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EXOGENOUS APPLICATION OF SALICYLIC ACID, INDOLEACETIC ACID AND TRYPTOPHAN PROTECTS GROWTH, CHLOROPHYLL SYNTHESIS AND ANTIOXIDANT ACTIVITIES AND GENE EXPRESSION IN *CATHARANTHUS ROSEUS*

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Abstract

Vinblastine (VBL) and vincristine (VCR) are produced exclusively and in small amounts in *Catharanthus roseus*. These secondary metabolites (terpenoid indole alkaloids) have immense medicinal value. In this study, the impact of different concentrations of salicylic acid (SA), indoleacetic acid (IAA), or tryptophan (TRP) on the biosynthesis of VBL and VCR in *C. roseus* plants was investigated. Using quantitative real-time PCR, gene expression levels of the transcription factor ORCA3 and expression levels of genes encoding the catalytic enzymes strictosidine synthase (STR), tryptophan decarboxylase (TDC), and desacetoxyvindoline 4-hydroxylase (D4H) were studied. Treatment with 0.5 mg L^{-1} IAA significantly increased the gene expression of ORCA3, STR, and D4H, compared to the control; however, there was no effect on TDC. Production of VBL and VCR was qualitatively and quantitatively analyzed by HPLC using the corresponding reference standards. The maximum production of VCR and VBL, 146.23 and 311.09 µg mL^{-1}, respectively, was achieved with 0.5 mg L^{-1} IAA. In addition, compared to the control plants, spraying *C. roseus* plants with 0.5 mg L^{-1} IAA resulted in the maximum dry weight, maximum content of photosynthetic pigments (Chl *a*, *b* and carotenoids), enhanced activity of antioxidant enzymes (catalase, superoxide dismutase, ascorbate peroxidase), and phenylalanine ammonia-lyase. This study demonstrated the concentration-dependent induction effect of IAA in the biosynthesis of VCR and VBL. Therefore, IAA can be used to compensate for the low production of VCR and VBL under natural conditions.

Key words: Indoleacetic acid, Lipid oxidation, ORCA3, Salicylic acid, Strictosidine synthase, Tryptophan.

Introduction

Catharanthus roseus (L.) G. Don, commonly known as Madagascar periwinkle or annual vinca, is a flowering herbaceous plant in the family Apocynaceae. It is of immense medicinal value and used in traditional medicine for the treatment of diabetes, cancer, hypertension, and fever (Singh *et al.*, 2001; Heijden *et al.*, 2004). It produces more than 130 secondary metabolites known as terpenoid indole alkaloids (TIAs). Some of the TIAs have pharmacological potential, including the potent anticancer agents, vinblastine (VBL) and vincristine (VCR), produced exclusively by *C. roseus* (Negi, 2011; Kellner *et al.*, 2015; Zhu *et al.*, 2015). Vincristine has been used for acute lymphocytic leukemia, and Hodgkin and non-Hodgkin lymphomas, while vinblastine has been used for germ cell, breast, bladder, and some types of brain malignancies ((Alam *et al.*, 2017). *C. roseus* produces a small amount of these TIAs. Despite concentrated efforts in the last few years, it has been possible to increase the production of TIAs by *C. roseus* by only a small amount (Pan *et al.*, 2016). Furthermore, commercial-scale production of TIAs is difficult due to its complicated molecular biosynthesis (Zhou *et al.*, 2010).

Biosynthesis of the TIAs, in *C. roseus*, have been induced using different physical or chemical inducers, such as light, plant growth regulators, or signaling molecules (Wei, 2010; Li *et al.*, 2011). Liu *et al*., (2011) reported that treatment with plastic film in the presence of low light/UV light induced the biosynthesis of vinblastine in *C. roseus* leaves. The influence of plant growth regulators and inducers, such as methyl jasmonate, salicylic acid, gibberellic acid, sodium nitroprusside, and abscisic acid, on the biosynthesis of TIAs has also been investigated by many researchers (Pan *et al.*, 2010; Liu *et al.*, 2014). Xing *et al*., (2011) studied the effect of different combinations of ethylene, chlormequat chloride, and salicylic acid on the biosynthesis of TIAs in *C. roseus*. The combination treatments significantly improved vinblastine biosynthesis, compared to the single treatments. The increase of the vinblastine content was up to 246% at 48 h.

In *C. roseus*, strictosidine is the central precursor for the biosynthesis of TIAs, which occurs under rigid environmental control. Although the biosynthetic pathway has been extensively studied, and numerous genes and catalyzing enzymes have been characterized, the entire process remains largely unclear (Zhu *et al.*, 2015). Strictosidine is produced by the condensation of tryptamine and secologanin, the products of the indole and terpenoid biosynthetic pathways, respectively. Strictosidine is further converted to vindoline and catharanthine (monomeric alkaloids), and their coupling leads to the synthesis of vinblastine and vincristine (bisindole alkaloids) (Zhu *et al.*, 2014). The genes encoding TIA biosynthetic enzymes, such as strictosidine synthase (STR), tryptophan decarboxylase (TDC), desacetoxyvindoline 4-hydroxylase (D4H), and strictosidine beta-glucosidase (SGD), have been characterized (Zárate & Verpoorte, 2007). The transcription factors, octadecanoid-responsive *Catharanthus* AP2/ERF-domain (ORCA2 and ORCA3), have a regulatory role in the biosynthetic pathway of TIAs in *C. roseus* (Liu *et al.*, 2011). Overexpression of these regulatory genes enhances the expression of some of the genes of the biosynthetic pathways of TIAs leading to increase in the biosynthesis of TIAs (Pan *et al.*, 2012).

The present study aimed to study the effect of spraying different concentrations of salicylic acid (SA), indoleacetic acid (IAA) or tryptophan (TRP) on the biosynthesis of vinblastine and vincristine in *C. roseus* plants. The effect on gene expression of the transcription factor ORCA3 and on the expression of genes encoding the catalyzing enzymes STR, TDC, and D4H were investigated. Growth, photosynthetic potential, and antioxidant activity of the *C. roseus* plants were also evaluated.

Materials and Methods

Plant seeds and growth conditions: Seeds of *C. roseus* cv. Cooler Orchid, previously grown in the experimental fields of King Saud University in Riyadh region of Saudi Arabia, were used in this study. The healthy seeds were surface sterilized by immersing in 1% sodium hypochlorite solution for 5 min, washed in sterilized water, and dried using filter papers. Disinfected pots (20 cm diameter) filled with sterile soil were used. Six seeds were sown in each pot. Fifty-day-old plants were singly sprayed with salicylic acid (SA), indoleacetic acid (IAA), or tryptophan (TRP) (Sigma-Aldrich, Germany) at different concentrations (SA; 0.2 and 0.4 mM, IAA or TRP; 0.5 and 1 mg L^{-1}) for another fifteen days. Phytohormones were separately dissolved in absolute ethanol and then filled up with distilled water to get the required concentrations. For each treatment, five replicates were used. Pots were kept in a greenhouse at 35/28°C day/night temperature and watered as needed. The pots were arranged in a completely randomized design. Growth, lipid peroxidation and enzyme assays were performed 24 hours after last spray i.e., sixteen days after start of phytohormones treatment. While as gene expression was observed 12 and 24 hours (fifteen and sixteen days after start of phytohormones treatment) after last spray, and phytohormones were estimated three days after last spray i.e., 19 days after start of phytohormones treatment).

Total RNA extraction and quantitative real-time PCR (qRT-PCR): After 12 and 24 h of the last spray, total RNA was extracted from *C. roseus* leaves using an RNA isolation kit (Qiagen, Germany), according to the manufacturer's instructions. The isolated RNA was dissolved in diethyl dicarbonate-treated water and then incubated with DNase I for 1 h at 37°C to remove any DNA residues. Synthesis of cDNA was carried out in a reaction mixture of 25 µL containing 3 µL RNA, 2.5 µL 10 mM dNTPs, 2.5 µL 10X buffer with $MgCl_2$, 4 µL oligo (dT) primer, and 0.2 µL reverse transcriptase enzyme. The PCR was carried out using a SureCycler 8800 thermocycler (Agilent Technologies, USA), at 37°C for 2 h and 65°C for 20 min.

Quantitative real-time PCR was carried out using TOPreal^{TM} qPCR 2X PreMIX SYBR Green (Enzynomics, Daejeon, Korea). Specific primers for STR, TDC, D4H, and ORCA3 were used (Table 1). The *β*-actine gene was used as the reference gene. Real-time PCR was run on a Rotor gene 6000 RT- PCR System (Qiagen, USA). Amplification of these genes was carried out in a 20 µL reaction mixture containing 0.05 µM of the forward and reverse primers, cDNA template, and 0.5 unit SYBR Green I. The amplification program was 95°C for 3 min, followed by 40 cycles at 95°C for 10 sec and 60°C for 40 sec. The CT of each sample was used to calculate ΔCT values. For each sample, three biological and technical replicates were carried out.

High-performance liquid chromatography (HPLC): Three days after the last spray, alkaloids were extracted from the plant leaves (0.5 g) according to the method described by Misra and Gupta (2006). Qualitative and quantitative analysis of the contents of VCR and VBL was performed using the corresponding reference standards (Sigma-Aldrich, St. MO, USA) in the YL9100 HPLC system (Young Lin, Seoul, Korea). The samples were loaded separately on a C18 reverse phase analytical cartridge and eluted at a flow rate of 1 mL min^{-1} using the elution buffer (methanol: acetonitrile: 0.01 M ammonium acetate + 0.1% triethyl amine, 1: 1: 2.25) at pH 8.2. To construct the calibration curves, stock standard solutions of VCR and VBL sulfate at 100 mg L^{-1} were prepared in methanol. Serial dilutions were made to obtain 10, 5, 1, and 0.5 mg L^{-1} and analyzed separately by HPLC. The calibration curves were then plotted between peak area and concentration.

Dry weight estimation: Three plants from each treatment group were uprooted, washed under tap water and oven dried for two days at 80°C for dry weight estimation.

Estimation of the contents of photosynthetic pigments: The photosynthetic pigment content (chlorophyll a, b, and carotenoids) was estimated according to the method described by Harborne (1998).

Table 1. Nucleotide sequences of the primers used in qRT-PCR.

Primer	Type	Sequence (5'-3')
Octadecanoid-responsive Catharanthus AP2/ERF-domain3 (ORCA3)	F	5′- CGAATTCAATGGCGGAAAGC-3′
	R	5′- CCTTATCTCCGCCGCGAACT-3′
Strictosidine synthase (STR)	F	5′- GCCTTCACCTTCGATTCAACTG-3′
	R	5′- GTGGCTAGTTGTGTGGCATACC-3′
Tryptophan decarboxylase (TDC)	F	5′- GGTCGAGGATGACGTGGCGGCCGG-3′
	R	5′- ACTCAGACTCAGTGAGTCAACTCGTT-3′
Desacetoxyvindoline 4-hydroxylase (D4H)	F	5′- GGAATTGCTTGCAATTGGAG-3′
	R	5′- TGTCCTGCATGTCATCAATG-3′
β-Actin	F	5′- GGCTGGATTTGCTGGAGATGAT-3′
	R	5′- TAGATCCTCCGATCCAGACACTG-3′

Analysis of lipid peroxidation: Lipid peroxidation was measured in terms of malondialdehyde content (MDA) according to the method described by Velikova *et al*., (2000).

Assay of the activity of antioxidant enzymes: Crude enzyme extract was prepared according to the method adopted by Misra *et al*., (2006). Assay of catalase enzyme (CAT), superoxide dismutase enzyme (SOD), and ascorbate peroxidase enzyme (APX) activity was done according to Aebi (1984), Giannopolitis & Ries (1977) and Rao *et al*., (1997) respectively.

Assay of phenylalanine ammonia-lyase enzyme (PAL): PAL activity was carried out according to the method of Whetten & Sederoff (1992).

Statistical analysis

The results were analyzed using the statistical analysis software CoStat (version 6.4). The Duncan's multiple range test was used to compare the means, which was significant at $p \leq 0.05$ (Duncan, 1955).

Results

qRT-PCR: To investigate the impact of salicylic acid, indoleacetic acid, and tryptophan treatment on the biosynthesis of VBL and VCR, mRNA of the transcription factor ORCA3 and the genes encoding the catalyzing enzymes STR, TDC, and D4H were amplified and quantified (Fig. 1A, B, C and D). Twelve hours after the last spray, increased expression of all the tested genes was observed, although the increase showed varied trend. Treatment of 0.5 mg L^{-1} IAA resulted in significant increase in the gene expression of ORCA3, STR, and D4H (2.28-, 1.24-, and 2.16-fold, respectively), except that of TDC compared to control. Treatment with 0.5 and 1 mg L^{-1} TRP increased the expression of TDC by 1.28- and 1.22-fold, respectively. In contrast, treatment with 0.4 mM SA did not influence the expression levels of the tested genes. Additionally, no changes were observed in the expression levels of ORCA3, STR, and D4H with 1 mg L^{-1} TRP, and in STR and D4H levels, with 0.5 mg L^{-1} TRP, compared to the control. Twenty-four hours after the last spray, treatment with 1 mg L^{-1} TRP led to increased expression of ORCA3, STR, and SDC, by 2.45-, 1.24-, and 1.17-fold, respectively, compared to control; while, D4H recorded a 2.53-fold increase upon treatment with 0.5 mg L^{-1} IAA. Treatment with 0.4 mM SA did not show any significant affect on the expression levels of the tested genes.

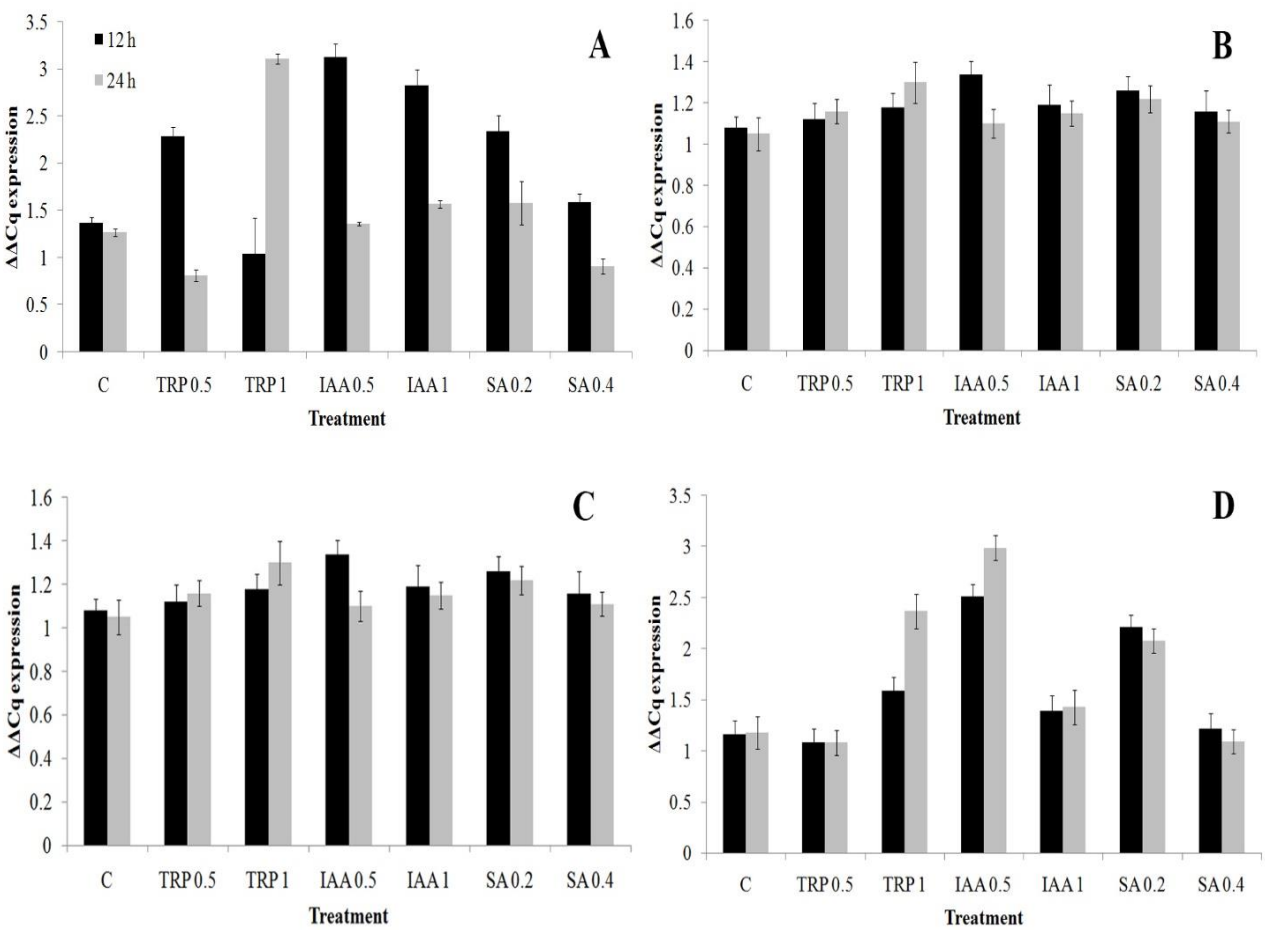

Fig. 1. Gene expression levels of the transcription factor ORCA3 (A), tryptophan decarboxylase (B), strictosidine synthase (C), and desacetoxyvindoline 4-hydroxylase (D) in *Catharanthus roseus* plants in response to the spraying of different concentrations of tryptophan (TRP), indoleacetic acid (IAA), and salicylic acid (SA) using qRT-PCR.

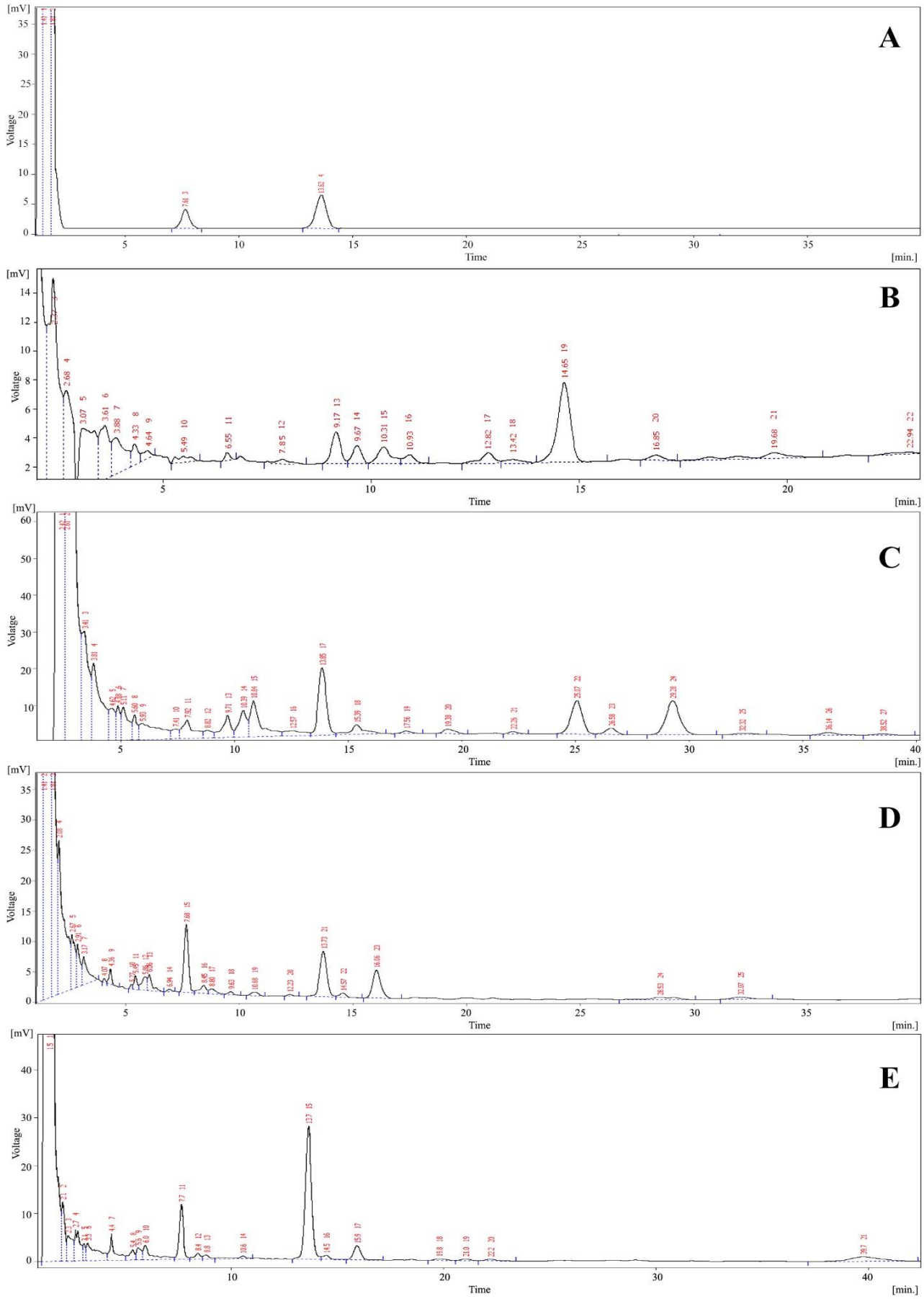

Fig. 2. HPLC chromatograms of vincristine and vinblastine in response to different treatments in *Catharanthus roseus* plants. A) reference standards, B) control, C) tryptophan (1 mg L^{-1}), D) salicylic acid (0.2 mM) and E) indoleacetic acid (0.5 mg L^{-1}).

HPLC analysis: Qualitative and quantitative analyses were performed using HPLC to study the influence of the treatments on the production of VBL and VCR in *C. roseus* plants. Based on their efficiencies in the qRT-PCR test, the effects of 1 mg L^{-1} TRP, 0.5 mg L^{-1} IAA, and 0.2 mM SA were only studied. Mass spectra of all the tested samples showed two ion masses 824 and 810 at retention times of 7.67 and 13.67 min, which are specific for VCR and VBL, respectively and their presence was confirmed by corresponding reference standards (Fig. 2A, B, C, D, and E). The results revealed a low production of VCR and VBL in the control plants. On the other hand, the treatments induced the biosynthesis of VCR and VBL. The maximum production of VCR and VBL was achieved with 0.5 mg L^{-1} IAA treatment showing 146.23 and 311.09 µg mL^{-1} respectively. However, treatment of *C. roseus* plants with 1 mg L^{-1} TRP showed 50.21 and 242.02 µg mL^{-1} of VCR and VBL concentrations respectively, while as 0.2 mM SA treated seedlings showed 136.38 and 87.21 µg mL^{-1} VCR and VBL respectively (Fig. 2).

Plant growth and photosynthetic activities: Results showing influence of treatments on dry weight and pigment contents in *C. roseus* plants are presented in Table 2. The results indicated that all the treatments led to significant increments in the dry weight of the treated plants compared to control. Maximum growth was observed with 0.5 mg L^{-1} IAA (2.67 g), followed by 2.39 g and 2.35 g for 1 mg L^{-1} IAA and 1 mg L^{-1} TRP, respectively. The lowest growth was observed after treatment with 0.4 mM SA. All the treatments significantly induced the production of photosynthetic pigments in *C. roseus* plants. The maximum content of the photosynthetic pigments, chlorophyll a, chlorophyll b, and carotenoids (8.6, 5.7, and 4.15 mg g^{-1} f.wt, respectively), was recorded in seedlings treated with 0.5 mg L^{-1} IAA treatment, while as 0.4 mM SA proved less effective compared to the control (Table 2).

Lipid peroxidation, PAL activity and antioxidant enzymes: The effects of the treatments on the activities of the antioxidant enzymes CAT, SOD and APX, and PAL and lipid oxidation were investigated. Results of enzyme activities (antioxidants and PAL) and the malondialdehyde content in treated *C. roseus* plants are presented in Table 3. Treatment with SA, IAA, and TRP significantly induced enzyme activity and lipid oxidation. Compared to the control, maximum enzyme activity and malondialdehyde content were recorded for the treatment with 0.5 mg L^{-1} IAA; 9.51, 20.51, and 13.25 units mg^{-1} protein for CAT, SOD, and APX enzymes, respectively, 7.95 µmol *t*-cinnamic acid g^{-1} fresh weight for PAL enzyme, and 0.92 µmol g^{-1} fresh weight for MDA level. Among the treatments the lowest activity was observed with 0.4 mM (Table 3).

Table 2. Dry weight (g) and photosynthetic activities (mg g^{-1} f.wt) of *C. roseus* plants in response to spraying of tryptophn, salicylic acid and indoleacetic acid at different concentrations.

Treatment*		Dry wt.	Chl. *A*	Chl. *b*	Carotenoids
Control		1.19 ± 0.24**e	5.72 ± 0.18f	1.15 ± 0.16f	2.92 ± 0.16e
TRP	0.5	1.97 ± 0.19c	6.91 ± 0.20e	2.88 ± 0.18d	3.39 ± 0.30cd
(mg L^{-1})	1	2.35 ± 0.26b	8.36 ± 0.22b	4.27 ± 0.10b	3.77 ± 0.23b
IAA	0.5	2.67 ± 0.20a	8.60 ± 0.26a	5.70 ± 0.34a	4.15 ± 0.31a
(mg L^{-1})	1	2.39 ± 0.22b	7.19 ± 0.24d	3.60 ± 0.09c	3.23 ± 0.22d
SA	0.2	1.86 ± 0.21c	7.80 ± 0.22c	3.79 ± 0.26bc	3.59 ± 0.15c
(mM)	0.4	1.58 ± 0.26d	6.74 ± 0.18e	1.83 ± 0.38e	3.38 ± 0.19cd

*TRP = tryptophan, IAA = indoleacetic acid, and SA = salicylic acid

**Values within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($p \leq 0.05$). Each value represents the mean of three replicates ± SD

Table 3. Lipid peroxidation (µmol g^{-1} f.wt.) and activities of the antioxidant enzymes CAT, SOD, APX (unit mg^{-1} protein) and PAL (µmol *t*-cinamic acid g^{-1} f wt) of *C. roseus* plants in response to spraying of tryptophan, salicylic acid and indoleacetic acid at different concentrations.

Treatment*		MDA	CAT	SOD	APX	PAL
Control		0.42 ± 0.02f **	2.60 ± 0.17f	15.17 ± 0.43e	9.60 ± 0.53e	3.65 ± 0.13f
TRP	0.5	0.57 ± 0.02d	5.71 ± 0.12d	17.19 ± 0.32cd	10.73 ± 0.45cd	5.24 ± 0.14c
(mg L^{-1})	1	0.69 ± 0.02c	8.11 ± 0.15b	18.46 ± 0.56b	11.03 ± 0.68c	7.63 ± 0.09b
IAA	0.5	0.92 ± 0.03a	9.51± 0.09a	20.51 ± 0.71a	13.25 ± 0.49a	7.95 ± 0.18a
(mg L^{-1})	1	0.74 ± 0.04b	8.41 ± 0.11b	17.80 ± 0.68bc	12.43 ± 0.33b	5.17 ± 0.11d
SA	0.2	0.66 ± 0.01c	6.29 ± 0.16c	18.24 ± 0.66bc	10.87 ± 0.58cd	5.14 ± 0.10d
(mM)	0.4	0.51 ± 0.01e	4.47 ± 0.14e	16.73 ± 0.53d	10.65 ± 0.51cd	4.39 ± 0.15e

*TRP = tryptophan, IAA = indoleacetic acid, SA = salicylic acid, MDA = malondiadehyde content, CAT = catalase enzyme, SOD = superoxide dismutase enzyme, APX = ascorbate peroxidase enzyme, and PAL = phenylalanine ammonia-lyase enzyme.

**Values within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($p \leq 0.05$). Each value represents the mean of three replicates ± SD

Discussion

Present study describes the influence use of chemical inducers to increase the production of VCR and VBL in *C. roseus* plants. In this regard, the effect of these chemicals on the expression of genes of the VCR and VBL biosynthetic pathways was investigated. Compared to the control (untreated), exogenous application of 0.5 mg L^{-1} IAA exerted the maximum influence post 12 h of treatment, whereas the influence of 1 mg L^{-1} TRP was maximum post 24 h of treatment. These results are in agreement with the study by El-Domyati *et al*., (2014), indicating the efficiency of these inducers in triggering the regulatory genes of the TIAs biosynthetic pathway. In addition, Pan *et al*., (2010) had reported an increase in VCR and VBL biosynthesis in response to treatment with salicylic acid. The transcription factor ORCA3 is a regulatory protein acting as DNA-binding protein which directly binds to the promoters of the structural genes involved in primary as well as secondary plant metabolic pathways thereby regulating their gene expression (Pan *et al.*, 2012). In this study, increased expression of ORCA3 led to up-regulation of the tested genes (TDC, STR, and D4H), which in turn modulated the biosynthesis of TIAs, resulting in the increased production of VCR and VBL. The TDC gene regulates the enzymatic conversion of L-tryptophan to tryptamine, and STR gene regulates the condensation of tryptamine and secologanin to produce strictosidine, which is the central precursor in the TIAs biosynthesis (Zhu *et al.*, 2014). Exogenous application of tryptophan or IAA led to the induction of VCR and VBL biosynthesis. In the treated plants, tryptophan is bio-converted into IAA, thus enhancing their growth, whereas L-tryptophan is first metabolized to indole-3-acetamide (IAM), which is then converted to IAA by IAM-hydrolase (Mohite, 2013). This explains the efficacy of 1 mg L^{-1} TRP in enhancing the biosynthesis of VCR and VBL, the plant dry weight and the total content of the photosynthetic pigments after 24 h, compared to treatment with lower concentrations as well as control after 12 h. In other words, TRP affects these metabolic processes through its role in IAA biosynthesis. Plant growth regulators, such as IAA, enhance the growth of the plants in a concentration-dependent manner; therefore, treatment with 0.5 mg L^{-1} IAA exhibited higher values than that recorded for 1 mg L^{-1} IAA. This result is in agreement with the report on buckwheat sprouts by (Park *et al.*, 2017). Indoleacetic acid acts as a co-enzyme and enhances various metabolic processes in plants, including activation of the cell division, formation of the photosynthetic pigments, and/or preservation of the chromoproteins (Sardoei, 2014; Majda and Robert, 2018). In addition, spraying of TRP or IAA led to a significant increase in the activity of the tested antioxidant enzymes as well as lipid peroxidation. These results is in agreement with that obtained by (Mohammadi & Khavari-Nejad, 2013), and may be attributed to the positive role of IAA as a co-enzyme and in quenching the reactive oxygen species (Lecube *et al.*, 2014). In addition the activities of antioxidant enzymes studied and PAL were greater in treated seedlings than control resulting in growth promotion. Antioxidants promote growth by reducing the accumulation of toxic reactive oxygen species (Ahanger *et al*., 2017; Ahanger *et al*., 2019). It has been reported that greater synthesis of secondary metabolites due to up-regulation of PAL strengthens the antioxidant system when enzymatic scavenging system weakens to eliminate reactive oxygen species (Ahanger & Agarwal, 2017; Ahanger & Agarwal, 2017). Therefore, exogenous application of tested phytohormones can be exploited to improve growth and production of VCR and VBL in *C. roseus*.

Conclusion

In conclusion, spraying of *C. roseus* plants with 0.5 mg L^{-1} IAA or 1 mg L^{-1} TRP resulted in the increased expression of transcription factor ORCA3 and the genes encoding the catalytic enzymes STR, TDC, and D4H therefore leading to significant induction of VCR and VBL biosynthesis, improvement of growth and activation of the antioxidant activity in the plants. This study may help in maximizing the production of VCR and VBL under natural conditions.

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References

Aebi, H. 1984. Catalase in vitro. In: Methods in Enzymology. Elsevier: pp: 121-126.

Ahanger, M.A. and R.M. Agarwal. 2017. Potassium up-regulates antioxidant metabolism and alleviates growth inhibition under water and osmotic stress in wheat (*Triticum aestivum* L). *Protoplasma*, 254(4): 1471-1486.

Ahanger, M.A. and R.M. Agarwal. 2017. Salinity stress induced alterations in antioxidant metabolism and nitrogen assimilation in wheat (*Triticum aestivum* L.) as influenced by potassium supplementation. *Plant Physiol. & Biochem.*, 115: 449-460.

Ahanger, M.A., C. Qin, Q. Maodong, X.X. Dong, P. Ahmad, E.F. Abd_Allah and L. Zhang. 2019. Spermine application alleviates salinity induced growth and photosynthetic inhibition in *Solanum lycopersicum* by modulating osmolyte and secondary metabolite accumulation and differentially regulating antioxidant metabolism. *Plant Physiol. & Biochem.*, 144: 1-13.

Ahanger, M.A., N.S. Tomar, M. Tittal, S. Argal and R.M. Agarwal. 2017. Plant growth under water/salt stress: ROS production; antioxidants and significance of added potassium under such conditions. *Physiol. & Mol. Biol. of Plants*, 23(4): 731-744.

Alam, M.M., M. Naeem, M.M.A. Khan and M. Uddin. 2017. Vincristine and vinblastine anticancer catharanthus alkaloids: pharmacological applications and strategies for yield improvement. In: Catharanthus roseus. Springer International Publishing: pp. 277-307.

Duncan, D.B. 1955. Multiple Range and Multiple F Tests. *Biometrics*, 11(1): 1.

El-Domyati, F., A. Abdel Azeiz, A. Atef, A. Elhalwagi, J. Sabir, A. Ramadan, N. Gadalla, S. Edris, M. Baeshen and N. Hajrah. 2014. Functional genomic analysis for overproduction of key terpenoid indole alkaloids in mature leaf of *Catharanthus roseus*. *Life Sci. J.*, 11(10): 1213-1220.

Giannopolitis, C.N. and S.K. Ries. 1977. Superoxide Dismutases. *Plant Physiol.*, 59(2): 309-314.

Harborne, A. 1998. Phytochemical methods a guide to modern techniques of plant analysis. springer science & business media.

Heijden, R., D. Jacobs, W. Snoeijer, D. Hallard and R. Verpoorte. 2004. The Catharanthus Alkaloids: Pharmacognosy and Biotechnology. *Curr. Med. Chem.*, 11(5): 607-628.

Kellner, F., J. Kim, B.J. Clavijo, J.P. Hamilton, K.L. Childs, B. Vaillancourt, J. Cepela, M. Habermann, B. Steuernagel, L. Clissold, K. McLay, C.R. Buell and S.E. O'Connor. 2015. Genome-guided investigation of plant natural product biosynthesis. *The Plant J.*, 82(4): 680-692.

Lecube, M.L., G.O. Noriega, D.M. Santa Cruz, M.L. Tomaro, A. Batlle and K.B. Balestrasse. 2014. Indole acetic acid is responsible for protection against oxidative stress caused by drought in soybean plants: The role of heme oxygenase induction. *Redox Report*, 19(6): 242-250.

Li, M., C.A.M. Peebles, J.V. Shanks and K.-Y. San. 2011. Effect of sodium nitroprusside on growth and terpenoid indole alkaloid production in *Catharanthus roseus* hairy root cultures. *Biotechnol. Prog.*, 27(3): 625-630.

Liu, J., J. Zhu, L. Tang, W. Wen, S. Lv and R. Yu. 2014. Enhancement of vindoline and vinblastine production in suspension-cultured cells of *Catharanthus roseus* by artemisinic acid elicitation. *World J. Microb. & Biotechnol.*, 30(1): 175-180.

Liu, Y., D.M. Zhao, Y.G. Zu, Z.H. Tang, Z.H. Zhang, Y. Jiang and D.Y. Shi. 2011. Effects of low light on terpenoid indole alkaloid accumulation and related biosynthetic pathway gene expression in leaves of *Catharanthus roseus* seedlings. *Bot. Stud.*, 52(2): 191-196.

Majda, M. and S. Robert. 2018. The Role of Auxin in Cell Wall Expansion. *Int. J. Mol. Sci.*, 19(4): 951.

Misra, N., M.S. Ansari and A.K. Gupta. 2006. Differential response of scavenging of reactive oxygen species in green gram genotype grown under salinity stress. *Amer. J. Plant Physiol.*, 1: 41-53.

Misra, N. and A.K. Gupta. 2006. Effect of salinity and different nitrogen sources on the activity of antioxidant enzymes and indole alkaloid content in *Catharanthus roseus* seedlings. *J. Plant Physiol.*, 163(1): 11-18.

Mohammadi, F. and R. Khavari-Nejad. 2013. Effect of indoleacetic acid on growth and antioxidant properties in *Glycine max* seedlings exposed to aluminium toxicity. *Genetics & Plant Physiol.*, 3(3-4): 179-190.

Mohite, B. 2013. Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. *J. Soil Sci. & Plant Nutr*., 13(3): 638-649.

Negi, R. 2011. Fast *In vitro* callus induction in *Catharanthus roseus*-A medicinally important plant used in cancer therapy. *Res. J. Pharm. Biol. Chem. Sci.*, 2(4): 597-603.

Pan, Q., Y. Chen, Q. Wang, F. Yuan, S. Xing, Y. Tian, J. Zhao, X. Sun and K. Tang. 2010. Effect of plant growth regulators on the biosynthesis of vinblastine, vindoline and catharanthine in *Catharanthus roseus*. *Plant Growth Regul.*, 60(2): 133-141.

Pan, Q., N.R. Mustafa, K. Tang, Y.H. Choi and R. Verpoorte. 2016. Monoterpenoid indole alkaloids biosynthesis and its regulation in *Catharanthus roseus*: a literature review from genes to metabolites. *Phytochem. Rev.*, 15(2): 221-250.

Pan, Q., Q. Wang, F. Yuan, S. Xing, J. Zhao, Y.H. Choi, R. Verpoorte, Y. Tian, G. Wang and K. Tang. 2012. Overexpression of ORCA3 and G10H in *Catharanthus roseus* Plants Regulated Alkaloid Biosynthesis and Metabolism Revealed by NMR-Metabolomics. *PLoS ONE*, 7(8): e43038.

Park, C., H. Yeo, Y. Park, A. Morgan, M. Valan Arasu, N. Al-Dhabi and S. Park. 2017. Influence of Indole-3-Acetic Acid and Gibberellic Acid on Phenylpropanoid Accumulation in Common Buckwheat (*Fagopyrum esculentum* Moench) Sprouts. *Molecules*, 22(3): 374.

Rao, M.V., G. Paliyath, D.P. Ormrod, D.P. Murr and C.B. Watkins. 1997. Influence of Salicylic Acid on H2O2 Production, Oxidative Stress, and H2O2-Metabolizing Enzymes (Salicylic Acid-Mediated Oxidative Damage Requires H2O2). *Plant Physiol.*, 115(1): 137-149.

Sardoei, A.S. 2014. Plant growth regulators effects on the growth and photosynthetic pigments on three indoor ornamental plants. *Europ. J. Exp. Biol.*, 4(2): 311-318.

Singh, S.N., P. Vats, S. Suri, R. Shyam, M.M.L. Kumria, S. Ranganathan and K. Sridharan. 2001. Effect of an antidiabetic extract of *Catharanthus roseus* on enzymic activities in streptozotocin induced diabetic rats. *J. Ethnopharmacol.*, 76(3): 269-277.

Velikova, V., I. Yordanov and A. Edreva. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants. *Plant Sci.*, 151(1): 59-66.

Wei, S. 2010. Methyl jasmonic acid induced expression pattern of terpenoid indole alkaloid pathway genes in *Catharanthus roseus* seedlings. *Plant Growth Regul.*, 61(3): 243-251.

Whetten, R.W. and R.R. Sederoff. 1992. Phenylalanine ammonia-lyase from loblolly pine: purification of the enzyme and isolation of complementary DNA clones. *Plant Physiol.*, 98(1): 380-386.

Xing, S., Q. Pan, Y. Tian, Q. Wang, P. Liu, J. Zhao, G. Wang, X. Sun and K. Tang. 2011. Effect of plant growth regulator combinations on the biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus*. *J. Med. Plants Res.*, 5(9): 1692-1700.

Zárate, R. and R. Verpoorte. 2007. Strategies for the genetic modification of the medicinal plant *Catharanthus roseus* (L.) G. Don. *Phytochem. Rev.*, 6(2-3): 475-491.

Zhou, M.L., H.L. Hou, X.M. Zhu, J.R. Shao, Y.M. Wu and Y.X. Tang. 2010. Molecular regulation of terpenoid indole alkaloids pathway in the medicinal plant, *Catharanthus roseus*. *J. Med. Plants Res.*, 4(25): 2760-2772.

Zhu, J., M. Wang, W. Wen and R. Yu. 2015. Biosynthesis and regulation of terpenoid indole alkaloids in *Catharanthus roseus*. *Pharmacog. Rev.*, 9(17): 24.

Zhu, X., X. Zeng, C. Sun and S. Chen. 2014. Biosynthetic pathway of terpenoid indole alkaloids in *Catharanthus roseus*. *Front. of Med.*, 8(3): 285-293.

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