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Influence of Differentiation State, Salt Stress and Methyl Jasmonate on *in Vitro* Production of Cucurbitacins from Tissue Cultures of *Ecballium elaterium* and *Cucumis prophetarum* Endemic to Egypt

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ABSTRACT

The best medium for callus proliferation from hypocotyl and cotyledon explants of aseptically grown seedlings of two Egyptian landraces of rare wild plants, *Ecballium elaterium* and *Cucumis prophetarum* was found to be Murashige and Skoog (MS) medium supplemented with 0.1-0.5 mg/l NAA in combination with BA at 2 mg/l. On the other hand, MS medium supplemented with 2 mg/l BA, 60 mg/l adenine sulfate and 170 mg/l KH_2PO_4 was the best medium for shoot proliferation after a phase of callus formation. The content of cucurbitacins in suspension cultures was elicited by application of different concentrations of NaCl (salt stress) and methyl jasmonate (MeJA). The highest yield of cucurbitacins (0.1898%) was reached after three days of culture in liquid medium containing 200 µM MeJA.

Keywords: callus culture, cucurbitacin B, cucurbitacin E, MeJA, regeneration

INTRODUCTION

The utilization of medicinal and aromatic plants as natural resources for pharmaceuticals is increasingly encouraged due to the hazardous effects of synthetic chemicals. Consequently, medicinal and aromatic plants have been a target of uncontrolled collection and over-consumption. In Egypt, these plants are also exposed to destruction by urbanization and reclamation of new lands (Saker *et al*. 2000). This has resulted in a biodiversity crisis and the loss of unredeemable genetic resources. At present, the most common method to preserve national plant resources depends on seed collections and in rare cases on whole plants remaining in the field. This strategy seems to be inefficient as whole plant collections in the field are exposed to natural disasters and attacks by pests and pathogens (Swanson 1996).

Plant cell and tissue culture is a technique in plant boiology utilized for many purposes, among which genetic and germplasm conservation and *in vitro* production of bioactive substances used in pharmaceutical industries is applied (Couceiro *et al*. 2005). Therefore, the use of plant tissue culture of wild plants as a source of natural bioactive resources could protect native germplasm from endangerment and does not affect their existence and genetic diversity (Saker *et al*. 2000).

Ecballium elaterium and *Cucumis prophetarum* are two important members of Egyptian wild cucurbit germplasm in the Mediterranean region. Their juice has been used in traditional medicines for various therapeutic treatments including hepatic conditions, sinusitis and rheumatic conditions (Ekici *et al*. 1998). The juice of the fruits and cucurbitacin B reduce carbon tetrachloride-induced hepatotoxicity as shown by the reduction in the SGPT enzyme level (Abo Setta 2002). *C. prophetarum* is a rare wild plant endemic to Egypt found in sandy plains in Saint Catherine, Egypt. The plant contains cucurbitacins in its fruits, which could be used as raw material for the production of cucurbitacins containing baits for controlling diabroticite beetles and as an antitumor drug (Rao *et al*. 1974; Arisawa *et al*. 1984; Agil *et al*. 1999). Tissue cultures of these plants open the opportunity of endless supply of active ingredients, which sometimes are in higher concentration than the parent plant (Attard and Scicluna-Spiteri 2001; Chen *et al.* 2005).

The optimization of medium composition for *in vitro* production of cucurbitacins from cell and tissue cultures of wild cucurbit germplasm is a tedious and time-consuming task involving the study of many variables. The accumulation of secondary metabolites by plant cells can be enhanced by stress factors such as biotic (pathogen homogenates) and abiotic stresses (salt, osmotic stresses and UV irradiation) (for review see Moreno *et al*. 1995). In addition to stresses, jasmonic acid (JA) and methyl jasmonate (MeJA) belong to the group of natural plant stress hormones, synthesized in response to stress, that can elicit synthesis of most active secondary metabolites (Poulev *et al*. 2003; Wang *et al*. 2004).

In this regard Attard and Scicluna-Spiteri (2001) used 5 mg/I NAA (α-naphthalene acetic acid) and 5 mg/I BAP (6-benzyl aminopurine) after using different concentrations of plant growth regulators for callus induction. The same authors used 5 mg/I NAA to optimize the yield of cucurbitacins.

The present study was conducted to establish a tissue culture system and optimize *in vitro* culture conditions affecting accumulation of cucurbitacins in cell and tissue cultures of *E. elaterium* and *C. prophetarum*.

MATERIALS AND METHODS

Plant material

E. elaterium and *C. prophetarum* were collected from their growing habitats in El-Arish and Saint Catherine mountains, respectively. The seeds of both plants were propagated at the Experimen-

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tal Farm of the Department of Medicinal and Aromatic Plants at Giza. Voucher specimens were deposited in the herbarium of the National Research Centre.

Callus induction and propagation

Seeds were surface sterilized with 70% ethanol for 2 min, followed by soaking in 10-15% sodium hypochlorite solution for 10 min and finally rinsed four times at 5-min intervals with sterile distilled water. Sterilized seeds were germinated on basal Murashige and Skoog (MS) medium (Duchefa Biochemie, Netherlands) supplemented with 3% sucrose and 7 $g1^{-1}$ agar (Alliance Bio, USA). The pH was adjusted to 5.8 with 1N KOH. The seeds were incubated in the dark at 25°C and relative humidity of about 95 ± 5% in culture jars (200 cm^3). Cotyledon explants (5 mm) were excised from three-weeks-old *in vitro*-grown seedlings. They were cultured on MS medium supplemented with various combinations of NAA and BA. Cultures were incubated at 25°C under a 16-h photoperiod (58 µmole/m^2/s) provided by tubular white fluorescent lamps (Philips 40 W LVF 6500 K) and regularly transferred to the same fresh medium at 3-week intervals to obtain stock callus cultures. Callus induction and maintenance medium was MS medium supplemented with 0.2 mg/l NAA + 2 mg/l BA. Data of callus frequency was recorded after 2 months of cultivation.

Salt stress

Proliferated calli were transferred to salt stress medium, i.e. callus induction media supplemented with different levels of NaCl, i.e. 0.0, 0.2, 0.4 and 0.6% (w/v) and grown for 4 weeks under the same conditions described before. Growth measurements and content of cucurbitacins were determined after four weeks of cultivation.

MeJA suspension culture media

Liquid MS medium (pH 5.8) contained basal MS salts (4.4 g/l) (Duchefa Biochemie), 30 g/l sucrose, 2 mg/l 2,4-dichlorophenoxy acetic acid + 1 mg/l kinetin and increasing concentrations of MeJA (Roth, Germany) (0.0, 100, 200 and 400 µM) and was inoculated with 0.1 g fresh callus of *C. prophetarum* and incubated on an incubating shaker (150 rpm, 28°C, with a 16-h photoperiod using cool white fluorescent lamps at a light intensity of 58 µmole/m^2/s for three days. Cultures were harvested daily: cells and cell aggregates were harvested, dried and cucurbitacins were extracted and measured spectrophotometrically.

Determination and identification of cucurbitacins

1. Extraction

Cucurbitacins were extracted from dried plant samples (2 g) with absolute ethanol (15 ml) for 2 h and then filtered. The filtrate was completed to 50 ml with absolute ethanol in a volumetric flask.

2. Preparation of standard curve

Cucurbitacin E (Sigma) was dissolved in absolute ethanol and serial dilutions ranging from 0.04 to 5.00 mg/ml were prepared. All samples (1 ml) were mixed with 1 ml of 2% phosphomolybdic acid in absolute ethanol. After 5 min the green colour was measured at 492 nm using a Shimadzu spectrophotometer according to the method reported by Attard and Scicluna-Spiteri (2001).

From calli and intact plants the major cucurbitacin was fractionated from the crude extract (total cucurbitacins) by TLC using different solvent systems (all v/v): benzene-ethyl acetate 85: 15, toluene-ethyl acetate 9: 1, and chloroform-acetone 8: 1 and detected with vanillin reagent. The R_f values are compiled in **Table 4**. The crystalline cucurbitacins separated from preparative TLC were subjected to spectral analysis using UV and MS techniques for structure elucidation, and compared with pure authentic cucurbitacin (Sigma). Instrumentation involved a Shimadzu UV 240 P/N 204-58000 connected with IBM computer for UV and A mass spectrometer (Finnigan SS Q 7000 Masses 50-500) for MS.

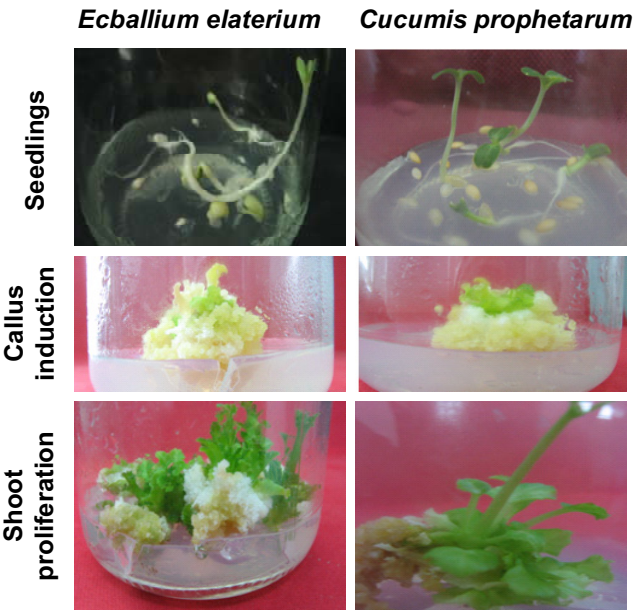

Fig. 1 Different stages of *in vitro* culture of Egyptian *Ecballium elaterium* and *Cucumis prophetarum* landraces.

Experimental design and statistics

All values were expressed as the mean of three measurements. Data were subjected to analysis of variance (ANOVA) (Snedecor and Cohchran 1967).

RESULTS AND DISCUSSION

Effect of differentiation on cucurbitacin content

Data from a pilot study indicated that MS medium containing NAA at 0.1 to 0.5 mg/l in combination with BA at 2 mg/l is the best medium for callus proliferation from hypocotyl and cotyledon explants of aseptically grown seedlings of *E. elaterium* and *C. prophetarum* plants. Stock of calli cultures were plated onto MS medium supplemented with 0.2 mg/l NAA + 2 mg/l BA. The proliferated calli were subcultured twice, at 4-week intervals, onto the same fresh medium to obtain sufficient calli from *E. elaterium* and *C. prophetarum.* To achieve shoot organogenesis from calli cultures, MS medium supplemented with 2 mg/l BA 60 mg/l adenine sulfate and 170 mg/l KH_2PO_4 was the best medium for shoot proliferation after a phase of callus formation. **Fig. 1** shows the different stages of the *in vitro* established culture system for *E. elaterium* and *C. prophetarum.*

Cucurbitacin content of different types of *in vitro* cultures at different differentiation and developmental stages is summarized in **Table 1**. The data obtained indicates clearly that freshly proliferated vigorous callus (1st subculture) of *E. elaterium* contained 3.086% total cucurbitacin and young regenerated shoots contained 3.396%. Thus, total cucurbitacin content of callus cultures of *E. elaterium* represents 144% of the content of the intact plant (3-weeks-old seedlings) and 69.3% of the intact flowering plant. Similarly, total cucurbitacin content of shoots represent 158.7 and 76.3% of intact plants at 3 weeks and flowering stages, respectively. In the case of C. *prophetarum* callus culture, the total cucurbitacin content (3.583%) represents 190 and 84.7% of intact plants at 3 weeks and flowering stages, respectively.

Effect of salt stress on cucurbitacin content

The data summarized in **Table 2** indicates that the level of salt stress applied led to a significant reduction in both growth, expressed as fresh and dry weight yield, and the

Table 1 Cucurbitacins contents of different types of *in vitro* culture.

Plant	Cucurbitacins (%)			
	Intact plant (3 week)	Intact plant (flowering)	Callus (1st subculture)	1st regenerated shoots
Ecballium elaterium	2.14	4.45	3.086	3.396
Cucumis prophetarum	1.88	4.23	3.583	0.0

Table 2 Accumulation of cucurbitacins in callus cultures of *Ecballium elaterium* and *Cucumis prophetarum* under salt stress.

NaCl (%)	*Ecballium elaterium*				*Cucumis prophetarum*			
	FW (g ± SE)	DW	Cucurbitacins (%)	Yield (mg/flask)	FW (g ± SE)	DW	Cucurbitacins (%)	Yield (mg/flask)
0.0	2.5 ± 0.7	0.18	2.46	4.40	2 ± 0.2	0.11	0.0	0.00
0.2	2.4 ± 0.1	0.19	1.79	3.40	1.9 ± 0.4	0.10	0.235	0.24
0.4	1.8 ± 0.3**	0.10	1.10	1.10	0.9 ± 0.11**	0.04	1.478	0.59
0.6	0.5 ± 0.26**	0.03	1.23	0.37	0.6 ± 0.10**	0.03	2.723	0.82
LSD 5% *	0.3				0.4			
LSD 1%**	0.5				0.5			

level of cucurbitacins in the case of *E. elaterium* callus cultures. Thus, the cucurbitacin content of calli cultures on 0.6% NaCl was about half of the content of calli growing on salt-free medium. The highest percentage of cucurbitacins (2.46%) and yield (4.4 mg), expressed as mg cucurbitacins per total yield of dry weight (flask), were recorded on salt-free medium. Both the percentage of cucurbitacins and yield decreased as the concentrations of NaCl in the culture media increased (**Table 2**). Unlike *E. elaterium*, the cucurbitacin content of *C. prophetarum* calli cultures on 0.6% NaCl was about 11-fold greater than the content of calli cultures on lower levels of salt or salt-free medium and the highest yield of cucurbitacins (0.82 mg) was recorded on 0.6% NaCl (**Table 2**).

A glance at the data presented in **Table 2** also indicates that the percentage cucurbitacins of *E. elaterium* callus cultures at high levels of NaCl (0.4 and 0.6%) is lower than that of *C. prophetarum*; however, this observation is misleading because the highest yields of cucurbitacins (4.4 and 3.4 mg/flask) were detected in *E. elaterium* callus cultures grown on salt-free and 0.2% NaCl-containing media, respectively. It could be concluded that salt stress can be applied successfully to elicit the accumulation of cucurbitacins in *C. prophetarum* callus cultures. The same strategy is not recommended for *E. elaterium* callus culture. The highest yield of cucurbitacins in *E. elaterium* (4.4 mg) is about 5-fold greater than the highest yield in *C. prophetarum* (0.82 mg).

Generally fast growing cells with active division accumulate only low levels of secondary products and an inverse relationship between the production of secondary metabolites and growth has been suggested (Lindsey *et al*. 1983). The results of the present study indicate that salt stress can be used as another strategy to increase the content of cucurbitacins in cell cultures of *C. prophetarum.* In this context, the addition of 200 mM sorbitol to cell culture medium resulted in a 63% increase in catharanthine content (Smith *et al*. 1987). Similar observations regarding the stimulating effect of salt and osmotic stress on secondary metabolites content of cell cultures *in vitro* were reported for *Catharanthus roseus* (Hernandez and Vargas 1991), for Egyptian henbane, *Datura* and *Atropa* (Saker and El-Ashal 1995; Saker *et al*. 1997) and for *Taxus chinensis* (Kim *et al*. 2001).

Effect of MeJA on cucurbitacin content

The data presented in **Table 3** indicates clearly that inclusion of MeJA in culture media of *C. prophetarum* suspension cultures at 100 and 200 µM resulted in accumulation of cucurbitacins. The highest yield of cucurbitacins (0.1898%) was reached after 3 days of culture in liquid medium containing 200 µM MeJA. This level of cucurbitacins was about 11-fold more than that of control cultures. The stimulating effect of MeJA on cucurbitacin biosynthesis was in accordance with the finding of Laskaris *et al*. (1999), who reported that 100 µM MeJA strongly increased the accumulation of taxanes in suspension cultures of *Taxus baccata*. It has been suggested that jasmonic acid and its derivatives (MeJA) could be an integral part of a general signal transduction system regulating inducible defense genes in plants. Through known mechanisms, it activates specific genes resulting in the synthesis of almost all classes of secondary metabolites (Gundlach *et al*. 1992). The positive relationship between the treatment with jasmonate and increased accumulation of secondary metabolites has been reported in the literature (Lee *et al.* 1997; Biondi *et al*. 1998; Mader 1999).

Table 3 Effect of MeJA on cucurbitacins content of *Cucumis prophetarum* suspension culture.

Conc.	1 Day	2 Day	3 Day
0	0.020	0.050	0.000
100	0.020	0.020	0.010**
200	0.050**	0.030**	0.190**
400	0.010**	0.020**	0.010**
LSD 1%**	0.014	0.014	0.006
LSD 5%**	0.010	0.010	0.005

Chromatographic investigation

Chromatographic examination of cucurbitacins from calli and the intact plants shows close similarities. Both *E. elaterium* and *C. prophetarum* contain cucurbitacin E and B as major compounds. On the other hand, *Cucumis* fruit juice and *Ecballium* fruit of intact plants contain cucurbitacin D. Cucurbitacin I was found in shoots of calli and intact plants (Toker *et al*. 2003).

To confirm the identification of the chromatographically fractionated cucurbitacins, they were subjected to an EI/MS spectrum beside UV analysis to elucidate their structures against authentics (**Table 4**).

Spectral data

Cucurbitacin E

The R_F values of this compound using the three solvent systems as mentioned before were 0.312, 0.116 and 0.712. The maximum absorption of Cucurbitacin E was $\lambda_{Max}^{Chloroform}$ 248.6. Mass spectrum of this compound shows a molecular ion at *m/z* = 539 corresponding to a molecular formula ($C_{32}H_{43}O_7$). The peaks at *m/z* = 496 (9.56%) and 479 (2.5%) were due to the loss of $CO\text{-}CH_3$ and OH group, respectively. The peaks at *m/z* = 400 and 383 may be due to loss of side chain by fission of the double bond between the carbon atoms 23 and 24. A very intense and characteristic peak, often the base peak was found at *m/z* = 96 (C_6H_8O). It is worth noting that the loss of either an OH or an acetate group and leaving isopropenyl group was also occurred in

Table 4 R_F values and colours with vanillin phosphoric acid reagent in different solvent systems.

Solvent	Isolated cucurbitacins				Authentic cucurbitacins			
	E	**B**	**I**	**D**	**E**	**B**	**I**	**D**
Benzene-ethylacetate	0.312	0.075	0.10	0.050	0.311	0.075	0.10	0.050
(85: 15 v/v)	violet	violet	brown		violet	violet	brown	
Toluene-acetone	0.116	0.023	0.012	0.034	0.116	0.023	0.012	0.034
(9: 1 v/v)	violet	violet	Reddish brown		violet	violet	Reddish brown	
Chloroform-acetone	0.712	0.026	0.20	0.085	0.710	0.025	0.20	0.085
(9: 1 v/v)	violet	violet	brown		violet	violet	brown	

compound I (Chen *et al*. 2005). The mass spectrum indicates the presence of peaks at *m/z* = 164 and 113, characteristic for $C_6H_{14}O_2$, the peak at *m/z* = 111 might be corresponding to the formula $C_6H_9O_2$. The strong peak at m/z = 96 (100%) suggests the occurrence of C25 acetyl derivative of this compound.

The aforementioned spectral data and R_F values indicate that the chemical structure of this compound is cucurbitacin E (Curtis and Meade 1971; Hatam *et al*. 1989; Stuppner and Wagner 1989).

Cucurbitacin B

The R_F values of this compound were 0.075, 0.023 and 0.026 in using the three solvent systems mentioned before. The maximum absorption of this compound was $\lambda_{Max}^{Chloroform}$ 243.0. Mass spectrum of this compound shows a molecular ion at *m/z* = 496 corresponding to molecular formula ($C_{30}H_{42}O_7$). The peaks at *m/z* = 190, 167, 149, 140, 127, 113, 95, 83, and 60 are characteristic of the saturated ring A and unsaturated ring.

The mass fragments of cucurbitacin B gave the same MS fragment pattern of authentic cucurbitacin B and with that reported in the literature (Curtis and Meade 1971; Hatam *et al*. 1989; Stuppner and Wagner 1989; Schenkel *et al*. 1992).

Cucurbitacin I

The R_F values were 0.010, 0.011 and 0.20, in using the three solvent systems mentioned before. The maximum absorption of compound III was $\lambda_{Max}^{Chloroform}$ 242.5. Mass spectrum of this compound shows molecular ion at *m/z* = 496 corresponding to molecular formula ($C_{30}H_{40}O_7$). The peak at 479 appeared due to loss of OH group. The peak at *m/z* = 401 may be due to loss of side chain. In this case, the very intense and characteristic peak, often the base peak was found at *m/z* = 96 (C_6H_8O). The peaks at *m/z* = 161and 113 were related to ($C_9H_{14}O_2$) and ($C_6H_{14}O_2$), respectively. The strong peak at *m/z* = 96 (100%) suggesting that it was the C25 derivative of cucurbitacin I. The peak at *m/z* = 314 is due to the cleavage between C5 and C6 after rearrangement of the 11 keto.

The aforementioned spectral data and R_F values indicate that the chemical structure of this compound is cucurbitacin I.

Cucurbitacin D

The R_F values of this compound were 0.050, 0.034 and 0.085 in using the three solvent systems mentioned before. The maximum absorption of cucurbitacin D was$\lambda_{Max}^{Chloroform}$ 216 nm. The mass spectrum of cucurbitacin D shows a molecular ion at *m/z* = 494 corresponding to molecular formula (C_{30} $H_{44}O_7$). The peak at 474 is appeared due to loss of OH group. The peak at *m/z* = 436 and 405 may be due to loss of side chain (C-C-C and C-C-C). The peaks at *m//z* = 190, 167, 149, 140, 127, 113, 95, 83, and 60 are characteristic of the saturated ring A and unsaturated ring.

In general, the mass fragments of this compound gave the same MS fragment pattern of cucurbitacin D authentic sample and with those reported in literature (Stuppner *et al*. 1990; Hatam *et al*. 1989; Schenkel *et al*. 1992).

CONCLUDING REMARKS

Culture medium supplemented with 2 mg/l BA, 60 mg/l adenine sulphate and 170 mg/l KH_2PO_4 is the best medium for shoot proliferation of endangered *Cucumis prophetarum.* Cucurbitacin content was elicited by methyl jasmonate.

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