Effect of NaCl and Sorbitol on the Production of Some Alkaloids of Fenugreek Cotyledons Derived Callus

Saeb A. Hassan* and Emad H. Jassim
Genetic Engineering and Biotechnology Institute for Post Graduate Studies,
University of Baghdad, Baghdad-Iraq.
* Corresponding author: ZhrNr1970@gmail.com.

Abstract

Alkaloids often had pharmacological effects. The aim at this study is to increase some alkaloids in fenugreek. Callus induction occurred on cotyledon segments 5 mm long. Murshige and Skoog medium (MS) supplied with 1 mg.l^{-1} of 2, 4 – D and 0.4 mg.l^{-1} of kinetin was prepared. The best medium maintained callus was using MS medium supplied with 0.5 mg.l^{-1} 2, 4-D and 0.5 mg.l^{-1} kinetin. Callus formation was allowed to take place for a period of thirty days in the dark at 25°C±2. Calli were analyzed using high performance liquid chromatography (HPLC).Methanol callus extract showed high concentrations of some alkaloids compared to the methanol extract of cotyledons. For increasing, the concentration of secondary metabolites, NaCl was added at concentrations 0, 1, 1.5 or 2 g.l^{-1}, sorbitol at concentrations 0, 7, 8 or 9 g.l^{-1}. NaCl at 2 g.l^{-1} led to significant increase in trigonelline reaching 1354.72 µg per 100 mg fresh weight of callus. NaCl at 1g.l^{-1} increased choline and carpaine reached 400.82, 483.92 µg.ml^{-1} per 100 mg respectively. Treatment with 9 g.l^{-1} sorbitol increased trigonelline and carpaine reached 1666.41, 742.67 µg per 100 mg respectively, while treatment 8 g.l^{-1} sorbitol significantly increased in choline recorded 588.87 µg per 100 mg. [DOI: 10.22401/JUNS.21.1.14]

Keywords: fenugreek, alkaloids, NaCl, sorbitol.

Introduction

Fenugreek (*Trigonella foenum-graecum* L.) is an important crop belonging to the leguminosae family and cultivated in semi-arid regions of the world. It is a native of Asia, Southern Europe and India [1]. The biological and pharmacological actions of fenugreek are attributed to the variety of its constituents. The plant contains active constituents such as pyridine alkaloids (mainly trigonelline), flavonoids, steroids, saponins, free amino acids (mainly 4-hydroxyisoleucine). Trigonelline, choline and carpaine have been isolated from fenugreek seeds and shown to be useful in diabetes [2]. Trigonelline is a hormone finding naturally in plant products and is a vitamin B6 derivative. Trigonelline may also had several therapeutic properties, such as anti-migraine, anti-carcinogenic (cervix and liver), antiseptic, hypoglycemic and hypocholesterolemic, activities [3]. As [4] explained that choline was also used in the treatment of alzheimer's disease and liver disorders. Some studies have suggested that choline was used in the treatment of hepatitis, atherosclerosis and possibly neurological disorders. The studies have shown that it was possible to increase the production of callus for active substances when using abiotic stress and represent this process is adding of sodium chloride and sorbitol. Many nitrogen compounds accumulate in the plants, that had been exposed to salt stress. These include amino acids, amides, proteins, quaternary ammonium compounds and polyamine, where they vary from plant type to control osmotic potential, protect macromolecules, storage components, maintain pH, anti-toxicity, and eliminate free radicals of the cell [5]. Plant cells, in response to osmotic stress, lead to accumulate sugars and compatible solutes in the cytoplasm [6]. Plenty of the stress induced compounds are useful secondary metabolites. For example, *in vitro* cultured plant cells were noted to synthesize extra amounts of trigonelline in callus of (*Trigonella foenum graecum* L.). [7] High amount of alkaloids of *Salvia officinalis* [8], indole alkaloids [9], [10] in response to various abiotic stress factors. The main aims at the present study are to improve biosynthesis of some alkaloids, in fenugreek callus by growing it under the effect

of water stress or salt stress which can be created in culture medium by the addition of sorbitol or sodium chloride.

Materials and Methods

Seeds sterilization and germination

The seeds of the Indian fenugreek plant were used and obtained from the agricultural garden centers Baghdad. Seeds were sterilized in 70 % ethanol for 60 sec. and 4.5 % sodium hypochlorite solution for 15 minutes. Seeds were then rinsed three times in sterile water. The seeds were then dried between two layers of sterile filter papers in a petri dish. Using sterile scalpel then transferred to screw glass tubes(85 x 28 mm) containing hormone free, half strength MS [11], allowed to germinate for one week at 25 ± 2°C and photoperiod of 16 hrs light provided by fluorescent tubes (with light intensity of 1000 lux) and 8 hrs dark.

Induction and maintenance of callus

Cotyledons of the sprouts were cut under aseptic conditions into 5 mm long for callus initiation. For induction of callus from 5 mm long cotyledon, MS containing 30 $g.l^{-1}$ sucrose supplemented with two plant growth regulator types were used to assess their effect on callus induction. Cotyledons explants were cultured in culture tubes containing MS medium supplemented with (0.0, 1.0, 2.0, 3.0,4.0 or 5.0 $mg.l^{-1}$) 2,4-dichloro phenoxy acetic acid (2,4-D) and (0.0,0.2,0.4 or 0.6 $mg.1^{-1}$) kinetin were applied in twenty-four combinations then distributed in to 10 replicates per treatment after 30 days of culture the fresh and dry weight of callus were recorded. PH value of the culture medium was adjusted to 5.7 prior to autoclaving with 7 $g.l^{-1}$ (w/v) agar, autoclaving at (15 min, 121°C and a pressure of 1.04 $kg.cm^{-1}$) was carried out. In this study, the maintenance of callus from 16 hormonal composition containing 2,4-D (0.0,0.5,1.0 or 1.5 $mg.l^{-1}$) and kin. (0, 0.3, 0.4 or 0.5 $mg.l^{-1}$) was used 0.5 $mg.l^{-1}$ (2, 4-D) and 0.5 $mg.l^{-1}$ kinetin combination was used for callus maintenance.

Stimulation of secondary metabolites

After obtaining the required amount of callus 150 mg of callus, was cultured on the callus maintenance medium plus NaCl at concentrations 0, 1, 1.5 or 2 $g.l^{-1}$ or sorbitol at concentrations 0, 7, 8 or 9 $g.l^{-1}$ then distributed in to 10 replicates per treatment after 30 days of culture. The fresh and dry weight of callus were recorded. For extracting secondary metabolites from callus, HPLC technique was used following the method [11] to estimate the increase or decrease of alkaloids.

Alkaloid extraction:

Plant samples (3g of cotyledons or 100 mg of callus) were homogenized, ground and dissolved in 3 % H2SO4 for 2 hours at room temperature. The supernatants were mixed with adjusted alkaline with 25 % NH4OH (pH 9.5) and applied to (Merk) columns. The alkaloids were eluted by CH2Cl2 (6 ml per 1 g extrelut) and the extracts were evaporated to dryness. Obtained residues were desolved in CH3OH for further analysis by HPLC according the optimum separation of authentic standard. Concentrations were determined according to the following equation. Then 20µl was injected with HPLC column. [12],[13].

Conc. of sample=Area of sample/Area of standard×conc. of standard× dilutions factor

Note: that the concentration of the standard solution=25µg/ml and dilutions factor = 4

The results were compared with the intact plant.

High Performance Liquid Chromatography (HPLC)

The separation occurred on liquid chromatography Shimadzu 10 AV-LC equipped with binary delivery pump model LC-10A Shimadzu, the eluted peaks were monitored by UV-Vis 10 A-SPD spectrophoto- meter. The alcoholic extracts were separated on FLC (Fast Liquid Chromatographic) column, 3µm particle size (50×4.5 mm I.D) C18-DB (debased) column.

Mobile phase: was 0.01M phosphate buffer PH 8.2: Acetonitrile (45:55, V/V respectively) detection UV set at 220 nm, flow rate 0.9 ml per min.

Statistical analysis: was performed using SAS (2012) Statistical Analysis System. A completely randomized design (CRD) was used compare the averages using the least significant difference L.S.D to show the

statistical differences between the coefficients and the probability level of 0.05[14].

Results and Discussion

Callusing: MS medium in each plant growth regulator and their interaction showed significant differences at 0.05. Tables (1 and 2) show the effects of 2, 4-D and kinetin on callus weight. The effect of 2,4-D and kinetin interactions was exhibited in Fig.(1). Maximum fresh and dry weight of callus induction on MS medium containing 1 $mg.l^{-1}$, 2.4-D and 0.4 $mg.l^{-1}$kin was obtained. Maximum fresh and dry weight of callus maintained on MS medium containing 0.5 $mg.l^{-1}$ of 2, 4-D and 0.5 $mg.l^{-1}$ kin was show in Tables (3 and 4). Suitable combination hormone kinetin and 2, 4-D led to increased callus induction [15].

Table (1)
Effects of 2.4-D and kinetin on fresh weight of callus induction.

Kinetin ($mg.l^{-1}$)	***2,4-D***$(mg.l^{-1})$						***average***
	0	***1***	***2***	***3***	***4***	***5***	
0.0	0.0	468.6	311.3	233.5	229.6	296.5	256.58
0.2	0.0	479.0	412.2	277.0	271.6	379.0	303.13
0.4	0.0	672.2	144.1	178.8	23.8	404.0	237.15
0.6	0.0	342.2	348.7	366.7	434.1	230.1	286.97
average	0.0	490.50	304.07	264.00	239.77	327.40	---
L.S.D. 0.05	2,4-D : 52.793 * kinetin:40.429* 2,4-D x kinetin :82.602*						

Table (2)
Effects of 2.4-D and kinetin on dry weight of callus induction.

Kinetin $(mg.l^{-1})$	***2,4-D***$(mg.l^{-1})$						***average***
	0	***1***	***2***	***3***	***4***	***5***	
0.0	0.0	29.3	19.5	14.6	14.4	18.6	16.06
0.2	0.0	30.6	26.2	17.5	17.0	23.6	19.15
0.4	0.0	42.0	9.0	11.4	1.6	25.2	14.80
0.6	0.0	21.8	21.8	23.0	27.2	14.4	18.03
average	0.0	30.92	19.12	16.62	15.05	20.45	---
L.S.D. 0.05	2,4-D :3.087* kinetin: 2.655* 2,4-D x kinetin : 5.169*						

Table (3)
Effects of 2.4-D and kinetin on fresh weight of maintained callus.

Kinetin ($mg.l^{-1}$)	2,4-D($mg.l^{-1}$)				average
	0.0	0.5	1.0	1.5	
0.0	169.0	214.5	356.3	194.3	233.52
0.3	240.9	170.0	173.9	151.5	184.07
0.4	320.0	290.9	210.6	203.6	256.27
0.5	313.2	422.0	341.9	160.1	309.30
average	260.77	274.35	270.67	177.37	---
L.S.D. 0.05	2,4-D :26.58* kinetin :26.58* 2,4-D x kinetin: 43.17*				

Table (4)
Effects of 2.4-D and kinetin on dry weight of maintained callus.

Kinetin ($mg.l^{-1}$)	2,4-D($mg.l^{-1}$)				average
	0.0	0.5	1.0	1.5	
0.0	10.0	13.4	22.6	12.1	14.52
0.3	15.0	11.2	11.2	9.6	11.75
0.4	20.3	16.8	13.2	12.5	15.70
0.5	19.9	26.1	20.1	10.4	19.12
average	16.30	16.87	16.77	11.15	---
L.S.D. 0.05	2,4-D: 2.972* kinetin: 2.972* 2,4-D x kinetin :4.315*				

Fig.(1): Callus induction from cotyledon segments on MS medium containing.
A- (4 $mg.l^{-1}$) 2,4-D, (0.6 $mg.l^{-1}$) kinetin.
B- (1 $mg.l^{-1}$) 2,4-D, (0.0 $mg.l^{-1}$) kinetin.
C- (1 $mg.l^{-1}$) 2,4-D, (0.2 $mg.l^{-1}$) kinetin.
D- (1 $mg.l^{-1}$) 2,4-D, (0.4 $mg.l^{-1}$) kinetin.

Effect of different concentrations of NaCl on alkaloid production in callus.

Fig.(2) shows the standard curve of alkaloids compared with the curves of compounds extracted from cotyledons of fenugreek grown under field conditions and extracted callus (Figures 3-A, 3-B, 3-C and 3-D) showed the HPLC curves resulted by using different concentrations of NaCl, which showed the presence of three alkaloids. Table (5) Callus gave the highest concentration of choline, carpaine, reached

400.82, 483.92 µg.ml^{-1} respectively at the concentration 1g.l^{-1} NaCl, while trigonelline significantly increased reached 1354.72 µg.ml^{-1} at the concentration 2 g.l^{-1} NaCl compared with control treatment which recorded of trigonelline, choline, carpaine 214.84, 87.62 and 70.69 µg.ml^{-1} per 100mg fresh weight of callus respectively while the lowest concentration recorded was in cotyledon (63.278, 59.35 and 26.175) µg.ml^{-1} per 3g fresh weight of intact plant respectively.

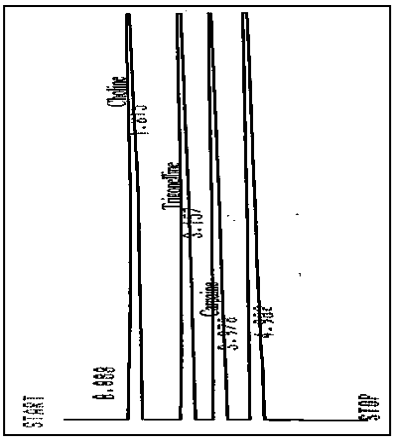

Fig.(2): Standard curve of three alkaloids in fenugreek using HPLC.

compound	*retention time (minute)*	*area*
choline	1.815	190362
trigonelline	3.157	203321
carpaine	3.978	189186

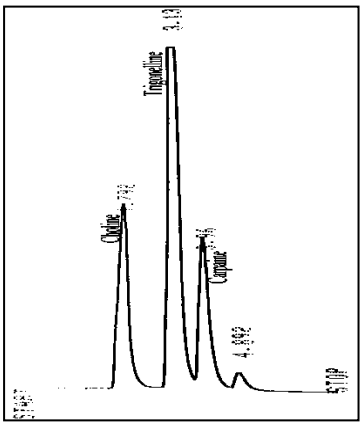

Fig.(3-A): HPLC analysis of the three alkaloids in callus of fenugreek.

compound	*retention time (minute)*	*area*
Choline	1.792	166802
Trigonelline	3.13	436818
Carpaine	3.96	133750

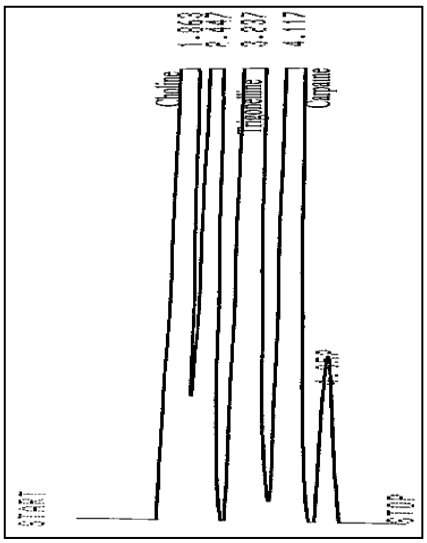

Fig.(3-B) :The three alkaloids that detected with HPLC at 1 g.l^{-1} of NaCl.

compound	*retention time (minute)*	*area*
choline	1.863	763007
trigonelline	3.237	2187918
carpaine	4.117	915508

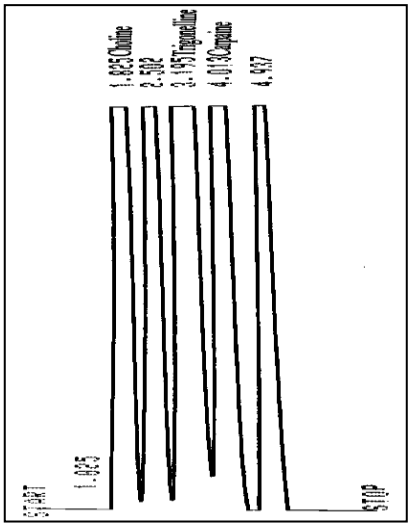

Fig.(3-C): The three alkaloids that detected with HPLC at 1.5 g.l^{-1} of NaCl.

compound	*retention time (minute)*	*area*
Choline	1.825	4902232
Trigonelline	3.195	2486944
Carpaine	4.013	664604

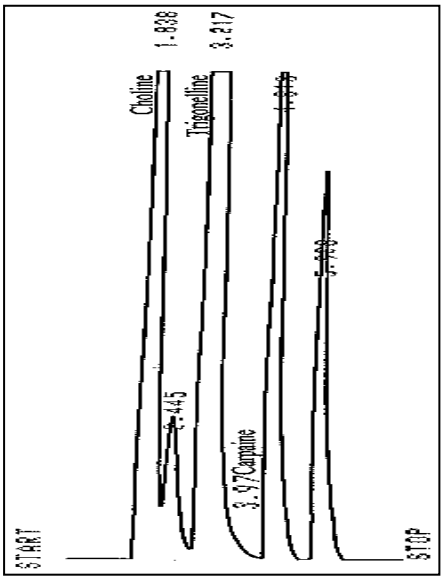

Fig.(3-D): The three alkaloids that detected with HPLC at 2 g.l⁻¹ of NaCl.

compound	retention time (minute)	area
choline	1.838	675624
trigonelline	3.217	2754419
carpaine	3.97	5000

Table (5)
Effect of NaCl concentration on the three alkaloids from callus of fenugreek.

NaCl(g.l⁻¹)	carpaine µg.ml⁻¹	trigonelline µg.ml⁻¹	choline µg.ml⁻¹
0	70.69 cd	214.84 c	87.62 d
1	483.92 a	1076.1 b	400.8 a
1.5	351.3 b	1223.16 ab	257.5 c
2	2.64 d	1354.72 a	354.9 b
L.S.D 0.05	82.97*	198.46*	12.64*

Effect of different concentrations of sorbitol on alkaloids production in callus.

Results Table (6) show that callus gave the highest concentration of trigonelline, carpaine reached 1666.41, 742.67 µg.ml⁻¹ respectively at the concentration 9g.l⁻¹ sorbitol. Choline significantly increased reached 588.87 µg.ml⁻¹ at the concentration 8 g.l⁻¹ sorbitol compared with control treatment and intact plant Table (7). The Figs. (4-A, 4-B, 4-C and 5) showed HPLC curves using different concentrations of sorbitol.

Table (6)
Effect of sorbitol concentration on the three alkaloids of callus.

sorbitol (g.l⁻¹)	carpaine µg.ml⁻¹	Trigonellin -e µg.ml⁻¹	choline µg.ml⁻¹
0	70.69 bc	214.84 d	87.62 b
7	79.54 b	1387.41 b	83.2 bc
8	16.53 c	288.54 cd	588.87a
9	742.67 a	1666.41 a	39.2 c
L.S.D. 0.05	61.58*	241.96*	12.55*

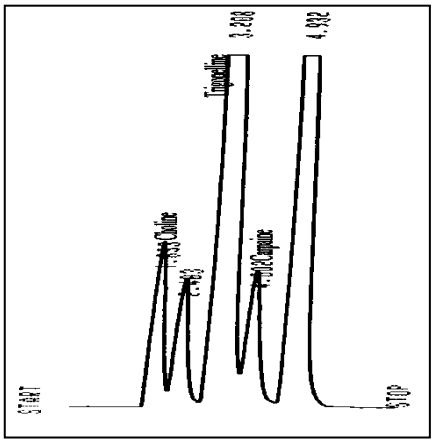

Fig.(4-A): The three alkaloids detected with HPLC at 7 g.l⁻¹ of sorbitol.

compound	retention time (minute)	area
Choline	1.833	158409
Trigonelline	3.208	3820891
Carpaine	4.002	150474

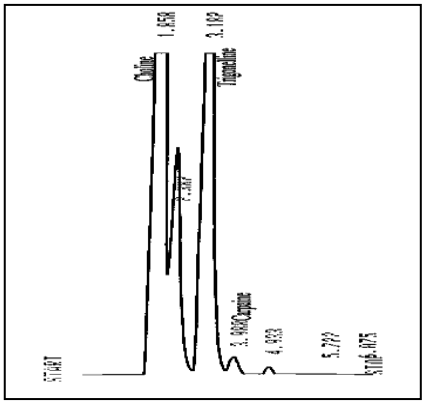

Fig.(4-B): The three alkaloids detected with HPLC at 8 g.l⁻¹ of sorbitol.

compound	retention time (minute)	area
choline	1.858	1120984
trigonelline	3.182	586656
carpaine	3.988	31265

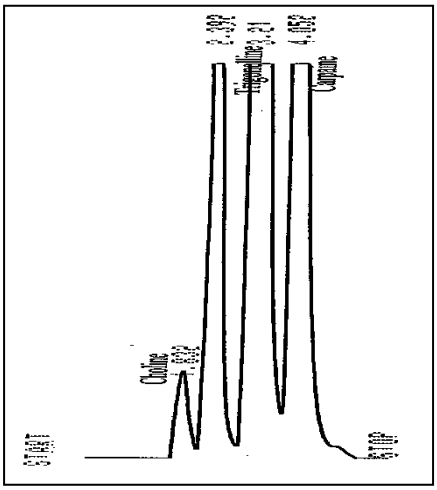

Fig.(4-C): The three alkaloids detected with HPLC at 9 g.l⁻¹ of sorbitol.

compound	*retention time (minute)*	*area*
choline	1.832	74643
trigonelline	3.21	3388156
carpaine	4.052	1405033

Table (7)
The three alkaloids detected and quantified (µg.ml⁻¹) in the cotyledon extract and callus using HPLC.

alkaloids (µgml⁻¹)	*per3000 mg cotyledon*	*per100 mg callus*
choline	59.35	87.62
trigonelline	63.278	214.84
carpaine	26.175	70.69

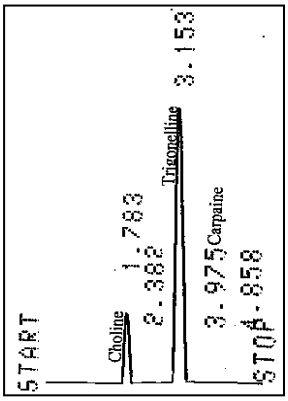

Fig.(5) :HPLC of the three alkaloids in cotyledon extract of fenugreek.

compound	*retention time (minute)*	*area*
choline	1.783	112977
trigonelline	3.153	128658
carpaine	3.975	49520

Results displayed in Table (5) agreed with the results of [16] who referred to increased secondary metabolites in callus of *Trigonella foenum-graecum* L. under NaCl stress. [17] reported that stress increases the various secondary metabolites in plants. Post-elicitation may induce cell to synthesize some amino acids such as proline, which regulates the osmotic pressure and makes it able to absorb water and nutrients within certain levels of salinity. The capability of plants to survive well under salinity stresses due to high ability of osmotic adaption [18]. The increase in sorbitol, caused an increase in these alkaloids[19] mentioned that total alkaloids accumulation of leaf derived calli cultures of ***Catharanthus roseus*** gradually increased by increasing the level of mannitol concentrations. [8] MS medium containing 40 g.l⁻¹ of sorbitol resulted in the production of high amount of thujone, camphor and 1, 8-cineol which amounted to 137.0, 126.3 and 118.5 micrograms per 1 g dry weight of callus, respectively [20]. Increased stress led to increasing amino acid [21]. Perhaps the reason for increasing alkaloids in callus, is the added growth regulators to the medium of callus induction and maintaining callus led to induce and increase the production of some alkaloids in callus, or because of continuous subculture led to the emergence of somatic variation in cells, led to increasing production of secondary metabolites [22], or the difference may be due to the concentration of some alkaloids to various factors as light, humidity and the growth stage of the plant in addition to the genetic factors that may influenced the physiological factors [23].

Conclusion

Some alkaloids in callus *Trigonella foenum-graecum* L. significantly increased after treatment with NaCl and sorbitol.

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