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Alkaloids production and antioxidant properties in *Catharanthus roseus* (L.) G. Don. shoots and study of alkaloid biosynthesis-related gene expression levels in response to methyl jasmonate and putrescine treatments as eco-friendly elicitors

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Introduction: This study aimed to determine the effects of methyl jasmonate (MJ) combined with putrescine as eco-friendly elicitors on secondary metabolism and gene expression of alkaloid biosynthetic pathway in *Catharanthus roseus in vitro*-propagated shoots. *Methods:* The expression of mitogen-activated protein kinase 3 and the transcription factor, octadecanoid-responsive Catharanthus AP2-domain3, upstream of plant alkaloids' biosynthetic pathway, and of key genes in the pathway (CrPRX1, STR, DAT, and GS) are investigated as well using qRt-PCR. Antioxidant enzyme (superoxide dismutase, peroxidase, and catalase) activities and non-enzymatic antioxidants (phenolics, flavonoids, and carotenoids) contents have studied to determine the stress levels of the plant by spectrophotometer. *Results:* Results showed increased contents of non-enzymatic antioxidants after 4–8 hr and enzymatic antioxidants activities after 24 hr. Alkaloids contents increased mostly after 1 week. The investigated signaling genes upregulated after 8 hr and biosynthetic genes after 24 hr of treatments. Combined treatments had more positive effects on gene expression levels, antioxidant responses, and secondary metabolite production than MJ individually. *Discussion:* Increased effects of combined elicitor on genes expression may be due to cross talks between their signaling pathways. Combination of MJ and putrescine can be used as an eco-friendly elicitor for enhancing the production of economically important alkaloids in *C. roseus*.

INTRODUCTION

Vinblastine and vincristine are two dimeric alkaloids that synthesized by *Catharanthus roseus* (L.) G. Don. from *Apocynaceae* family. It is one of the significant pharmaceutical plants creating more than 130 terpenoid indole alkaloids (TIAs). In addition, these two alkaloids are known as antineoplastic factors and indispensable elements of most cancer chemotherapies; two precursors of them, catharanthine and vindoline, are also of great importance. Furthermore, this plant produces an antihypertensive alkaloid, ajmalicine, which is one of the main ingredients of medicines applied for controlling hypertension and different kinds of cardiovascular disorders (Ncube & Van Staden, 2015).

Research and study on this plant has always been a concern for several researchers. The hard process of synthesis, low amounts of TIAs, and being the unique source of these alkaloids are some reasons that can be mentioned for the importance of this subject. It can be attractive to use molecular and physiological tools to manage the secondary metabolites. One alternative strategy for enhancing these TIAs is using

abiotic elicitors like methyl jasmonate (MJ) and putrescine (Wojciak-Kosior et al. 2016). Putrescine is an aliphatic low molecular weight diamine that acts as a mediator in defense responses against biotic and abiotic stresses in addition to its variable roles in cellular processes. It is a cellular signal and has a cross talk with hormonal pathways (Gill & Tuteja, 2010). The plant hormone jasmonic acid (JA) and its methylated form (MJ) are key elements of the plant's immune system that regulate the protective reactions against stresses. Previous studies showed the positive effect of MJ on signal transduction chain and accumulation of valuable TIAs in *C. roseus* (Van Moerkercke et al., 2015).

The separate impacts of putrescine and MJ on amounts of TIA's production have already been studied, but the effects of joint treatment on the expression levels of key elements of the pathway, such as strictosidine synthase (STR), deacetylvindoline-4-O-acetyltransferase (DAT), geissoschizine synthase (GS), and PRX1, have not been performed yet. STR, DAT, and PRX1 are three of the main bottleneck steps. STR condenses the tryptamine and secologanine to form strictosidine, the first monoterpene indole alkaloid. DAT acetylates deacetylvindoline to form vindoline, and then PRX1 dimerizes the vindoline and catharanthine to produce dimeric TIAs. GS is a novel gene that forms 19E-geissoschizine from 4, 21-dehydrogeissoschizine, one of the intermediate steps in stemmadenine biosynthesis. GS is identified by Qu et al. (2018) and, to date, the expression of this gene has not been studied in response to any treatment. MAPK cascades and transcriptional factors (TFs), which are upstream of the genes, are important in the regulation of the pathway and production of main TIAs in this condition. One of the TFs that regulates many key genes in TIA pathway is *ORCA3* (APETALA2/ethylene-responsive factor3) with an AP2/ERF domain, which is inducible by jasmonate and other elicitors (Raina et al., 2012).

Transgenic approaches demonstrate that polyamines play essential roles in plant stress tolerance (Alkazar et al., 2006). Exogenous usage of MJ is also one of the most important quick ways that induces plant secondary metabolism and MJ is required to stimulate the production of TIAs (Akula & Ravishankar, 2011). Combined effects of these two stress responsive elements have not been investigated in *C. roseus* yet. Although there have been reported many interactions and cross talks between different signaling molecules leading to defense responses in plants, many of these relationships are unknown and elicitor responsive gene expression investigations can open new horizons to our knowledge in this area. Therefore, we have treated *in vitro*-propagated shoots of *C. roseus* with MJ and its combination with putrescine for evaluating their combined effects on the production of secondary metabolites. We have also measured expression levels of *CrMAPK3* and *ORCA3* genes, as upstream signaling molecules trigger *C. roseus* secondary metabolism. Moreover, we have investigated production of medicinal alkaloids, such as vincristine, vinblastine, catharanthine, vindoline, and ajmalicine, and their important biosynthetic genes (*CrPRX1*, *STR*, *DAT*, and *GS*) to know that how the employed treatments affect alkaloid biosynthesis in *C. roseus*. *GS* is a novel gene identified in 2018 and this is the first report of elicitor-induced *GS* expression changes. For investigating the plant oxidative stress level, we have also evaluated enzymatic and non-enzymatic antioxidants in treated shoots.

MATERIALS AND METHODS

Plant materials, growth condition, and elicitor preparation

Seeds of *C. roseus* (Pacifica X P cherry red halo) were procured from Pan American seed Company (USA). After surface sterilization, seeds were germinated in B5 medium. Germinated seeds were transported to Murashige and Skoog (MS) medium in a growth chamber with 25 ± 2 °C temperature and 16 hr photoperiod with 400 μm · m^{-2} · s^{-1} photon flux density. After 5 weeks, shoot explants were moved to MS medium augmented with 100 μM of MJ (Sigma-Aldrich, USA) alone and in combination with 0.5 and 1 mM of putrescine (Merck, Germany). For the control group, explants were cultured in basal MS medium. Treated and control samples were then harvested after 0.5, 4, 8, and 24 hr and 1 week after elicitation, chilled in liquid N_2, and then kept at −80 °C until analyzing.

Lipid peroxidation

The content of malondialdehyde (MDA), which is evaluated by thiobarbituric acid reaction, represents lipid peroxidation in plant tissues that was measured by the method of Heath and Packer (1968).

Total phenolic flavonoid and carotenoid contents

To evaluate total phenolic content, the Folin–Ciocalteu method was used. Determination of flavonoid content was carried out by a colorimetric method explained by Dewanto et al. (2002). Carotenoids contents were determined according to the method of Lichtenthaler (1987) at the wavelength of 470 nm and calculated using the following equation:

$$\text{Carotenoid} = [(1000\,A_{470} - 1.82\,\text{Chl.}a - 85.02\,\text{Chl.}b)/198] \times V/100\,W(\text{g}).$$

Alkaloid extraction and analysis

Alkaloid extraction was performed according to Miranda-Ham and Islas-Flores (2007). For determination of TIAs contents, a quantitative high-performance liquid chromatography (HPLC) by a Knauer GmbH HPLC system was used. A 5-μm C18 vertex column (125 mm × 4 mm ID) was applied for the separation of samples. About 20 μl of each alkaloid extract was injected and the column temperature was set at 25 °C. The mobile phase was composed of a blend of 5 mM Na_2HPO_4 (pH adjusted to 6 with H_3PO_4) (solvent A) and acetonitrile (solvent B). Flow rate was 1.0 ml/min. Ultraviolet (UV) detection process of the HPLC system was done at 258 nm. The calibration curve was illustrated using standard ajmalicine, vindoline, catharanthine (Sigma Chemical Co., USA), vincristine, and vinblastine (Sobhan Oncolgy Co., Iran), and co-chromatograms of the standards and samples gained. Alkaloids were computed as μg/g DW. In addition, total alkaloid content was counted at 280 nm by UV-VIS spectrophotometer (Vario 2600, Germany).

Protein content and assays of antioxidant enzyme activity

Bradford's method was used to consider the protein content. Catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) activities were determined by standard methods as previously described in Sanchez-Rojo et al. (2015).

RNA extraction, cDNA synthesis, and gene expression

Total RNA was extracted from *in vitro*-cultured *C. roseus* plantlets (0.1 g) using RNX plus (Cinnaclon, Iran). The qualities and concentrations of the extracted RNA were checked with agarose gel electrophoresis and spectrophotometer, respectively. After DNase I treatment, the first strand of cDNA was synthesized from 6 µg of total RNA using an oligo-d (T) primer. Reverse transcription was performed using the following program: 37 °C for 15 min, 85 °C for 5 s, and 4 °C as a final hold. The sequence of oligonucleotide primers used for study was as follow:

F: *MAPK3* (5′-CGAAAACATAATTGCCATAA-3′),
R: *MAPK3* (5′-TGACAATGCTCCTCAGATAGA-3′),
F: *ORCA3* (5′-CAGGAGGATTCTGTTGTGG-3′),
R: *ORCA3* (5′-CTGGATCCTTTCTTTTTCG-3′),
F: *PRX1* (5′-TCACTTCTGACCAAGATTTGTA-3′),
R: *PRX1* (5′-CTTGATTCCCCGTTAACAC-3′),
F: *RBCL* (5′-GCTGCTGAATCTTCTACTGG-3′),
R: *RBCL* (5′-GTCTAAGGGGTAAGCTACATAAG-3′),
F: *STR* (5′-GGTTCTACACTTCCGTCCA-3′),
R: *STR* (5′-CAATGGTCTTTTCTCTGGATC-3′),
F: *DAT* (5′-CCAAGCTATTAATTTACGTCC-3′),
R: *DAT* (5′-CTTTCCTTAGCTCATTAATCACT-3′),
F: *GS* (5′-GTGAACGGGATGTGAAGAT-3′),
R: *GS* (5′-TCTCTACTTTGCTGCCAACT-3′).

Real-time quantitative RT-PCR amplification was accomplished using PrimeScript™ RT Reagent Kit (Takara, Japan) according to the manufacturer's instructions. PCR conditions consisted of a 95 °C for 2.5 min, 40 cycles of 95 °C for 15 s, 78 °C for 15 s, and 72 °C for 20 s. The abundance of targeted gene transcripts was normalized to *rbcl* mRNA and it was determined by the standard $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001).

Statistical analysis

Averages of three replicates for all data in this study were used. Standard deviations are given as bars. One-way analysis of variance using Duncan's test was applied for comparison of mean, which was performed using statistical software SPSS v. 16.0 (IBM SPSS statistics, USA).

RESULTS

Alkaloid contents

The present investigation found that, after 0.5-hr treatment, there was not any significant difference between groups. After 4-hr treatment, only 100 µM of MJ + 1 mM putrescine caused a significant increase in vincristine content. After 8-hr treatment, MJ in combination with both concentrations of putrescine caused a significant increase in vincristine content but this increase for vinblastine occurred only in 100 µM of MJ + 1 mM putrescine. After 24-hr treatment, MJ separately and in combination with two concentrations of putrescine significantly elevated the vincristine content compared to control but the content of vinblastine and catharanthine significantly increased only in joint treatment. With regard to vindoline, only 100 µM of MJ + 1 mM putrescine increased it significantly compared to control. The maximum total yield of ajmalicine was 2.53-fold more than control in response to 100 µM of MJ + 0.5 mM putrescine. This increase was 1.82-, 3.72-, 2.02-, and 1.85-fold for vincristine, vinblastine, vindoline, and catharanthine, respectively, in response to 100 µM of MJ + 1 mM putrescine. MJ alone and combined with 0.5 and 1 mM putrescine significantly increased total alkaloids after 4, 8, and 24 hr, and 1 week of treatment (Fig. 1a–f).

Gene expression analysis

As seen in Fig. 2a–d, 100 µM of MJ combined with 0.5 and 1 mM of putrescine caused a significant increase in *STR*, *GS, DAT*, and *PRX1* transcript levels, which was elevated with duration of elicitor contact. The maximum expression level of *STR*, *GS, DAT*, and *PRX1* genes was obtained after 24-hr treatment and it was 5.83-, 4.94-, 6.06-, and 3.6-fold, respectively, in response to 100 µM of MJ + 1 mM putrescine. *ORCA3* and *MAPK3* expression levels increased within 8 hr of all treatments and then decreased slightly but remained significantly higher than control. Our results showed that the highest level of *MAPK3* expression was 9.71-fold higher than control and it appeared 8 hr after 100 µM of MJ + 1 mM putrescine treatment. The highest level of *ORCA3* expression was 6.29-fold higher than control and it appeared 8 hr after 100 µM of MJ + 0.5 mM putrescine treatment. However, about *ORCA3* and *MAPK3*, two concentrations of putrescine did not show any significant difference with each other (Fig. 2e and f).

Lipid peroxidation

The amount of MDA was examined as shown in Fig. 3a. The results suggest that 100 µM of MJ + 1 mM putrescine after 0.5 hr caused a significant increase in lipid peroxidation compared to control. After 4 hr of treatment, 100 µM of MJ combined with two concentrations of putrescine significantly increased lipid peroxidation. The presence of 100 µM of MJ alone shows a significant increase in the amount of lipid peroxidation after 8 hr and 1 week of treatment.

Phenol, flavonoid, and carotenoid contents

After 0.5-hr treatment, there was not any significant difference in phenol and flavonoid contents compared to control but slowly over time, variations happened and the combination of two concentrations of putrescine and MJ caused significant elevation in total phenol and flavonoid contents. After 4-hr treatment, MJ alone and in combination with two concentrations of putrescine increased the contents of total phenol and flavonoid compared to control. After 8-hr

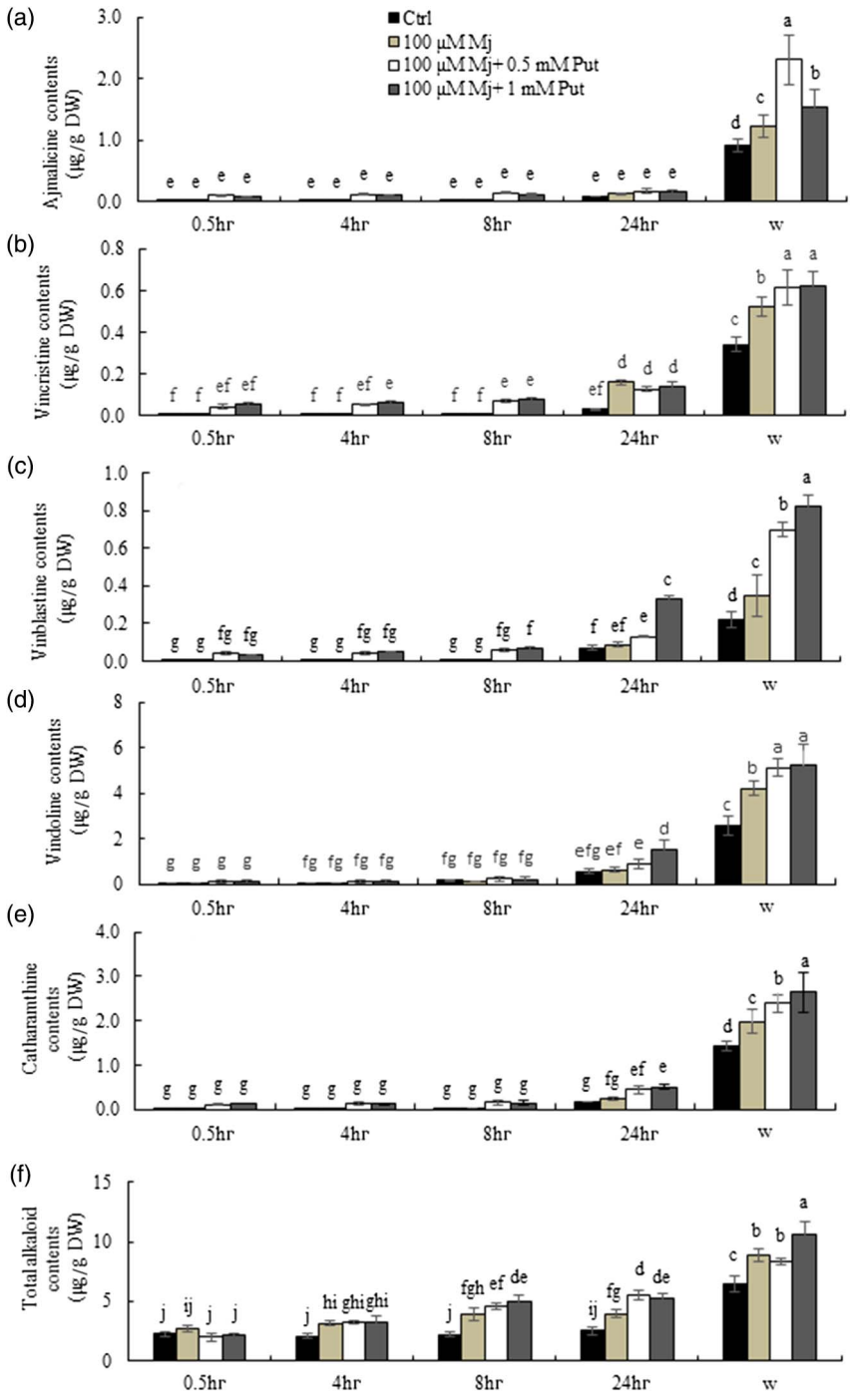

Fig. 1. Effects of MJ alone and in combination with put on ajmalicine (a), vincristine (b), vinblastine (c), vindoline (d), catharanthine (e), and total alkaloid (f) contents on *in vitro*-propagated *C. roseus* shoots after 0.5, 4, 8, 24 hr, and 1 week (w) treatment. Different letters indicate significant differences at $p < .05$ according to the Duncan's test

treatment, all of the treatments caused a significant increase in flavonoid contents but just joint treatment increased the total phenol contents compared to control, whereas in 24-hr treatment, the results were reverse. Finally, the highest contents of total phenol and flavonoid (1.34 and 1.271 mg/g FW, respectively) were observed in the 100 μM of MJ + 1 mM putrescine treatments after 1 week (Fig. 3b and c).

As shown in Fig. 3d, after 0.5- and 4-hr treatment, there was not any difference between carotenoid contents. After 8-hr treatment, MJ alone and in combination with 0.5 putrescine caused a significant increase in carotenoid content. After 24-hr treatment, all of the treatments showed an increase compared to control. The highest content was 0.493 mg/g FW and observed in the 100 μM of MJ + 1 mM putrescine treatments after 1 week (Fig. 3d).

Enzymatic analysis

After 0.5-hr treatment, there was not any significant difference in enzyme activities. The combination of MJ + putrescine significantly increased the SOD, POD, and CAT

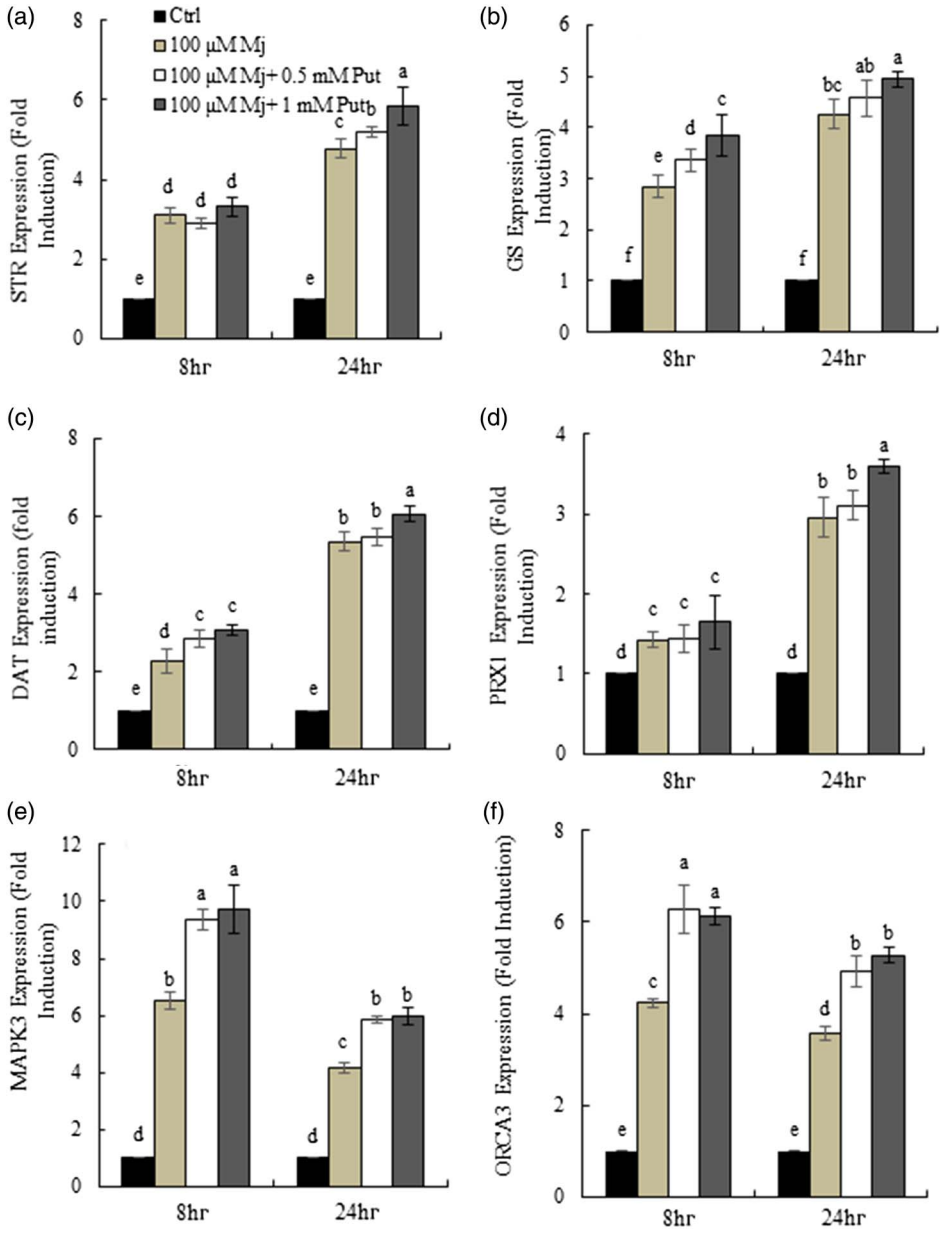

Fig. 2. Effects of MJ alone and combined with put on expression patterns of *STR* (a), *GS* (b), *DAT* (c), *PRX1* (d), *MAPK3* (e), and *ORCA3* (f) after 8 and 24 hr. Different letters indicate significant differences at $p < .05$ according to the Duncan's test

activities after 4, 8, and 24 hr of treatment. In addition, 100 µM of MJ alone increased SOD activity after 4 hr of treatment. Furthermore, significant progression in SOD, POD, and CAT activities of all groups was showed after 1 week (Fig. 4a–d). In this work, 100 µM of MJ combined with two concentrations of putrescine increased the protein contents after 24 hr of treatment. This increase was shown after 1 week of treatment in response to MJ separately and in combination with 1 mM putrescine.

DISCUSSION

TIA production is enhanced under abiotic stress conditions because of their role as a chemical defense. This is a complex matter that involves various regulatory components. Polyamines are non-toxic molecules that act as compatible solutes dealing with stress conditions. Their cellular functions and roles are diverse in these conditions. They can ameliorate the causes of stress but on the other hand, their own catabolic products may be the origins of stress destruction. These contradictory responses have been studied over the years and proved that in all cases (especially co-treatment with another stress), exogenous application of polyamines increased the levels of endogenous counterparts, resulting in the activation of the antioxidant defense system (Hussain et al., 2011). The diamine putrescine alone can be a precursor for numerous kinds of alkaloids reviewed by Ghosh (2000) and enzymes involved in secondary metabolism (Minocha et al., 2014). Jasmonates are a type of plant hormones with a variety of biological effects that participate in signal transduction chain and lead to accumulation of secondary metabolites in plants like TIAs in *C. roseus* (Verma & Mishra, 2005). Previous investigations

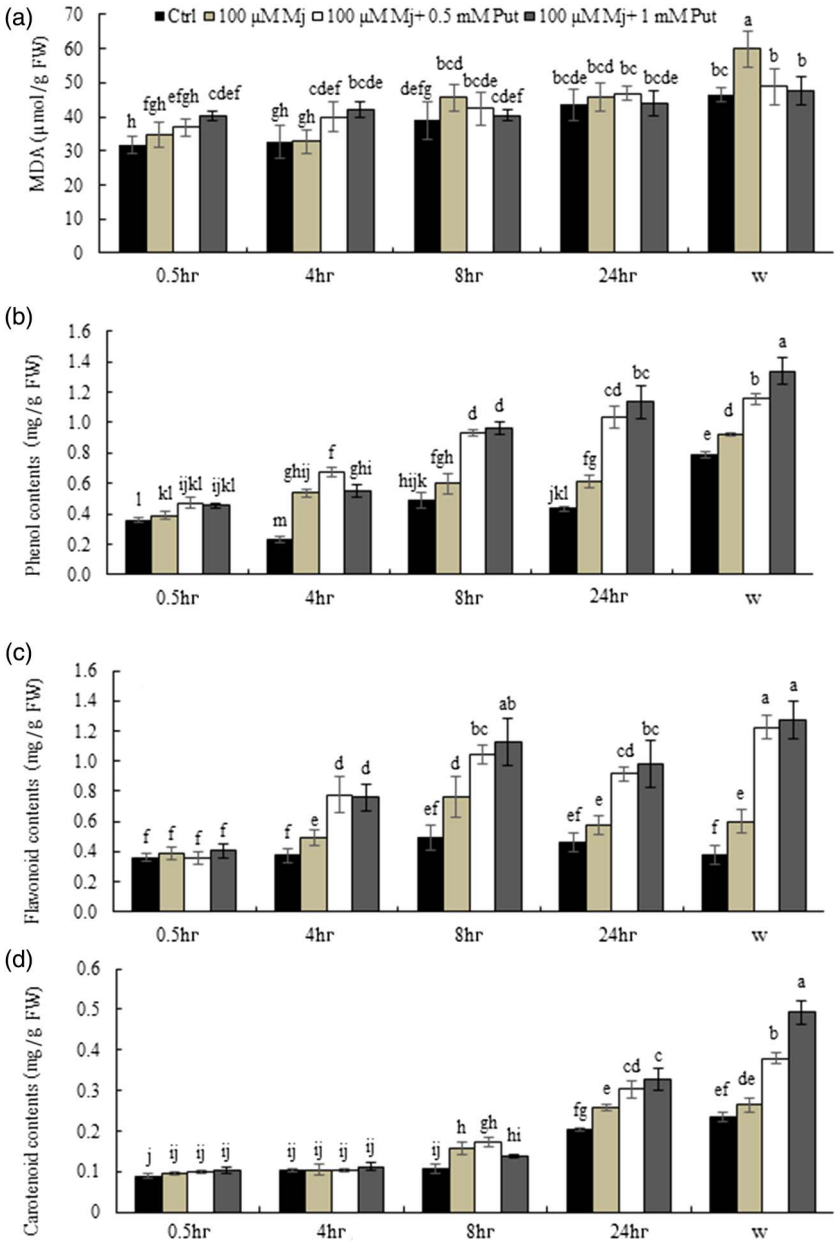

Fig. 3. Effects of MJ alone and combined with put on MDA (a) phenol (b), flavonoid (c), carotenoid (d) contents on *in vitro*-propagated *C. roseus* shoots after 0.5, 4, 8, 24 hr, and 1 week treatment. Different letters indicate significant differences at $p < .05$ according to the Duncan's test

(Walters, 2003) showed that using exogenous JA can change the distribution of polyamines and in this way they can affect the polyamines metabolism. Therefore, it was proposed that there may be a cross talk between polyamines and JA signal transduction. From our results, it may be discussed that putrescine can improve the MJ function, so that they can elevate TIA production.

Generally, these incremental effects by MJ and putrescine can be due to inducing the expression of biosynthetic genes in the TIA pathway. MJ controls the expression of many genes recognized as being stress activated, directly and indirectly (Verma & Mishra, 2005). Increased expression levels of *STR*, *GS, DAT*, and *PRX1* in this study are in agreement with the study of Pan et al. (2016) that showed vincristine and vinblastine were accumulated significantly in plants with *PRX1*, *DAT*, and *STR* overexpression. *GS* expression under stress conditions has never been investigated, but it seems to have a pattern similar to *PRX1*, *DAT*, and *STR*. Zhang's report has also proved that exogenous MJ causes an upregulation of many genes such as *G10H*, *TDC*, *STR*, *D4H*, etc., in the TIA pathway (Zhang et al., 2011). Moreover, exogenous putrescine stimulates the abiotic stress-responding genes (Gill & Tuteja, 2010), so it may have a similar inducing role on the activation of these genes. From these results, we infer that both of these two exogenous treatments, MJ and putrescine, participate in signal transduction pathways that cause the accumulation of TIAs in stress conditions in *C. roseus.*

All of defense mechanisms involved in abiotic stresses are controlled by the MAPK signaling cascade. MAPKs are a group of protein kinases that have several critical tasks, such as activation in defense responses to many stresses that

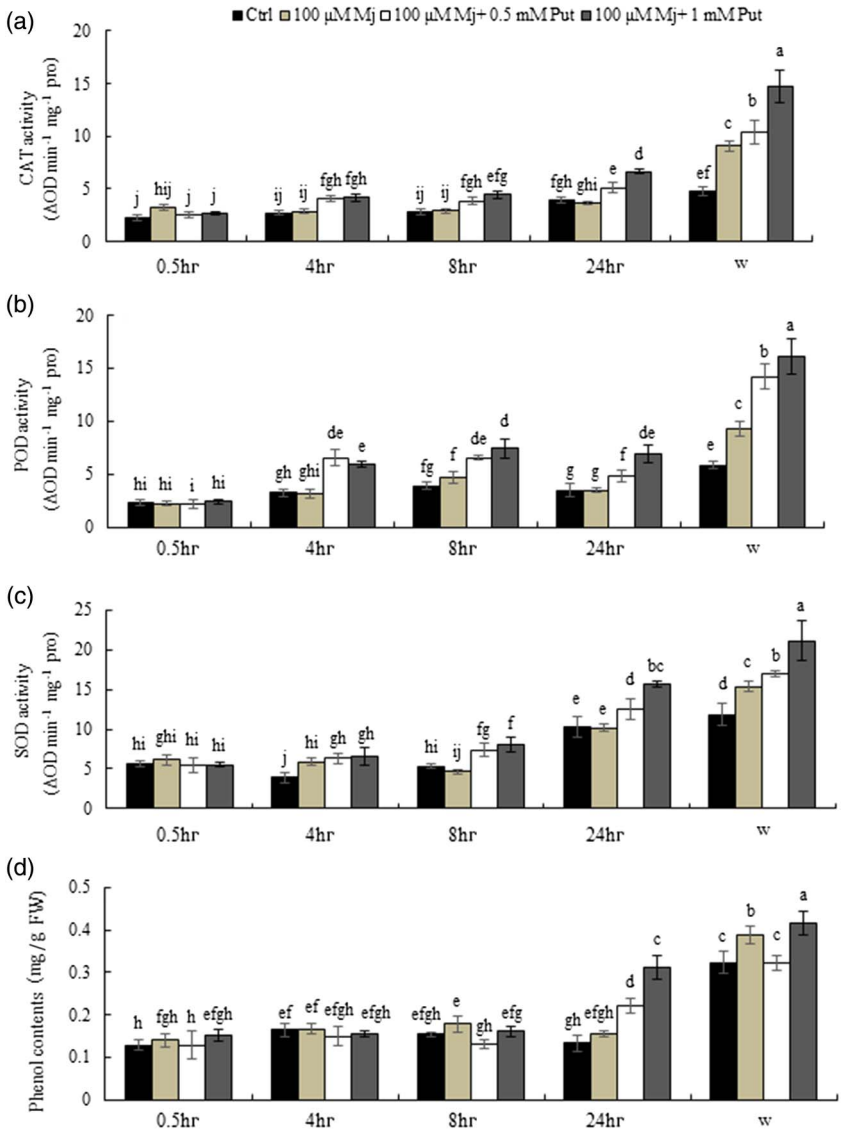

Fig. 4. Effects of MJ alone and combined with Put on activities of CAT (a), POD (b), SOD (c), and protein contents (d) on *in vitro*-propagated *C. roseus* shoots after 0.5, 4, 8, 24 hr, and 1 week treatment. Different letters indicate significant differences at $p < .05$ according to the Duncan's test

include reactive oxygen species (ROS) signaling, expression of jasmonate-inducible genes and responding to hormones (Gao et al., 2010), and have the ability to activate or repress TFs. ORCA3 is a TF that directly regulates many downstream stress-related gene expressions in *C. roseus* in response to jasmonates. The mechanism may be performed through connecting to a jasmonate- and elicitor-responsive element in the promoter region, including a GCC box reported by Zhang et al. (2011). Polyamines can act as a direct stress-protecting compound or as a stress-signaling regulator that mediate a signal transduction pathway. They increase the DNA-binding activity of TFs and have cross talk with hormonal pathways (Hussain et al., 2011). Our results showed upregulation of *ORCA3* and *MAPK3* in response to MJ alone and in combination with putrescine, which is consistent with Gao et al. (2010) and Hussain et al. (2011).

The current results also showed that it takes a longer time to raise *STR*, *GS*, *DAT*, and *PRX1* transcription levels compared to *MAPK3* and *ORCA3*. This is evidence of the fact that *MAPK3* and *ORCA3* are at the beginning of the signaling pathway, activated at an early stage immediately after the induction of stress. They may influence the other defense responses and biosynthetic genes of secondary metabolites. One of the most important points about *ORCA* and *MAPK* is that these genes interact with each other. They may also have reciprocal regulation roles between them, which elevate the expression of the TIA pathway genes to combat abiotic stress (Pan et al., 2016).

Polyamines show a multifaceted nature, although they produce ROS, ultimately, they act as a free radical scavenger, a membrane stabilizer and an antioxidant and trigger-signaling pathways that activate plant defense responses (Minocha et al., 2014). MJ has a controversial role in different conditions. When it applied exogenously, it can inhibit or activate physiological, morphological, and biochemical alterations in plants in a different way depending on the plant growth phase, the application time, and its concentration (Abdelgawad et al., 2014). Kupper et al. (2009)

showed that MJ has a strong potential to stimulate ROS production and oxidative stress with the strongest response at 100 mM reported by Kumari et al. (2015). Increased ROS production creates an oxidative damage in cell membranes and motivates lipid peroxidation (Mandal et al., 2013). In this study, putrescine application, initially (0.5 and 4 hr) in combination with MJ, increased lipid peroxidation and ROS production, but over time, slightly reduced both oxidative damages and lipid peroxidation. The increased lipid peroxidation at the beginning of the treatment (0.5 and 4 hr) may be due to catabolization of lipids by polyamine oxidases, which causes the production of ROS triggering a signaling pathway activated by putrescine (Kubis, 2008). Our results are also consistent with Kumari et al. (2015) who reported the MJ-induced lipid peroxidation. Tadolini (1988) reported the inhibitory effect of polyamines on lipoperoxidation and concluded that this effect is the major reason for polyamine-mediated inhibition of ROS generation.

Plants possess two kinds of antioxidants including enzymatic and non-enzymatic to relieve and renovate the ROS damages. Carotenoids, phenolic, and flavonoid compounds are non-enzymatic antioxidants. They could potentially scavenge the ROS and free-radical activity and preserve unstable macromolecules. Mandal et al. (2013) reported that phenol and flavonoid contents increased under different concentrations of H_2O_2 treatment supplemented with putrescine in *Salvinia natans* Linn. In addition, Ozturket al. (2015) worked on effects of pre-harvest MJ and reported similar results. Increased contents of phenolic and flavonoid compounds in the current work are in agreement with those studies. Moreover, the same results obtained for carotenoids required factors for stress tolerance in plants. *C. roseus* showed increased carotenoid contents as a defense strategy in consistence with Verma and Mishra (2005), who reported the increased carotenoids in leaf tissues of salt stressed *Brassica juncea* (L.) Czern. in response to putrescine.

Another kind of defense antioxidants is enzymatic including enzymes like SOD, CAT, and POD having potential ability to control ROS. Our findings demonstrated that combined treatment with MJ and putrescine promotes ROS scavenging by elevating the activities of CAT, POD, and SOD. The effects of exogenous putrescine on the activities of antioxidant enzymes along with a decline in lipid peroxidation indicate that exogenous putrescine has antioxidant properties that are the causes of its positive effects (Verma & Mishra, 2005).

The protein increase was shown after 1 week of treatment in response to MJ separately and in combination with 1 mM putrescine. This elevation by jasmonates may be due to the induction of gene expression leading to the biosynthesis of many proteins presumably including defense-related proteins. This finding is in agreement with Poonam et al. (2013) who reported the accumulation of proteins induced by MJ in *Cajanus cajan* (L.) Huth. Polyamines also can affect protein synthesis at translational and transcriptional levels. It was similar to our results and also was reported by Talaat et al. (2005).

This study is a small-scale assay aimed at revealing the signal transduction mechanism of *C. roseus* and to provide a safe way to produce significant values of these anticancer metabolites, the only precursors of anticancer drugs. However, full recognition of the regulatory mechanisms of this biosynthetic pathway requires further studies in this regard.

CONCLUSION FOR FUTURE BIOLOGY

Looking for eco-friendly elicitors, which can stimulate plant defense system against abiotic stresses, is paramount due to a necessity of molecular and physiological tools in crop management. The results of this study have demonstrated that the use of low and suboptimal concentrations of MJ combined with two concentrations of putrescine can provoke antioxidant defense system, leading to production of antioxidant secondary metabolites and enzymes, as a part of the general stress responses. Furthermore, it may be a commercial way to enhance the potential to overproduce medicinal valuable chemicals with high pharmaceutical values in *C. roseus*. Although laboratory scale results are hardly applicable in cultivation, controlled concentrations of MJ and putrescine can be used in cultivations of *C. roseus* in future studies. Moreover, investigating the plant responses to different elicitors results in a better understanding of the response components and helps identify more ways to increase the plant stress resistance.

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Authors' Contributions: EK performed the experiments, analyzed data, and wrote the manuscript. FK and KR designed the experiments, supervised the study, and reviewed the article. All authors read and approved the final version of the manuscript to be published.

REFERENCES

Abdelgawad, Z. A., Khalafaallah, A. A., Abdallah, M. M. (2014) Impact of methyl jasmonate on antioxidant activity and some biochemical aspects of maize plant grown under water stress condition. *Agric. Sci. 5,* 1077.

Akula, R., Ravishankar, G. A. (2011) Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal. Behav. 6,* 1720–1731.

Alkazar, R., Marco, F., Cuevas, J. C., Patron, M., Ferrando, A., Carrasco, P., Tiburcio, A. F., Altabella, T. (2006) Involvement of polyamines in plant response to abiotic stress. *Biotechnol Lett. 28,* 1867–1876.

Dewanto, V., Wu, X., Adom, K. K., Liu, R. H. (2002) Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *J. Agric. Food Chem. 50,* 3010–3014.

Gao, F., Su, Q., Fan, Y., Wang, L. (2010) Expression pattern and core region analysis of AtMPK3 promoter in response to environmental stresses. *Sci. China Life Sci. 53,* 1315–1321.

Ghosh, B. (2000) Polyamines and plant alkaloids. *Indian J. Exp. Biol. 38,* 1086–1091.

Gill, S. S., Tuteja, N. (2010) Polyamines and abiotic stress tolerance in plants. *Plant Signal. Behav. 5,* 26–33.

Heath, R. L., Packer, L. (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys. 125,* 189–198.

Hussain, S. S., Ali, M., Ahmad, M., Siddique, K. H. (2011) Polyamines: natural and engineered abiotic and biotic stress tolerance in plants. *Biotechnol. Adv. 29,* 300–311.

Kubis, J. (2008) Exogenous spermidine differentially alters activities of some scavenging system enzymes, H_2O_2 and superoxide radical levels in water-stressed cucumber leaves. *J. Plant Physiol. 165,* 397–406.

Kumari, P., Reddy, C. R., Jha, B. (2015) Methyl jasmonate-induced lipidomic and biochemical alterations in the intertidal macroalga *Gracilaria dura* (Gracilariaceae, Rhodophyta). *Plant Cell Physiol. 56,* 1877–1889.

Kupper, F. C., Gaquerel, E., Cosse, A., Adas, F., Peters, A. F., Müller, D. G., Kloareg, B., Salaün, J. P., Potin, P. (2009) Free fatty acids and methyl jasmonate trigger defense reactions in *Laminaria digitata*. *Plant Cell Physiol. 50,* 789–800.

Lichtenthaler, H. K. (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol. 148,* 350–382.

Livak, K. J., Schmittgen, T. D. (2001) Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta C}$T method. *Methods 25,* 402–408.

Mandal, C., Ghosh, N., Adak, M. K., Dey, N. (2013) Interaction of polyamine on oxidative stress induced by exogenously applied hydrogen peroxide in *Salvinia natans* Linn. *Theor. Exp. Plant Physiol. 25,* 223–230.

Minocha, R., Majumdar, R., Minocha, S. C. (2014) Polyamines and abiotic stress in plants: a complex relationship. *Front. Plant Sci. 5,* 175.

Miranda-Ham, L., Islas-Flores, I. (2007) Accumulation of monoterpenoid indole alkaloids in periwinkle seedlings (*Catharanthus roseus*) as a model for the study of plant–environment interactions. *Biochem. Mol. Biol. Educ. 35,* 206–210.

Ncube, B., Van Staden, J. (2015) Tilting plant metabolism for improved metabolite biosynthesis and enhanced human benefit. *Molecules 20,* 12698–12731.

Ozturk, B., Yildiz, K., Ozkan, Y. (2015). Effects of pre-harvest methyl jasmonate treatments on bioactive compounds and peel color development of "Fuji" apples. *Int. J. Food Prop. 18,* 954–962.

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

Poonam, S., Kaur, H., Geetika, S. (2013). Effect of jasmonic acid on photosynthetic pigments and stress markers in *Cajanus cajan* (L.) Millsp. seedlings under copper stress. *Am. J. Plant Sci. 4,* 817.

Qu, Y., Easson, M. E., Simionescu, R., Hajicek, J., Thamm, A. M., Salim, V., De Luca, V. (2018) Solution of the multistep pathway for assembly of corynanthean, strychnos, iboga, and aspidosperma monoterpenoid indole alkaloids from 19E-geissoschizine. *Proc. Natl. Acad. Sci. U. S. A. 115,* 3180–3185.

Raina, S. K., Wankhede, D. P., Jaggi, M., Singh, P., Jalmi, S. K., Raghuram, B., Sinha, A. K. (2012) CrMPK3, a mitogen activated protein kinase from *Catharanthus roseus* and its possible role in stress induced biosynthesis of monoterpenoid indole alkaloids. *BMC Plant Biol. 12,* 134.

Sanchez-Rojo, S., Cerda-García-Rojas, C. M., Esparza-García, F., Plasencia, J., Poggi-Varaldo, H. M., Ponce-Noyola, T., Ramos-Valdivi, A. C. (2015) Long-term response on growth, antioxidant enzymes, and secondary metabolites in salicylic acid pre-treated *Uncaria tomentosa* microplants. *Biotechnol. Lett. 37,* 2489–2496.

Tadolini, B. (1988) Polyamine inhibition of lipoperoxidation. The influence of polyamines on iron oxidation in the presence of compounds mimicking phospholipid polar heads. *Biochemic. J. 249,* 33–36.

Talaat, I. M., Bekheta, M. A., Mahgoub, M. H. (2005) Physiological response of periwinkle plants (*Catharanthus roseus* L.) to tryptophan and putrescine. *Int. J. Agric. Biol. 7,* 210–213.

Van Moerkercke, A., Steensma, P., Schweizer, F., Pollier, J., Gariboldi, I., Payne, R., Bossche, R. V., Miettinen, K., Espoz, J., Purnama, P. C., Kellner, F. (2015) The bHLH transcription factor BIS1 controls the iridoid branch of the monoterpenoid indole alkaloid pathway in *Catharanthus roseus*. *Proc. Natl. Acad. Sci. U. S. A. 112,* 8130–8135.

Verma, S., Mishra, S. N. (2005) Putrescine alleviation of growth in salt stressed *Brassica juncea* by inducing antioxidative defense system. *J. Plant Physiol. 162,* 66977.

Walters, D. R. (2003) Polyamines and plant disease. *Phytochemistry 64,* 97–107.

Wojciak-Kosior, M., Sowa, I., Blicharski, T., Strzemski, M., Dresler, S., Szymczak, G., Wnorowski, A., Kocjan, R., Świeboda, R. (2016) The stimulatory effect of strontium ions on phytoestrogens content in *Glycine max* (L.) Merr. *Molecules 21,* 1–9.

Zhang, H., Hedhili, S., Montiel, G., Zhang, Y., Chatel, G., Pré, M., Gantet, P., Memelink, J. (2011) The basic helix-loop-helix transcription factor CrMYC2 controls the jasmonate-responsive expression of the *ORCA* genes that regulate alkaloid biosynthesis in *Catharanthus roseus*. *Plant J. 67,* 61–71.