

Importance of Hormonal Elicitors in Inducing Morphine Biosynthesis in the Cell Culture of (*Papaver bracteatum* Lindl.)

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ABSTRACT

Plant cells have enough capacity to produce many of secondary metabolites, similar to the whole plants. Elicitation is one of the most significant methods to increase the synthesis of secondary metabolites in the medicinal plants. The purpose of this study was to investigate the effect of three different hormones on alkaloids production in suspension culture of *Papaver bracteatum* Lindl., in order to identify the relationship between the alkaloid biosynthesis and gene expression. Inducible factors initiate Benzylisoquinoline Alkaloids (BIAs) biosynthesis in Opium poppy. The current study investigated the accumulation of alkaloids content and Tyrosine/Dopamine Carboxylase (TYDC), Berberine Bridge Enzyme (BBE), Salutaridinol Acetyl Transferase (SAT), and Codeinone Reductase (*COR*) gene transcripts in suspension culture of *P. bracteatum*. Indole-3-Acetic Acid (IAA), Indole Butyric Acid (IBA) and Gibberellic Acid (GA) were used as hormonal elicitors in the suspension cultures with three different doses and two timings along with the control. This research showed the induction of morphine alkaloid in the suspension culture of *P. bracteatum*. Elicitation by 20 mg L⁻¹ concentration of IAA after 48 h indicated significant increase in morphine amount. Comparison among genes revealed that the expression levels of *COR* dramatically increased while TYDC, BBE and SAT had no significant difference compared to the control. After elicitation by IAA, IBA, and GA, the highest levels of morphine were measured as 243.2, 207.2, and 178.1 mg g⁻¹, respectively. The results demonstrated that timing had a significant effect on the hormonal elicitation: 48 h treatment could induce more morphine alkaloids compared to 24 hours treatments.

Keywords: Elicitation, Gene expression, Gibberellic acid, Indole butyric acid, Indole-3-acetic acid.

INTRODUCTION

Iranian Poppy (*Papaver bracteatum*) is an important medicinal plant that is the main source of the opium alkaloids. In addition, opium and the isoquinoline alkaloids morphine, codeine, noscapine, papaverine, and thebaine are isolated from the dried material. Some accessions of *P. bracteatum* contain 98 percent thebaine of whole alkaloids. Therefore, this species is an outstanding source of basic materials to produce narcotics, 0.7 to 1.3 percent in the roots, 26 percent in dry latex or 3.5 percent in the dry, ripe capsules (Day *et al.*, 1986). The original habitat of *P.*

bracteatum is the mountainous areas of Iran (the Alborz Mountains and Mahabad regions), Turkey and Transcaucasian Russia. The flowers of *P. bracteatum* are red with long petals along with big blackish spots in the middle. According to the cytogenetic researches, this species is diploid with the chromosomal base number 2n= 14 (2X) (Balažová *et al.*, 2008; Cline and Coscia, 1988).

Alkaloid biosynthesis in plants involves many catalytic steps, catalyzed by enzymes that belong to a wide range of protein families (Bohm, 1967; Rostampour *et al.*, 2009; Sharafi *et al.*, 2013 Nasiri *et al.*, 2016). Thebaine, as one of the alkaloid components,

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is produced in the cell culture of *P. bracteatum* at the early stages of cell growth, but the concentration of thebaine in the cells is diminished. It is interesting to note that, unlike the prediction of having substantial amounts of thebaine in the tissue culture, L-stylopine and protopine are formed in the media (Kamimura et al., 1967). A very small amount of oxysanguinarine and sanguinarine were detected in suspension culture of *P. bracteatum*, however, dihydrosanguinarine was produced as principal alkaloid (Hook et al., 1988). The research revealed that morphinan collect in the existence of laticifer cells, but these kinds of cells are not essential for benzophenanthridine alkaloids or their precursors (Rush et al., 1985). The regeneration of *P. bracteatum* from tissue culture was developed and regenerated plants were transferred to soil successfully. Regeneration of *P. bracteatum* can be helpful to produce improved lines because this plant is self-incompatible (Day et al., 1986).

In other word, elicitation is the procedure of induced biosynthesis of secondary metabolites, which is comparable to resistance responses in plants against pathogen contamination (Doke, 1983). Several parameters, such as elicitor concentration and selectivity, duration of elicitor exposure, age of culture, cell line,

growth regulation, nutrient composition, quality of cell wall materials, and substantial enhancement of product accumulation, have been reported among which elicitor concentration plays a very important role in the elicitation process (Namdeo et al., 2007).

The conversion of L-Tyrosine to dopamine and 4-hydroxyphenylacetaldehyde via a lattice of ortho-hydroxylations, deaminations, and decarboxylations is the first step of Benzylisoquinoline alkaloid biosynthesis. The role of Tyrosine/DopaDecarboxylase (TYDC) is in transformation of l-tyrosine and l-dopa to tyramine and dopamine, respectively (Figure 1). The conversion of (S)-reticuline to (S)-scoulerine is catalyzed by Berberine Bridge Enzyme (BBE) which is the first step in sanguinarine pathway (Facchini et al., 1996). Salutaridinol Acetyl Transferase (SAT) catalyzes the conversion of (7S)-salutaridinol to Salutaridinol-7-O-acetate for the spontaneous production of thebaine (Samanani et al., 2006). Codeinone Reductase (COR) converts codeinone to codeine as the critical role in morphine biosynthesis (Unterlinner et al., 1999).

Codeinone reductase gene, which has a main role in the creation of morphine and codeine, was used to produce transgenic plants. Alkaloid content in transgenic plants had a

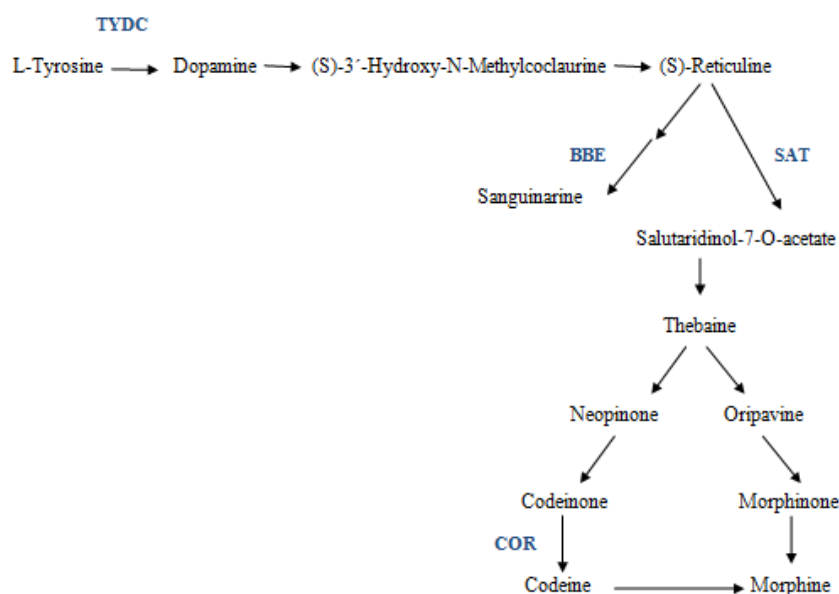


Figure 1. Biosynthesis pathway of thebaine, codeine, morphine, papaverine in *P. somniferum* TYDC, BBE, SAT and COR.

significant increase in comparison to the control group. The results indicated a 10-fold increase in morphinan alkaloids content in comparison with the control plants (Larkin *et al.*, 2007). We propose research design to increase the amount of alkaloid in cell culture by using three different hormones, namely, Indole-3-Acetic Acid (IAA), Indole Butyric Acid (IBA) and Gibberellic Acid (GA) as elicitors. In this project, we aimed to establish the cell cultures and induce gene activation using elicitors. Finding effective elicitors to induce more alkaloids and their influence on the gene expression of alkaloid biosynthesis was also evaluated. Additionally, the current study investigated the accumulation of alkaloids content and *TYDC*, *BBE*, *SAT* and *COR* gene transcripts in suspension culture of *P. bracteatum*.

MATERIALS AND METHODS

Plant Material

Seeds used as experimental material were obtained from the Research Institute of Forest and Rangelands, Tehran, Iran (Polur, 14305 accession number). The ethanol 70% (v/v) was utilized to sterilize the seeds for 1 minute (3 times) and sodium hypochlorite solution 50% for 8 minutes, then, washing with double sterile distilled water. Modified MS medium was used for germination of seeds (Rostampour *et al.*, 2009; Zare *et al.*, 2014). The cell cultures of *P. bracteatum* were grown at 16 hours photoperiod cool white fluorescent light. Seeds germinated after adding 7.5 g L^{-1} agar in the dark room at 25°C (Kamo *et al.*, 1987). The explants were separated from roots after germination during four weeks of the planting.

Cell Suspension Culture

The explants of *P. bracteatum* were transferred to the modified MS medium including 0.5 mg L^{-1} BAP, 0.5 mg L^{-1} 2-4-D and 7.5 g L^{-1} agar were added in the solid medium for callus induction.

Callus culture was the subculture of the fresh medium every 21 days. In the next step, to initiate the suspension cultures, friable calli (50 mg) was transferred to 25 mL fresh medium with the same concentration of hormones in 100 mL flasks were incubated on the shaker at 120 rpm, in a dark room at $25 \pm 2^\circ\text{C}$. The suspension cultures were established after three different subcultures. The percentage of volume and weight cells was measured. The cells entered the growth phase and, gradually, the rate of cell growth diminished (Farjaminezhad *et al.*, 2013). Elicitations on samples were performed after 23 days of inoculation when the cell cultures were in the exponential phase of growth. To investigate the effects of IAA, IBA and GA, hormones elicitors were added in three different concentrations (5 , 10 , and 20 mg L^{-1}) to the 25 mL suspension culture of *P. bracteatum*. Additionally, an equal amount of medium with no elicitation was used as the control. Then, all cells were collected by filter papers after 24 and 48 hours treatments to evaluate the optimal time and elicitors for the highest level of alkaloid production.

Alkaloid Extraction and HPLC Analysis

Freeze-drier (Lyotrap, Fisher Scientific, USA) was used to dry the cells. Alkaloids were extracted by the mixture of methanol and HCL. Alkaloids were measured by HPLC system along with standard compounds. HPLC analysis was performed on a Knauer Smartline HPLC system (UV detector). A volume of 25 mL of samples was injected in a C-18 reverse-phase Knauer Smartline column (201Tp54 VDAC). The mobile phase for alkaloid elution was 50% methanol and 50% water. (Tisserat and Berhow, 2009). In addition, thebaine, morphine, codeine, papverine, noscapine, and sanguinarine as standards were obtained from Temad Chemical Co (Tehran, Iran).

Gene Expression

Approximately 100 mg of cells treated with elicitors were applied for the expression on the analysis of alkaloid biosynthetic genes. Then, they were grounded to a powder in



liquid nitrogen, and the total RNAs were isolated with the RNeasy Plant Mini Kit (Qiagen). The quality and quantity of the RNAs were estimated by spectrophotometry and Nano drop. Reverse transcription was performed using a First Strand cDNA Synthesis Kit (Fermentas), and Elongation Factor 1 alpha (EFa1) was the reference gene with primers generated for it according to the method used in Zhang's study (2008). RT-PCR was performed by *TYDC*, *BBE*, *SAT*, and *COR* in genes to analyze the expression levels of the gene. These primers were designed using Primer3 and Perlprimer software programs. The primers are listed in Table 1. The Real-Time PCR on SYBR Green was conducted using an iQ5 (BIORAD) machine under the following conditions: the process was performed at 95°C for 90 seconds followed by 40 cycles of 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 20 seconds. In addition, data were normalized using the levels of *EFa1* transcripts (Ranasinghe et al., 2008).

Statistical Analysis

The statistical significance of data was evaluated by one-way Analysis Of Variance (ANOVA). Each treatment contained three independent replicates and SAS Statistic performed the experiments three times. Furthermore, mean comparisons were carried out using Duncan's multiple range tests at a probability level of 0.05.

RESULTS AND DISCUSSION

The results of this study revealed that the main component was morphine for all of the elicitation treatments and the control. In addition, morphine was recognized as a major alkaloid and a little thebaine and codeine in the different samples after treatment by indole-3-acetic acid, indole butyric acid and gibberellic acid hormones. Our analyses demonstrated that timing had a significant effect on the elicitation treatments of hormones, since 48 hours of treatment produced more morphine alkaloids compared to the 24 hours treatments after using elicitors. On the contrary, the evaluation among three hormones indicated that Indole-3-acetic acid induced more morphine alkaloid. In the concentration of 20 mg L⁻¹ and 48 hours of the Indole-3-acetic acid and Gibberellic acid, the level of morphine increased, but the highest level of morphine was obtained after using 10 mg L⁻¹ in 48 hours by Indole butyric acid. The treatment of cells with Indole-3-acetic acid at a concentration of 20 mg L⁻¹ in 48 hours raised the amount of Morphine, whereas the addition of this elicitor at concentration of 10 mg L⁻¹ at 24 hours decreased the quantity of Morphine. Morphine yield of the cells at 48 hours after elicitation in the concentration of 20 mg L⁻¹ enhanced 2.287-fold in comparison with the control. Also, the effect of Indole-3-acetic acid on morphine production was dose and time dependent. Morphine at 24 hours after using indole-3-acetic acid decreased to half amount compared to the control (Figure 2-

Table 1. Primer sequences designed for real-time amplification of alkaloid biosynthetic genes of *P. bracteatum*.

Primer	Sequences
COR-F	CCACCATTTGACCAGCATTAG
COR-R	GGGCTCATCTCCACCTTAATC
SAT-F	GGGCTCATCTCCACCTTAATC
SAT-R	GCTGGTAAGAACGCCGAAAC
TYDC-F	AACCCACTAGACCCTGATGA
TYDC-R	GACCTGGCTTCTAACTGGATAAC
BBE-F	GGGTTTACTGCTGGTTGGT
BBE-R	ATCGCATCCACGACGTTATC
Elf1a-F	AGATGATTCCAACCAAGCCCA
Elf1a-R	CCTTGATGACACCAACAGCAACT

a). Using indole butyric acid as elicitor on cell culture of Iranian poppy indicated that morphine enhanced after 48 hours treatment. The elicitation of cells with 10 mg L⁻¹ Indole butyric acid after 48 hours treatment had significant effect on morphine production. At this level, morphine reached the 2.03-fold level of the control. The highest amount of morphine alkaloid was 9.09-fold more than the lowest level treatment with Indole butyric acid (Figure 2-b).

Gibberellic acid elicitation with 20 mg L⁻¹ after 24 hours treatment produced more morphine compared to other concentrations. The elicitation of cells with Gibberellic acid at a 20 mg L⁻¹ dose after 48 hours treatment had a significant effect on morphine production. The highest morphine yield obtained at the 20 mg L⁻¹ concentration enhanced this alkaloid 1.75-fold in comparison with the control. The application of Gibberellic acid at the 20 mg L⁻¹ dose after 48 hours increased the quantity of morphine 2.81-fold more than the treatment by

10 mg L⁻¹ in 24 hours. The lowest morphine yield was obtained at the 10 mg L⁻¹ concentration in 24 hours after treatment with all the three elicitors; indole-3-acetic acid, indole butyric acid and gibberellic acid hormones (Figure 2-c).

We used Real-time PCR to recognize the responses of the genes *TYDC*, *BBE*, *SAT*, and *COR* to test whether IAA, IBA and GA hormones could influence transcription of the prominent genes in alkaloid biosynthesis. The current study revealed that indole-3-acetic acid increased the accumulation of morphine through the mechanism shown in Figure 1, in which the morphine content continuously increases until 48 hours after the application of 20 mg L⁻¹. Additionally, the main difference between the highest and the lowest morphine concentration for those plants treated with indole-3-acetic acid is related to the *COR* gene. At the 48 h mark, the expression levels of *COR* up-regulated in comparison to the control, but *TYDC*, *BBE* and *SAT* did not show

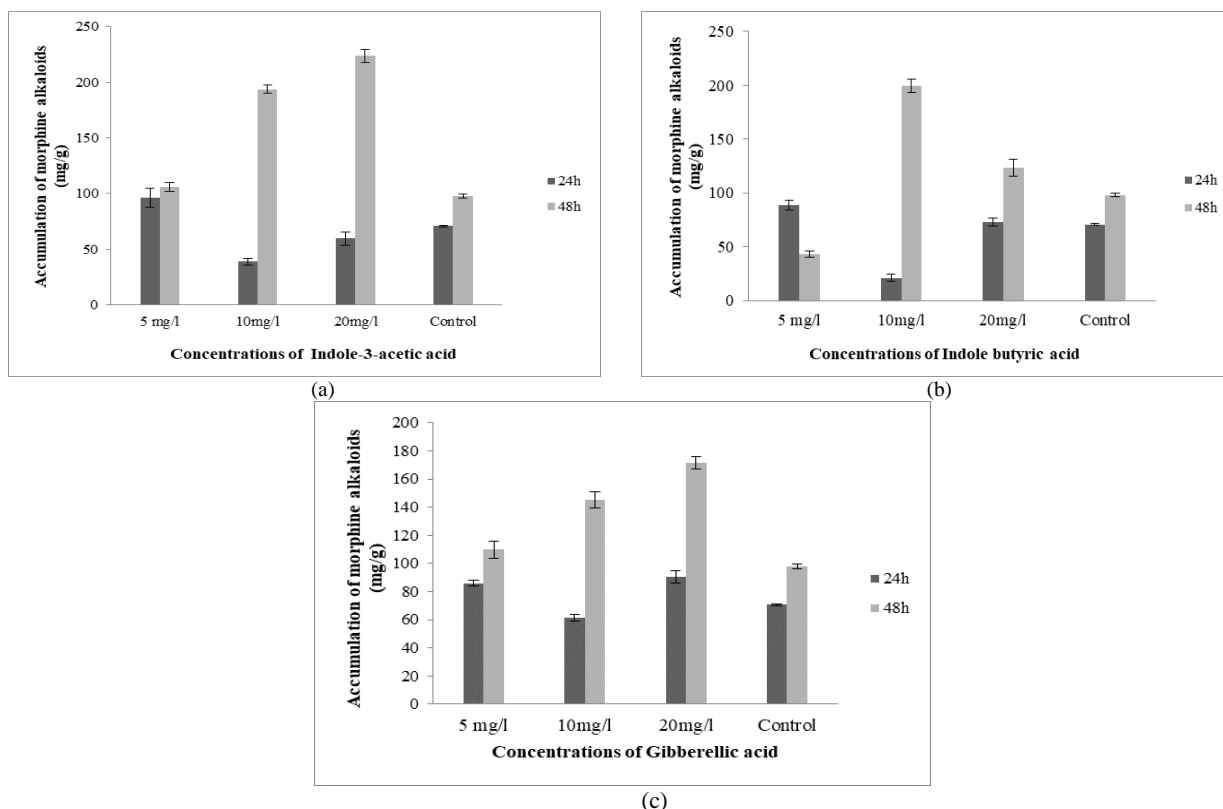


Figure 2. Accumulation of morphine alkaloids in cell culture of *P. bracteatum* after (a) using Indole-3-acetic acid, (b) Indole butyric acid and (c) Gibberellic acid elicitor along with the control.



any significant difference with the control. On the other hand, in the lowest amounts of morphine after 24 h elicitation with 10 mg/l of aforementioned hormone, no difference between the transcript levels of TYDC and SAT in comparison with the control group was observed. There was no difference between the transcript levels of TYDC and SAT in comparison with the control group. Also, the gene expression of BBE and COR down-regulated (Figure 3-a). As a result, Plants treated with Indole butyric acid with 10 mg L⁻¹ after 48 hours treatment showed only significant changes in the transcription levels of BBE, SAT and COR compared to the control. Additionally, in the lowest amounts of morphine after elicitation by Indole butyric acid, transcriptional levels of TYDC and BBE showed down-regulation. However, the

expression level of COR did not change significantly in comparison with the control, and SAT transcript levels were up and down regulated at this concentration (Figure 3-b).

The transcript levels of genes in those plants exposed to Gibberellic acid with the highest level of morphine and lowest level of this alkaloid were more variable. At 48 hours treatment with 20 mg L⁻¹ hormone, the expression levels of BBE and SAT up-regulated, but TYDC and COR had no significant difference compared to the control. In contrast, gene expression levels for TYDC, BBE, SAT and COR in the 10 mg L⁻¹ at 24 hours, considered as the lowest production of morphine alkaloid, did not significantly alter in comparison with the control (Figure 3-c).

In some other researches, different

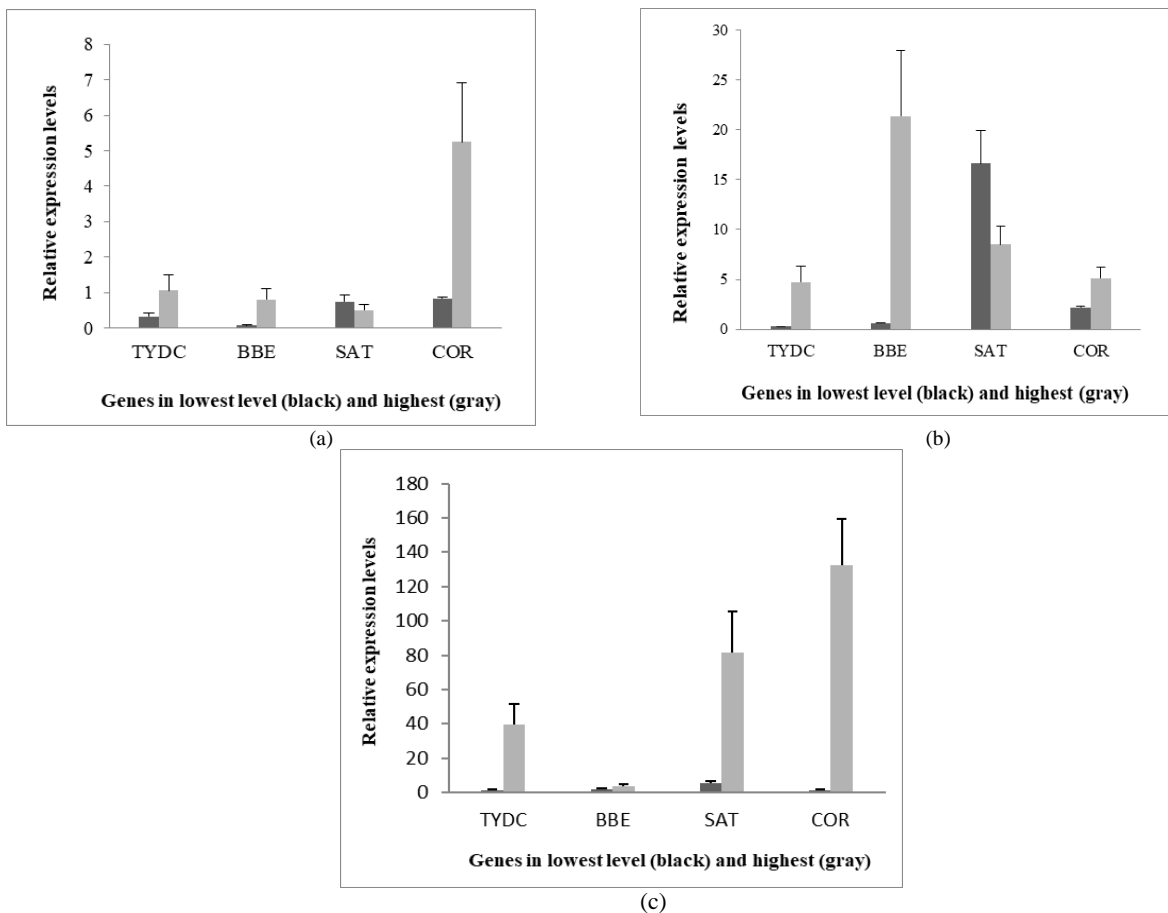


Figure 3. Changes in TYDC, BBE, SAT and COR genes in *P. bracteatum* treated with (a) Indole-3-acetic acid, (b) Indole butyric acid and (c) Gibberellic acid at the lowest and highest levels of morphine concentration.

accessions contained various alkaloids as the major secondary metabolites. Comparing the distinct accessions of *P. bracteatum* indicated that some strains, such as West Germany, only had thebaine as the main alkaloids, while the other seven strains in this report contained isothebaine as the major alkaloid (Kamimura *et al.*, 1967). Research on the natural abundance of morphinan alkaloids in *P. bracteatum* along with the results from feeding codeinone-[16-3H] and codeine-[16-3H] indicated that this species could reduce codeinone to the codeine (Hodges *et al.*, 1977). In another study on the tissue culture of *P. bracteatum*, accumulation of large amounts of dopamine and a small quantity of thebaine and sanguinarine were reported (Larkin *et al.*, 2007). Even though in most studies thebaine has been found as a major alkaloid in the *P. bracteatum* (Farjaminezhad *et al.*, 2013; Zare *et al.*, 2014, Rostampour *et al.*, 2009), there are some reports that show the other alkaloids can be detected from the cell culture of *P. bracteatum*. For instances, sanguinarin accumulated in the cell suspension of *P. bracteatum* after using fungal elicitors and hormones and thebaine were produced in the media without hormones (Cline and Coscia, 1988). Brochmann-Hanssen and Wunderly (1978) confirmed that codeine could be found in *P. bracteatum*, though morphine was not found. This result emphasized that the biosynthesis O-6-demethylation of thebaine to neopinone can be considered restrictive step. The existence of neopinone and codeinone can be caused by acidic condition in the plant, which hydrolyzes nonenzymatically the enol ether group of thebaine. Therefore, these two compounds can be reduced to the related alcohols in the plant quickly (Brochmann-Hanssen and Wunderly, 1978). Moreover, codeine oxidation could produce 14-hydroxycodine and norcodeine in *Streptomyces griseus* (Kunz *et al.*, 1985). It can be concluded that, morphine can be produced by two substantial genes, 6-O-demethylase and codeine O-demethylase and/or codeinone reductase genes, which can proceed the genetic pathway of morphinan alkaloid thebaine to the codeine and morphine.

According to the special patent, genetically modified *P. bracteatum* with nucleic acid and amino acid sequence variants of the aforementioned genes are capable of producing more quantity of an alkaloid selected from codeine, oripavine and/or morphine in comparison with a wild type of this plant. Also, salutaridinol 7-O-acetyltransferase gene converts the phenanthrene alkaloid salutaridinol to salutaridinol-7-O-acetate, which is an important enzyme to biosynthesize morphine in *P. somniferum*. The transcripts of this gene have been identified in *P. bracteatum* in addition to *P. somniferum* (Grothe *et al.*, 2001). Over expression of codeinone reductase in Opium Poppy, which is essential to produce morphine, codeine and various semi-synthetic analgesics, could increase morphinan alkaloids (Larkin *et al.*, 2007).

Unlike primary data, which supported the hypothesis that *P. bracteatum*, has outstanding potential to produce thebaine alkaloid, our results demonstrated that morphine alkaloid can be detected in the cell culture of this plant. In addition, the investigations of gene expression elucidate genetic pathway and molecular basis of alkaloids. The current study revealed that using Indole-3-Acetic Acid (IAA), Indole butyric acid, and Gibberellic acid hormones treatments as elicitors can enhance alkaloid production in the cell culture of *P. bracteatum* with significant changes in the expression of the studied genes.

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اهمیت الیستورهای هورمونی در القای مورفین در کشت سلولی (*Papaver bracteatum* Lindl.)

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چکیده

سلول های گیاهی ظرفیت تولید متابولیت های ثانویه را همانند تولید این ماده در کل گیاه دارند. الیستورها ابزار مناسبی برای افزایش تولید متابولیت های ثانویه در گیاهان دارویی هستند. هدف از این مطالعه بررسی اثر سه الیستور هورمونی به منظور افزایش تولید آلکالوئیدهای گیاه خشخاش ایرانی و همچنین بررسی همزمان ارتباط سنتر آلکالوئیدها و بیان ژن های مربوطه می باشد. در تحقیق حاضر میزان تجمع آلکالوئیدها و هم چنین میزان رونوشت ژن های Tyrosine/dopadecarboxylase (TYDC), Berberine bridge، Codeinone reductase و Salutaridinol acetyl transferase (SAT), (BBE) enzyme (COR) در گیاه *P. bracteatum* در محیط کشت سوسپانسیون سلولی بررسی شد. سه الیستور هورمونی ایندول تری استیک اسید (IAA)، ایندول بوتیریک اسید (IBA) و جیبرلیک اسید (GA) در سه غلظت و زمان متفاوت مورد استفاده قرار گرفتند. نتایج حاصل از این تحقیق، مورفین را بعنوان آلکالوئید غالب در این گیاه نشان داد و بالاترین میزان مورفین در استفاده از الیستور IAA در غلظت ۲۰ میلی گرم بر لیتر در زمان ۴۸ ساعت بدست آمد. هم چنین، مقایسه ی ژن ها نشان داد که سطح بیان ژن COR به مقدار زیادی افزایش یافت، در حالی که بیان ژن های SAT و TYDC, BBE تغییر معنی داری در مقایسه با شاهد از خود نشان ندادند. میزان مورفین در بالاترین سطح تولید شده پس از استفاده از IAA, IBA و GA به ترتیب ۲/۲۴۳،



۲۰۷/۲ و ۱۷۱/۱ میلی گرم بر گرم بدست آمد. نتایج حاصل از این تحقیق نشان می دهد که در استفاده از الیستورهای هورمونی عامل زمان نقش مهمی در القای مورفین ایفا می کند، بطوریکه تیمار در بازه ی زمانی ۴۸ ساعت، مورفین بیشتری را نسبت به ۲۴ ساعت تولید نمود.