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BIOPRODUCTION OF BENZYL ISOTHIOCYANATE IN SALVADORAPERSICA L. CALLUS TISSUE

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Abstract
In vitro production of valuable secondary metabolites is the best suited alternative over traditional field cultivation and chemical synthesis, for mass production of natural pure chemicals more cheaply and rapidly, independently from climate change and negative biotic and abiotic influences on secondary metabolites production in naturally grown plants. Benzyl isothiocyanate (BITC), a major compound in Salvadorapersica L., is one of the naturally occurring isothiocyanates, and has antioxidant and anticancer properties. In this study, an in vitro method was developed for the production of BITC from callus cultures of S. persica, a rare medicinal plant in Egypt. Two types of explants; leaf and stem sections were cultured on Murashige and Skoog (MS) medium, supplemented with different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) independently or in combination with kinetin (Kn), for callus induction and mentainance. MS medium supplemented with 1 mg/l 2,4-D gave the maximum mean fresh and dry weights of yellowish white friable callus obtained from stem sections. Friable and beige coloured callus was induced when leaf sections were cultured on MS medium supplemented with 0.5 mg/l of each of 2,4-D and Kn, with the maximum fresh and dry weights of callus. The effect of different concentrations of two amino acid precursors; phenylalanine (Phe) and cysteine (Cys), on the callus growth and BITC 3content was determined. Gas liquid chromatography (GLC) analysis showed the presence of high BITC content in the callus when compared to the intact plant. Phe at the concentration of 12.5 mg/l increased the amount of BITC in callus cultures (29.88 µg/g dry weight of callus), produced from stem sections, by about 4.5 times that in the stem of the intact plant and 8 times that in the leaves of the intact plant. This protocol is a stepping stone for the in vitro production of one of the chemo protective agents without exploiting natural plant resources.

Keywords: in vitro; secondary metabolites; anticancer compound; Salvadoraceae

Introduction

Medicinal plants are the most exclusive source of life saving drugs for the majority of world's population. Production of plant secondary metabolites by cultivation of plants and chemical synthesis are important agronomic and industrial objectives. The evolving commercial importance of secondary metabolites has resulted in a great interest in secondary metabolism, particularly in the possibility of altering the production of bioactive plant metabolites by means of tissue culture technology (Vanisreeet al., 2004). Since, the synthesis of bioactive compounds chemically is difficult because of their complex structure and

high cost (Anonymous, 2001). Production of valuable secondary metabolites through plant tissue culture technology has many advantages over traditional field cultivation and chemical synthesis, particularly for many natural compounds that are either derived from slow growing plants or difficult to be synthesized with chemical methods (Taha**,** ElBahr, and Seif-El-Nasr, 2009). To increase production of secondary metabolites in in vitro systems, feeding precursors or intermediate metabolites is an obvious and popular approach (Parale, Rarmukh, and Nikam, 2010).

Benzylisothiocyanate (BITC) is classified as one of the chemo-preventive agents that are thought to prevent carcinogenic and other genotoxic compounds from reaching or reacting with the target sites on the treated tissue (Ezmirly and El-Nasr, 1981). Higher intakes of BITC correlate with reduced risk of cancers of the lung, breast, and colon while blocking cancer development. BITC is one of the isothiocyanates with the strongest anticancer effects. Studies have shown that it helps to prevent lung cancer and esophageal cancer, and can also lower the risk of other cancers, including gastrointestinal cancer. BITC efficiently inhibits several cancer promoting**-**cytochrome enzymes, helping to prevent carcinogenesis (Von Weymarn, Chun, and Hollenberg, 2006).Srivastava and Sahu (2009) reported that BITC induces apoptosis in human pancreatic cancer cells. Also, it inhibits growth of cultured and xenografted human breast cancer cells (Sehrawat and Singh, 2011).

SalvadorapersicaL. From family Salvadoraceae, is one of the most important members in natural plant life of Egypt. It is an evergreen shrub or tree, facultative halophytes found in dry and arid regions. The plant is native to the Arabian Peninsula, Egypt, and India (Khalil, 2006). Traditionally, the wood sticks of S. persicahave been used for cleaning the teeth and so named toothbrush tree or Miswak, which possess antibacterial, anti-viral, anti-diabetic, anti-fungal, anti-cancer, anti-ulcer, anti-plaque, anti-caries, anti-plasmodial activity (Arora and Gupta, 2011). The other parts of the tree have therapeutic values as corrective, deobstruent, liver tonic, diuretic, analgesic, anthelmintic, astringent, lithontriptic, carminative, diuretic, aphrodisiac, and stomachic (Akhtaretal., 2011). Recently, Ahmad and Rajagopal (2013) reported that the extracts and preparations from this species have been found to possess a broad spectrum of biological and pharmacological effects, such as antidepressant effects, wound-healing, antiviral and antimicrobial activity. Moreover, it exhibited various additional biological effects like anti-gingival irritation, hypoglycemia, antioxidant, anti-fever, anti-ulcerogenic, anti-caries and antiplatelet-agression effect.

BITC is the major active constituents of S. persica, it was isolated from the roots and is claimed to be responsible for antiviral activity against HSV**-**1(Herbs Simplex Virus-1) (Al-Bagieh, 1992) as well as controlling dental caries (Al-Bagieh and Weinberg, 1988).S. persica extracts and volatiles contained BITC, one major antibacterial component, with rapid bactericidal effect against all gram-negative bacteria including periodontal pathogens (Sofrataet al., 2011). Moreover, recently it was that it induces G2/M phase arrest and apoptosis in human melanoma A375.S2 cells through reactive oxygen species (ROS), and both mitochondriadependent and death receptor-mediated multiple signaling pathways (Huang et al., 2012).

Until now, no publications on the in vitro production of BITC from callus cultures of S. persica were found. The objective of the present study was to induce in vitro production of the important anticancer compound; BITC, from callus cultures of the leaf and stem sections of S. persica to exceed that in the intact plant, as an easy tool to produce a pure compound and to avoid exploiting the natural plant resources.

Materials and Methods

This study was conducted in Tissue Culture Unit, Plant Genetic Resources Department, Desert Research Center, El-Matarya, and Cairo, Egypt.

Plant material and explant source

Actively growing shoots of S. persicabearing eight to ten nodes with terminal buds were excised from young selected uniform plants derived from seeds obtained from Gabal Elba natural habitat and brought to the Egyptain Botanical Garden (Orman). Explants were moistened and wrapped in wet papers untill used.

Plant surface sterilization

Leaf and stem sections were cut, separated and washed under running tap water for 10 min. Surface sterilization of the explants was performed under complete aseptic conditions in a Laminar Air Flow Hood. Leaf sections were surface sterilized by soaking in 3% Clorox (containing 5.25% NaOCl) for 5 min, followed by dipping in 0.1% (w/v) $HgCl_2$ plus 1 ml of 10% hydrogen peroxide (H_2O_2) for 30 sec with gentle shaking. Stem sections were surface sterilized by submerging in 30% Clorox for 10 min, followed by dipping in 0.1% (w/v) $HgCl_2$ for 45 sec. Then, both explants were thoroughly rinsed 5 times with double sterilized distilled water.

Culture medium, conditions and callus induction

After surface sterilization, the ends of each explant were trimmed. Both explants (0.1-0.5 cm long) were cultured on solid Murashige and Skoog (MS) basal medium (Caisson, USA) (Murashige and Skoog, 1962) supplemented with 100 mg/l myo-inositol, 3% w/v sucrose and 0.7% w/v agar. Different PGRs (Sigma Cell Culture, min. 98%, St. Louis, USA) were tested for callus induction. An auxin; 2,4-dichlorophenoxy acetic acid (2,4-D), at concentrations of 0.5, 1, 2.5 and 5 mg/l, were added to the media independently or in combination with a cytokinin; kinetin (Kn) at concentrations of 0.1, 0.5 and 1 mg/l. Also, β-naphthalene acetic acid (NAA) independently or combined with either benzyl adenine (BA) or thiadiazuron (TDZ) were examined, at different concentrations and combinations, for callus induction. The pH of the media was adjusted to 5.7-5.8 with 0.1 N NaOH and 0.1 N HCl prior to gelling with 0.7% w/v agar. Media (40-45 ml volumes) were dispensed into large jars. Jars were sealed with autoclavable polypropylene caps and autoclaved for 15 min at 121°C under 1.1 kg/cm^2 pressure, then left to cool and stored at room temperature till used.

All cultures were incubated in an air conditioned incubation room at a temperature of 25±2°C under a 16/8-h (light/dark) photoperiod provided by cool-white fluorescent tubes (F 140t9d/38, Toshiba). Each treatment consisted of 40 replicates. After 10 weeks, the percentage of callus induction (%), mean fresh and dry weights of callus (mg/explant), color and texture of callus were recorded.

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Precursor's supplementation

About 0.1-0.5 g from the obtained callus was cultured on MS medium supplemented with 1 mg/l 2,4-D combined with the precursor amino acids (Sigma, USA); L-phenylalanine (Phe) at concentrations of 12.5, 25, 50 and 100 mg/l, or L-Cysteine (Cys) at concentrations of 50, 100, 150 and 200 mg/l. The percentage of callus induction (%), mean fresh and dry weights of callus (mg/explant), and both color and texture of callus were recorded after 8 weeks of culture.

5. Quantitative estimation of BITC Effect of PGRs and precursor's supplementations on BITC content in the mother plant and produced callus was determined by using gas-liquid chromatography (GLC).

1. Extraction of BITC

A weight of 250 mg crushed, undefatted callus tissues, fresh leaves and stem sections from the mother plant were taken and extracted. Tissues were mixed with 15 ml distilled water and left for autolysis overnight (17 h) at 27±2°C. Dichloromethane (20 ml) was added and the mixture was shaken for 30 min. then centrifuged for 5 min at 3500 rpm for the separation of the organic solvent. The dichloromethane layer was dried over anhydrous sodium sulfate and then concentrated under nitrogen to 100 µl and examined for the presence of BITC (Songsak and Lockwood, 2004).

5.2. GLC analysis

One µl of the concentrated extract was chromatographed by capillary GLC. A Hewlett Packard 5890 GC, fitted with a flame ionization detector, was used for the analysis of BITC. The column used was high performance fused silica linked 5% methyl silicon capillary column, 30 m×0.22 mm, 0.25 µM film thickness. The results were recorded on a Hewlett Packard 3392A integrator. The operating condition was 50–280°C at 5°C/min, and then isothermal for 5 min. The injector and detector temperatures were 250°C and 280°C, respectively. Quantification of glucosinolate hydrolysis product (BITC) was carried out by the production of a calibration curve with a standard BITC, and was estimated assuming standard detector response (Songsak and Lockwood, 2004).

Data analysis

The experiments were subjected to completely randomized design. Variance analysis of data was carried out using Anova program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using Duncan's multiple range test (Duncan, 1955) as described by Snedecor and Cochran (1990).

Results and Discussion

1. Induction of callus from leaf sections

The results of callus induction from leaf sections of S. persicaare presented in **Table 1**. The percentage of callus induction and its mean fresh and dry weights varied with the variation in the PGRs concentrations and combinations.

It is noticed from the obtained data that addition of Kn to the media containing 2,4-D has a noticeable effect on percentage of callus induction as well as both mean fresh and dry weights of callus. Addition of 2,4-D individually greatly affected percentage of callus induction. The highest mean fresh weight of callus was

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obtained on the MS medium containing 0.5 mg/l of each of 2,4-D and Kn, it reached 1.72 g/explant. This medium produced 100% piege friable callus, and gave also significantly the highest mean dry weight of callus (0.12 g/explant) **(Fig. 1A)**. Themean dry weight of callus reached also its highest value of 0.13 g/explant on MS medium containing 2.5 mg/l 2,4-D and 0.5 mg/l Kn. The control medium without PGRs failed to induce any callus formation.

It was concluded that both auxins and cytokinins played an important role in regulating the induction and proliferation of callus generated from leaf sections of S. persica. In callus induction of Withaniacoagulans, callus initiation from leaf segments was observed best on MS medium fortified with 2,4-D (2 mg/l) and Kn (0.5 mg/l); and from internode segments on MS medium contained 2,4-D (4 mg/l) with combination of BA (0.25 mg/l) (Valizadeh and Valizadeh, 2009). Also, MS medium supplemented with Kn and 2,4-D has been reported to be effective in inducing and maintaining callus formation from Cleome chelidonii seedlings (Songsak and Lockwood, 2004). It is well known that auxins play an import role in the callus induction, and different types of auxins had various effects (Baskaran, Raja, and Jayabalan, 2006). Furthermore, the cytokinins facilitated the effect of auxin in callus induction (Raoet al., 2006).

Table 1. The induction of callus from leaf sections of S. persica. Data were recorded after 10 weeks of culture.

PGRs concentration (mg/l)			% of callus induction	Mean fresh weight of callus (G/explant)	Mean dry weight of callus (G/explant)	Colour of Callus	Texture of Callus
2,4-D	Kn						
0.0	0.0	0	0.00	-	-	-	
0.5	0.0	0	0.00	-	-	-	
1.0	0.0	100	0.21^{de}	0.053^{cd}		Greenish yellow	Granulated, friable
					Piege, greenish	Friable, lose 2.5	0.0 100 0.77^{bc}
	0.100^{ab}		spots				
5.0	0.0	100	0.26^{de}	0.089^{a-c}		White	Less compact
0.5	0.1	100	0.34^{de}	0.054^{cd}		Yellowish piege	Friable
1.0	0.1	0	0.00	-	-	-	
2.5	0.1	80	0.58b-e		0.061^{b-d}	White	Friable
5.0	0.1	40	0.31^{de}	0.059^{b-d}		White	Powdery
0.5	0.5	100	1.72^{a}	0.120^{a}	Piege	Friable	
1.0	0.5	50	0.61b-d		0.067^{b-d}	White	Friable
2.5	0.5	80	0.90^{b}	0.130^{a}	Brownish	Granulated	
Yellow							
5.0	0.5	0.0	0.00	-	-	-	Loose and less
0.5	1.0	50	0.15^{e}	0.030^{d}		White	granulated
1.0	1.0	80	0.38^{c-e}		0.066^{b-d}	Piege	Friable and granulated

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					Piege with	Friable and
2.5	1.0	90	0.43^{c-e}	0.063^{b-d}	green spots	granulated
5.0	1.0	50	0.16^{e}	0.043^{cd}	Piege	Friable and
Granulated						

Mean values followed by the different letter under different treatments within a column are significantly different from each other at $p< 0.05$.

2. Induction of callus from stem sections

Callus induced from stem sections ofS. persica under the influence of different PGRs concentrations is represented in **Table 2**. Data clearly show that 100% of stem sections induced callus on all tested MS media containing either 2, 4-D individually or in combination with Kn. The mean fresh weights of callus ranged between 0.2 and 1.83 g/explant and the mean dry weights ranged between 0.041 and 0.17 g/explant. MS medium containing 1 mg/l 2, 4-D gave the maximum mean fresh and dry weights of yellowish white friable callus **(Fig. 1B)**. The medium containing 0.5 mg/l 2, 4-D was insignificantly different in its effect on the mean fresh and dry weights of callus (1.45 and 0.13 g/explants, respectively) comparing to the previous medium.

On the other hand, the minimum response was obtained on MS medium supplemented with 5 mg/l 2,4D and 0.1 mg/l Kn. It was also noticed that 2, 4-D at low concentrations positively affected the growth of callus, and the addition of Kn decreased both fresh and dry weights of callus. Control medium without PGRs did not give any callus induction.

Table 2. The induction of callus from stem sections of S. persica. Data were recorded after 10 weeks of culture.

PGRs Concentration (Mg/l)		**% of callus induction**	**Mean fresh weight of callus (G/explant)**	**Mean dry weight of callus (g/explant)**	**Colour of callus**	**Texture of callus**
2,4-D	Kn					
0.0	0.0	0	0.00	0.000	-	-
0.5	0.0	100	1.45^{a}	0.130^{ab}	Piege	Compact and granulated
1.0	0.0	100	1.83^{a}	0.170^{a}	Yellowish white	Less friable
2.5	0.0	100	0.90^{b}	0.100^{bc}	Greenish yellow	Friable
5.0	0.0	100	0.50^{b-d}	0.070^{c-e}	Greenish white	Powdery
0.5	0.1	100	0.53^{b-d}	0.090^{b-d}	Greenish white	Friable
1.0	0.1	100	0.23^{e}	0.047^{e}	Yellowish green	Powdery
2.5	0.1	100	0.78^{bc}	0.070^{c-e}	Piege	Loose and granulated
5.0	0.1	100	0.20^{e}	0.041^{e}	White	Powdery
0.5	0.5	100	0.35^{cd}	0.051^{de}	Piege	Friable and granulated
1.0	0.5	100	0.81^{bc}	0.093^{b-d}	Piege	Friable and powdery

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2.5	0.5	100		0.66^{b-d}	0.074^{c-e}	White Powdery
5.0	0.5	100	0.36^{cd}	0.054^{de}	White	Compact and powdery
0.5	1.0	100	0.53^{b-d}	0.075^{c-e}	Piege Less friable	
1.0	1.0	100	0.31^{cd}	0.060^{de}	Piege Less friable	
2.5	1.0	100	0.34^{cd}	0.056^{de}	Yellowish white	Powdery
5.0	1.0	100		0.58^{b-d}	0.076^{c-e}	Yellowish white Powdery and loose

Mean values followed by the different letter under different treatments within a column are significantly different from each other at $p< 0.05$.

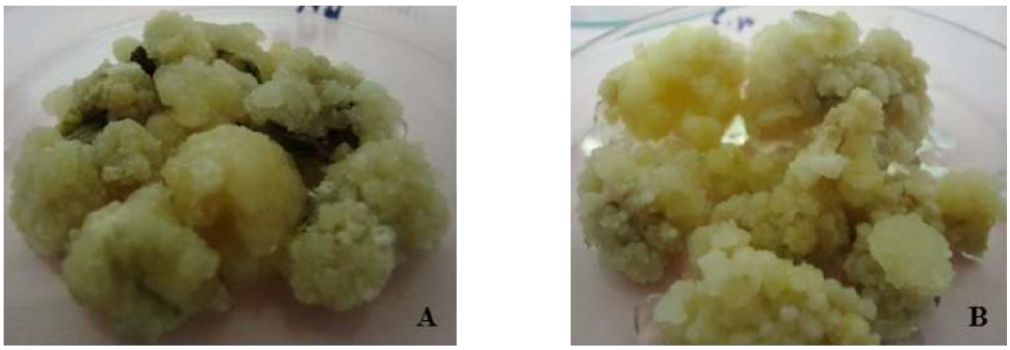

Figure 1. Callus of *S. persica*.**A.** Callus obtained from leaf sections of *S. persica*Induced on MS medium Containing 0.5 mg/l 2, 4-D and 0.5 mg/l Kn. **B.** Callus obtained from stem sections of S. persica induced on MS medium containing 1 mg/l 2, 4-D.

In the present investigation, the different explants showed differences in callus induction as regard to Percentage and weight of callus. The variation in the response of explants to callus induction and the various colours and textures of callus may be attributed to that the cultured explants comprise of various tissues, either meristematic, parenchymatous or others less differentiated cells. So, the type of cells exposed to growth regulators perhaps determines the nature of callus. Diverse group of cells comprising callus, on the other hand, are also triggered differently by the same PGRs (Aftab, Akram, and Iqbal, 2010). It could be noticed from the obtained data that stem sections were the most suitable explants for callus Initiation and maintenance in S. persica. They were found to precede leaf sections in their response in callus formation with higher mean fresh and dry weights of callus on the best medium.

3. Effect of precursors supplementation on the BITC content

The effect of different concentrations of two amino acids precursors; Phe and Cys, on the growth of callus induced from stem sections of S. persica and their capability for elevating the amount of BITC is represented in **Table 3.** The medium contained 100 mg/l Phe gave the highest mean fresh weight of callus (1.83 g/explant).

Table 3. Effect of feeding of Phe and Cys on biomass and BITC accumulation in the callus of S. persicaderived from stem sections. Data were recorded after 8 weeks of culture.

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Precursors conc. (mg/l)		Mean fresh weight of callus	Mean dry weight of callus	Conc. of BITC (µg/g dry
Phe	Cys	(g/explants)	(g/explant)	callus)
00.0	0	1.78[a]	0.139[a-d]	29.61
12.5	0	1.44[a]	0.100[cd]	29.88
25.0	0	1.82[a]	0.212[ab]	3.21
50.0	0	1.54[a]	0.149[a-d]	3.51 100
00.0	50	1.38[a]	0.136[a-d]	7.20
00.0	100	1.13[a]	0.122[b-d]	3.69
00.0	150	1.43[a]	0.234[a]	2.88
00.0	200	0.65[a]	0.070[d]	3.12
0	0	1.83[a]	0.176[a-c]	7.74

Mean values followed by the different letter under different treatments within a column are significantly different from each other at $p< 0.05$.

The dry weight of callus varied significantly with the different concentrations of the two tested amino acids. The highest mean dry weight of callus reached 0.234 g/explant on the medium contained 150 mg/l Cys.

GLC analysis revealed that BITC could be produced from callus of S. persica cultured on the control medium devoid of the precursor amino acids as well as and the media incorporated with the different concentrations of Phe and Cys. The amount of BITC in callus cultures varied from 2.88 to 29.88 µg/g dry weight of callus. The maximum concentration of BITC was obtained on the medium contained 12.5 mg/l Phe. The accumulation of BITC at this concentration was shown to be more or less as the control medium without precursors. These results are compatible with those obtained by Konczacet al. (2000) and Simõset al. (2009) who found that the supplementation of 2,4-D proved to be essential to support biomass increase as well as high secondary products accumulation.

It was found that dry leaves and stems of the intact plant of S. persica contained 3.66 and 6.6 µg BITC/g dry weight of plant tissue, respectively **(Table 4)**. Application of 2,4-D at a concentration of 1 mg/l independently, or with the lowest concentration of Phe (12.5 mg/l) increased the amount of BITC in callus cultures by about 4.5 times that in the stems of the intact plant and 8 times that in the leaves of the intact plant. On the other hand, biosynthesis of aromatic glucotropaeolin (benzyl glucosinolate) in Tropaeolummajus strictly depends on Phe and Cys, as a source of sulfur in β-D-thioglucoside moiety (Wielanek and Urbanek, 2006).

Table 4.A comparison between the amount of BITC in the leaves and stems of the intact plant and callus cultures of S. persica.

Sample	Conc. of BITC (µg/g dry weight)
Leaves of intact plant	3.66
Stems of intact plant	6.60

Callus derived from stem segments on MS medium + 1 mg/l 2,4-D	29.61
Callus derived from stem segments on MS medium + 1mg/l 2,4-D + 12.5 mg/l Phe	29.88

Pintão and Pais (1994) found thatfriable callus, obtained from Tropaeolummajusleaves on Gamborg B5 medium supplemented with 1.5 mg/l 2,4-D, 0.1 mg/l Kn, 2 g/l casein amino acid and 100 mg/l cysteine. Under these conditions, the cells produced the secondary metabolite; BITC in vitro, in amounts that reached about 10% of the leaf content. These results are in contrast with those detected byWielanek and Urbanek (2006), who revealed that supplementation of Tropaeolummajus hairy root cultures both with Phe and with Cys increased glucotropaeolin content, but the effect was more pronounced when the precursors were applied in combination. A stimulating effect of precursors (Phe, Cys) on the BITC content could be reported, but that effect was correlated with a decreased biomass growth that may reduce BITC yield.

In conclusion, these results demonstrated that callus cultures of S. persicacould be useful for BITC production, and the manipulation of medium by adding PGRs and amino acids precursors can enhance the production of this valuable compound. Further studies should be conducted to produce BITC in a large scale, with a focus on its application in health-related research.

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