

Short Communication:

The impact of Gamma radiation on *Tdc* and *Str* gene expressions in *Catharanthus roseus* regenerated plantlets

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Abstract. Noormohammadi Z, Taban M, Farahani F. 2018. Short Communication: The impact of Gamma radiation on *Tdc* and *Str* gene expressions in *Catharanthus roseus* regenerated plantlets. *Biodiversitas* 19: 1805-1810. *Catharanthus roseus* L.G. Don, is the essential medicinal plant with considerable attention. This plant is a rich source of terpenoid indole alkaloids (TIAs). The main alkaloids in *C. roseus* are vinblastine, vincristine, and ajmalicine. The tryptophan decarboxylase (*Tdc*) and *Strictosidine* synthase (*Str*) are key enzymes in TIA biosynthesis. In the present study, *Tdc* and *Str* gene expressions, as well as vinblastine production were evaluated in tissue culture regenerated plantlets in 4 groups; control, 60 Gy irradiation, 50 mg/L putrescine and 60 Gy irradiation + 50mg/L putrescine treatments. The results revealed significant increase in *Tdc* and *Str* gene expressions in 60 Gy irradiation + 50mg/L putrescine treated plantlets in comparison with control samples by using qPCR methods. HPLC analysis showed a higher amount of vinblastine in 60 Gy + 59 mg/L putrescine treated plantlets. Gamma radiation and putrescine as elicitor and polyamine, respectively, are able to improve vinblastine production in *C. roseus*.

Keywords: Gene expression, periwinkle, radiation, secondary metabolism, tissue culture

INTRODUCTION

The medicinal plant *Catharanthus roseus* L.G. Don, comprises more than 130 terpenoid indole alkaloids (TIAs) in different organs. They are used in treatments of hypertension and Hodgkin's disease, testicular cancer, ovarian cancer, breast cancer and head and neck cancer (Nejat and Vadmalai 2012; Almagro et al. 2015; Sears and Boger 2015). The main alkaloids in *C. roseus* are vinblastine, vincristine, and ajmalicine (Xing et al. 2011). They act as inhibitors during the metaphase of the cell cycle by binding to the microtubules (Keglevich et al. 2012). The tryptophan decarboxylase (*Tdc*) and *Strictosidine* synthase (*Str*) are key enzymes in TIA biosynthesis. The *Tdc* converts tryptophan into tryptamine. The *Strictosidine* synthase (*Str*) catalyzes the tryptamine with secologanin to become *Strictosidine*, that leads to production of vindoline and catharanthine, which are precursors of vinblastine and vincristine (Ginis et al. 2012; Almagro et al. 2015).

The tissue and cell cultures provide a large amount of alkaloids with medicinal properties and active compounds for pharmaceutical industries (Sidhu 2011; Naz et al. 2015). Different tissue cultures including callus, cell, and shoot of Madagascar periwinkles are currently considered for vinblastine and vincristine productions (Moreno et al. 1995; Datta and Srivastava 1997; Miura et al. 1998; Ataei-Azimi et al. 2008). Using plant growth regulators like jasmonate, methyl-jasmonate, and salicylic acid, have also been used as elicitors to increase secondary metabolites

(El-Sayed and Verpoorte 2007; Verma et al. 2012; Antonio et al. 2013).

Physical elicitors like gamma irradiation have been attentive as a new process to improve the secondary metabolites in plants (Fulzele et al. 2015). Irradiated *Atropa belladonna* L. seeds with various doses of gamma rays can produce altered plant phenotypes and can also increase the alkaloids percentage in the different organs of a plant, particularly in the leaves (Abdel-Hady et al. 2008). The only report showed an increase of vinblastine in roots and leaves of *C. roseus* by use of 0.5 and 5 Gy gamma irradiation (Sadowska et al. 1989).

Polyamines, such as putrescine, have roles in plant growth regulation, DNA replication, gene transcription, and translation, as well as membrane stabilization. The polyamines may vary in different species, tissue, and developmental stages. Abiotic stresses can also induce it. Recently, exogenous polyamines are used to study relationship between endogenous polyamine and plant tolerance under abiotic stress (Zhang et al. 2009; Xu et al. 2011; Tabart et al. 2015).

The *Str* and *Tdc* gene expressions have been studied in different tissue culture treatments (Goddijn et al. 1992; Moghazee et al. 2014), as well as UV-B inducing *C. roseus* plantlets (Ramani et al. 2007; Aslam et al. 2010). The raising of vinblastine yield in cell culture of various tissues has also been reported through somatic embryogenesis by HPLC (Aslam et al. 2010).

The aim of this study was (i) to investigate the effect of gamma radiation (60Gy) and putrescine treatments on *Str* and *Tdc* gene expressions, and the measurement of

vinblastine in the leaves of *C. roseus*; (ii) the effect of putrescine on gamma radiation damages.

MATERIALS AND METHODS

Plant materials and treatment

F₁ seeds of *Catharanthus roseus* L.G. Don were provided by Pan American Company. The seeds divided to four groups for planting in MS media as following groups: (i) control (0 Gamma radiation + 0 mg putrescine), (ii) 60 Gy dose radiation + 0 mg putrescine, (iii) 0 gamma radiation + 50 mg/μL putrescine and (iv) 60 Gy radiation + 50 mg putrescine (El-Sharnouby et al. 2016). The irradiation was carried out by Gamma Cell, GC-220 in ⁶⁰Co gamma source with 9587 Ci activity and 2/28 Gy/s dose rate (Partonegaran-Saba company, Tehran, Iran).

Tissue culture and morphological characteristics

The *C. roseus* seeds sterilized under aseptic laminar flow hood with 75% Ethanol for 30 sec, then 5% NaClO for 3 min and finally washed 4-5 times with aseptic water. The seeds were cultured on Murashig and Skoog (MS) medium (Murashige and Skoog 1962) with 30 g/L sucrose and pH = 5.7 for 40 days. The explants were transformed to new MS medium and incubated under light treatments: illumination-5000 lx, photoperiod-16 h, temperature 25 ± 2°C for 45 days. The morphological characters, including surface area of leaves, stem length, root length, and fresh weight were measured for 4 groups by using digitizer software.

RNA extraction and qPCR assay

The fresh young leaves (3-5 leaves of each group) were used for RNA extraction. The RNA extraction protocol was based on CTAB method with modifications (Ghangal et al. 2009). The leaves (0.1 g) were milled in liquid nitrogen and transferred in 1 mL of pre-warmed CTAB extraction buffer [2% CTAB, 100mM Tris-HCl (pH 8.0), 2M NaCl, 25mM EDTA (pH 8.0)] containing 2% PVP and 25 μL 2-mercaptoethanol. After removing proteins with phenol/chloroform/isoamyl alcohol (24: 1: 1), 0.2 volume of glacial acetic acid (1 M), 0.1 volume of sodium acetate (3M) and 2 volume of ethanol absolute (98%) were added to supernatant. They were incubated at -20 °C for 2h, then NaCl (5M) was added and centrifuged at 12000 rpm for 15 min at 4°C. The 70% ethanol solution was used for washing. After drying pellet, 50 μL of Diethyl Pyrocarbonate (DEPC) treated water was added. DNA contamination was removed by RNase-free DNase I treatment according to manufacturer protocol (Thermo Fisher Scientific, USA). The quantity and quality of RNAs were checked by UV spectrophotometer (260/280 nm) and 1% agarose gel electrophoresis.

The cDNA was synthesized from 1 μg of total RNA, using the RevertAid First Strand cDNA synthesis reagent (Thermo Fisher Scientific, USA) according to manufacturer instructions, using oligo (dT) and random hexamer as primers according to the manufacturers.

The *Tdc* and *Str* gene expressions, as well as *Elf1-α* gene as reference gene, were assayed in four group

samples. At least 3 pooled samples of each treatment with two technical replicas were used for qPCR assay.

Real-time PCR (qPCR) was performed in 20 μL containing 1 μg cDNA, 1X Master Mix SYBR Green (TaKaRa, Japan), 0.5 μM of each primer. The following designed primer pairs were used: *Str* forward: 5'-GCCTTCACCTTCGATTCAAC-3' and *Str* reverse: 5'-GATGCGTAGGCGAAGTCACT-3', *Tdc* forward: 5'-CGCCTGTATATGTCCCGAGT-3' and *Tdc* reverse: 5'-GTTGCGATTTGCCAATTTTT-3', *Elf1-α* forward: 5'-TGGGCTACTGGTCTTACTAC-3' and *Elf1-α* reverse: 5'-ACATACCCACGATTTCAGATCCT-3'.

The qPCR thermal program was conducted in Rotor-Gene Q-pure Detection (Qiagen, USA) as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 40 sec, 88°C for 30 sec and 72°C for 30 sec. Melting curves were generated at 65-95°C after 40 cycles to check specificity of primers.

Alkaloids extraction and HPLC assay

The young leaves of samples of each group studied were lyophilized for 24h. The dried sample (1 g) was added to 1 mL Methanol. They were mixed by vortex and sonicated for 30min in an ultrasonic bath (DL-60D). Then samples were centrifuged at 12,000 rpm for 10 min. The supernatant was filtered with 0.45 μm needle type PTEE membrane filter (Pan et al. 2016). The standard curve of 2 mg of vinblastine sulfate of over 97% purity (Sigma-Aldrich (St. Louis, MO), was depicted after preparation of it in the same way similar to sample preparation. The Agilent Technologies 1200 series in National Center for Genetic and Biological Resources, Karaj, Iran was used for HPLC assay. The column was an Agilent Eclipse XDB-C18 column. The mobile phase consisted of a mixture of 5 mM Na₂HPO₄ and Methanol at a flow rate of 1.5 mL per min. The injection volume was 30μL.

Statistical analysis

The morphological characteristics and vinblastine amounts were analyzed by ANOVA (Analysis of variance), followed by LSD test to study significant difference among the samples. The level of *Str* and *Tdc* gene expressions were measured by a relative quantification based on the relative expression of target genes versus a reference gene (*Elf1-α*).

RESULTS AND DISCUSSION

Morphological characteristics analysis

Morphological comparison of regenerated plantlets of four groups (control, 60 Gy dose radiation + 0 mg putrescine, 0 gamma radiation + 50 mg/μL putrescine and 60 Gy radiation + 50 mg putrescine 60 Gy irradiations) were studied. Morphological characters showed that regenerated plants treated by putrescine showed the highest means in stem and root lengths and putrescine + 60 Gy irradiation plantlets showed the highest amounts in fresh weigh, surface area of leaves in comparison with other treated plantlets while plantlets of control group revealed

the lowest mean values studied. The analysis of variance ANOVA test (Table 1) showed the significant differences in surface area of leaves and stem length between 4 groups studied ($P < 0.01$). In detail, LSD test showed significant differences between all 4 groups in surface area of leaves except between putrescine and putrescine + 60 Gy irradiation plantlets. Length of stem was significant difference between control and other treated regenerated plants including 60 Gy irradiated plantlets, putrescine and 60 Gy irradiated plantlets, putrescine and putrescine + 60 Gy irradiated plantlets.

Str and Tdc gene expressions and vinblastine production

Relative comparison of *Tdc* and *Str* gene expressions plantlets of 4 groups were analyzed by qPCR method. Our finding showed up-regulating of *Str* gene transcripts in 60 Gy irradiation, putrescine, 60 Gy + putrescine in comparison to control samples significantly ($p < 0.05$, Figure 1). Meanwhile, the expression of *Str* gene was the highest in 60 Gy + putrescine treated plantlets. However, no significant differences were detected between treated groups in *Str* gene expression.

The *Tdc* gene expression was only up-regulated significantly ($p < 0.05$, Figure 1) in 60 Gy + putrescine treated plantlets against control samples. The 60 Gy + putrescine treated plantlets were also up-regulated against 60 Gy irradiated as well as putrescine treated plantlets significantly. The *Tdc* gene expression level in three groups including control, 60 Gy irradiation and putrescine treated plantlets showed no significant differences ($P > 0.05$).

The amount of vinblastine in leaves was measured by HPLC with about 24 min retention time based on standard

curve (Figure 2, Table 2). The highest mean value of vinblastine was detected in control samples (233.86 $\mu\text{g/g}$ plant) while the lowest mean belonged to 60 Gy irradiated plantlets (11.99 $\mu\text{g/g}$ plant). Apart from control samples, leaves of 60 Gy + putrescine treated plantlets (155.88 $\mu\text{g/g}$ plant) showed higher vinblastine production than other treated samples. ANOVA and LSD tests showed significant differences between all 4 groups studied (data not shown).

Discussion

In the present study, we have used MS medium lack of any phytohormones and found proper plant growth in control and 3 treated groups studied. The main reason was to eliminate any hormone effects on morphological traits and gene expression. Morphological study on *C. roseus* plantlets showed higher value in length of stem and roots in putrescine treated plantlets (Noormohammadi et al. personal communication). This supported impact of exogenous putrescine on plant growth. Several reports have shown that exogenous polyamines can restore the metabolism of endogenous polyamines (Zhang et al. 2009; Xu et al. 2011). On the other hands, 60 Gy + putrescine plantlets showed higher mean value of fresh weight and surface area of leaves than 60 Gy gamma irradiation *per se*. It may prove the impact of putrescine on plantlets to relieve effect of gamma radiation. However, control samples showed the lowest mean value of all morphological traits. These results show improvement of morphological characters by putrescine even in plantlets which exposed to gamma radiation.

Table 1. ANOVA test based on morphological traits between 4 treated plantlets

Morphological traits		Sum of squares	df	Mean square	F	P value
Fresh Weight	Between Groups	.002	3	.001	1.924	.166
	Within Groups	.005	16	.000		
	Total	.007	19			
Surface area of Leave	Between Groups	194.891	3	64.964	22.915	.000
	Within Groups	45.359	16	2.835		
	Total	240.250	19			
Stem length	Between Groups	34.973	3	11.658	3.773	.032
	Within Groups	49.429	16	3.089		
	Total	84.402	19			
Root length	Between Groups	61.067	3	20.356	2.214	.126
	Within Groups	147.097	16	9.194		
	Total	208.164	19			

Table 2. The amounts of vinblastine in 4 groups with retention time, peak area, concentration (ppm) based on HPLC analysis

Sample	Retention time(min)	Peak area	Con. (ppm)	Mean $\mu\text{g} / \text{g}$ plants
Control (10000 ppm)	24.925	46.07479	2.33864	233.864
Putrescine (10000 ppm)	24.918	10.9613	0.33101	33.101
60 Gy (10000 ppm)	24.916	7.2697	0.11994	11.994
60 Gy + putrescine (10000 ppm)	24.774	32.43533	1.5588	155.88

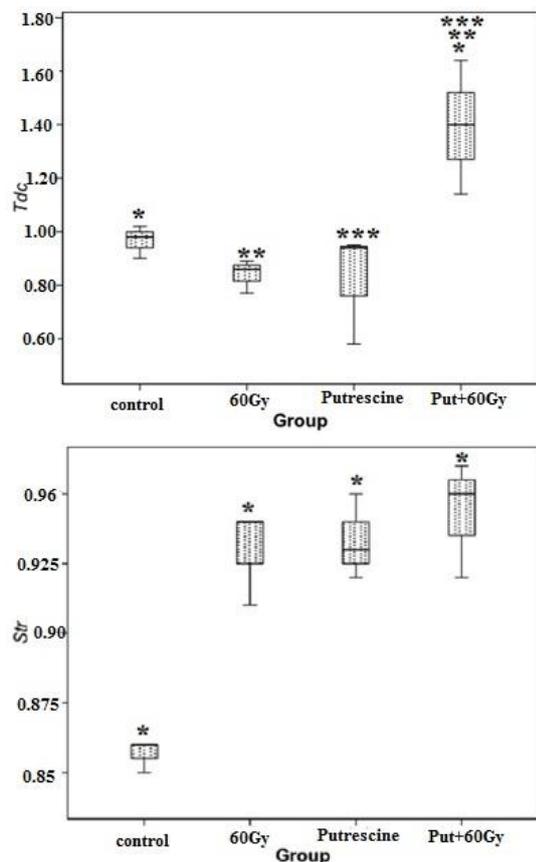


Figure 1. Bar plots of *Tdc* and *Str* gene expressions in 4 groups studied. X axis is 3 treated groups as well as control studied. Y axis is *Tdc* and *Str* gene expressions/reference gene ratio. * indicates significant differences between groups ($P < 0.05$).

El-Sharnouby et al. (2016) also emphasized a negative effect of 30 Gy gamma irradiation on seed germination, number of shoots, shoot length of *C. roseus* plantlets while leaf area, fresh weight of leaves were increased. However, morphological characters are multigenic and multi-factorial traits which are affected by genotypes and environmental conditions. The impact of gamma irradiation on plant growth may be related to the compounds involved in multiplication of genomes and cell division (Elangovan and Pavadai 2014).

Previous studies have shown that physical elicitors like UV-B induced *Tdc* and *Str* gene expressions in *C. roseus* (Ouwerkerk et al. 1999 a,b). Ramani and Chelliah (2007) worked on cell suspension culture of *C. roseus* and exposure low dose of UV-B irradiation to evaluate the amount of catharanthine and transcription of genes encoding *Tdc* and *Str*. Their results demonstrated that UV-B signaling leading to stimulation of *Tdc* and *Str* genes and the accumulation of catharanthine in *C. roseus* cell suspension cultures. The UV-B may induce ROS production and accumulation of catharanthine. The ROS generation could be induced by different elicitors like yeast and fungal elicitors in different plants (Dietrich et al. 1990; Felix et al. 1998; Tebayashi et al. 2001).

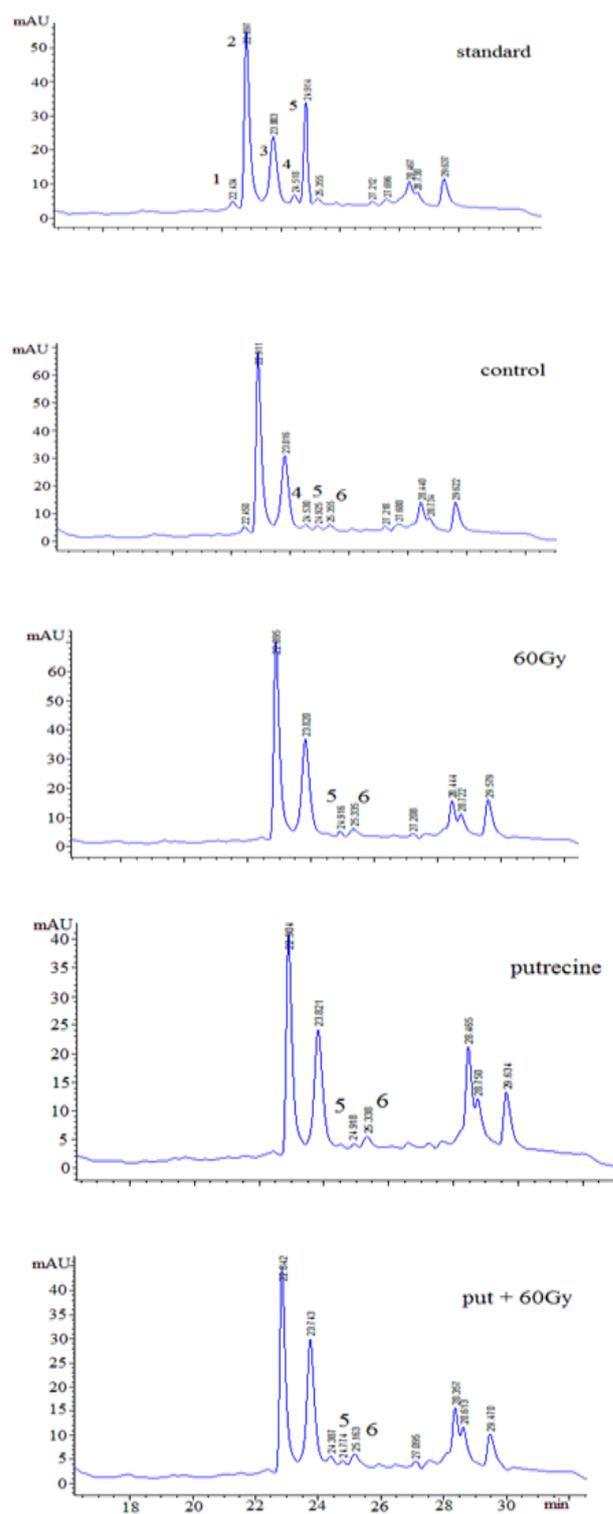


Figure 2. HPLC chromatographs of vinblastine of 4 groups studied and its standard. X axis is time (min) and Y axis is mAU/mA.

In our findings, gamma irradiation increased *Tdc* and *Str* gene transcripts in comparison to control samples. Meanwhile, gamma radiation with putrescine treatment showed highest gene expressions studied. It seems gamma radiation could act as elicitor and it would be boosted by

putrescine as a polyamine. Both may share elements in signal transduction for initiating TIA pathway.

The production of vinblastine in 3 treated groups also showed increase in 60 Gy irradiation + putrescine support gene expression results. Aslam et al. (2010) reported that somatic embryo-derived plantlets contained more vinblastine that leaves developed *ex vitro*. The amount of vinblastine in present study in gamma radiation and putrescine treated plantlets are more than those have been reported by Aslam et al. (2010). However, the differentiation and development of tissues are main factors which regulate the biosynthesis of alkaloids like vinblastine. Our findings suggest that polyamine and physical elicitor could have impact on terpenoid indole alkaloid pathway.

In this present investigation, we observed increase of mean values of morphological traits, *Tdc* and *Str* transcripts in treated plantlets in comparison to control plantlets. The vinblastine production in leaves showed that gamma radiation and putrescine as a polyamine could be change produce of this valuable alkaloid. The controversy data in this study between amount of *Str* and *Tdc* gene transcripts and vinblastine in different groups studied, may come from complicated biochemical pathway of vinblastine in *C. roseus* plants. However, the further studies are necessary to survey the biochemical pathways which gamma radiation may influence on them.

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