Journal of Medicinal Plants and By-products (2021) 1: 69-75

Original Article

Enhanced Production of Thebaine in Cell Suspension Cultures of *Papaver bracteatum* by overexpression of *SalAT gene*

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Article History: Received: 25 November 2019/Accepted in revised form: 25 July 2020

Abstract

The Iranian poppy by the scientific name of *Papaver bracteatum* Lindl. has been long propounded as a source for thebaine. Thebaine is among secondary metabolites that due to its wide spread and unique medicinal properties play a valuable role in production of herbal medicines and pharmaceutical industries on the whole.The objective of the present study is to investigate the enhanced production of thebaine by over expression of salutaridinol 7-o-acetyltransferase (*SalAT*) gene, a key gene in thebaine biosynthesis pathway, in *Agorobacterium*-mediated transformed cell suspension culture of *P. bracteatum*. In this study, the cDNA of *SalAT* with *P. bracteatum* origin was cloned in pBI121 expression vector under CaMV35s promoter. In order to transfer the cloned gene *Agrobacterium tumefaciens* of LBA4404 strain and hypocotyl–cotyledon explants were used. For recognition of transformed calli obtained, PCR and Southern blot analysis were employed and high performance liquid chromatography analysis (HPLC) was applied for determination of thebaine content. The comparison of callus induction and thebaine content between transformed and non-transformed cell culture was studied on completely randomized design with three replications. According to HPLC analysis which revealed a significant 1.7 fold (0.58 mg/g) enhancement in thebaine content in transformed suspension cell culture comparing to non-transformed cell culture (0.34 mg/g), it can be concluded that the over expression of key genes such as *SalAT* gene in the cellular suspension culture of *P. bracteatum* could be a promising potential for mass production of this valuable alkaloid.

Keywords: Cell suspension culture, Over expression, *Papaver bracteatum*, *SalAT* gene, Thebaine

Introduction

Papaver bracteatum Lindl. is a medicinal plant that naturally is scattered and distributes in 1500 up to 2500 m of the skirts of Alborz Mountains, Iranian Kurdistan and northern slope of the Caucasus [1]. Thebaine developed and enhanced in *P. bracteatum* is could be considered as a major alkaloid [2-3] and Because of the low activity of enzymes involved in demethylation to codeine and morphine, could be employed as a non-addictive substitute for morphine as a medical painkiller [4].

In terms of yield, the perennial nature of *P. bracteatum* makes it somehow uneconomical as it cannot be harvested until the second growingseason [5]. This delay in harvest has stimulated research objectives into the potential of in vitro cell culture technologies for Benzylisoquinoline alkaloid production [6]. In vitro culture of plant cells is a promising source for the production of highly valuable secondary metabolites from medicinal plants. Instead of growing the plants in their natural habitats where they are subjected to environmental barriers and obstacles such as climate and temperature change, plant cell cultures produce valuable secondary metabolites under controlled and defined conditions [7]. Considering the fact that the most practical synthetic routes to prepare important pharmaceuticals including oxycodone, naloxone, naltrexone, nalbuphine and buprenorphine have utilized the alkaloid, thebaine, as a starting material, its production on the scale of commercial is important [8]. Therefore, cell suspension culture systems couldbe used an appropriate approach for large-scale, defined, continuous and reliable source of bioactive natural products [9]. The aim of the current study was to evaluate the possibility of increasing thebaine content in

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cell suspension culture of *P. bracteatum* by over expression of salutaridinol 7-o-acetyltransferase (*SalAT*, EC 2.3.1.150) gene. *SalAT* is a key gene to produce thebaine in *P. bracteatum* by converting the salutaridinol to salutaridinol-7-0-acetate.

Material and Methods

Plant Material, Strains and Plasmid

Papaver bracteatum seeds were provided by Medicinal Plant Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran). *Escherichia coli* DH5α used in all molecular experiments and *A. tumefaciens* strain LBA4404 used for transformation procedure. The binary vector pBI121 was used for cloning the target sequence to generate the binary vector containing the salutaridinol 7-o-acetyltransferase gene (*SalAT*) isolated from *P. bracteatum.*

General Procedures

Bacteria were grown in Luria-Bertani (LB) medium (Bacto-tryptone 10g/l, Bacto-yeast extract 5 g/l, NaCl 10 g/l) at 37 ºC and *A. tumefaciens* strain was grown in YEP medium (LB supplemented with rifampicine (25.5 mg/L) at 28 ºC with shaking (150 rpm).

Plasmid DNA preparation was performed by routine procedures. *E. coli* DH5α was transformed by using the $CaCl_2$ method and the recombinant plasmid was then mobilized into *Agrobacterium* by freez-thaw method [10].Enzymatic treatments of DNA molecules were carried out as recommended by the manufacturer (Fermentas, Canada).

Plasmid Construction

One hundred nanogram of RNA was used as template for reverse transcription using cDNA synthesis kit (Invitrogen, USA). A poly-T oligonucleotide (oligodT) primer was used which hybridizes onto the poly-A tail of the mature mRNA template. For reverse transcription, the reaction mixture was incubated for 1 h at 42 ºC. Following cDNA synthesis, RT-PCR reactions were performed using cDNA as the template. The forward and reverse primers used were SalATF/SalATR primers (Table. 1) containing *BamH*I and *Sac*I sites at their 5´ ends. The PCR product was subjected to electrophoresis on 1% agarose gel and then, it was cloned into pBI121 at the homologous sites (*BamH*I and *Sac*I sites). The recombinant plasmid contains, within the T-DNA region, neomycin phosphotransferase II (*NPTII*) gene as a selectable marker that is kanamycin-resistant gene (Fig. 1).

Preparation of the Explants and Bacterial Strain for Transformation

The seeds were washed thoroughly with tap water and surface-sterilized with 70% (v/v) ethanol for 1min and sodium hypochlorite solution 1.5% (w/v) for 8 min, and then rinsed 3 times (5 min each) with sterile distilled water to remove any traces of the surfactants.

Table 1 Oligonucleotides (primers) used in PCRand Southern blot analysis

Name	Oligonucleotides sequence
SalATF	5'- TTGGATCCGCCATGGCAACAATGTATA-3'
SalATR	5'- GAGAGCTCTCAAAATTCAAGGATTTCA-3'
35S	5'-GCGAACAGTTCATACAGAGTCT-3'
NOSR	5'-GTGAAGCTTCCCGATCTAGTAACAT-3'
virGf	5'-ATGATTGTACATCCTTCACG-3'
virGr	5'-TGCTGTTTTTATCAGTTGAG-3'
CaMV35SF	5'-GGACTAACTGCATCAAGAACACAG-3'
CaMV35SR	5'-GAAGGATAGTGGGATTGTGCGTC-3'

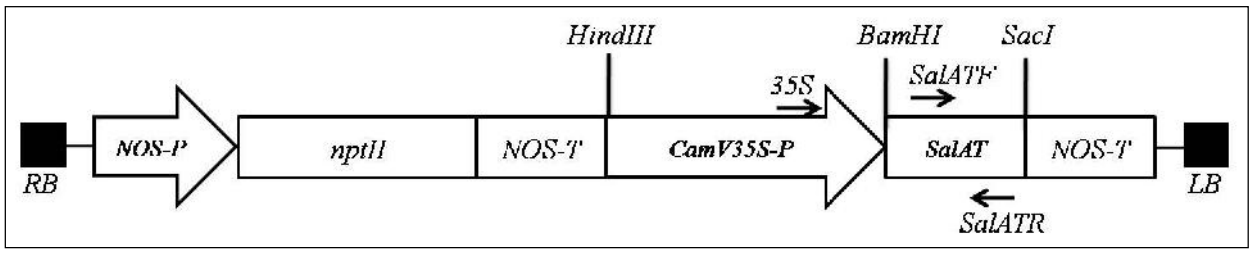

Fig. 1 Schematic representation of the T-DNA region of recombinant plasmid. The position of primers and recognition sites of restriction enzymes are indicated. RB, right border; LB, left border; CamV35S-P, cauliflower mosaic virus 35S promoter; NOS-P and NOS-T, nopaline synthase promoter and terminator; *npt*II, neomycin phosphotransferase

The seeds were then germinated on MS medium [11] solidified with 6/5 g/L plant agar, and maintained in a growth chamber at 25 ± 2 °C with a 16/8 h (light/dark) photoperiod under a photon flux of 6000 Lux, provided by fluorescent lamps. After germination, hypocotyl–cotyledon explantswere cut and placed on the MS solid medium with 2 mg/L 2, 4-Dichlorophenoxyacetic (2.4-D) along with 0.2 mg/L kinetin and 15 mg/L ascorbic acidfor pre-culture. After 2 days, the explants were used for transformation. Single colonies of the *A. tumefaciens* harboring *SalAT* gene were grown in the LB medium supplemented with 20mg/l kanamycin, and allowed to grow overnight at 28 ºC with constant shaking (180 rpm) to mid-log phase. The bacterial culture was then transferred to a fresh medium cultivated till OD600 = 0.4 with liquid medium. The bacterial cells were collected by centrifugation (6000 rpm for 5 min) and re-suspended in ½MS medium for the subsequent inoculation step.

Papaver bracteatum Transformation and Selection Procedure

The explants were immersed in the bacterial suspension for 5 min with constant shaking, and then placed on the sterile filter paper to remove the excessive moisture. Then, they were placed on the MS solid medium 3 mg/L 2,4-D and 0.5 mg/L PAP in the Petri dishes for co-cultivation at 25 ºC for 3 days in the dark. After co-cultivation, the explants were washed with the sterile water containing 400 mg/l cephatoxime to inhibit the growth of *A. tumefaciens* attached to the explants.

Callus induction and Cell Suspension Culture

The non-transformed explants (5 ± 1 mm segments) from 2-weeks-old in vitro grown plants were cultured on solid MS medium fortified with 2 mg/L 2, 4-D, 0.2 mg/L BAP and 15 mg/L ascorbic acid. The transformed explants were also cultured on selective medium (the same medium supplementing with 20 mg/l kanamycin and 400 mg/l cephatoxime). Both transformed and non-transformed cultures were maintained in the dark at 25 ± 2 °C and were incubated for 21 days. Prior to autoclaving (121°C, 20 min), all media were adjusted to pH 5.6–5.8.

Cell suspension culture was initiated by transferring friable root callus (80–100 mg) to a 50-mL Erlenmeyer flask containing 15 mL of liquid 3/4 MS medium supplemented with 3 mg/L 2, 4-D and 0.5 mg/L BAP. The suspension cultures were incubated on a rotary shaker at 120 rpm and 25 ± 2 °C under 16-h photoperiods with a light intensity of 4000 lux. Every 10 days, the established suspension cells (5.0 g fresh weight) were sub-cultured to 250-mL Erlenmeyer flasks containing 50mL of the same previous medium.

Molecular Analysis

Aliquots obtained from transformed and non-transformed control callus were collected and centrifuged at 12500 *g* for 10 min. Then, 200 mg cells from the pellet were used for extracting genomic DNA by the CetylTrimethyl Ammonium Bromide (CTAB) method [12]. PCR amplification was used for initial evidence of the transgene presence in the transformed calli. DNA fragment was amplified by PCR using the genomic DNA and 35S/NOSR primers for amplifying *SalAT* (Table 1). In the PCR experiments one assay contained 50–100 ng genomic DNA, 10 pmol of each primer, 16.0 mM $(NH4)_2SO4$, 50.0 mM Tris-HCl pH 8.8, 0.01%(v/v) Tween 20, 2.0 mM magnesium chloride, 0.2 mM of each dNTP (Roth, Karlsruhe, Germany) and 1 unit *Taq* polymerase (InViTek, Berlin, Germany) in a total reaction volume of 50 mL.PCR was carried out as follows: an initial denaturation at 94 ºC for 15 min followed by 40 cycles of denaturation at 94 ºC for 1 min, annealing at 58 °C for 1 min, extention at 72 ºC for 1 min and a final extension at 72 ºC for 10 min. The resulting PCR products were separated by electrophoresis on 1% (w/v) agarose gel.For more confirmation of transgene integration, southern blot analysis was applied. Genomic DNA (20 µg) was digested with *Hind*III. The digested genomic DNAs were fractioned on 0.8% (w/v) agarose gels, transferred onto a nylon membrane (AmershamHybond NTM+; Amersham International Plc, Amersham, UK) and hybridized to the Dig-dUTPlabelled CaMV35S promotor probe. A partial internal fragment (631 bp in size) was obtained from PCR amplification by using CaMV35SF/ CaMV35SR primers (Table 1) and plasmid pBI containing the CaMV35S promoter as template and subjected to DIG DNA labelling (Roche Applied Science GmbH, Mannheim, Germany) and used as a probe in hybridization experiments.

Alkaloid Extraction and HPLC Analysis

Papaver bracteatum extracts were prepared by mixing freeze-dried cell cultures (0.05 g) with 1mL methanol solution containing 0.5% (v/v) HCl, treated with 60 min sonication. The extracts were then centrifuged at 12,000rpm at 4°C for 20min. The supernatant was then collected and filtered prior to HPLC analysis. In addition, 1 mLmedium was freeze-dried and analyzed for extracellular benzophenanthridine alkaloid content HPLC analysis [13].

Benzophenanthridine alkaloids were quantified by their optical density peaks at 283 nm, using thebaine (Temad Chemical Co. Tehran, Iran) as standard compound for calibration. For standard sample preparation, 20 mg of standard thebaine powder was dissolved with 100 mL absolute methanol. 50, 75 and 100 ppm of this solution

were applied as standard. HPLC analysis was performed on Knauer HPLC system (1200 series, UV detector K-2501) equipped quaternary gradient pump and spectrophotometric photodiode array detector (DAD) and connected to a18 reverse-phases Phenomenex column (Gemini NX-C18, 5mm, 4.6 × 250 mm). A volume of 50 µL of samples and also standard thebaine were injected in C18 column. The mobile phase for alkaloid elution was 90% methanol and 5% deionized water and the retention time was 34 min. The extraction samples of each treatment were prepared and analyzed three times.

Statistical Analysis

The experiments were setup on an independent sample T-Test with three replicates and ten explants per replication. Differences were considered significant at a probability level of $p<0.05$. The values are expressed as the mean ± standard error of mean (SEM).

Results and Discussion

Molecular Analysis of the Transgenic Plant

The kanaomycin-resistant callus was initially screened by PCR for the presence of T-DNA region of recombinant plasmid. Transformed callus showed the amplification of 1733bp fragment using 35S/NOSR primers (Table1). No amplification was detected in wild type plants (Figure 2A). A set of virG primers (Table1) used for detection of Agrobacterium contamination if any that might have escaped the selection. The PCR of transgenic plants DNA with virG primers showed no band (data was not shown). Integration and the copy number of transgene in PCR positive callus were defined through Southern blot hybridization. Genomic DNA of both transformed and non-transformed calli was digested with *Hin*dIII. The results of Southern blot hybridization with CaMV35S promoter probe indicated that transformed callus had one copy and no hybridization signal occurred in the non-transformed control callus (Fig. 2B). Comparison of callus cultures in transformed and non-transformed explants

Both transformed and non-transformed explants induced callus cultures with differences in morphology. Most of transformed calli were light brown and hard while non-transformed calli were cream and friable (Fig. 3).

On the basis of T-Test analysis, transformation also significantly influenced the frequency of callus induction, fresh and dry weights (Table2). According to mean comparison, the frequency of callus induction, fresh and dry weights related to non-transformed explants were higher than transformed explants (Fig. 4).

Several factors influence the morphology and growth indexes of calli such as genotype differences, callus induction media, growth condition [14-15]. Here, *Agrobacterium* inoculation was the only factor which was led to observed differences in some characteristics of transgenic calli (callus induction, fresh and dry weights) compared to non-transgenics. Such differences have been also seen in callus cultures of *Rubia cordifolia* transgenic for the 35S-*rolB* and35S-*rolC* genes in comparison with a non-transformed callus culture. Similarly, the control *R. cordifolia* cultures were more watery and less colored than the transgenic ones and differently, percentage of dry biomass of the transgenic callus cultures were higher than non-transgenic cultures [16].

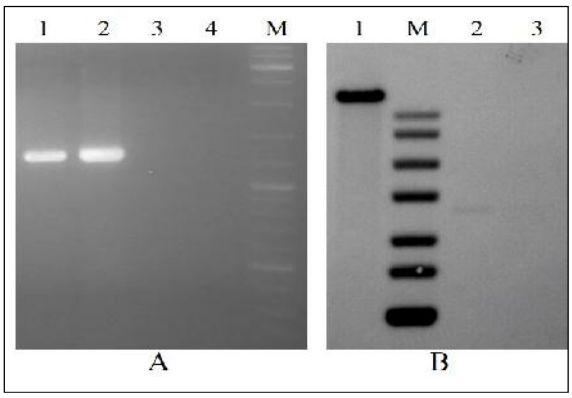

Fig. 2 Molecular analysis of transformed and non-transformed calli of *Papaver bracteatum* Lindl. A) PCR analysis; 1, transformed callus; 2, positive control (recombinant plasmid); 3, non-transformed callus; 4 negative control (PCR master Mix without DNA) M, molecular marker. B) Southern blot analysis; 1, positive control (recombinant plasmid); 2, transformed callus; 3, non-transformed callus; M, molecular marker.

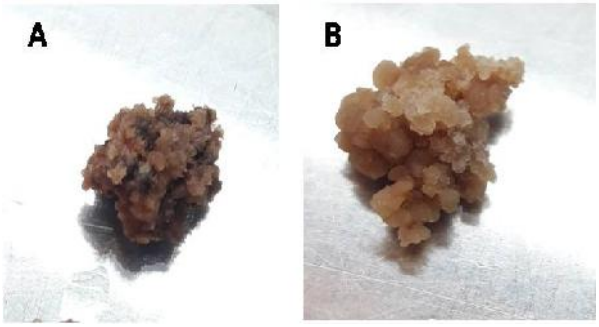

Fig. 3 Callus morphology from *Agrobacterium*-mediated transformed and non-transformed explants of *Papaver bracteatum* Lindl. A) Brown and hard callus obtained from transformed explants B) Cream and friable callus obtained from non-transformed explants

Table 2 T-Test analysis of *Agrobacterium*-mediated transformation on callus formation of *Papaver bracteatum* Lindl.

	Mean and SEM Transformed explants	Control explants	Mean Difference	t	df	Sig. (2-tailed)
Callus Induction	50.00 ± 5.000	70.00 ± 5.000	-20.000	-4.899	4	0.008
Fresh Weight	0.550 ± 0.0100	0.700 ± 0.0500	-0.1500	-5.095	4	0.007
Dry weight	0.50 ± 0.0020	0.60 ± 0.000	-0.0100	-8.660	4	0.001

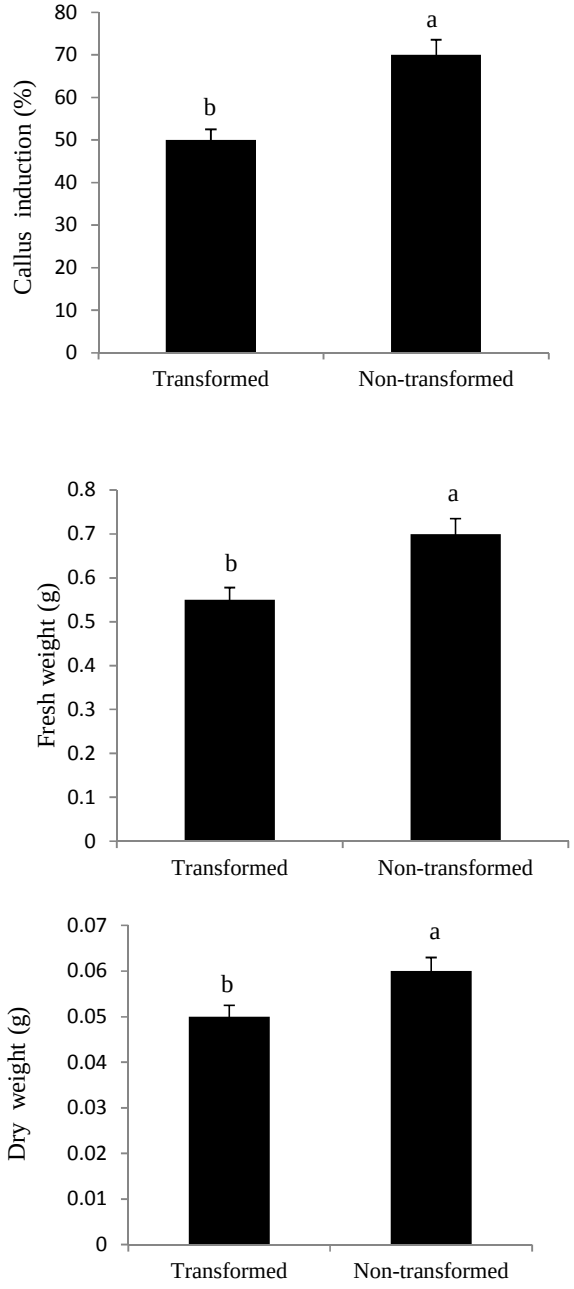

Fig. 4 Effect of *Agrobacterium*-mediated transformation on callus formation of *Papaver bracteatum* Lindl. A) frequency of callus induction B) fresh weight C) dry weight. Values followed by different letters in each trait are significantly different at $P \leq 0.05$. The values are expressed as standard error of mean (SEM).

Comparison of the Baine production in Transformed and Non-transformed Suspension Cultures The results obtained from T-Text analysis related to the extracted thebaine detecting by HPLC method showed a significant difference for thebaine concentration (mg/g of dried weight) between calli obtained from transformed explants and calli obtained from non-transformed explants (Table 3).

The accumulated amount of thebaine in transformed suspension culture was about 1.7 times higher than non-transformed cells which were 0.58mg/g and0.34 mg/g of dried weight cells, respectively (Fig. 5). The presence of thebaine in reco mbinant cells was validated by the confirmatory spiking HPLC analysis with the commercial standards (Fig. 6).

Several strategies for increasing the production of secondary metabolites such as morphinan alkaloids in plants and plant cell cultures have been employed including media and environmental optimization, differentiated cell cultures, elicitation, RNA mediated suppression of pathway enzymes, and overexpression of rate limiting pathway enzymes [17-20]. Here, we applied the last strategy to enhance the baine content in cell suspension culture of *P. barctetatum*.

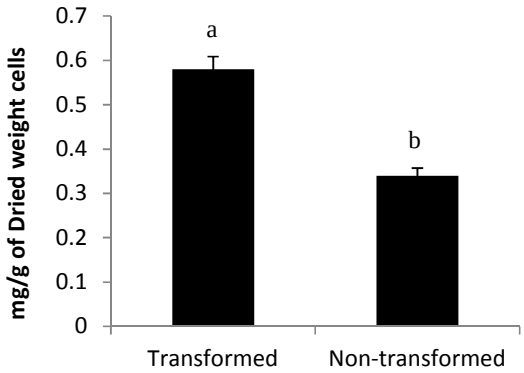

Fig. 5 Effect of *Arobacterium*-mediated transformation on thebaine concentration of *P Papaver bracteatum* Lindl. cell suspension culture. Values followed by different letters in each trait are significantly different at $P \leq 0.05$. The values are expressed as the mean ± standard error of mean (SEM).

The synthesis of the desired compound can be increased by designing the genetic constructs and introducing them to the calli c ultures. Transgenic cultures have been

found to be safe, efficient and cost-effectivesources of valuable secondary metabolites for medicine and industry [21]. At the current study, significant 1.7-fold enhancement in thebaine content was obtained in the recombinant cell suspension cultures.

This demonstrates that increase of secondary metabolite s levels can be achieved by increased expression of a single pathway enzyme. Similar investigations which have been down previously confirm it.

For example, overexpression of *P. somniferum* BBE in hairy root cultures resulted in a five-fold increase in total alkaloid content [22]. The overexpression of codeinonereductase in opium poppy plants did in fact result in an increase in morphine, codeine and thebaine compared to the control plant [23]. Inducing the overexpression of three stilbene synthase (STS) genes of *Picea jezoensis* (Siebold & Zucc.) Carrière in calli cultures of *Vitis amurensis* Rupr, resulting in an increase in the content of stilbene [24].

Another example is the overexpression of1-deoxy-d-xylulose-5-phosphate synthase 1 (SrDXS1) and kaurenoic acid hydroxylase (SrKAH) in *Stevia* spp., which increases the production of steviol glycosides [25]. Cell suspension cultures of *Silybum marianum* (L.) Gaertn. with the *Vitis vinifera* L. stilbene synthase gene, allowing increased accumulation of t-resveratrol [26].

Table 3 T-Test analysis of *Agrobacterium*-mediated transformation on thebaine concentration of *Papaver bracteatum* Lindl. cell suspension culture

	Mean and SEM Transformed explants	Control explants	Mean Difference	t	df	Sig. (2-tailed)
Thebaine concentration	0.580±0.1000	0.340±0.1000	0.2400	2.939	4	0.042

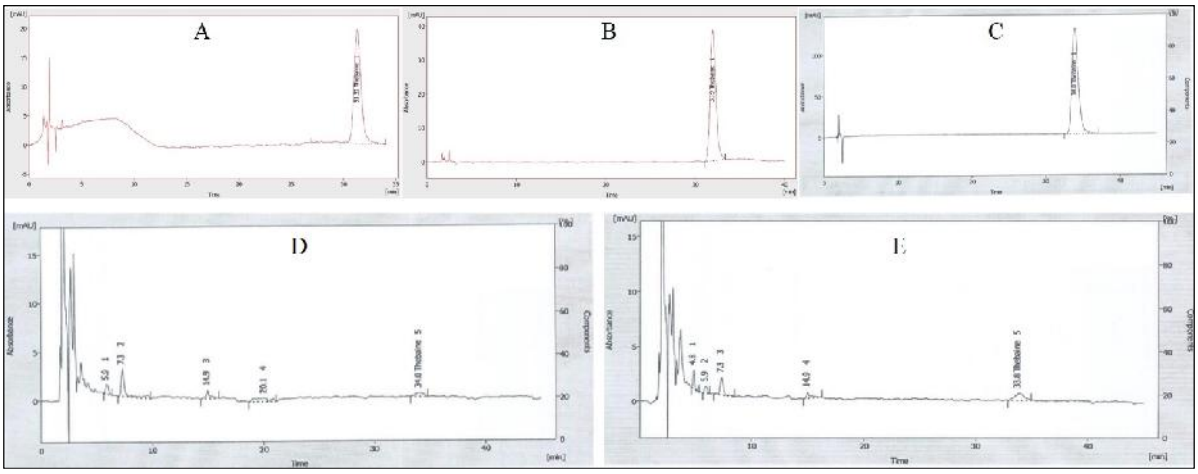

Fig. 6 HPLC chromatograms of *Papaver bracteatum* Lindl. cell suspension cultures. (A) Standard thebaine (50 ppm) (B) Standard thebaine (75 ppm) (C) Standard thebaine (100 ppm) (D) non-transformed cells (E) transformed cells

In addition, overexpression of the neutral / alkaline invertase (NINV) gene *in Taxus chinensis* (Pilg.) Rehder cell suspension significantly enhances the expression of the taxadiene synthase (TAS) gene, and the biosynthesis of seven individual taxanes [27].

In conclusion, this study is promising the ultimate prospect of large-scale bio-production of one of the pharmacologically important secondary metabolites; thebain.

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