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Effect of different plant growth regulators and elicitors on the production of cucurbitacins in *Ecballium Elaterium* callus

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Abstract

The effect of different growth regulators and elicitors on the *in vitro* production of its main constituents; cucurbitacins E and I, in the callus of *Ecballium elaterium* growing in Egypt was investigated. The suspension culture was supplemented with biotic stress substance like chitosan, abiotic elicitors like acetic acid, citric acid and salicylic acid and drought stress agents like mannitol. The accumulation of cucurbitacins E and I in callus and media were analyzed using high performance liquid chromatography. The highest content of Cu E (3.6 mg/g DW) and Cu I (1.3 mg/g DW) were achieved when the media supplemented with a combination of 5.0 mg/L NAA + 1.0 mg/L BA and 0.1 mg/L NAA +1.0 mg/L BA, respectively. The use of chitosan (1g/L) was found to give the highest concentration of cucurbitacin E (2.6 mg % w/w) in the media, while the use of 0.5 mM of salicylic acid yielded 1.3 mg/g dry weight of cucurbitacin E in the callus. On the other hand; the highest concentration of cucurbitacin I released to the media (2.4 mg % w/w) and in the callus (0.5 mg/g DW) was obtained by using mannitol 20% as elicitor. Hereby we report the studying of the effects of different plant growth regulators and elicitors on the production of cucurbitacins in *E. elaterium* plant. Elicitation studies have a promising results in increasing yields of the secondary metabolites along with decreasing the production costs.

1. Introduction

Ecballium elaterium (L.) A. Rich. (squirting cucumber) is an important perennial herb of the family *Cucurbitaceae* indigenous to the Mediterranean region. The plant has a long history of use as analgesic, anti-inflammatory, antiphlogestic and cathartic agent. Most of the activities are attributed to the presence of cucurbitacins, a group of tetracyclic triterpenes. Anticancer and anti-inflammatory activities of cucurbitacins from *Cucurbita andreana* were reported and cucurbitacins E and I were found to inhibit cyclooxygenase (COX)-2 enzymes with no effect on COX-1 enzymes [1]. Also, cucurbitacin E was found to inhibit tumor angiogenesis through VEGFR2-mediated Jak2-STAT3 signaling pathway [2], and cucurbitacin I showed significant inhibition of HIV replication in H9 lymphocytes [3]. This diverse group of compounds may prove to be important lead molecules for future research. Using elicitation factors such as drought stress or the change of growth regulators levels in the culture medium are seen to be an effective strategy to enhance the production of secondary metabolites such as terpenoids, alkaloids, flavonoids, phytoalexins and phenolic compounds [4, 5].

The present study was carried out to investigate the effect of different growth regulators as well as biotic and abiotic elicitors on calli formation from different explants of *E. elaterium* and production of cucurbitacins E and I in calli cultures and in the media.

2. Material and Methods

2.1 Plant material

The seeds were obtained from immature fruits of *E. elaterium* (L.) A. Rich. growing at north Sinai, Al-arish, el-sheikh Zowaid, Egypt in July 2008. Identity of the seeds was confirmed by Professor Souad el-Gengeihi, Department of Medicinal and Aromatic Plants, National Research Centre, Egypt. A voucher specimen (number 2012.050) was deposited at the herbarium of Faculty of Pharmacy, Cairo University, Egypt.

2.2 Materials and reagents

The Murashige and Skoog (MS) medium was purchased from Duchefa Biochemie (Netherlands). The plant growth regulators; benzyladenine (BA) and naphthalene acetic acid (NAA), chitosan, salicylic acid, and mannitol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Authentic samples of cucurbitacins E and I were purchased from Carl Roth GmbH and Co KG. (Karlsruhe, Germany). Gelrite was purchased from Alliance bio (Bothell, WA, USA). Methanol and acetonitrile used were of HPLC grade purchased from Sigma-Aldrich Co. LLC (Bellefonte, PA, 16823, USA).

2.3 Instruments

A High Performance Liquid Chromatograph (HPLC) Agilent 1200 series (Waldbronn, Germany) equipped with a G1311A quaternary pump, a G1329A degasser, G1316A Columm Compartment, a Diiodo Array Detector G1315B DAD, a Zorbax ODS column (5 µm 4.6× 150 mm, USA), gyratory shaker: 311 DS (Labnet international, inc, Germany), Rotary evaporator: heidolph, Germany were used in the present study.

2.4 Media Preparation with different growth regulators

Different concentrations of the growth regulators BA and NAA (in 0.5 % of 0.1N KOH) were prepared and added to MS medium to give five different media; **M1** (5 mg/l NAA), **M2** (5 mg/l NAA + 0.1 mg/l BA), **M3** (NAA 5 mg/l + 1 mg/l BA), **M4** (0.1 mg/l NAA+ 1 mg/l BA), **M5** (0.1 mg/l NAA + 5 mg/l BA). The media were sterilized by autoclaving at 121^{o}C for 15 min.

2.5 In vitro seeds sterilization and germination

Seeds of *E. elaterium* were decoated under sterile conditions. The decoated seeds were sterilized by immersion in 70% ethanol for 30 sec, and then in 20% NaOCl solution for 30 min. Seeds were then rinsed thrice with sterile distilled water and germinated on MS medium containing 3% sucrose and 4.4 g/l basal MS salts without growth regulators for 48 hours in darkness. All cultures were then incubated at 27±1C° for 4 weeks under a 16/8 light/dark cycle using cooling white fluorescent lamp (2000 Lux).

2.6 Callus induction

Different explants *viz.* leaves, stems and roots were excised from the *in vitro* raised 15 days old seedlings and were used as the source material for callus induction. About 3 segments; 2.3 mm per jar were cultured on full strength solidified MS medium which prepared as described by Murashige and Skoog [6] and supplemented with different combinations of growth regulators to give 5 media; M1 to M5. All cultures were incubated in the growth chamber at 27±1 ºC under a 16/8 light/dark cycle at light intensity 2000 Lux, produced from cooling white fluorescent lamps. After 4 weeks of culturing, percentage of callus formation was calculated. The type and concentration of growth regulators that give the highest and healthy callus proliferation from different explants were determined. Percentage of callus formation was calculated from the following equation:
Number of explants produced callus / Number of all cultured explants × 100.

2.7 Regeneration via organogenesis from apical shoot tips

In this experiment, the two week-old seedlings of *E. elaterium* were used as source of apical meristem-explants because it has the highest efficiency on direct regeneration when compared with other explants. The cultivation of apical-shoot tip explants was done on the five different media (M1, M2, M3, M4 and M5) which were prepared as described above.

2.8 Effect of some growth regulators on production of cucurbitacin E and I

Different concentrations of growth regulators BA and NAA as described above were used to give 5 different media (M1 to M5) in order to investigate their effects on callus formation and accumulation of cucurbitacins E and I in calli cultures of *E. elaterium* initiated from leaf, stem and root explants. The calli obtained from different treatments were dried and extracted with a mixture of chloroform-ethanol-methanol (1:1:1), the extraction details and the contents of cucurbitacin E and I were analyzed by HPLC technique as mentioned below.

2.9 Dry weight determination

Dry weights of *in vitro* calli cultures were determined after drying the samples at 50ºC for 3 days in the oven till constant weight. The dried samples were grounded and the fine powder was stored in brown bottles till used. Furthermore, the liquid medium was dried using rotary evaporator.

2.10 Effect of some precursors on enhancement and accumulation of cucurbitacin E and I

In this experiment, 3 g of fresh callus as transferred to 250 ml Erlenmeyer flasks containing 100 ml of liquid MS medium supplemented with 1 mg/l BA + 5 mg/l NAA (M3). The incubation was carried out at 27±1°C; in gyratory shaker at 100 rpm with a 16/8 light/dark cycle using cool white fluorescent lamps at the light intensity of 2000 Lux. The experiment was done with each of the following elicitors at three different concentrations as described below. All the experiments were analyzed in triplicate.

2.10.1 Effect of chitosan

Chitosan solution was prepared according to the method described by Alvarez-Pitta *et al.* [6] by dissolving it in 1 % v/v acetic acid. The pH was adjusted to 5.5 with 1 N NaOH and the solution was sterilized by autoclaving at 120ºC for 25 minutes. Aliquots of concentrated chitosan solution were added at the following concentrations: 0, 10, 100, and 1000 mg/l to liquid MS medium containing stem suspension cultures of *E. elaterium*. The callus was exposed for a period of 48 hours.

2.10.2 Effect of salicylic, acetic and citric acids.

Solutions of each acid were prepared according to Alvarez-Pitta *et al.* [7] and Hernandez *et al.* [8] by dissolving acids in distilled water and the pH was adjusted to 5.5 using 1N NaOH. Solutions of acetic and citric acids were sterilized using 0.22 µM filter membrane, however salicylic acid solution was sterilized by autoclaving at 120ºC for 20 minutes. Aliquots from the prepared acetic, citric and salicylic acid solutions were added to MS medium containing 5 g fresh stem calli of *E. elaterium* and transferred to 250 ml Erlenmeyer flasks containing 100 ml of M3 medium in final concentrations of 0, 0.16, 1.60 and 16.00 mM in case of acetic and citric acids .However, with the salicylic acid, the final concentrations were 0, 0.5, 10 and 20 mM. For acetic and citric acid, the callus was exposed for 48 hours, however, in case of salicylic acid; it was exposed for 72 hours. The incubation was carried out at a temperature of 27ºC, in gyratory shaker at 100 rpm with a 16/8 light/dark cycle using cooling white fluorescent lamps at a light intensity of 2000 Lux.

2.10.3 Effect of mannitol

Mannitol at different concentrations (0, 1%, 2%, and 3%) was added to MS medium supplemented with 1 mg/l BA + 5 mg/l NAA. Then the MS medium was sterilized by autoclaving at 120 ºC for 20 minutes. Five grams of stem calli were inoculated in the fresh medium. The callus was exposed for a period of 20 days. The incubation was carried out at a temperature of 27±1 Cº, in gyratory shaker at 100 rpm with a 16/8 light/dark cycle using cooling white fluorescent lamps at a light intensity of 2000 Lux.

2.11 Qualitative and quantitative determination of cucurbitacin E and I using HPLC

2.11.1 Extraction of cucurbitacins

Extraction of cucurbitacins from the dried samples was carried out according to the method prescribed by Greige *et al.* 2007 [9]. The dried material; the callus or the medium was extracted with a mixture of chloroform-ethanol-methanol (1:1:1) at 60ºC for 2 hours and filtered. The obtained filtrate was centrifuged for 15 minutes at 10,000 rpm at 4°C. The supernatant were collected, evaporated under *vacuum* and subjected to HPLC under the condition described below.

2.11.2 HPLC Condition

High performance liquid chromatograph (HPLC) coupled with DAD was used for qualitative and quantitative determination of cucurbitacin E and I in the extract of each sample using a Zorbax ODS column (5 µm 4.6× 150 mm, USA) and a gradient elution system for a mobile phase consists of 2% acetic acid in water (solvent A) and acetonitrile (solvent B). The gradient elution program was as follows: from 0-3 minutes with 90% solvent A; 3-5 minutes from 90% to 80% solvent A; 5-10 minutes from 80% to 60% solvent A; and from 10-40 minutes with 60% solvent A. Flow rate of the mobile phase was 1mL/min, sample injection volume was set to 20 µL and column temperature was fixed at 30°C. The detector was set at 229 nm for the quantification of cucurbitacin E and cucurbitacin I. Both compounds were identified and quantified by comparison of their retention times and UV spectra with those of authentic samples.

2.11.3 Qualitative and quantitative determination of cucurbitacin E and I

A standard calibration curve of five different injection volumes of each standard at a concentration of 1mg/ml (5, 10, 30, 60, 100 µl) were plotted. The extract of each sample was dissolved in acetonitrile-water (1:1) and analyzed by HPLC. Cucurbitacins E and I were detected using a DAD detector at λ 229. Retention time (t_R) for cucurbitacin E was 7.2 min and 2.7 min for cucurbitacin I at 229 nm [10].The concentrations of cucurbitacins E

and I in each extract were calculated using the regression equation of its peak area to the peak area of a known concentration of standard from the calibration curve. The results were expressed as milligrams per g of dry weight (mg/g callus or media). Concentration of cucurbitacin E or I in each injected sample = RF x A; where RF = response factor and A is the peak area [11].
Regression equation (Y= ax + b) for cucurbitacin E: Y= 1308.8X + 9370.6, R2= 0.9754, and for cucurbitacin I: Y= 1562.5X + 2878.7, R2= 0.9754

2.12 Statistical analysis
All data were expressed as the mean± standard deviation. Data were subjected to analysis of variance ANOVA to test the significance in all experiments. The least significant difference (LSD) at P= 0.05 level was calculated using SPSS 10.0 for Windows package according to the statistical analysis method described by [12]. The smallest p value that should be expressed is $p < 0.001$ and the largest p value that should be expressed is $p > 0.99$

3. Results and discussion

3.1 Effect of different growth regulators on the production of calli cultures from different explants of E. elaterium

3.1.1 Callus induction:
The germination percentage reached about 40% and the percentage of callus formation was scored after four weeks of cultivation on MS medium supplemented with different combinations of growth regulators (as previously mentioned). The frequency of callus formation varied from 20% to 100% and differed significantly among different explants and different combinations of growth regulators (Figure 1).

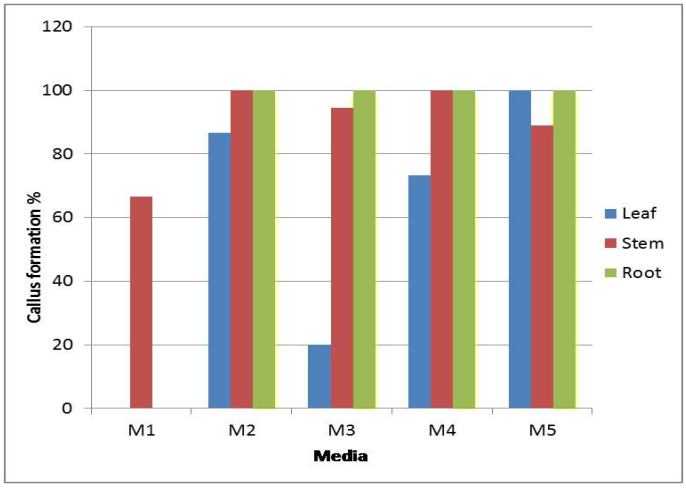

Figure 1: Percentage of the callus formation of leaf, stem, and root explants after four weeks of culturing on MS media supplemented with different combinations of growth regulators; **M1**: 5 mg/l NAA, **M2**: 5 mg/l NAA + 0.1 mg/l BA, **M3**: 5 mg/l NAA + 1 mg/l BA, M4: 0.1 mg/l NAA + 1 mg/l BA, and **M5**: 0.1 mg/l NAA + 5 mg/l BA.

Concerning calli culture derived from stem explants all the tested media; M1 to M5 were effective in inducing greenish yellow friable calli over the entire explants, while M2 and M4 showed the best callus biomass. In case of calli culture derived from leaf explants M2 and M5 gave the best callus biomass and the color was variable from greenish yellow to green (Figure 2). In case of calli culture derived from root explants, M2 and M3 gave pale yellow friable calli, however, M1 failed to induce callus from leaf and root explants. Based on the above mentioned findings, M2 and M5 media were found to be the best among all tested media, giving healthy and rapidly growing callus proliferation for all type of explants.
Media M5 showed the highest mean of callus formation followed by M2, and maximum callus percentage was observed with stem followed by root and leaf explants.

3.1.2 Regeneration via organogenesis from apical shoot tips
The highest values of regenerated shoots were recorded with M4 and M3. However, M1 and M5 gave only callus while M2 gave callus and regenerated shoots. The highest number of regenerated leaves was recorded with M4, which produced about 26 leaves per jar (Table 1, Figure 2D and 2E).

Table 1: Effect of MS medium supplemented with different combination of growth regulators on percentage of direct regeneration from apical shoot explants of *Ecballium elaterium* after 4 weeks of cultivation under light condition 16 hour/day.

Culture media	M2	M3	M4
Regenerated shoots (%)	33.30	66.60	70
No of leaves/Jar	12	16	26

Cultural media M2, M3and M4 is a basal MS media contains: 5mg/l NAA + 0.1 mg/l BA (M2), 5mg/l NAA + 1mg/l BA (M3) and 0.1mg/l NAA + 1mg/l BA (M4). M1and M5 didn't give any regenerated shoots.

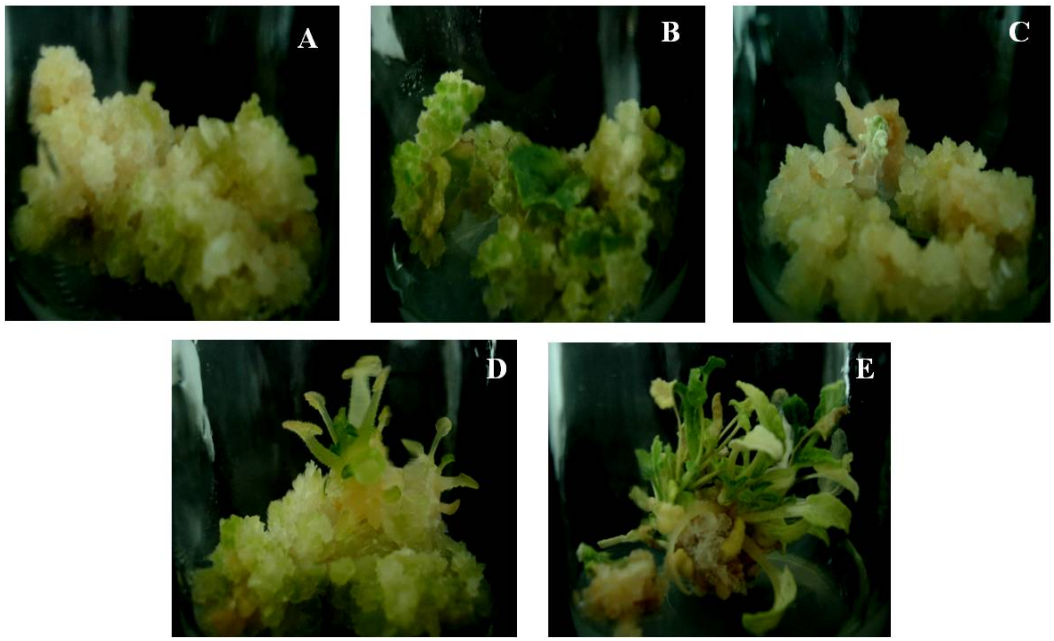

Figure 2: Photographs of calli and regenerated shoots induced from *E. elaterium* explants. A, B and C :Callus induction from *Ecballium elaterium*; stem explant on M2 medium (A), leaf explant on M5 medium (B) and root explants on M2 medium (C) after 4 weeks of culturing on selected media. D and E: Apical regenerated shoots of *E. elaterium* after 4 weeks of culturing on M2 medium (D) and on M4 medium (E). Where M2: MS medium supplemented with 5 mg/l NAA + 0.1 mg/l BA, M4: MS medium supplemented with 0.1 mg/l NAA + 1 mg/l BA and M5: MS medium containing 0.1 mg/l NAA + 5 mg/l BA.

3.2 Effect of some growth regulators on production of cucurbitacin E and I

3.2.1 In calli cultures

In this experiment, different concentrations of growth regulators BA and NAA were used in order to enhance the bioprocess of secondary metabolites production in calli cultures of *E. elaterium* initiated from leaf, stem and root explants. The production of cucurbitacins E (CU-E) and I (CU-I) in the obtained calli from different treatments was analyzed by HPLC-DAD (Figure 3).

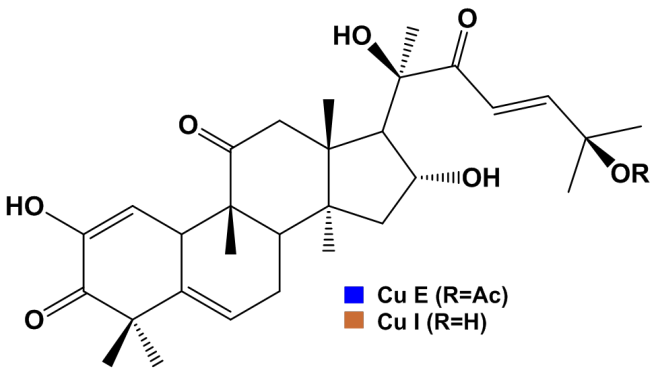

Figure 3: Chemical Structure of Cucurbitacin E (CU-E) and Cucurbitacin I (CU-I)

The highest value of cucurbitacin E (2.38 mg/g DW) was produced from calli cultures derived from root explant with M3 medium, followed by calli culture derived from leaf explant with M5 (2.04 mg/g DW) (Table 2). On the other hand, the highest value of cucurbitacin I was recorded with calli culture derived from stem explant with M1 medium (3.11 mg/g Dw), followed by calli culture derived from leaf explant with M3 and M5 media (1.44 and 1.48 mg/g DW, respectively) and calli culture derived from root explant with M3 medium (1.31 mg/g DW) (Table 2).

Table 2: Cucurbitacin E and I contents (mg/g DW) derived from calli cultures of leaf, stem and root explants of *E. elaterium* after 4 weeks of culturing on MS medium supplemented with different combinations of growth regulators

Compound	Explant	MS Culture Media				
		M1	M2	M3	M4	M5
Cucubitacin E (CU-E) (mg/g DW)	Leaf	-	1.13±0.001	1.39±0.003	1.19±0.002	2.04±0.005
	Stem	0.18±0.005	0.765±0.0005	1.44±0.005	1.105±0.005	0.18±0.001
	Root	-	0.399±0.003	2.38±0.001	0.94±0.01	0.276±0.006
Cucubitacin I (CU-I) (mg/g DW)	Leaf	-	0.17±0.0016	1.44±0.008	0.586±0.003	1.48±0.001
	Stem	3.11±0.003	-	0.48±0.006	-	0.62±0.008
	Root	-	0.38±0.0014	1.314±0.001	0.36±0.001	0.187±0.0012

The culture media M1 to M5 are basal MS media containing 5 mg/l NAA (M1), 5 mg/l NAA + 0.1 mg/l BA (M2), 5 mg/l NAA + 1 mg/l BA (M3), 0.1 mg/l NAA + 1 mg/l BA (M4), 0.1 mg/l NAA + 5 mg/l BA (M5). Data is the mean of three different experiments ± S.E.

3.2.2 In regenerated shoots

In this experiment, different concentrations of the growth regulators, NAA and BA were used in order to investigate its effect on the production of secondary metabolites in regenerated cultures of *E. elaterium* initiated from shoot apix. Extracts of the regenerated shoots from different treatments were analyzed for their contents of cucurbitacins E and I using HPLC-DAD. The highest value of cucurbitacin E was produced by using M2 medium (3.65 mg/g DW) followed by M4 (1.3mg/g DW), while the highest value of cucurbitacin I was recorded with M2 medium (0.865 mg/g DW) followed by M3 medium (0.66 mg/g DW) (Table 3).

Table 3: Cucurbitacin E and I contents (mg/g DW) derived from regenerated shoots of apical meristem after 4 weeks of culturing on MS medium supplemented with different combinations of growth regulators

Compound	M2	M3	M4
Cucurbitacin E (Cu-E)	3.650±0.001	0.085±0.003	1.320±0.001
Cucurbitacin I (CU-I)	0.390±0.005	0.663±0.005	0.865±0.006

The culture media M2 to M4 are basal MS media containing 5 mg/l NAA + 0.1 mg/l BA (M2), 5 mg/l NAA + 1 mg/l BA (M3), 0.1 mg/l NAA + 1 mg/l BA (M4). M1 and M5 didn't show any regenerated shoots. Data is the mean of three different experiments ± S.E.

Mahzabin et al. (2008) reported that multiple shoots were regenerated from shoot tips of *Cucurbita maxima* Duch. using MS medium containing NAA and BA, at various concentration [13], and the best response was obtained with BA at a concentration of 3.0 mg/l. The direct shoot regeneration from nodal segments and shoot tips from *in vitro* grown seedlings of *Momordica charantia* L. was initiated when cultured on MS medium supplemented with different concentrations and combinations of cytokinins (BA, Kin) and auxins (NAA, IAA) [14]. Bottino (1981) reported that extensive callus formation can be induced by hormonal treatments or growth regulators on tissue culture media and actively growing callus can be initiated using balance between auxins and cytokinins [15]. Cytokinins produce two direct effects on undifferentiated cells; they increase cell division and stimulate DNA synthesis, while auxins stimulate cell expansion, in particular cell elongation [16].

When compared to explants from different plant species, *E. elaterium* explants exhibited a rate of more than 5 shoots developing within 1 month especially in media containing a combination of NAA and BA [17]. Attard and Scicluna (2001) found that undifferentiated callus of *E. elaterium* in media containing 5 mg/l of NAA produced (2.970% w/w) of cucurbitacin E (CU-E) [18]. Also, supplementation of the media with a combination of NAA (0.1 mg/l) and BA (1.0 mg/l) led to the highest concentration of cucurbitacin B (1.126%) in the first subculture calli from stem nodes of *E. elaterium*, relative to that obtained from raw plant material (0.01%) [19]. From the present investigation we found that both combination and balance of NAA and BA were essential for callus formation and regeneration of shoots from calli of *E. elaterium*; highest callus formation was obtained from M2, and M5 media (both supplemented with NAA and BA in a ratio of 5.0 mg/L: 0.1 mg/L, and 0.1 mg/L: 5.0 mg/L, respectively).

On the other hand, the balance between NAA and BA significantly affected the accumulation of Cu E and CU-I in the callus; highest content of CU-E (3.6 mg/g DW) was achieved in the presence of a combination of (5.0 mg/L NAA+ 1.0 mg/L BA) (as in M2), and CU-I (1.3 mg/g DW) as in M4 (with a combination of 0.1 mg/L NAA +1.0 mg/L BA), respectively.

3.3 Effect of some elicitors on enhancement and accumulation of cucurbitacins E and I in different E. elaterium calli cultures

3.3.1 Effect of chitosan

In this experiment, no change in color and texture of all tested stem calli was observed by increasing the concentration of chitosan. The effect of chitosan on the production of CU-E and CU-I (expressed in mg/g DW) in callus culture and in the medium are shown in (Figure 4A). Relative to that of the control, rate of the accumulation of cucurbitacins E and I in stem calli was decreased significantly in the presence of chitosan at all tested concentrations (10, 100 and 1000 mg/l). However, in the liquid media, contents of CU-E and CU-I were significantly increased, in the presence of 1000 mg/ml chitosan, to reach 2.6 mg % and 0.8 mg %, respectively. (Figure 5A).

3.3.2 Effect of acetic acid

No change in the color and texture of all tested calli was observed by increasing acetic acid concentrations. The effect of acetic acid on the production of CU-E and CU-I in the stem calli and in the medium was shown in Figure 4B and 5B, respectively. The amount of CU-E in the stem calli was decreased markedly by elicitation with all concentrations added of acetic acid (0, 0.16, 1.60 and 16.00 mM), while the concentration of CU-I was found to be similar to that of the control when using high concentration of acetic acid; 16.00 mM (Figure 4B). However, in the liquid media the concentration of both CU-E and CU-I was markedly increased. In the presence of 1.6 and 16 mM acetic acid, the concentration of CU-I reached 0.7 mg% and 2.3 mg%, respectively, while that of CU-E reached 0.20% in the presence of 16.00 mM acetic acid (Figure 5B). We found that addition of a high concentration (16 mM) of the stress agent, acetic acid, to the medium significantly increased the weight of the callus as well as the concentration of CU-E and CU-I.

Alvarez-Pitta and Giulietti (2000) previously used MS medium supplemented with acetic acid (16 mM) to increase the content of hyoscyamine and scopolamine and to promote the release of both alkaloids into the medium [6]. Gagnon and Ibrahim (1997) reported that white lupin roots are sensitive to the presence of acetic acid and found that its addition to the media led to significant increase in the content of isoflavonoids in lupin [20].

3.3.3 Effect of citric acid

In this experiment, no change in color and texture of all tested calli was observed by increasing the concentrations of citric acid. Relative to that of the control, the presence of citric acid led to marked decrease of CU-E in the stem calli. However, the amount of CU-I was increased to reach its maximum value (0.08 mg/g DW) by using 0.16, 1.6 mM of citric acid (Figure 4C). On the other hand, the presence of citric acid in the liquid media led to increase in the concentration of CU-E and CU-I, where maximum concentration of CU-E (0.90 mg% DW) was obtained in the presence of 16 mM citric acid, while 0.30 mg% of CU-I was obtained when citric acid was added in a final concentration of 0.16 mM (Figure 5C).

3.3.4 Effect of mannitol

Mannitol has been proposed to enhance tolerance to water deficit stress primarily through osmotic adjustment. In the present experiment, different concentrations of mannitol; 10%, 20% and 30% were used as an osmotic agent. Relative to that of the control, no change in color and texture of all tested calli was observed by increasing mannitol concentrations. The amount of CU-E in stem calli was gradually increased by increasing the concentration of mannitol to reach a maximum value of 0.5 mg/g DW in the presence of 30% mannitol.

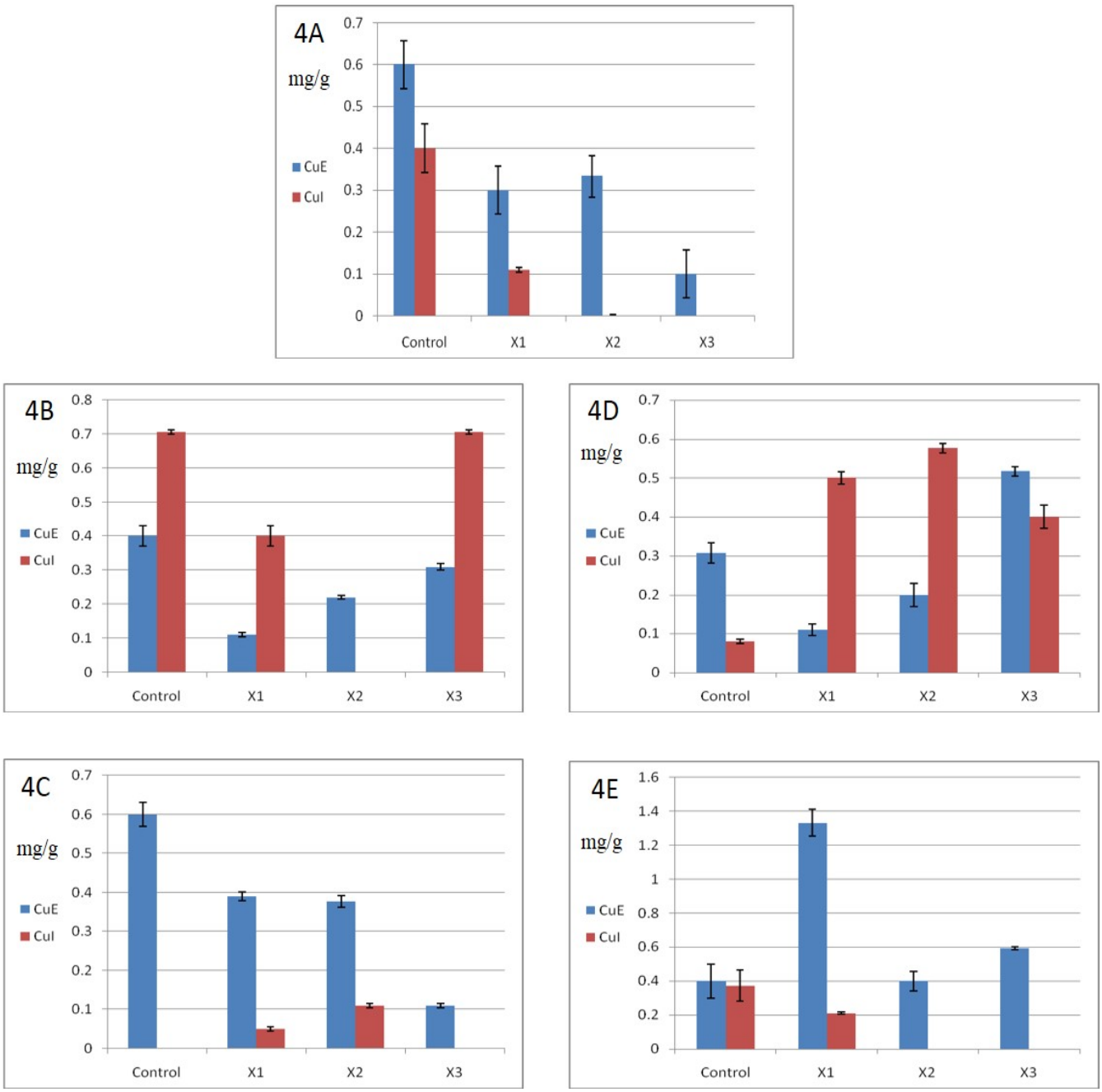

Figure 4: Effect of different elicitors in enhancement and accumulation of CU-E and CU-I in callus culture: 4A: Effect of chitosan at concentration of 10 mg/l (X1), 100 mg/l (X2), 1000 mg/l (X3). 4B: Effect of acetic acid at concentration of; 0.16 mM (X1), 1.6 mM (X2), 16 mM (X3). 4C: Effect of citric acid at concentration of 0.16 mM (X1), X2 = 1.6 mM (X2), X3 = 16 mM (X3). 4D: Effect of mannitol at concentration of 0.5 mM (X1), 10 mM (X2), 20 mM (X3). 3E: Effect of salicylic acid at concentration of 0.5 mM (X1), 10 mM (X2), 20 mM (X3).

Similarly, CU-I was increased by increasing the concentration of mannitol and reached its maximum value (0.5 mg/g DW) in the presence of 20% mannitol (Figure 4D). However, accumulation of both CU-E and CU-I in the liquid media reached their maximum values (0.42 mg% and 2.4 mg% w/w, respectively) in the presence of 20% mannitol and then decreased when using higher concentration of mannitol (30%) as shown in (Figure 5D).

We found that supplementation of the media with only low concentrations of mannitol significantly increased the weight of calli and content of CU-E and CU-I. This is in agreement with Saker *et al.* (1997) who previously reported that addition of mannitol stimulated the accumulation of tropane alkaloids in the suspension cultures of the leaves of *Hyoscyamus muticus, Datura stramonium* and *Atropa belladonna*. Maximum production of the alkaloids were obtained using mannitol concentrations of 0.027 M, 0.082 M, and 0.11 M. However, higher concentration of mannitol caused a marked decrease in the accumulation of the alkaloids in

suspension cultures [21]. Also, production of indole alkaloid in *Catharanthus roseus* cell cultures was increased 4 fold by addition of mannitol [8, 22]. Supplementation with mannitol is known to cause mild osmotic stress to cell cultures and may activate an adaptive mechanism which makes the cells tolerant to more severe treatments. Though mannitol is considered as an osmoprotectant agent and a compatible solute, its accumulation may have adverse effects and the target plant may not tolerate high levels of mannitol [23, 24].

3.3.5 Effect of salicylic acid

The amounts of CU-E and CU-I in stem calli were increased and reach its maximum values (1.3 mg/g DW and 0.2 mg/g DW, respectively) in the presence of 0.5 mM of salicylic acid (SA), but then decreased by increase of its concentration (Figure 4E). However, the amount of CU-E and CU-I in the liquid medium reached its maximum values (0.19 mg% and 2.04 mg%, respectively) in the presence of 10 mM SA (Figure 5E). SA is known as a secondary metabolism elicitor which induces a systemic acquired resistance (SAR) in plants to many pathogens [25-27] and the production of certain secondary metabolites [28-30]. SA elicitation was reported to enhance the production of diterpene alkaloid in the cell suspension cultures of genus *Taxus* [31-33]. Supplementation with SA significantly increased the weight of *E. elaterium* calli but its stimulating effect on accumulation of CU-E and CU-I was marginal at only very low concentration (0.5 mM).

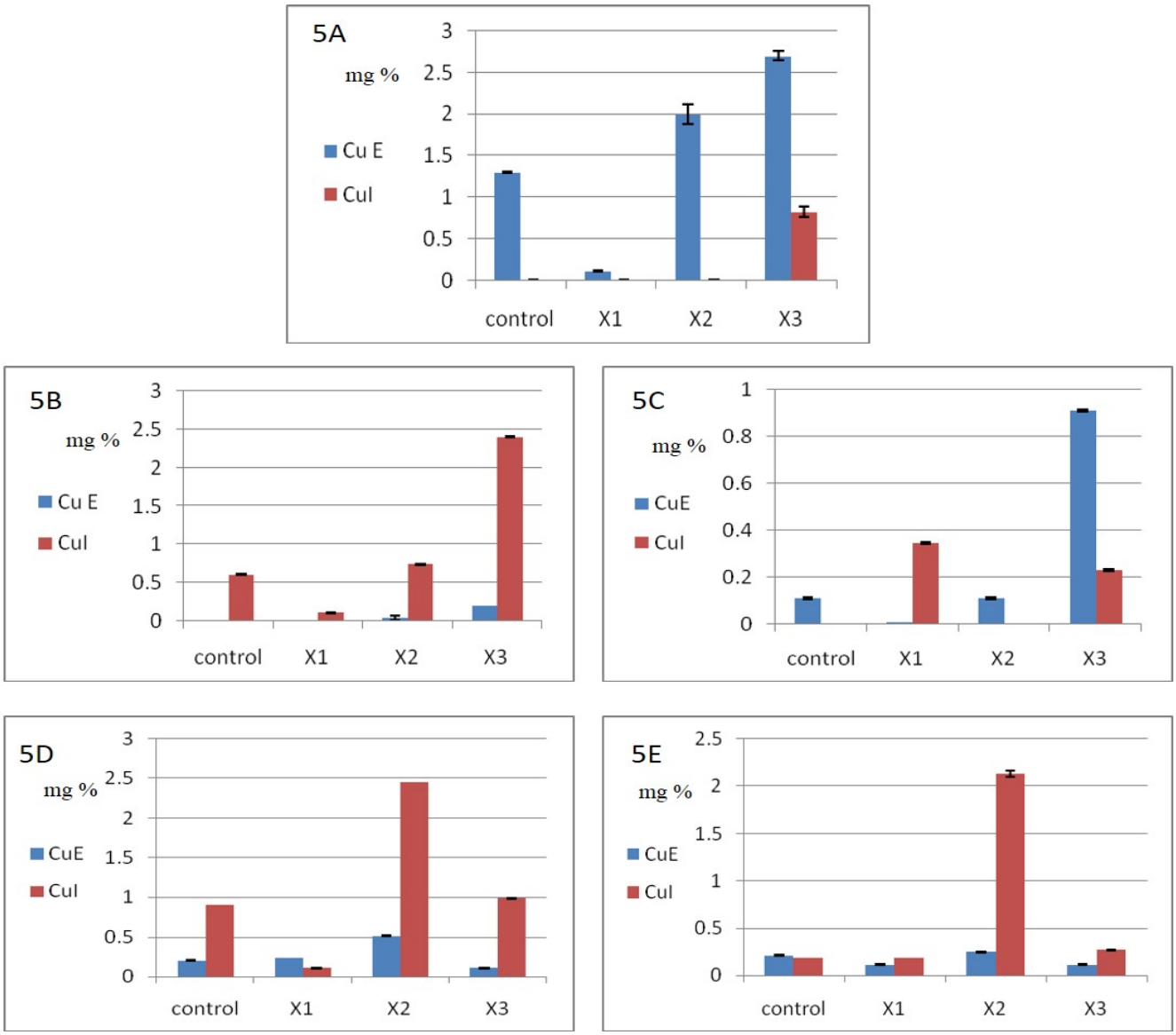

Figure 5: Effect of different elicitors in enhancement and accumulation of CU-E and CU-I in liquid medium: 5A: Effect of chitosan at concentration of 10 mg/l (X1), 100 mg/l (X2), 1000 mg/l (X3). 5B: Effect of acetic acid at concentration of; 0.16 mM (X1), 1.6 mM (X2), 16 mM (X3). 5C: Effect of citric acid at concentration of 0.16 mM (X1), X2 = 1.6 mM (X2), X3 = 16 mM (X3). 5D: Effect of mannitol at concentration of 0.5 mM (X1), 10 mM (X2), 20 mM (X3). 4E: Effect of salicylic acid at concentration of 0.5 mM (X1), 10 mM (X2), 20 mM (X3).

4. Conclusion

The present study indicated that enhancement of callus induction and accumulation of cucurbitacins E and I in *E. elaterium* callus cultures were higher than those obtained from organs from the normally grown plant and suggested that *in vitro* production of cucurbitacins using callus cultures is an appropriate approach. Supplementation of the media with plant growth regulators and elicitors at specific concentrations could significantly increase yield of cucurbitacins, decrease production costs, and has economic impact on its bio-industry. The observations that the concentration of cucurbitacins in the culture medium were higher than that in the calli cells suggest that these compounds released inside the membrane vesicles rather than stored in the cellular vacuole. Further investigation is in progress to clarify the effect of these plant growth regulators and elicitors on the biosynthesis of cucurbitacins and the intermediates.

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