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Expression Analysis of Gene Encoding 1-Deoxy-d-Xylulose 5-Phosphate Reductoisomerase (DXR) and Cardenolide and Digitoxin Production in *Digitalis purpurea* L. Using Polyamines and Methyl Jasmonate as Elicitors

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ABSTRACT

Digitoxin is a glycoside isolated from leaves of various species of Digitalis genus that are commonly called foxgloves. Digitoxin is used for chronic cardiac insufficiency, tachyarrhythmia form of atrial fibrillation, paroxysmal ciliary arrhythmia, and paroxysmal supraventricular tachycardia. 1-Deoxy-d-Xylulose 5-phosphate Reductoisomerase (DXR) is an important rate-limiting enzyme in 2-*C*-Methyl-d-Erythritol 4-Phosphate (MEP) pathway, and expression level of DXR plays a critical role in control of plant isoprenoid biosynthesis pathway. The effects of different elicitors including putrescine, spermine, and spermidine at concentrations of 50 and 100 mg L^{-1} and also Methyl Jasmonate (MJ) at 50, 100, and 200 µM concentrations were explored on transcript levels of *DXR* gene in cell suspension culture of foxglove (*Digitalis purpurea*). *DXR* transcription levels were assessed by the semi-quantitative RT-PCR. Results showed that elicitors had significant effects on the expression level of *DXR* and contents of cardenolide and digitoxin. The highest digitoxin (61.3 µg g^{-1} DW) and cardenolide (1.48% mg^{-1} DW) content was observed in 100 mg L^{-1} spermidine treatment. In all treatments, the expression of *DXR* gene was increased compared with the control sample. The highest transcription levels of *DXR* gene was observed in 50 µM MJ and 100 mg L^{-1} putrescine elicitors, which was nine folds higher than the control condition, and the lowest levels were observed for putrescine treatments at concentrations of 50 and 100 mg L^{-1}, respectively.

Keywords: Elicitor, Foxglove, Gene expression, Tissue culture.

INTRODUCTION

Medicinal plants are the source of secondary metabolites and bioactive compounds that are used as food additives, natural pigments, insecticides, flavors, aromas, fragrances, bio-based fuels, plastics, enzymes, preservatives, cosmetics (cosmeceuticals), bioactive compounds, and fine chemicals (Mulabagal and Tsay, 2004). *Digitalis purpurea* (foxglove, common foxglove, purple foxglove) is a species of medicinal flowering plant in the family Plantaginaceae (Olmstead *et al*., 2001). The plants are well known as the original source of the heart medicine digoxin (Olmstead *et al*., 2001). Plant tissue culture is the

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technique of maintaining and growing plants on artificial medium under defined conditions *in vitro* (Bunsupa *et al*., 2018). Cell cultures have been established for many plants, but often they do not produce sufficient amounts of the required secondary metabolites. However, in many cases the biosynthesis of secondary metabolites can be enhanced by treatment of undifferentiated cells with elicitors such as methyl jasmonate (Poulev *et al*, 2003). An 'elicitor' may be defined as a substance which, when introduced in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds (Gorelick and Bernstein, 2014). It is well known that the treatment of plants with elicitors causes accumulation of a range of plant defensive secondary metabolites in intact plants or in plant cell cultures (Poulev *et al*, 2003). There are many reports on Methyl Jasmonate (MJ) effect on secondary metabolite production such as Wang *et al*. (2015) who enhanced production of secondary metabolites by MJ elicitation in cell suspension culture of *Hypericum perforatum.* Khan *et al.* (2019) reported that elicitors had significant effects on the production of pharmacologically attractive secondary metabolites in callus cultures of *Fagonia indica.* It was reported that MJ had effects on the production of secondary metabolites in plant cell suspension cultures of *Thevetia peruviana* (Mendoza *et al.,* 2018).

Terpenoids constitute one of the most diverse groups of natural products and have different roles in growth regulation, cell signaling, inducing defense mechanisms, membrane structures, and redox chemistry (Rodriguez-Concepcion *et al*., 2001). All isoprenoids are derived from a common building unit, Isopentenyl diPhosphate (IPP) and its isomer Dimethylallyl diPhosphate (DMAPP), and plastidial isoprenoids are derived from a mevalonate-independent pathway currently known as the 2-C-Methyl-d-Erythritol 4-Phosphate (MEP) pathway (Rodriguez-Concepcion *et al*., 2001). The initial reaction is formation of 1-Deoxy-d-Xylulose 5-Phosphate (DXP) from pyruvate and d-glyceraldehyde 3-phosphate, and it is catalyzed by DXP synthase (DXS) (Lange *et al*., 1998). *1-DeoxyXylulose-5-phosphate isomerase-Reductase* gene (DXR), catalyzes the NADP-dependent rearrangement and reduction of 1-Deoxy-d-Xylulose-5-Phosphate (DXP) to 2-C-Methyl-D-Erythritol 4-Phosphate (MEP), which is the key element for MEP-pathway that supports the main C5 units for the formation of mono and diterpenes (Takahashi *et al*., 1998; Banerjee *et al*., 2013). Plastidial MEP pathway is considered as the main important source of precursors for essential plastid isoprenoids (Wright *et al*., 2014). Most isoprenoids including volatile compounds are involved in plant resistance responses to biotic and abiotic stresses and made from precursors produced by the MEP pathway (Gershenzon and Dudareva, 2007). Because of the pivotal role of *DXR* for providing the downstream products of the MEP pathways, in the past two decades, there has been increasing interest in exploring the DXR function for manipulating biosynthetic pathways to increase the yield of terpenoids for different purposes. By altering the expression of *DXR*, the beneficial metabolite compounds have increased in mint plant (Mahmoud and Croteau, 2002). Constitutive expression of *DXR* in Arabidopsis seedlings has resulted in the accumulation of high levels of isoprenoids such as chlorophylls, tocopherols, and carotenoids (Carretero-Paulet *et al*., 2006). Over-expression of *DXR* gene also resulted in high increase of diterpene yield in transgenic bacteria (Morrone *et al*., 2010). Biotic elicitors have significant role in production of pharmaceutical terpenoids by increasing the amount of the enzymes involved in biosynthetic pathways via regulating the expression of biosynthetic genes (Kang *et al*. 2009; Lu *et al*., 2016). Xing *et al*. (2010) reported a high expression of DXR under the drought as an abiotic stress. Application of MJ increased the expression level of *DXR* and increased triptophenolide content in cell

culture suspension of *Tripterygium wilfordii* (Hook. f.) (Tong *et al*., 2015). Because of the key role of DXR as an upstream gene involved in MEP pathways for producing secondary metabolites in plants, we evaluated the expression of the *DXR* in *Digitalis purpurea* L. as an important medicinal plant in responses to polyamines and MJ (biotic elicitors) treatments under *in vitro* condition.

MATERIALS AND METHODS

Sterilization and Culture Medium

The seeds of the *Digitalis purpurea* L. with code number 411Dp*MPISB were provided from Medicinal Plants Institute, Academic Center for Education, Culture and Research (ACECR). The seeds were disinfected under laminar flow hood with 70% ethanol for one minute, then sterilized with 1% sodium hypochlorite for 10 minutes and washed three times with autoclaved distilled water. Also, all instruments were sterilized by autoclave at 121°C and 1.5 atmospheres for 20 minutes. The macro and micro elements and vitamins were added to the medium according to Murashig and Skoog's consideration.

The most effective treatments for callus induction was stablished based on our previously study (BAP 2 mg L^{-1}; 2,4-D 0.5 mg L-1). The levels of elicitors included four levels of MJ (distilled water as control, 50, 100, and 200 µM, abbreviated as MJ0, MJ50, MJ100, MJ200) and 7 levels of polyamine (50 and 100 mg L^{-1} of putrescine; 50 and 100 mg L^{-1} of spermine; 50 and 100 mg L^{-1} of spermidine, Treatments abbreviation: Ctrl- Control, Put50- 50 mg L^{-1} Putrescine, Put100- 100 mg L^{-1} Putrescine, SPM50- 50 mg L^{-1} Spermine, SPM100- 100 mg L^{-1} Spermine, SPD50- 50 mg L^{-1} Spermidine, SPD100- 100 mg L^{-1} spermidine). The research was performed based on factorial experiment in RCBD design with three replications.

Cardenolide and Digitoxin

Cardenolide and digitoxin concentration in treated plants were measured by HPLC as described by Desta and McErlane (1982).

Measuring Cardenolide

Methanol (MeOH) with 1.8 mL value was used to extract 50 mg of callus and the samples were homogenized at a speed of 6 $m.s^{-1}$ for 45 seconds using the Fast Prep Tool (MP Biomedicals, Solon, Ohio, USA). After centrifugation, the supernatant solution was collected and filtered through a pressure of 0.45 mµ. Fifteen µL of the extract was injected into an HPLC of the Agilent 1100 series and the compounds were discarded on the reverse phase column of Gemini C18. Extraction of cardenolides was performed with a constant flow of 0.7 mg min^{-1} with phosphoric acid 0.25% of acetonitrile on a gradient of water: 0 to 5 minutes 20% of acetonitrile, 20 minutes 70% of acetonitrile, 20-25 minutes 70% of acetonitrile, 30 minutes 95% of acetonitrile, 30-35 minutes 95% of acetonitrile. The UV absorption spectrum from 200 to 400 nm was recorded by a diode array detector. Courtesy with a maximum absorption of 217 and 222 nm was recorded as Cardenolide (Bingham and Agrawal, 2010).

Measuring Digitoxin

Calli were dried in an oven at 40°C for 48 hours, then, 50 mg of dried calluses was treated with 1 milliliter of 70% methanol for 30 minutes in ultrasonic bath at 65-70-65°C and immediately placed on ice for 3 minutes. It was centrifuged at room temperature for 10 minutes at 13,000 rpm. Then, the supernatant was centrifuged with 0.25% of 15 m/v lead acetate solution, lead Acetate Isolated was discarded separately.

Half a milliliter of 4 m / v monosodium phosphate was added and centrifuged.

Supernatant was mixed with 0.5 milliliters of chloroform/ isopropanol solution in a ratio of 3 to 2 and centrifugation was performed for 5 minutes. The lower phase was transferred to the 2 mL tube as the first extract. The remaining methanolic solution was used for secondary extraction. Chloroform/isopropanol was added and then centrifuged at room temperature for 5 minutes at 13,000 rpm. Both extracts were combined under the laminar hood and injected to HPLC apparatus (Desta and McErlane, 1981).

Semi-Quantitative RT-PCR

Semi quantitative RT-PCR reaction was used to determine the relative expression of *DXR* in treated samples with different polyamine stimuli. Primer sequences for *DXR* gene and *18srRNA* as reference gene are shown in Table 1.

Total RNA was extracted from 200 mg of upper leaves of treated and un-treated *Digitalis lanata* L. according to the manufacturer's protocol (Sinaclon, Iran). DNase treatment was performed using the RNase-Free DNase Set (Promega, Promega UK, Southampton, UK) at the end of the extraction procedure. cDNA synthesis was performed using M-MLV Reverse Transcriptase (Fermentas, Germany) following the manufacturer's protocol. The yield of cDNA was measured based on the PCR signal generated using *18SrRNA* as a reference gene amplified from 30 to 35 cycles starting with 2 µL of the cDNA template. Semi-quantitative RT-PCR was carried out using published protocol (Spencer and Christensen, 1999) to measure the expression level of *DXR* in treated cells. To ensure that no false-positive PCR fragments would be generated from pseudogenes in contaminating genomic DNA, primer sequences were designed to span intron regions whenever genomic sequence data were available (Table 1). All RT and PCR were performed with reagents from Fermentas; Inc. PCR in 30 and 35 different cycles was performed independently as follows: 1 cycle of 95 ºC for 3 minutes; 94 ºC for 1 minute, 55 ºC for 40 seconds and 72ºC for 60 seconds; 1 cycle of 72 ºC for 7 minutes. Each set of PCR reaction included cDNA from non-treated plant as template for negative control and cDNA template from the digitoxin treated sample as a positive control. PCR products were resolved in 1.5% agarose gel and stained in GelRed^{TM} (Biotium). A 100 bp DNA ladder molecular weight marker (Fermentas, Germany) was used to confirm the expected molecular length of the amplified products.

Expression level of the *DXR* gene was assessed according to the published protocol (Antiabong *et al*., 2016). The amplicon images (PCR bands) in the gel were captured under Ultraviolet (UV) light and documented using a DigiDoc gel documentation system (Bio-rad; Australia). All the parameters and experimental conditions used were kept constant throughout the study. Image analysis was done using ImageJ software version 1.4.3u (Abramoff *et al*., 2004). The PCR band density was automatically generated by the ImageJ software using the specified "area".

Statistical Design and Analysis

Factorial (methyl jasmonate and

Table 1. Primer sequences for *DXR* and *18srRNA* genes.

Primers		sequnces	amplicon size (bp)	Accession No.	eferences
DXR	F	5'-GTGCGGGACTAAAGCCTACG-3'	101	NM_001203671.1	Mohammadi Faresani (2015)
	R	5'-CGGAAGCACGAAAGGACCAC-3'			
18srRNA	F	5'-AACGGCTACCACATCCAAG-3'	461	JQ612131.1	Nematollahi *et al*. (2012)
	R	5'-TCATTACTCCGATCCCGAA-3'			

Table 2. Means of square for digitoxin and cardenolide in response to experimental treatments.

SOV	Digitoxin	Cardenolide	Dry weight	Fresh weight
Polyamine	658.51**	0.49**	2199.37**	548498.96**
methyl jasmonate	727.48**	0.58**	3975.09**	1077929**
Polyamine×Methyl jasmonate	181.25**	0.18**	1462.21**	431637.24**
Error	2.79	0.001	24.55	8643.61
CV (%)	4.16	4.75	3.78	6.21

**significant effect at 1% statistically level.

polyamine) experiments were conducted based on completely randomized design with three replications. The SAS (9.1 v) was applied for data analysis, also means comparisons were done with Duncan test at 5% probability level. Image j software was used for analysis of molecular section in relation to electrophoresis product.

RESULTS AND DISCUSSION

Application of Elicitors

It was found that interaction effect of polyamine and methyl jasmonate had significant effect on fresh, dry, and fresh/dry weights of callus (Table. 2). The highest means of fresh (2204 mg), dry (168.3 mg), and fresh/dry (13.10) weights was obtained by the application of 100 mg L^{-1} Spermine+50 µM MJ treatments. In addition, lowest means of fresh (877.3 mg) and dry (86.27 mg) weights was obtained by the application of 200 µM MJ without polyamine. In this regard, El-Mekkawy *et al*. (2018) reported that application of elicitors led to induction of *Ecballium Elaterium* callus and resulted in increasing yields of the secondary metabolites.

Digitoxin and Cardenolide

According to analysis of variances, treatments had significant effects on Digitoxin and Cardenolide traits: the highest means of cardenolide and digitoxin was observed by 100 mg L^{-1} spermidine+50µM MJ and this treatment showed 4.1 and 2.4 folds increase of cardenolide and digitoxin compared to the control, respectively. In addition, lowest means of cardenolide and digitoxin were obtained by 200 µM MJ without polyamine (Figure 1). Treatment of plant cells with biological elicitors (glycemic polymers, glycoproteins, small organic acids, or extracts from fungal cells) and non-toxic elicitors (US, UV, salts, heavy metals and substances such as salicylic acid, Methyl jasmonate, fungal eucalyptus, silver nitrate, arachidonic acid and vanadyl sulfate) is always one of the most effective tools that increases the synthesis of secondary metabolites. The elicitors, through induction of defensive responses, cause biosynthesis and accumulation of secondary metabolites (Perez-Alonso *et al*., 2016).

Gene Expression of DXR in Response to Different Elicitors

Grecian foxglove plants treated with any kind of polyamine compound expressed the DXR *gene* transcript levels more than the untreated control for 3 to 7 days after treatment (Figures 2 and 3). However, *DXR* gene transcribed in cells treated with 100 mg L^{-1} putrescine and methyl jasmonate 50 µM + putrescine 100 mg L^{-1} transcribed to lower and highest levels compared to the negative control. Samples treated with the 50 µM methyl jasmonate+100 mg L^{-1} putrescine or 50 µM methyl jasmonate+50 mg L^{-1}

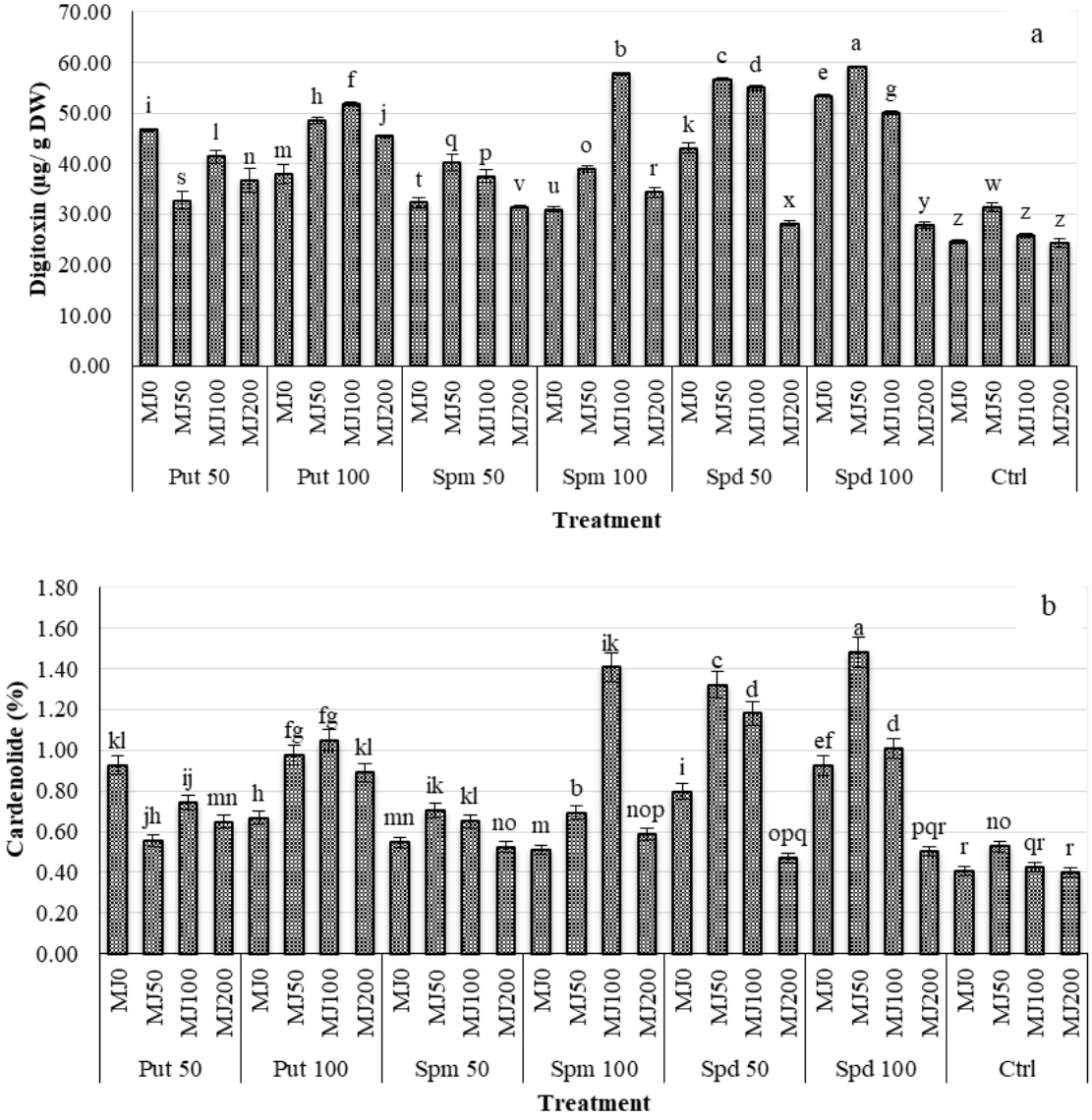

Figure 1. Mean comparisons for interaction of polyamines and methyl jasmonate on digitoxin (a) and cardenolide (b).

spermine mg accumulated DXR gene transcripts about 7.5 and 7 fold more than the negative control, respectively. DXR smilingly transcribed to a 2-fold level compared to the negative control in plants treated with the 50 µM methyl jasmonate+50 and 100 mg L^{-1} of spermidine as well as 100 µM methyl jasmonate+100 mg L^{-1} putrescine, polyamine compounds. Results showed that with increase in the concentration of polyamines in methyl jasmonate 200µM+putrescine 50 mg treatment, DXR transcript level did not significantly increase and it was about the level of the later treatments. Dabiri *et al*. (2017) showed that DXS was up-regulated in response to methyl jasmonate elicitor. Fan *et al.* (2016) reported that *DXS* and *DXR* were differentially expressed in roots, stems, leaves, and protocorms, with higher transcript levels in roots and stems compared with other organs in *Dendrobium*

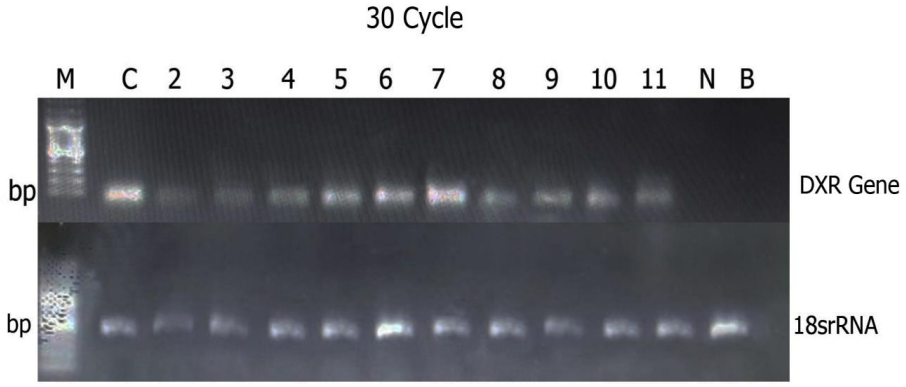

Figure 2. Semi-quantitative RT-PCR of *RDX* gene in digitoxin treated and un-treated grecian foxglove plants, 3 to 7 days after treatment. Tissue of grecian foxglove plants treated with digitoxin is the positive control and different concentrations of polyamines compounds. The levels of DXR messenger RNA accumulation are compared with that of the housekeeping gene *18SrRNA*. Numbers indicate the different concentrations of treated polyamines as follows: putrescine (50 and 100 mg), spermidine (50 and 100 mg), methyl jasmonate 50 mg+ putrescine 100 mg, methyl jasmonate 50 mg+Sprmin 50 mg, methyl jasmonate 50 mg+ sprmidin (50 and 100 mg), methyl jasmonate 100 mg+putrescine 100 mg, and methyl jasmonate 200 mg+putrescine 500 mg, respectively. C, positive control; N, negative control; L, 100 bp DNA ladder. Amplified products for *DXR* and *18SrRNA* genes are 101 and 461 bp in size for 30 cycles of PCR, respectively.

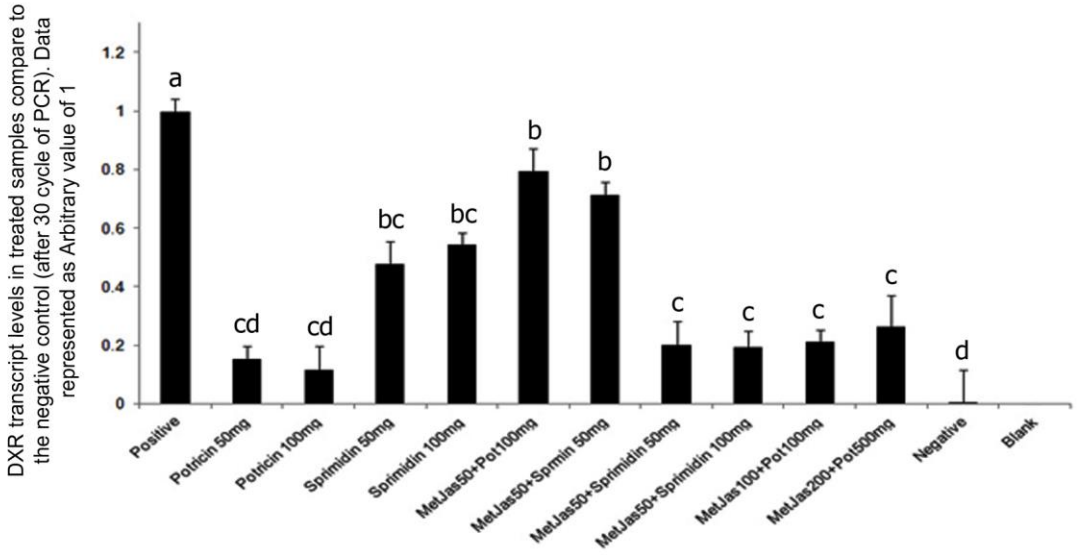

Figure 3. Assessment by RT-PCR of DXR gene transcript levels in tissue of grecian foxglove plants treated with digitoxin as positive control and different concentrations of polyamines including: putrescine (50 and 10 mg), Sprimidin (50 and 100 mg), methyl jasmonate 50 mg+ putrescine 100mg, Methyl Jasmonate 50 mg+Spermin 50 mg, Methyl Jasmonate 50 mg+ Spermidin (50 and 100 mg), methyl jasmonate 100 mg+putrescine 100 mg, methyl jasmonate 200mg+putrescine 500 mg. Densitometry analysis results are shown as bar charts whose values appear relative to the mean intensity of the positive controls (arbitrary value 1). Bars: Standard error.

officinale. As a result, transcript levels of *DXS* and *DXR* were induced by abscisic acid, methyl jasmonate, and salicylic acid, whereas their transcript levels were not regulated by brassinolide. According to the fact that DXR plays a pivotal role in the control of plant isoprenoid biosynthesis, application of different elicitors may affect

the plant isoprenoid quality and quantity. It has been reported that there are correlations between the expression of this gene and isoprenoid biosynthesis, Of course, this correlation depends on the type of plant, tissue, and stage of development (Schmiderer *et al*., 2010). Mahmoud and Croteau (2002) reported that in transgenic peppermint, the increasing of DXR expression led to the increase in the monotropenes compared to wild plants, and depletion caused a significant decrease in monotropenes. Such a correlation was reported by McConkey *et al*. (2000) on peppermint.

CONCLUSIONS

Results of the present study suggested that foxglove and its callus were a potential source of digitoxin and cardenolide content. Elicitors, namely, putrescine, spermine, spermidine, and methyl jasmonate significantly influenced the digitoxin and cardenolide. This research provides valuable insights into the potential manipulation of foxglove callus on the production of secondary metabolite for pharmacological, cosmetic, and agronomic industries. Therefore, the elicitation of cultured tissues is necessary to improve the production of phytochemical compounds. Likewise, DXR interestingly transcribed to a 7.5 to 8 fold levels compared to the negative control in plants treated with the methyl jasmonate 50 mg+ Spermine 50 mg as well as methyl jasmonate 50 mg+putrescine 100 mg, polyamine compounds.

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آنالیز بیان ژن DXR و تولید کاردنولید و دیجیتوکسین تحت تاثیر الیسیتورهای پلی آمین و متیل جاسمونات در گیاه گل انگشتانه ارغوانی (*Digitalis purpurea* L.)

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چکیده

دیجیتوکسین ترکیبی گلیکوزیدی است که از برگ گونه‌های مختلف جنس Digitalis استخراج می‌شود. دیجیتوکسین در کنترل و درمان نارسایی مزمن قلبی، حمله‌های قلبی ناگهانی و آریتمی‌های قلبی مختلف کاربرد دارد. ۱- دئوکسی- دی- گزیلولوز ۵- فسفات ردوکتاز ایزومراز(DXR) یک آنزیم مهارکننده مهم در مسیر بیوسنتزی MEP (-c-2متیل -d-اریتریتول ۴- فسفات) است و سطح بیان ژن DXR نقش مهمی در کنترل بیوسنتز ترکیبات ایزوپرونوئیدی در گیاه دارد. در این تحقیق اثرات محرک‌های زیستی پلی‌آمینی شامل پوترسین ، اسپرمین و اسپرمیدین در غلظت های ۵۰ و ۱۰۰ میلی گرم در لیتر همراه با متیل جاسمونات (MJ) در غلظت های ۵۰ ، ۱۰۰ و ۲۰۰ میکرومولار بر بیان ژن DXR در شرایط کشت سوسپانسیون سلولی گل انگشتانه ارغوانی (*Digitalis purpurea*)

مورد بررسی قرار گرفت. تغییرات در سطح رونویسی ژن DXR توسط روش RT-PCR ارزیابی گردید. نتایج نشان داد که محرک‌های زیستی اثر معنی‌داری بر مقدار بیان ژن DXR و بیوسنتز کاردنولید و دیجیتوکسین دارند. بالاترین میزان دیجیتوکسین (۶۱/۳ میکروگرم بر گرم وزن خشک) و کاردنولید (۱/۴۸ درصد در میلی گرم وزن خشک) در تیمار۱۰۰ میلی گرم در لیتر اسپرمیدین مشاهده شد. در کلیه تیمارها بیان ژن DXR در مقایسه با نمونه شاهد افزایش یافت. بالاترین سطح رونویسی ژن DXR در غلظت ۵۰ میکرومولار متیل جاسمونات و ۱۰۰ میلی گرم در لیتر پوترسین وجود داشت که نه برابر بیشتر از شرایط کنترل بود وهمچنین کمترین میزان در تیمار پوترسین در غلظت های ۵۰ و ۱۰۰ میلی گرم در لیتر مشاهده شد.