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Molecular analysis of *Peganum harmala* L. callus to determine the gene expression of beta-carboline alkaloids harmine and harmaline

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Abstract

The external signals are used as elicitors that can modify the levels of secondary metabolites production and trigger the biotechnological enforcements to improve plant production. In this study, sodium chloride NaCl was used as a stimulating factor for the production of beta-carboline alkaloids, harmine and harmaline, in *Peganum harmala*. The *in vitro* induced callus of *P. harmalain* was used as a source for alkaloid production in the stimulating experiments with sodium chloride. The results illustrated that 2 $mg.l^{-1}$ of NaCl increased the fresh and dry weight of callus with an average of 944.30 and 72.0 mg, respectively. In a comparative analysis through Gas Chromatography (GC), high concentrations of harmine of 58.55 $\mu g.g^{-1}$ from the root and 56.50$\mu g.g^{-1}$ from stem callus were recorded upon treatment with 4 $mg.l^{-1}$ of NaCl. Treatment with 4 $mg.l^{-1}$ NaCl also showed an increased amount of harmaline concentration in both root and stem, with values of 2.72 and 2.65 $\mu g.g^{-1}$, respectively. The analysis and calculation of gene expression by real-time PCR of RNA showed that the induced stem callus had a high gene expression with a copy number of 229,030. While in the root, the effect of higher salinity increased the percentage of alkaloids without increasing the copy number of gene expression.

Keywords: *Peganum harmala*, callus, harmine, harmaline, GC, RT-PCR, RNA

التحليل الجزيئي لكالس نبات الحرمل *Peganum harmala* L. لتحديد التعبير الجيني لقلويدات بيتا كاربولين حرمين وحرمالين.

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الخلاصة

تعمل الاشارات الخارجية والمستخدمة كمحفزات على تحوير مستويات انتاج مركبات الايض الثانوي، وتعتبر كزناد في تطبيقات التقانات الاحيائية لتحسين الانتاج النباتي. وقد استخدم ملح كلوريد الصوديوم كعامل تحفيزي لانتاج المركبات القلويدية الحرمين والحرمالين لنبات الحرمل. استخدم كالس نبات الحرمل و المستحث خارج الجسم الحي كمصدر لتجارب التحفيز بملح كلوريد الصودوم. حيث بينت النتائج ان 2 ملغم.لتر$^{-1}$ من NaCl ادت الى زيادة الوزن الطري والجاف للكالس بمعدل 944.30 و 72.0ملغم على التوالي. بينت نتائج تحليل كروماتوغرافيا ان اعلى تركيز لمركب الحارمين بلغ 58.55 و56.50 مايكروغم.غم$^{-1}$ في الكالس المستحث من الجذر والساق على التوالي عند تركيز 4 ملغم.لتر$^{-1}$ من NaCl بالاضافة الى زيادة انتاج مركب الحرمالين بمعدل2.72 و 2.65 مايكروغم.غم$^{-1}$ للجذر والساق على التوالي

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عند التركيز الملحي نفسه. كما بينت نتائج التحليل المتضاعف المتسلسل RT–PCR لمادة RNA ان الكالس المستحث من الساق قد اعطى اعلى عدد نسخ من التعبير الجيني للجين المسؤول عن انتاج مركبي الحارمين والحرمالين بلغ 229,030,في حين ان التأثير الملحي على الجذر ادى الى زيادة تركيز القلويدين ولم يؤدي الى اي زيادة في عدد نسخ التعبير الجيني.

Introduction

The medicinal plant *Peganum harmala*, from Nitrariaceae family, is a wild perennial plant distributed in the middle and southern areas of Iraq in deserts and dry soil [1][2]. The isolation of different types of chemical ingredients from *P. harmala* such as alkaloids (mostly β-carbolines such as harmine, harmaline, harmalol, harmol and tetrahydroharmine), tannins, steroids, sterols, saponins, flavonoids, anthraquinones, amino acids, and polysaccharides was studied through phytochemical studies and qualitative analyses [3]. The β-carbolines, harmine and harmaline, were found to be the main substances responsible for the antimicrobial activities [4], with their extracts showing antioxidant [5], vasorelaxent [6], and anti-inflammatory effects [7], as well as being used as antiviral agents for the treatment of genital herpes HSV-2 [8].The biosynthesis of secondary compounds can be enhanced and improved by the addition of trace amounts of elicitors *in vitro* or *in vivo* [9]. Different environmental stress factors, including abiotic factors such as salinity, drought stress, thermal stress, light and others often increase the accumulation of secondary metabolites in plant *in vitro* cell cultures [10]. Sodium chloride is considered as one of the abiotic stress factors; it is an ionic compound rwith a 1:1 ratio of sodium and chloride ions that have molar masses of 22.99 and 35.45 g/mol, respectively. Hence, 100 g of NaCl contains 39.34 g of Na and 60.66 g of Cl[11]. The aim of this study was to determine the chemical ingredients through Gas chromatography (GC) to estimate the quantity and quality of secondary products, along with utilizing Real-time Polymerase chain reaction (RT-PCR) for amplification and quantification of RNA responsible for the associated genes expression.

Materials and methods

Callus source media

The callus was taken from media prepared from full strength Murashige and Skoog (1962) ready-made MS medium from Himedia Company. pH was adjusted to 5.8 by using 0.1N NaOH or 0.1N HCl, with 2 $mg.l^{-1}$ of kinetin and 0.5 $mg.l^{-1}$ of 2, 4-D hormones with 7 $g.l^{-1}$ agar added to the medium. The volume was completed to 1 liter with water. After four weeks, the callus was transferred into the salt induction media.

Stock solution NaCl salt preparation

The stock solution of NaCl salt was prepared by dissolving 1 g from pure NaCl in 100 ml of distilled water and kept in the refrigerator. Different concentrations (0.0, 0.5, 1, 2 or 4 $mg.l^{-1}$) were then prepared from the stock in order to be used in the stimulating experiments.

Preparation of induction media

Full strength Murashige and Skoog (1962) MS medium was adjusted to 5.8 pH using 0.1N NaOH or 0.1N HCl with 2 $mg.l^{-1}$ of kinetin and 0.5 $mg.l^{-1}$ of 2,4-D hormones. Then 0.0, 0.5, 1, 2 or 4 $mg.l^{-1}$ of NaCl stock and 7 $g.l^{-1}$ agar were added to the medium. The volume of the medium was completed to 1 liter water. The medium was dissolved by heating on a hot plate with a magnetic stirrer until boiling. The medium was dispensed equally into cultural tubes (50 ml/tube) which were autoclaved at 121 C° under pressure of 1.04 $kg.cm^{-2}$ for 15 minutes, then allowed to cool at room temperature.

The measurement of callus fresh & dry weight

The callus fresh weight was measured after 6 weeks of callus sub-culturing onto salt media by cleaning the callus from the medium, placing it on a filter paper, and then measuring it by sensitive electronic balance. The fresh callus was dried in the oven at 60 C° for 48 hrs. The steady weight was taken and considered as a callus dry weight. The fresh callus was then taken for GC detection and molecular analysis.

GC analysis

The chemical compounds that include alkaloids were diagnosed by Gas chromatography (GC) model SHIMADZU 2010. The separation column type was DB5 with 30 mm × 0.25 mm × 0.25 mm dimensions. The temperature began in 90c and gradually was brought to 220 C° with an increasing rate of 10 C°/min, while the temperatures of the injection area and detector area were 280 and 340 C°, respectively. In the detector area, the indicator type was the flammable ionized detector for active compound indication, while nitrogen was used as a transporter gas [12].

RNA extraction

RNA was isolated from callus samples according to the protocol of TRIzol™ Reagent. For each tube, 1 ml from TRIzol™ Reagent was added per 50-100 mg of sample (callus tissue samples) and gently mixed by vortex. The separation phases were performed with the addition 0.2 ml of chloroform to each tube of the lysate then the tube cap was secured. All mixes were incubated for 2-3 minutes then centrifuged for 10 minutes at 12,000 rpm until the mixture was separated into a lower organic phase, interphase, and a colorless upper aqueous phase. The aqueous phase containing the RNA was transferred to a new tube. Isopropanol was added in 0.5 ml to the aqueous phase, the mixture was incubated for 10 minutes, and then centrifuge for 10 minutes at 12,000 rpm. Total RNA precipitated and formed a white gel-like pellet at the bottom of the tube. The supernatant was discarded, ethanol 70% was added as 0.5 ml for each tube, and the sample was briefly mixed by vortex. Then, the samples were centrifuged for 5 minutes at 10000 rpm. Ethanol was aspirated and the pellet was air-dried. Pellet was rehydrated in 100 µl of Nuclease-Free Water then incubated in a water bath or heat block set at 55–60 C° for 10–15 minutes [13].

Detection of harmaline gene sequence

The gene of harmaline production and mRNA sequence were obtained from the National Center for Biotechnology Information (NCBI) by providing access to biomedical and genomic information [14]**.** The primer sequence of the Harmala gene was: Forward (5`-CGGTACGGTGGTTTCAGCAT-3`) and Reverse (5`-GTTGGTGGAGGTGCTGATCT-3`).

Quantitation of RNA, cDNA

Quantus fluorometer was used to detect the concentration of extracted RNA and cDNA in order to detect the goodness of samples for downstream applications. With 1 µl of RNA or cDNA, 199 µl of diluted Quanty Flour Dye was mixed. After 5 min incubation at room temperature in dark place, RNA concentration values were detected.

Statistical analysis

The experiments were implemented by augmented factorial and completely randomized design (CRD). The data were analyzed by the GenStat software program V. 12.1, and the means were compared by the least significant difference (LSD) at p 0.05 [15].

Results and discussion

The results presented in a Tables-(1 and 2) as well as Figure-1 show that there were differences between the different concentrations of NaCl treatments and the control in the percentage of callus fresh and dry weight. As it shown in Table-1, there were significant differences among the four treatments of NaCl. The callus treated with 2 $mg.l^{-1}$ of NaCl (A3) showed significant differences with other concentrations, with an average fresh weight of 944.30 mg, followed by 873.3 mg on 4 $mg.l^{-1}$ medium of NaCl.

The fresh weight of callus induced from the root explants was increased with raising NaCl concentration. The highest fresh weight of the induced root callus was recorded as 1114.3 mg in 4 $mg.l^{-1}$ NaCl concentration, while in stem explants, the highest value was 818.3 mg recorded in 1 $mg.l^{-1}$ of NaCl concentration. The root showed significant preponderance over the stem in increasing the fresh weight of callus, with means of 790.32 and 608.52 mg, respectively.

In respect to the dry weight, there were no significant differences among the treatments with different salinity levels, although A3 treatment caused the highest mean value (72.0 mg) followed by A4 treatment (64.8 mg) (Table-2).

On the other hand the A1 treatment caused the lowest average in dry weight compared with the higher concentrations of NaCl treatments. The A4 treatment caused a high value of callus dry weight (81.4 mg) from root explants. In general, there was an increase in dry weight that was induced from root explants, as compared to the control group, due to the effect of NaCl salt on the ingredients of the tissue culture in this study.

In vitro, the combination levels of sodium chloride salt NaCl are necessary to stimulate the vital activities required for growth. The osmotic stress triggers a series of changes of endogenous hormone metabolism, sensing, and signal transduction, which leads to increasing sucrose uptake and starch accumulation and provides sufficient carbon source for further shoot regeneration [16].

In plant tissue, the cells are stimulated to absorb the water and the ions necessary for growth, leading to decreased water voltage of the cells and thus maintaining the pressure and ionic balance. The increasing absorption of nutrients is a means of adaptation for saline stress conditions [17]. The

decrease in fresh and dry weight under the high salt concentrations in the stimulating media is due to osmotic and ionic effects. The increased salinity level leads to reduced water availability, affects cell growth, and reduces the osmotic pressure in the growth media [18]. Salinity affects the synthesis of proteins needed for cell growth by inhibiting certain enzymes responsible for building proteins. In addition, decreasing the RNA necessary for protein synthesis might inhibit metabolic processes in the cell [19]. Previous *in vivo* experiments showed that salinity reduced the primary and lateral root length and weight, density, root diameter, shoot dry weight, shoot/root ratio and root water content, but increased the root length/ root weight ratio [20].

Table 1-The effects of NaCl treatments on fresh weight of *P. harmala* root and stem callus

Media code	Fresh weights (mg)		Mean
	Root	**Stem**	
Cont.	500.0	400.0	450.0
A1 (0.5 mg/l)	603.0	376.7	489.8
A2 (1 mg/l)	661.0	818.3	739.7
A3 (2 mg/l)	1073.3	815.3	944.3
A4 (4 mg/l)	1114.3	632.3	873.3
LSD	205.8		37.41
Mean	790.32 a	608.52 b	
LSD	102.9		

Table 2-The effects of NaCl treatments on dry weight of *P. harmala* root and stem callus

Media code	Dry weights (mg)		Mean
	Root	**Stem**	
Control	35.00	30.00	32.5
A1 (0.5 mg/l)	44.6	41.2	42.9
A2 (1 mg/l)	50.0	66.5	58.2
A3 (2 mg/l)	79.5	64.4	72.0
A4 (4 mg/l)	81.4	48.3	64.8
LSD	N.S		18.86
Mean	58.1	50.08	
LSD	N.S		

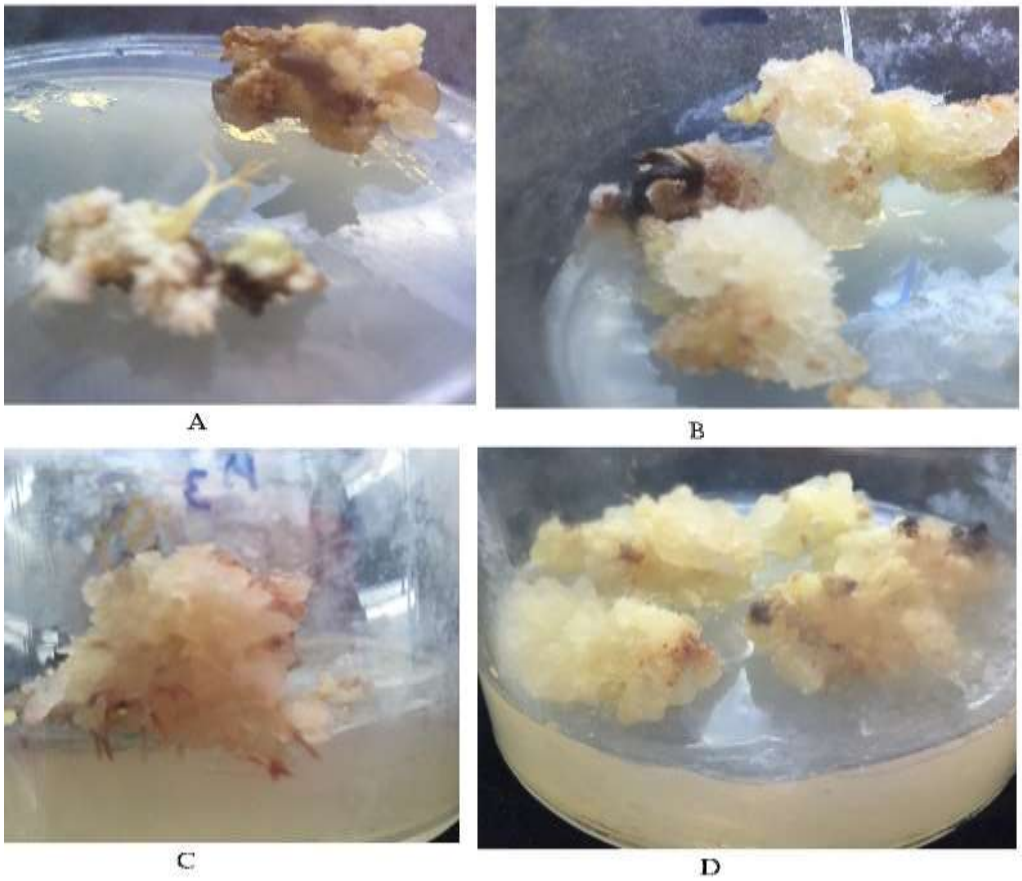

Figure 1-Callus stimulating media of NaCl treatments, A: induced stem callus on media with 0.5 mg NaCl, B: induced root callus on media with 1 mg NaCl, C: induced root callus with hairy roots on media with 2 mg NaCl and D: induced root callus with 4 mg NaCl.

The results shown in Table-3 illustrate the effect of salinity on harmine growth after GC-analysis of callus. The Control 1 in the table represents the stem and the root of field plant (explanted on pots) while Control 2 represents the callus without any treatment. The highest concentration of harmine (58.55 µg/g) was recorded for the root on A4 media (4 mg/l of NaCl) which was significantly different from those of A1, A3, and Cont.2, but insignificantly different from those of Cont. 1 and A2 treatment. The concentration of harmine was increased with increasing salinity, which proved the eliciting effect of salinity on plant cells in more production of secondary metabolites. This agreed with the finding of [21] in their work on Radish sprouts (*Raphanus sativus* L.).

Similar results were found with stem explant callus, where the highest amount of harmine (56.50µg/g) was obtained from A4 treatment which was very close to that from field stem intact plant (Cont.1).

In the current study, the root explants show higher ability than the stem explants to produce the β-Carboline alkaloid harmine. The treated callus is more likely to produce the metabolites in larger quantities than in their natural accumulation.

In Table-4, the A4 treatment showed an increase in harmaline concentration in both root and stem (2.72 and 2.65 $\mu g.g^{-1}$, respectively), although harmaline content in the root was higher than that in the stem. Hoever, there were no significant differences between natural production of harmaline in the field and that under salinity treatments.

Table 3-The concentration of harmine alkaloid in root and stem after NaCl elicitation

Sample code	Harmine µg.g^{-1} in root	Harmine µg.g^{-1} in stem
Cont.1 (field)	40.95	56.13
Cont.2 (lab.)	03.00	03.69
A1(0.5 mg/l)	03.13	03.36
A2 (1 mg/l)	43.74	19.43
A3 (2 mg/l)	38.61	16.39
A4 (4 mg/l)	58.55	56.50
Mean	31.33	25.92
SE±	9.38	9.97
P-Value¥	N. S.	

Table 4-The concentration of harmaline alkaloid in root and stem after NaCl elicitation

Sample code	Harmaline µg.g^{-1} in root	Harmaline µg.g^{-1} in stem
Cont.1 (field)	2.67	2.71
Cont.2 (lab.)	0.27	0.84
A1(0.5 mg/l)	1.43	0.78
A2 (1 mg/l)	1.63	1.49
A3 (2 mg/l)	1.52	0.18
A4 (4 mg/l)	2.72	2.65
Mean	1.70	1.44
SE±	0.37	0.42
P-Value¥	N. S.	

Table-5 shows the concentration of harmine and harmaline with their gene expression copy number in the root and stem tissues treated with high concentrations of salt. In the root, the effect of salinity increased the percentage of alkaloids without increasing the copy number of the gene responsible for the production of harmine and harmaline.

This state may be due to the protective mechamisms in root cells against environmental stresses. Here, the salinity stress induced and increased production of the secondary metabolites as a normal defense mechanism without impacts on genetic content. This suggests that the nature of the root cells of *P. harmala* tolerated the high concentration of the salinity stress, as previously shown in the roots of the field [22]. Deletions and duplications of chromosomal segments (copy-number variations or CNVs) constitute a major source of such variation [23].

On the other hand, in the stem cells, the highest copy number of the gene expression was recorded within high concentrations of salts. The results recorded a high concentration of alkaloids which may

be due to the stimulating effect of salinity as an abiotic factor on the production of alkaloids in the stem.

It is expected that a better understanding of the signal transduction pathways, linking plant cell stimulation and biosynthesis of natural compounds, may help to develop new strategies to alter the production of target compounds, by either activation or suppression of certain metabolic pathways [24].

While in the stem explant, the high gene expression copy number was recorded in control treatment in laboratory 229,030 but in very low production of alkaloid comparison with other treatments, may be the gene did not encode for alkaloids or there were other factors that affected the production of harmine or harmaline.

Another suggested explanation is the presence of many other genes that synergistically affect production. It is also possible that other factors might genetically impact as suppressors for the encoding of the gene of interest. Single-molecule full-length complementary DNA (cDNA) sequencing was able to facilitate genome annotation by revealing transcript structure and alternative splice forms of two plant genomes (*Arabidopsis thaliana* and *Oryza sativa*) [25].

Table 5-The highest values of the concentration of harmine and harmaline by GC-analysis and the copy number of gene that produce this alkaloids in root and stem of *P. harmala*

	Samples	Harmine µg.g^{-1}	Harmaline µg.g^{-1}	Gene expression Copy no.
Root	Root field	40.95	2.67	927
	Root callus	03.00	0.27	744
	NaCl 4 mg/l	58.55	2.71	631
Stem	Stem field	56.13	2.71	1,092
	Stem callus	03.69	0.84	229,030
	NaCl 4 mg/l	56.50	2.65	1,313

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