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Transcriptional profiling of three key genes of terpenoid indole alkaloid pathway in *Catharanthus roseus* under different tissue culture conditions

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The response of three key genes: strictosidine synthase (*str1*), tryptophan decarboxylase (*tdc*) and secologanin synthase (*cyp72A1*) of the wild plant species, *Catharanthus roseus* to different plant tissue culture treatments was studied. These genes encode enzymes acting early in the biosynthetic pathway of terpenoid indole alkaloids. *In vitro* culture system conditions involved the application of three sucrose (40, 50 and 60 g/L), three benzyl adenine (0.1, 0.2 and 0.4 mg/L) and two jasmonic acid (10 and 100 µM) concentrations. Quantitative RT-PCR (qRT-PCR) using SYBR Green I was used to analyze the changes in expression of the three genes in response to different media recipes. The maximum folding of *str1* expression (1.9x) between treated and untreated callus was obtained under ba2 (0.2 mg/L benzyl adenine) treatment. Relatively high folding values of 1.8x and 1.7x were obtained in S2 (50 g/L sucrose) and ba1 (0.1 mg/L benzyl adenine), respectively. Two-fold increase in gene expression of *tdc* was obtained when *C. roseus* callus was treated with 10 µM jasmonic acid (ja1), while only 1.5x was obtained when callus was treated with 100 µM jasmonic acid (Ja2). The maximum expression of *cyp72A1* gene (4.6x) was observed under ba2 treatment, when the callus was treated with 0.2 mg/L benzyl adenine. This emphasized the influence of BA on up-regulation of this gene.

Key words: Strictosidine synthase, tryptophan decarboxylase, secologanin synthase, *Catharanthus roseus*, terpenoid indole alkaloids.

INTRODUCTION

Catharanthus roseus (L.) is one of the most extensively investigated medicinal plants and is known mainly for its pharmacologically important alkaloids (Verpoorte et al., 1997). At present, the *Catharanthus* alkaloids comprise a group of about 130 terpenoid indole alkaloids (TIAs) (van der Heijden et al., 2004). The plant is particularly known

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for its economically important leaf-specific bisindole alkaloids, vinblastine and vincristine, which are potent anti-neoplastic agents (Svoboda and Blake, 1975) and indispensable constituents of most cancer chemotherapies. The lack of knowledge on the fully functional pathways required for the production of target molecules is pointed out as the main reason why the attempts to use plant tissue and cell cultures as alternative sources of natural products have been problematic (Zhou et al., 2010).

More than 100 *C. roseus* alkaloids that have been identified share many common biosynthetic steps. Early monoterpene indole alkaloid biosynthesis begins with the condensation of tryptamine, which is derived from the decarboxylation of tryptophan by tryptophan decarboxylase (*tdc*) with secologanin, which is derived from the iridoid pathway by secologanin synthase (*cyp72A1*) that converts loganin to secologanin (De Luca, 1993). These two moieties are enzymatically condensed by strict-sidine synthase (*str1*). The strictosidine, then, serves as universal precursor of the two secondary compounds: Vinblastine and vincristine (De Luca et al., 1986).

The production of secondary metabolites by tissue culture system has become an active field of study because of its potential as a source of valuable pharmaceutical compounds. In this context, *in vitro* cultures of plant cells or tissues look promising for the large scale production of secondary metabolites (Tikhomiroff and Jolicoeur, 2002; Mulabagal and Tsay, 2004).

Real-time PCR, which combines the advantages of conventional PCR with quantitative capability (Simpson et al., 2000) is one of the most sensitive and accurate methods for the detection and quantification of gene expression (Giulietti et al., 2001). Its high sensitivity allows the quantification of rare transcripts and small changes in gene expression (Schmittgen and Zakrajsek, 2000; Rajeevan et al., 2001, Vandesompele et al., 2002; Kim et al., 2003; Radonic et al., 2004).

This study aimed at showing the response of three key genes: Strictosidine synthase (*str*1), tryptophan decarboxylase (*tdc*) and secologanin synthase (*cyp72A1*) that act early in the biosynthetic pathway leading to terpenoid indole alkaloids, to different tissue culture treatments.

MATERIALS AND MATHODS

Seeds of red purple variety of Egyptian *C. roseus* were kindly obtained from Institute of Horticulture Research, Agricultural Research Centre, Giza, Egypt.

Preparation of plant material, media and culture conditions

Seeds were surface sterilized under aseptic conditions of laminar flow hood, using 12% H_2O_2 for 5 min. Then they were aseptically germinated on half-strength solid basal Murashige and Skoog (MS) medium including micro and macro elements and vitamins (Murashige and Skoog, 1962) in tissue culture incubator. After germination, the seedlings were transferred to growth chamber in pots containing soil at 25 ± 3°C. Leaves were excised from 4 to 6 weeks old seedlings and used as a source of explant materials for calli production.

Callus initiation and treatments

Callus cultures were initiated from fresh *C. roseus* leaf fragments. Transversal leave sections (2 mm) were transferred to jars containing 30 to 40 ml of solid medium. MS medium supplemented with sucrose at a concentration of 30 and 1 g/L of 2,4 dichlorophenoxy acid (D) + 0.1 mg/L of kinetin (Kin) was routinely used for *C. roseus* callus induction and growth and as a control medium in the different treatment experiments. Cultures were incubated at 16 h light and 8 h dark and 22 to 25°C. All culture media used in this study were adjusted to pH = 5.6 to 5.8 before solidification with 0.2% GELRITE.

After four weeks from callus initiation, the callus was weighted according to the study of Zhao et al. (2001) and sub-cultured to the treatment media. In this study, three different sucrose concentrations (40, 50 and 60 g/L) and three concentrations of benzyl adenine (0.1, 0.2 and 0.4 mg/L) in addition to two concentrations of jasmonic acid (10 and 100 µM) were applied to determine their influence on the expression of three key genes of TIAs pathway in *C. roseus* tissue culture.

Primer design for real time PCR analysis

Both forward and reverse primers, for recovering the full-length of the three *C. roseus* genes, were designed based on the known nucleotide sequences obtained from the GenBank database. These genes are strictosidine synthase (*str1*) (GenBank Acc. No. X61932), tryptophan decarboxylase (*tdc*) (GenBank Acc. No. X67662) and secologanine synthase (*cry72A1*) genes (GenBank Acc. No. L10081). In designing these primers, one of the most important criteria to take into account is the targeting of relatively small amplicon size of not more than 250 bp. DNASTAR V. 7.0.0 software and Primer-3 V.4 software were used to design the primers. The designed primers were synthesized by Metabion, Germany. Primer pairs of candidate endogenous reference gene *CrActin* (no accession number available) were designed according to the study of Jiao and Deng (2007). Primer sequences of the three target as well as the endogenous gene used for real-time PCR and their annealing temperatures are shown in Table 1.

Real time quantitative RT-PCR

Total RNA was extracted by TRI Reagent® RNA isolation (Sigma T9424) as describe in the manufacturer's manual. RevertAid™ H⁻ M-MulV reverse transcriptase (thermo scientific, Fermentas, Lithuania) was used in the reverse transcription and real time PCR. Total RNA from both treated and untreated *C. roseus* calli were DNase-treated prior to cDNA synthesis. Five hundred nanograms of RNA from each of the treatments were reverse transcribed according to the manufacturer's protocol. The 20 µl reaction consisted of 2x RT reaction mix (2.5 µM Oligo(dT)18, 10 mM $MgCl_2$ and 0.2 uM dNTP), 2 µl RT Enzyme Mix (RevertAid™ H⁻ M-MulV RT and RNaseOUT) and DEPC-treated water. All the components were mixed and incubated at 25°C for 10 min. Further incubation at 42°C for 50 min was carried out. The reaction was terminated by incubating at 85°C for 5 min and then chilled on ice.

Real-time PCR was carried out using the Agilent Mx3000P QPCR systems (Agilent technology, USA). All cDNA samples synthesized were diluted 1:10 prior to amplification. Each reaction was performed in a 25 µl volume. The reaction components were 12.5 µl Maxima™ SYBR Green/ROX qPCR Master Mix, 0.2 µM of

Table 1. Primer sequences of the three target genes and the endogenous gene used for real-time PCR.

Gene ID	Primer sequences	Annealing temperature (°C)
tdc	F 5'-TTC TTC ACC AGC CGC CAC CG-3' R 5'-GCG CTT CCC GCA TAA GCA GC-3'	62
str1	F 5'-TGA GGC CAC CTA CCA TCC CGT-3' R 5'-GCA GCA GAC ACT CAA AAT CTC CTC C-3'	62
CYP72A1	F 5'-ACC GGA GTT GGA AGC TTT GAG GGT-3' R 5'-TCC TGC AGG GAT TGT GTA CGA CC -3'	64
CrActin	F 5'-GGC TGG ATT TGC TGG AGA TGA T -3' R 5'-TAG ATC CTC CGA TCC AGA CAC TG -3'	57.6

each forward and reverse primer, and PCR-grade water to make up the volume to 22.5 µl. Finally, 2.5 µl of diluted cDNA template was added to the reaction mix. Amplification for each sample was carried out in triplicate along with a no-template control (NTC) in which PCR-grade water was used as template. The thermal cycling conditions consisted of 1 cycle at 95°C with a 2 min hold for denaturation (hot-start) and followed by 40 cycles of denaturation at 95°C for 15 s, annealing as shown in Table 1 for 30 s and extension at 72°C for 30 s. Data were collected and amplification plots of ΔRn versus cycle number were generated for analysis. To confirm that only one PCR product of the expected size was amplified in the treated and untreated samples, all the reaction products were further analyzed by agarose gel electrophoresis as described by Liu et al. (2011).

Data analysis

The data were analyzed and amplification plots were generated using the software program, MxPro QPCR software which is part of the Agilent Mx3000P QPCR systems. The standard curves were generated using both the software programs, and Microsoft Excel. The $\Delta\Delta C_T$ calculation for the relative quantification of target gene was used as follows $\Delta\Delta C_T = (C_T$, target gene - C_T, *CrActin*) χ - (C_T, target gene - C_T, *CrActin*) y, where χ = treated sample and y = control sample. After validation of the method, results for each sample were expressed in N-fold changes in χ target gene copies, normalized to *CrActin* relative to the copy number of the target gene in control, according to the following equation: amount of target = $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

RESULTS AND DISCUSSION

Researchers aim to produce substances with antitumor, antiviral, hypoglycaemic, anti-inflammatory and antimicrobials properties through tissue culture technology. However, establishment of tissue culture system is required prior to further exploration of the biosynthetic capabilities of various cell cultures. Tissues from various organs such as stem and leaf of the axenic plantlets can be induced to form callus. Callus tissue can serve as an experimental system to investigate the biological activities using specific bioassays. However, many factors contribute to the ability of a specific tissue to form callus such as medium and plant growth regulators.

Establishment of tissue culture system for Egyptian *C. roseus*

The red purple variety of Egyptian *C. roseus* was used in the present study as a source of explants for callus initiation. In a preliminary experiment, three explant types were used: hypocotyl (undeveloped lower stem), cotyledons and mature leaves. Transversal leaf sections proved to be the best explant, which resulted in production of healthy callus with good size, shape and color on MS medium (data not shown).

Effect of different sucrose concentrations on *C. roseus* callus growth

Increased sucrose concentration usually results in increased biomass and secondary metabolite production of plant cell cultures (Zhao et al., 2001). Five different sucrose concentrations were tested in the present study for their effect on callus growth. In response to increased sucrose concentrations (from 20 to 60 g/l) calli changed most of their characteristics such as color, size and the degree of compaction (decreased ratio of FW/DW). As shown in Table 2 and Figure 1, the ratio of fresh weight (FW) to dry weight (DW) of the calli varied from 3 to 25.24. The ratio of FW/DW can reasonably represent the degree of compaction of the callus structure. More organized and compacted callus usually gave lower ratios of FW/DW (Zhao et al., 2001).

Effect of phytohormone balance on *C. roseus* callus growth

Plant hormones, like animal hormones, are relatively small molecules that are effective at low tissue concentrations. The two types of plant hormones used in

Table 2. Effect of sucrose concentrations on *C. roseus* callus characteristics

Sucrose (g/L)	Callus characteristic				
	Color	Texture	(FW) mg*	(DW) mg*	FW/DW
20	Off white	Loose	85.8	3.4	25.24
30	Off white	Loose	19.94	1.61	12.39
40	dark yellow	Compact	23.02	2.18	10.56
50	dark yellow	Compact	23.4	2.2	10.63
60	Greenish yellow	Hard	18.6	6.2	3

*Mean of three calli, FW = fresh weight, DW = dry weight.

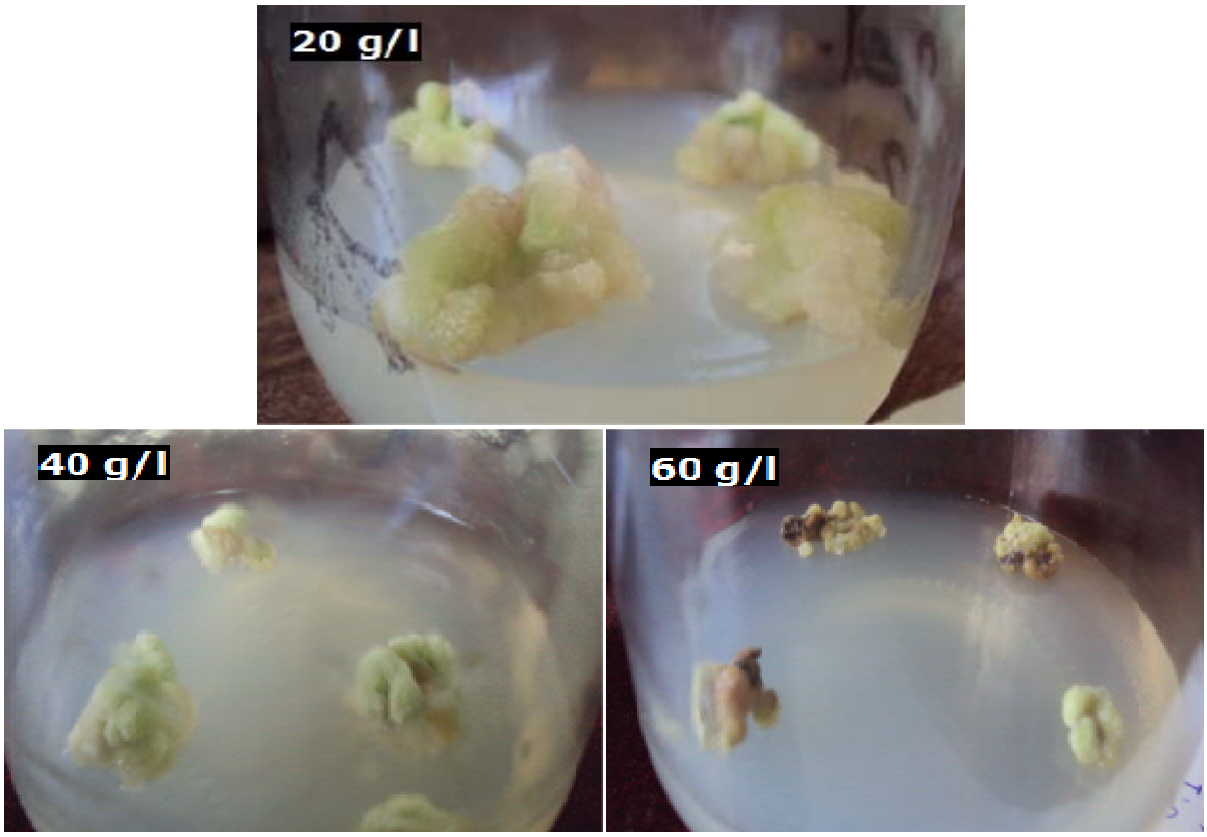

Figure 1. Effect of different sucrose concentrations on color, size and the degree of compaction of *C. roseus* callus.

this experiment are cytokinins and auxins. Cytokinins are derived from adenine and produce two immediate effects on undifferentiated cells: the stimulation of DNA synthesis and increase cell division (Ting, 1982). Auxins are indole or indole-like compounds that stimulate cell expansion, particularly cell elongation. Auxins also promote adventitious root development. Plant hormones do not function in isolation within the plant body, but, instead, function in relation to each other. Hormone balance is apparently more important than the absolute concentration of any one hormone. Both cell division and cell expansion occur in actively dividing tissue, therefore cytokinin and auxin balance plays a role in the overall growth of plant tissue (Xing et al., 2011). Four plant hormones were used in this study, two auxins (2,4 D and NAA) and two cytokinins (kinetin (Kin) and benzyl adenine (BA)). Four combinations of these plant hormones (M1 to M4) were tested for their effects on callus induction and growth. Qualitative and quantitative differences were observed and the results are shown in Table 3 and Figure 2. Based on morphological appearance, different types of calli were observed. Among the different combination, best results were obtained on the medium supplemented with 1 mg/L 2,4D + 0.1 mg/L Kin (M1) after four weeks of callus initiation. It was also observed that replacing 2,4 D with NAA gave the same result, but the callus took about

Table 3. Effect of four media containing different combinations of plant growth regulators on *C. roseus* callus characteristics.

Media	Growth regulator				Callus characteristic		
	Auxin (mg/L)		Cytokinine (mg/L)				
	2,4D	NAA	Kin	BA	Color	Texture	Texture
M1	1	0	0.1	0	Off white	Friable	Friable
M2	1	0	0	0.1	Dark yellow	Compact	Compact
M3	0	1	0.1	0	Dark yellow	Compact	Compact
M4	0	1	0	0.1	Dark yellow	Compact	Compact

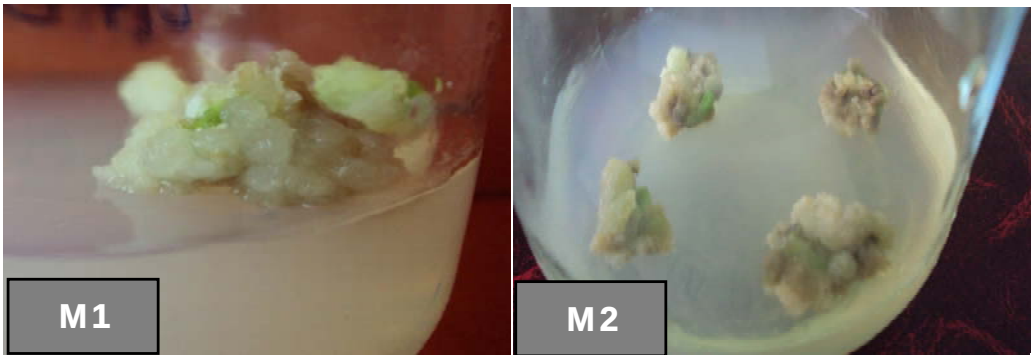

Figure 2. Effect of two combinations: M1 (1mg/l 2,4D + 0.1 mg/L kin) and M2 (1 mg/l 2,4D + 0.1 mg/L BA) of plant growth regulators on callus characteristics.

6 weeks to reach the desired size and shape.

Callus culture treatments

The significant advances in plant tissue culture techniques have led to the use of callus and cell suspension culture (undifferentiated cells) of some plant species for the study of biological activities and production of valuable secondary metabolites (Mulabagal and Tsay, 2004). In efforts to improve the production of alkaloids, cell cultures of *C. roseus* received considerable attention. The terpenoid indole alkaloids pathways are not only regulated tissue specifically and developmentally (Facchini, 2001), but also affected by external factors (Facchini, 2001; Zhao et al., 2001; Xing et al., 2011).

These factors include media components, phytohormones (growth regulators), pH, temperature, aeration, agitation, light, etc. (Moreno et al., 1995; Xing et al., 2011). Based on the results of the previous experiments, MS medium supplemented with sucrose at a concentration of 30 g/L and 1 mg/L of 2,4D + 0.1 mg/L of kin proved to be more appropriate for callus induction and growth of the Egyptian *C. roseus* and routinely used in this study for callus production and as a control medium in the different treatment experiments. Three sucrose concentrations (S1 = 40, S2 = 50 and S3 = 60 g/L) and three concentrations of benzyl adenine (ba1 = 0.1, ba2 = 0.2 and ba3 = 0.4 mg/L) in addition to two concentrations of jasmonic acid (ja1 = 10 µM and ja2 = 100 µM) were applied to determine their influence on regulation of gene expression of three key genes in TIAs pathway in *C. roseus*. Sucrose and glucose are the preferred carbon source for plant tissue cultures. The concentration of the carbon source affects cell growth and yield of secondary metabolites in many cases. Among a number of other components in the medium phytohormones are auxins and kinetins which have shown the most remarkable effects on growth and productivity of plant metabolites (Yahia et al., 1998; Doran, 2000).

The effects of plant growth regulators on the contents of *C. roseus* TIAs had been extensively studied (El-Sayed and Verpoorte, 2007; Zhao and Verpoorte, 2007; Pan et al., 2010). Jasmonic acid (JA) proved to have significant influence on TIAs production and enzymes activities of the biosynthesis pathways in *C. roseus* cell suspension cultures, hairy roots and seedlings (El-Sayed and Verpoorte, 2005; Peebles et al., 2009).

Quantification of gene expression using real-time PCR

Real-time PCR were carried out by using primers that produced single bands (Figure 3). For amplification of each target gene from mRNA, the *CrActin* gene was also

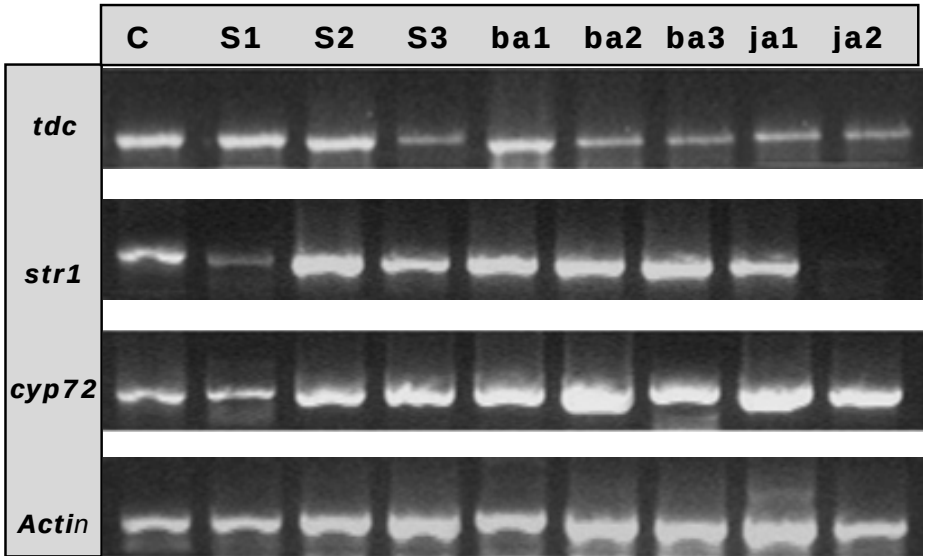

Figure 3. qPCR reaction products for the target genes (*str1*, *tdc* and *cyp72A1*) and the reference gene (*CrActin*) under treated and untreated calli. S = sucrose treatment; ba = benzyl adenine treatment; ja = jasmonic acid treatment.

Table 4. The level of *str1* gene expression between treated and untreated (control) *C. roseus* determined by the comparative $\Delta\Delta C_T$ method.

Treatment	C_T *str1*	C_T actin	ΔC_T	$\Delta\Delta C_T$	Folding
Control	21.54	26.24	-2.61	0	1
S1	23.92	26.14	-2.22	0.39	0.7
S2	23.82	27.29	-3.47	-0.86	1.8
S3	22.52	25.4	-2.88	-0.27	1.2
BA1	22.9	26.26	-3.36	-0.75	1.7
BA2	23.68	27.19	-3.51	-0.9	1.9
BA3	20.48	23.52	-3.04	-0.43	1.3
Ja1	24.79	27.71	-2.92	-0.31	1.2
Ja2	26.78	27.65	-0.87	1.74	0.3

included as an endogenous reference. An amplification plot was generated for each target gene and *CrActin* gene pair and C_T values were then obtained. Based on the C_T values, the ΔC_T treated and ΔC_T control values which have been normalized to the *CrActin* gene were determined. The $\Delta\Delta C_T$ value which represents the difference in transcript level between the treated and untreated callus was then calculated. The amount of transcript in the treated callus, normalized to an endogenous reference and relative to the untreated callus is expressed as $2^{-\Delta\Delta C_T}$. A positive $\Delta\Delta C_T$ value indicated down-regulation, while a negative $\Delta\Delta C_T$ value indicated an up-regulation of gene transcription (Radonic et al., 2004). The fold differences in the expression level of normalized treated callus relative to different untreated cDNA are shown in Tables 4, 5 and 6.

Transcriptional profiling of str1 gene under different treatments

Table 4 and Figure 4 represent the folding levels of *str1* gene expression for treated and untreated (control) calli of *C. roseus* determined by the comparative $\Delta\Delta C_T$ method. Positive values of $\Delta\Delta C_T$ were detected in two cases only among the eight treatments: S1 (40 g/L sucrose) and ja2 (100 µM jasmonic acid) indicating down-regulation of this gene in the two treatments. On the other hand, the maximum folding of *str1* expression (1.9x) between treated and untreated callus was obtained under ba2 (0.2 mg/L benzyl adenine) treatment as shown in Table 4 and Figure 4. Relatively high folding values of 1.8x and 1.7x were also observed in S2 (50 g/L sucrose) and ba1 (0.1 mg/L benzyl adenine), respectively.

Table 5. The level of *tdc* gene expression between treated and untreated (control) *C. roseus* determined by the comparative $\Delta\Delta C_T$ method.

Treatment	C_T *tdc*	C_T actin	ΔC_T	$\Delta\Delta C_T$	Folding
Control	23.66	26.24	-2.61	0	1
S1	24.32	26.84	-2.52	0.09	0.9
S2	19.54	22.29	-2.75	-0.14	1.1
S3	19.34	22.61	-3.27	-0.66	1.6
BA1	18.61	20.56	-1.95	0.66	0.6
BA2	20.7	23.79	-3.09	-0.48	1.4
BA3	22.4	25.52	-3.12	-0.51	1.4
Ja1	19.09	22.71	-3.62	-1.01	2.0
Ja2	22.93	26.15	-3.22	-0.61	1.5

Table 6. The level of *cyp72A1* gene expression between treated and untreated (control) *C. roseus* determined by the comparative $\Delta\Delta C_T$ method.

Treatment	C_T *cyp*	C_T *actin*	ΔC_T	$\Delta\Delta C_T$	Folding
Control	23.63	26.24	-2.61	0	1
S1	23.47	26.54	-3.07	-0.46	1.4
S2	14.64	18.29	-3.65	-1.04	2.0
S3	15.05	19.1	-4.05	-1.44	2.7
BA1	15.07	19.56	-4.49	-1.88	3.7
BA2	14.97	19.79	-4.82	-2.21	4.6
BA3	15.56	19.52	-3.96	-1.35	2.5
Ja1	15.04	19.71	-4.67	-2.06	4.1
Ja2	20.44	24.65	-4.21	-1.6	3.0

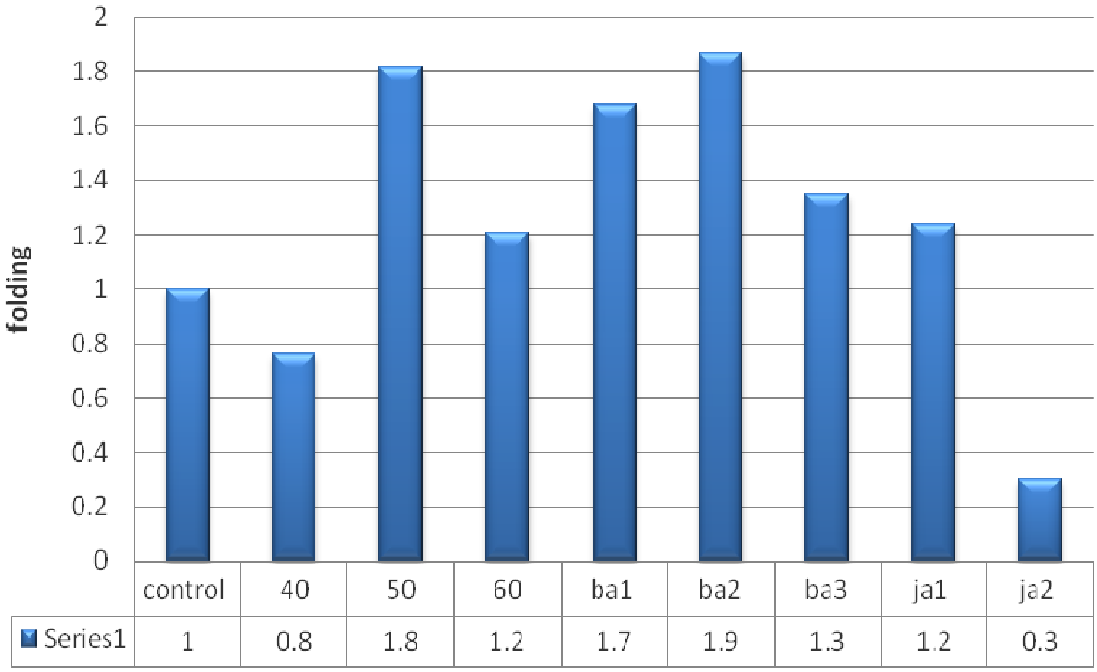

Figure 4. The folding levels of *str1* gene expression between treated and untreated (control) *C. roseus*. 40, 50 and 60 = sucrose treatments (g/l); ba = benzyl adenine treatment; ja = jasmonic acid treatment.

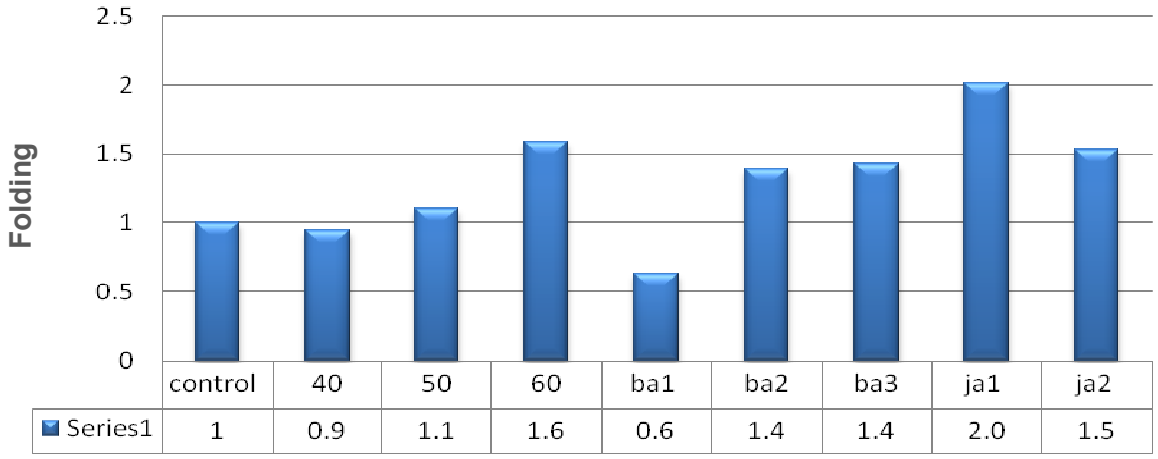

Figure 5. The folding levels of *tdc* gene expression between treated and untreated (control) *C. roseus*. 40, 50 and 60 = sucrose treatments (g/l); ba = benzyl adenine treatment; ja = jasmonic acid treatment.

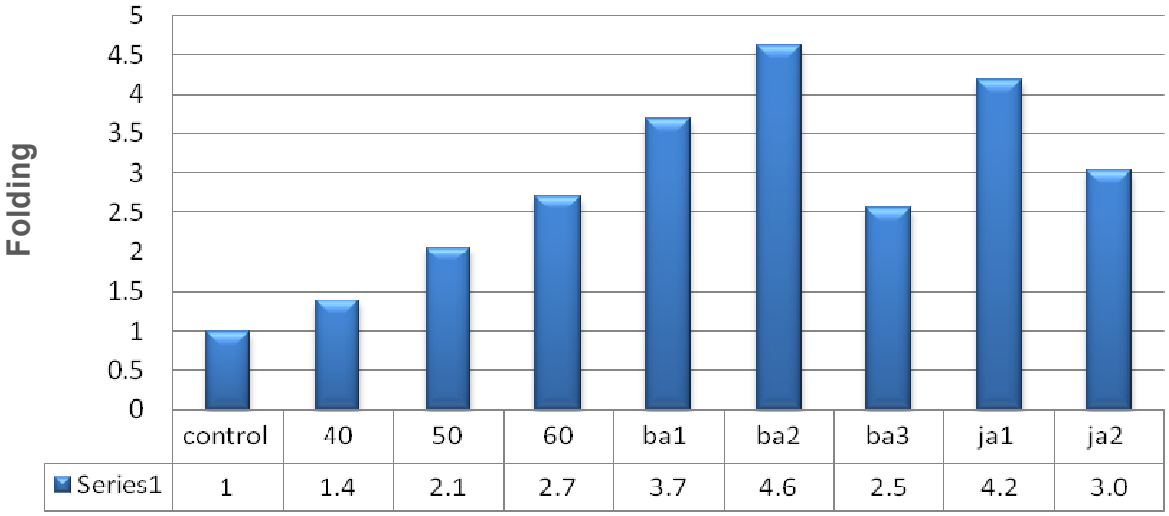

Figure 6. The folding levels of *cyp72A1* gene expression between treated and untreated (control) *C. roseus*. 40, 50 and 60 = sucrose treatments (g/l); ba = benzyl adenine treatment; ja = jasmonic acid treatment.

Transcriptional profiling of tdc gene under different treatments

Levels of *tdc* gene expression between treated and untreated (control) *C. roseus* determined by the comparative $\Delta\Delta C_T$ method are shown in Table 5 and Figure 5. Positive values of $\Delta\Delta C_T$ were detected in two cases only among the eight treatments: S1 (40 g/L sucrose) and ba1 (0.2 mg/L benzyl adenine) indicated down-regulation of this gene under these two treatments. Two-fold increase in gene expression of *tdc* was obtained when *C. roseus* callus was treated with 10 µM jasmonic acid (ja1) and just only 1.5x value was obtained when callus was treated with 100 µM (Ja2).

Transcriptional profiling of cyp72A1 gene under different treatments

Table 6 and Figure 6 represent the folding levels of *cyp72A1* mRNA between treated and untreated (control) *C. roseus* calli. Negative $\Delta\Delta C_T$ values were obtained in all treatments indicating an up-regulation of *cyp72A1* gene transcription. The three sucrose treatments S1, S2 and S3 (40, 50 and 60 g/l, respectively) resulted in the increase in folding levels (1.4, 2.0 and 2.7, respectively). The *cyp72A1* gene seems to respond to sucrose treatments in a dose response manner. The maximum expression of *cyp72A1* gene (4.6x) was observed in ba2, when the callus was treated with 0.2 mg/l benzyl adenine.

jasmonic acid resulted in up-regulation of *cyp72A1* gene in its two concentrations: ja1 and ja2 (10 and 100 µM, respectively). The folding levels were 4.1x and 3.2x, respectively.

Results of this study showed that the three genes respond differentially to the eight treatments. *cyp72A1* showed maximum folding of expression (4.2) between treated and untreated callus under ba2 treatment. This showed the influence of benzyl adenine in up-regulating this gene. The remaining genes represented comparable expression in all treatments. *str1* gene was up-regulated in all treatments except 4% sucrose treatment (0.7) and ja2 treatment (0.3), and with *tdc* gene, it was up-regulated in all treatments except 4% sucrose (0.9), while *cyp72A1* gene was up-regulated in all treatments.

Knowledge of the regulation of these biosynthetic genes will be helpful for metabolic engineering of terpenoid indole alkaloid productivity. Further studies would focus on investigating the correlation between the over expression (up-regulation) of the studied genes and the metabolic flow during TIAs biosynthesis in *C. roseus*.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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