content of inulinase activity (0.395 u/ml) were resulted from elicitation of augmented MS-medium with *A. niger* extract at the level of 0.2% in combination with Methyl-Jasmonate (150 μM) as compared with other concentrations after 2 weeks of cultivation. The chemical analyses of the different cell lines were spectro-photometerically performed. This study clearly indicates that combining of *A. niger* and Methyl-Jasmonate elicitors plays a critical role on inulin process and its accumulation in Jerusalem artichoke cell cultures.



* Corresponding author. Fax: +20 237607159.
E-mail address: Hussein.taha2@yahoo.com (H.S. Taha).
[1] Current address: Biology Department, Faculty of Science, Jazan University, Saudi Arabia.

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## 1. Introduction

Jerusalem artichoke Family (Asterceae) it is a perennial species known for its tubers rich in inulin, a valuable source of fructose for diabetics. It is also called the sunchoke, or earth apple [39]. In addition it is native to temperate regions of North America and has been grown in Europe since the 17th century [34]. Now it is also cultivated widely in China for it's highly adaptability and multiple tuber usability options [43]. The medicinal uses of *Helianthus tuberosus* were reported [6]. *H. tuberosus* contain many compounds, including coumarins, unsaturated fatty acids, polyacetylenic derivatives [19], and

sesquiterpenes [3]. Moreover, their extract has antimicrobial and antifungal activities which indicated significant activity *in vitro* against Ehrlich ascites carcinoma cells [2]. Moreover, it was found that crude extract of calluses; have a strong activity for hemagglutination [25]. On other hand, it was stated that *H. tuberosus* have aperient, aphrodisiac, cholagogue, diuretic, spermatogenic, stomachic, and tonic effects and has been utilized as a folk medicine for the treatment of rheumatism for the presence of inulin, which can be converted into fructose [15]. Inulins are a group of naturally occurring polysaccharides produced by many types of plants and belong to a class of fibers known as fructans [30]. Further, it suggested that the inulin extract may play a role in modulation of intestinal characteristics, blood metabolites and liver enzymes [44]. In addition it mentioned that wounds of tubercan be excrete the bioactive metabolites resulted a good resistance to some human tumor cell lines and especially the human mammary tumor cells MDA-MB-231 [12].

Plants cell and tissue culture systems represent a potential renewable source of valuable medicinal compounds flavors, fragrances and colorants, which cannot be produced by microbial or chemical synthesis. Further, suspension cultures could be used for the large-scale production of plant cells from which biologically active agents can be extracting [5]. Moreover, suspension culture is often an effective system to study the biological significance of bioactive metabolites under *in vitro* conditions, as well as for producing natural products for bio-processing applications [18]. Further, plant cell culture has been viewed as a promising alternative to whole plant extraction for obtaining valuable compounds.

Elicitors are molecules that stimulate defense or stress-induced responses in plants [29]. However the broader definition of elicitors is include both substances of pathogen origin and compounds released from plants by the action of pathogen (endogenous elicitors). Further, the nature elicitors can be divided into two types; biotic and abiotic. The biotic elicitors have biological origin, derived from the pathogen or from the plant itself while abiotic elicitors haven't a biological origin and are grouped in physical factors and chemical compounds [17]. Moreover, it had been reported that, using combined elicitor treatment of an *Aspergillus niger* mycelium and tetramethyl ammonium bromide with *Catharanthus roseus* (L.) Don cell cultures, enhanced the accumulation of ajmalicine content as compared with control medium [47].

On other hand, it was reported that jasmonic acid (JA) and Methyl-Jasmonate (MeJA) are plant hormones involved in chemical and physiological defense responses [11]. Moreover, it was stated that JA and MeJA 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 [4]. Elicitation or stress stimulus leads to a rapid release of α-linolenic acid from the lipid pool of the plant cell which through an intracellular signal cascade elicits secondary metabolite production important for plant defense [20]. 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 reeducates and three rounds of β-oxidation [22,21].

The main objective of this comparative study was too highlighted on the effect of different concentrations among of *A. niger* and Methyl-Jasmonate as biotic elicitors on leaf cell growth parameters and, achieves the accumulation rate of inulin compound in suspension cultures of Jerusalem artichoke (*H. tuberosus*).

## 2. Materials and methods

### 2.1. Plant materials

Tubers of Jerusalem artichoke (*H. tuberosus* L.) were secured from the Centre of Agriculture Research, Giza, Egypt, and used as plant materials. Then they were carefully cleaned with sop and tap water and kept under dark conditions. After few days, the obtained sprouting tubers were surface sterilized by immersion in 70% ethanol for 10 s, followed by three washes using sterile distilled water, then immersed in 50% of commercial Clorox solution containing a drop of Twin 20 for 15 min. The sprouts were subsequently rinsed several times with sterile distilled water. These sprouts were then cultured aseptically on basal solid MS-medium [24]. Cultures were solidified using 0.7% agar which was added prior autoclaving at 1.2 $kg/cm^2$ for 15 min. The pH of the medium was adjusted to 5.8 by addition of 0.1 N HCL or 0.1 N KOH. The cultivation was done in 300 ml glass jars containing 50 ml of basal MS-medium, i.e., hormones free. In a few days segment of leaves were excised from the obtained shootlets and used as a source material for callus production.

### 2.2. Callus production

Calli were produced from leaf explants on MS medium supplemented by 1 mg/l each of Naphthalene acetic acid (NAA) and 6-benzylamino purine (BAP) according the best results obtained by Taha et al. [38]

### 2.3. Cell cultures induction

The obtained calli from leaves explants were re-suspended according to the described method in an agitated liquid MS-medium supplemented with 1 mg/l NAA + 1 mg/l BA [40].

### 2.4. Effect of two types of biotic elicitors at different concentrations on enhancement of leaf cell growth patterns

#### 2.4.1. Fungus elicitor preparation

The fungus *A. niger* was obtained from The Department of Plant Pathology of the National Research Centre. *A. niger* was grown in malt extract (20 g/l) in shake flask (1000 ml) with 200 ml medium on a rotary shaker (120 rpm) at room temperature. After 7 days the cell suspension was autoclaved, and filtrated (on Whatman no. 1) filter paper. The mycelium was washed several times with sterilized distilled water and suspended in 100 ml water. This mixture was homogenized, autoclaved again and measured through the (P.C.V.) and used without purification.

#### 2.4.2. Effect of *A. niger*

In this experiment, the following concentrations 0%, 0.1%, 0.2% and 0.3% of suspended *A. niger* (P.C.V) were added to the culture media

#### 2.4.3. Effect of Methyl-Jasmonate

In this experiment, the following concentrations 0.1, 0.15 and 0.2 mM of Methyl-Jasmonate were used.

*2.4.4. Measurement of cell cultures growth parameters*

1-Fresh weight (g/250 ml flask)
2-Dry weight (g/250 ml flask)
3-Dry matter content (%)

*2.5. Determination of inulinase activity*

Determination of inulinase activity had been done through the following steps:

*2.5.1. Determination of total carbohydrate*
The total carbohydrate was determined according the described method [13]. Assay of inulinase activity. The inulinase activity was tested with the inulin authentic (Sigma, MW 4000) [28]. The reduce carbohydrates was analyzed by the 3,5-dinitrosalicylic acid [7]. One unit of inulinase activity was defined as the amount of enzyme that liberate 1 µmol of fructose equivalent from inulin/min

The design of all experiments was completely randomized and the obtained data were statistically analyzed using standers error (SE) according to the method described by Snedecor and Cochran [35].

## 3. Results

*3.1. Effect of two types of biotic elicitors on growth parameters of **H. tuberosus** leaf cell cultures*

Leaf explants of *H. tuberosus* were cultured on MS medium supplemented with 1 mg/l each of BA and NAA, the best medium for callus production as described by Taha et al. [38] as shown in Fig. 1(A). Friable calli were saved to obtain leaf suspension cultures as shown in Fig. 1(B). Concerning the effect of *A. niger* (AS) *as* biotic elicitor on growth parameters of *H. tuberosus* leaf cell cultures, it was found that as shown in Fig. 2 the highest values of cell fresh weight 7.58 (g/flask) and dry weight 0.68 were recorded with augmentation of modified MS medium containing 1 mg/l each of BA and NAA with 0.2 (%) of AS compared with other concentrations. However, the maximum values of cell growth parameters 8.45 and 0.75 (g/flask) were recorded with fortified of MS medium with 0.15 mM of Methyl-Jasmonate (MJ) for fresh and dry weights, respectively. However in contrast the highest percentage of dry matter contents 9.29 and 9.06 were recorded with MS medium supplemented with 0.3% of AS or 0.1% of MJ, respectively.

*3.2. Inulinase activity*

Suspension derived calli of leaf explants were subjected to determination of inulinase activity as indicator for enhancement of inulin accumulation. Further, powder of Jerusalem artichoke tubers was used as comparative study for inulinase activity whereas the inulinase activity was (14.95 u/ml). Data Illustrated in Fig. 3 clearly show that the highest value of inulinase activity produced from treated of MS-medium with different concentrations of AS (0.225 u/ml) was produced from augmented of MS medium with 0.2% of AS. However it was recorded 0.286 (u/ml) from supplemented of modified MS medium with 0.15 mM of MJ.

*3.3. Effect of incorporated of 0.2% of AS with 0.15mM of MJ on leaf cell growth parameters*

The previously best results clearly indicated that supplementation of modified MS medium with 0.2% of AS or 0.15 mM of MJ gave the best result of leaf cell growth parameters. Further, in this experiment the AS at the concentration of 0.2% and 0.15 mM of MJ were incorporated together to maximization of cell growth parameters. As shown in Fig. 4 the combination of both biotic elicitors; AS at 0.2% and MJ at 0.15 mM with modified MS medium enhanced of cell fresh, dry weights and dry matter contents compared with untreated cell suspension. Further, modified MS-medium recorded 10.13, 1.05 (g/flask) and 9.65 (%) for fresh, dry weights and dry matter contents, respectively. However, those recorded 4.13, 0.35 (g/flask) and 8.35 (%) with the untreated MS-medium.

*3.4. Effect of combination of 0.2% of AS with 0.15 mM of MJ on inulinase activity (u/ml) of leaf suspension*

Concerning the effect of these combinations (AS at the level of 0.2% and MJ at the level of 0.15 mM) on enhancement of inulinase activity it was found that the incorporated of these two biotic elicitors achieved of inulinase activity to about three folds (0.395 u/ml) compared with the untreated MS medium (0.113 u/ml) at the 15th day of cultivation (Fig. 5). However these concentrations were reduced consequently may be that due to the degradation of inulin concentrations.

## 4. Discussion

One of the methods frequently used to increase the productivity of plant cell culture is use of so-called elicitors [33]. Elicitors can

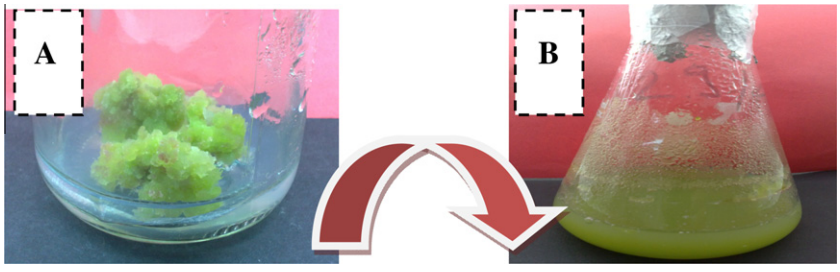


**Figure 1** (A) Leaf calli of *H. tuberosus* derived from culturing of leaf explants on MS-medium supplemented with 1 mg/l each of BAP and NAA, (B) leaf suspension cultures.

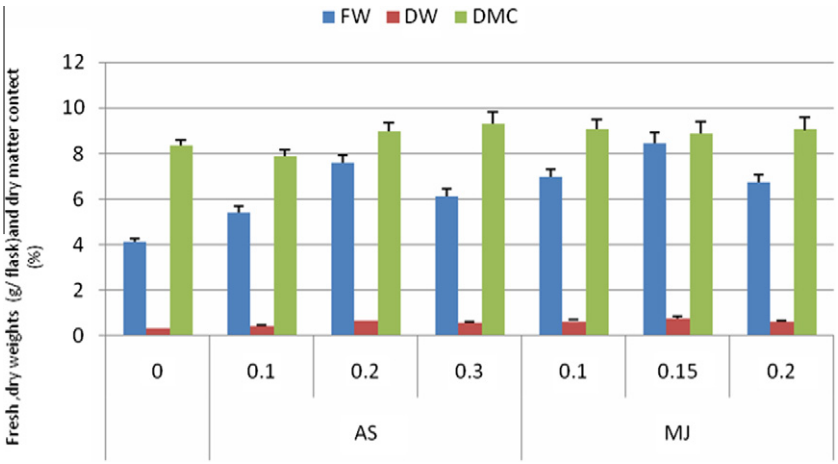


**Figure 2** Effect of different concentrations 0.1%, 0.2% and 0.3% of *A. niger* or 0.1, 0.15, 0.2 mM of Methyl-Jasmonate augmented to MS medium containing 1 mg/l each of BA and NAA on fresh, dry weights (g/flask) and dry matter content (%) of *H. tuberosus* leaf cell cultures.

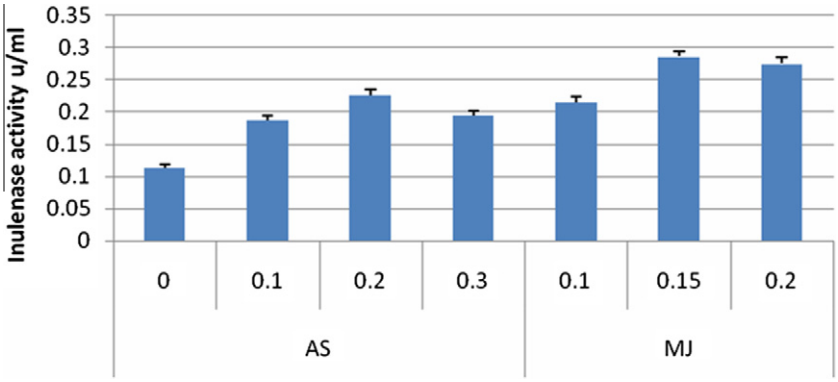


**Figure 3** Inulinase activity (u/ml) of leaf suspension cultures of Jerusalem artichoke induced from MS-medium supplemented with 1 mg/l each of BA and NAA and fortified with 0.1%, 0.2% and 0.3% of *A. niger* or with 0.1, 0.15, 0.2 mM of Methyl-Jasmonate.

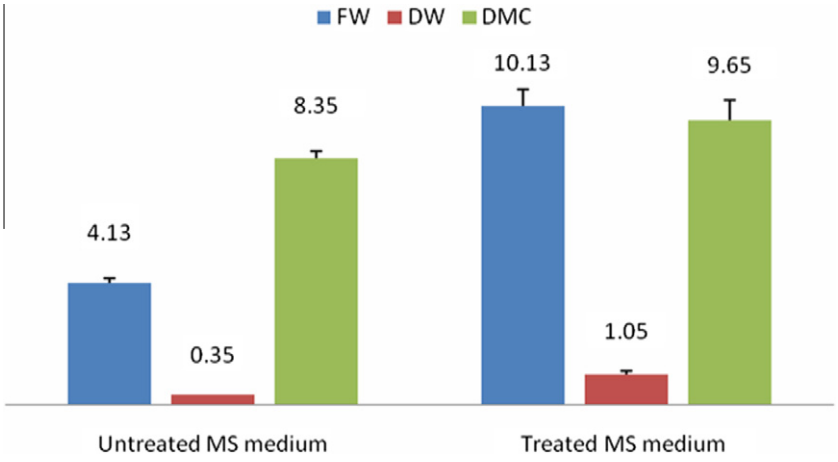


**Figure 4** Effect of treated MS medium with 0.2% of AS in combination with 0.15 mM of Methyl-Jasmonate on fresh, dry weights (g/flask), dry matter content (%) compared with untreated MS medium (control) of leaf suspension cultures of *H. tuberosus*.

be all types of compounds, that provoke (the increase of) the production of phytoalexins [23,16]. Phytoalexins are antibiotically active compounds, and by that important factors in the resistance of plants to microbial attack [8,9]. Many secondary metabolites belong to the group of phytoalexins. So, if the right elicitor can be found, it is possible to enhance the production of

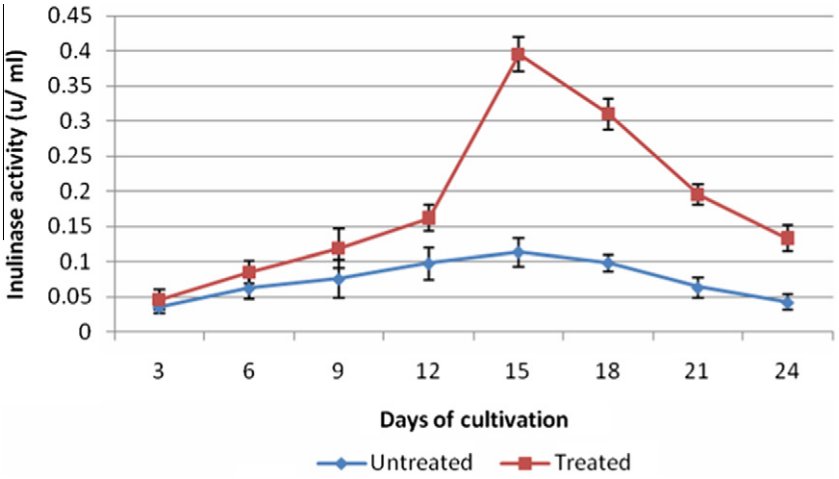


**Figure 5** Effect of treated MS medium with 0.2% of AS in combination with 0.15 mM of Methyl-Jasmonate on inulinase activity (u/ml) compared with untreated MS medium (control) of *H. tuberosus* leaf suspension cultures.

the desired secondary metabolite [10]. In addition, it was reported that the anthraquinons in *Cinchona ledgeriana* cell cultures were increased, when the cells were treated with 0.5 mg/ml of *A. niger* as elicitor [42]. JA actives stress response in cell by 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 activates synthesis of the hormone in systemic tissues [1].

There is a few literature discussed the inulinase activity in suspension cultures of *H. tuberosus*. Hase [14] reported that ATPase activity and the level of a polypeptide with a molecular weight of 97 KDa had increased more than 3.5 fold in calli derived from tuber tissue disks of Jerusalem artichoke when cultured onto MS-medium containing auxin. Moreover, it was reported that $H^+$ translocation activity of tonoplast vesicles increased about 8 fold in Jerusalem artichoke tuber calli cultures after 3 days of cultivation in the presence of 2,4-D in the culture medium [41].

Furthermore and in close of the obtained results Zabetakisa et al. [46] mentioned that elicitation through MJ increased tropane alkaloid from *Datura sramonium* more in comparison with fungal elicitor and oligoalacturonide. As well as Taha [37] established an efficient protocol for enhancement of total alkaloids production from suspension cultures of *A. belladonna* L. using various concentrations of *A. niger*. He reported that the optimum augmentation of liquid MS-medium was 1 mg/l of NAA and BA and extract of *A. niger* at the 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 following 10 days of cultivation. Moreover and in same direction, many researchers reported that MJ and salicialic acid proved to be more efficient to enhance yield of secondary metabolite *viz* hyoscyamine alkaloid ~1200% in *Brugmansia candida* [36] alkamide production in *Echinacea purpurea*, *Echinacea pallida* and *Echinacea angustifolia* [31], phytoestrogenic isoflavones in *Psoralea corylifolia* L. [32], ginsenoside in *Panax ginseng* [45,27] etc. As well as it reported that spectacular was increased in yield of anthraquinone content along with high lucidin primveroside, ruberithic acid and pseudopurpurin in the cell suspensions of *Rubia tinctorum* elicited by fungal polysaccharides [26].

In general, the obtained results may be due to enhancement and achievement of scaling up and inulin production from different Jerusalem artichoke cell lines through bioreactors.

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