doi:10.14720/aas.2019.113.1.09 Original research article / izvirni znanstveni članek

Chromium-induced alkaloid production in *Catharanthus roseus* (L.) G.Don in vitro cultured shoots and related gene expression patterns particularly for the novel gene *GS*

Elham KHATAEE [1], Farah KARIMI *[2], Khadijeh RAZAVI [3]

Received August 01, 2018; accepted January 27, 2019.
Delo je prispelo 01. avgusta 2018, sprejeto 27. januarja 2019.

ABSTRACT

This study aimed to determine the effects of methyl jasmonate (Mj) combined with chromium (Cr) as elicitor on production of medicinal alkaloids, its antioxidant potential, and its effects on the expression of signaling and biosynthetic enzymes. Combined treatment had positive effects on secondary metabolism and changed genes expression levels of mitogen-activated protein kinase 3 (*MAPK3*), a transcription factor (TF) known as octadecanoid-responsive *Catharanthus* AP2-domain 3 (*ORCA3*) upstream of plant alkaloids biosynthetic pathway. Maximum expression levels of peroxidase1 (*PRX1*), geissoschizine synthase (*GS*) (24 h-treatment), *MAPK3* and *ORCA3* (8 h-treatment), were 6.25−, 4.87-, 7.67-, and 5.38-fold higher than control, respectively, in response to 100 µM Mj + 50 µM Cr. This value was 5.92-fold for strictosidine synthase (*STR*) in response to 100 µM Mj + 100 µM Cr after 24 h. The maximum total yield of vincristine was 1.52-fold more than control in response to 100 µM Mj after one week. This increase was 2.16, 4.01, 2.39 and 1.97-fold for ajmalicine, vinblastine, vindoline and catharanthine respectively, in response to 100 µM Mj + 50 µM Cr. Mj + Cr can elevate alkaloid production by induction of *MAPK3* and *ORCA3* signaling pathway, which induces expression of downstream terpenoid indole alkaloids (TIAs) biosynthetic enzymes.

Key words: antioxidative responses; chromium; *GS*; *MAPK3*; *ORCA3;* real time PCR

IZVLEČEK

S KROMOM VZPODBUJENA PRODUKCIJA ALKALOIDOV PRI VRSTI *Catharanthus roseus* (L.) G.Don V *IN VITRO* GOJENIH POGANJKIH IN Z NJO POVEZANI VZORCI IZRAŽANJA GENOV, ŠE POSEBEJ NOVEGA GENA *GS*

Namen raziskave je bil določiti učinke metil jasmonata (Mj) v povezavi s kromom (Cr) kot elicitorjev v produkciji medicinskih alakaloidov, njun antioksidacijski potencial in njune učinke na ekspresijo signalizacije in biosinteze encimov. Kombinirano obravnavanje je imelo pozitivne učinke na sekundarni metabolizem in spremenilo ravni izražanja genov mitogen-aktivirane protein kinase 3 (*MAPK3*), transkripcijskega faktorja (TF) poznanega kot oktadekanoid-odzivne *Catharanthus* AP2-domene 3 (*ORCA3*), ki vzpodbuja biosintezo rastlinskih alkaloidov. Največje ravni izražanja peroksidaze1 (*PRX1*), geisošizin sintaze (*GS*) (24 h-obravnavanja), *MAPK3* in *ORCA3* (8 h-obravnavanja) so bile 6,25−, 4,87-, 7,67-, in 5,38-krat večje kot pri kontroli kot odziv na hkratno obravnavanje s 100 µM Mj + 50 µM Cr. Ta vrednost je bila za striktozidin sintazo (*STR*) 5,92-kratna kot odziv na 100 µM Mj + 100 µM Cr po 24 h. Največji celokupni pridelek vinkristina je bil za 1,52-krat večji kot pri kontroli kot odziv na 100 µM Mj po enem tednu. Enako povečanje je bilo 2,16, 4,01, 2,39 in 1,97-kratno za ajmalicin, vinblastin, vindolin in katarantin, kot odziv na 100 µM Mj + 50 µM Cr. Mj + Cr lahko povečata produkcijo alkaloidov z indukcijo *MAPK3* in *ORCA3* signalne poti, ki inducira izražanje encimov za biosintezo terpenoid indolnih alkaloidov (TIAs).

Ključne besede: antioksidacijski odziv; krom; *GS*; *MAPK3*; *ORCA3;* realni čas PCR

[1] Dep. of Biology, Faculty of Basic Sciences, Shahed University, 3319118651, Tehran, Iran

[2] Medicinal Plant Research Center, Shahed University, 3319118651, Tehran, Iran; *corresponding author: to fkarimi@shahed.ac.ir.

[3] National Institute of Genetic Engineering and Biotechnology, 1497716316 Tehran, Iran

1 INTRODUCTION

Catharanthus roseus L. (Apocynaceae) is a significant pharmaceutical plant that contains more than 130 alkaloids named terpenoid indole alkaloids (TIAs), 25 of which are found in nature in dimeric form and have antidiabetic, bactericide, antihypertensive, and anticancerous activities. Vincristine and vinblastine are two dimeric alkaloids that are potent antineoplastic factors and indispensable elements in most cancer chemotherapies. Additionally, two precursors of them, catharanthine and vindoline, are also of great importance. Furthermore, *C. roseus* is the source of ajmalicine which has been identified as an antihypertensive agent (Ncube & Van Staden, 2015).

The pharmacological significance of TIAs and their low amounts in the plant which is the unique source of them (around 0.0005 % of dry mass) have motivated broad research on the TIA pathway for manipulating plant metabolism to enhance alkaloid amounts. One important technique for raising secondary metabolite contents is the utilization of heavy metals in plant cell, tissue, and organ cultures not only to enhance the generation of secondary metabolites, but also to promote the *de novo* biosynthesis of them (Wójciak-Kosior et al., 2016). Chromium (Cr) is a special example of a heavy metal that, in hexavalent (Cr^{+6}) form, is soluble and highly mobile (Eleftheriou et al., 2015). Methyl jasmonate (Mj) is a key element of plant's immune system that regulates the protective reactions against stresses, also participates in signal transduction chain and results in high production of TIAs (Van Moerkercke et al., 2015). The effects of Cr combined with Mj on the expression levels of key elements of the biosynthetic pathway, such as strictosidine synthase (STR), deacetylvindoline-4-O-acetyltransferase (DAT), geissoschizine synthase (GS) and a peroxidase1 (PRX1), has not yet been determined. STR, DAT, and PRX1 are three main bottleneck steps. STR condenses 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 make dimeric TIAs. GS is a novel gene that forms 19E-geissoschizine from 4, 21-dehydrogeissoschizine, one of the intermediate steps in stemmadenine biosynthesis. GS was identified by Qu et al. (2018), and to date, the expression of this gene in response to any treatment has not been studied.

The critical enzymes of the pathway and important transcription factors (TFs) affecting their activity and regulatory pathways have always been considered in elicitation studies. A manifest example of this is the octadecanoid-responsive *Catharanthus* AP2-domain *3 (ORCA3)*, a jasmonate-inducible TF that regulates many important jasmonate and elicitor responsive genes in the TIA pathway by attaching to an area in their promoter (Raina et al., 2012). In addition to TFs, mitogen-activated protein kinase (MAPK) cascades, upstream of these factors, are important in the regulation of the pathway.

Little information is available on how the plant responds to this kind of stress (Mj + Cr) at the gene expression level. Thus, the purpose of this assay was to determine the joint effects of Cr and Mj synchronically as a kind of elicitor on the production of the medicinal alkaloids vincristine, vinblastine, catharanthine, vindoline and ajmalicine; its antioxidant potential; its effects on the expression of *CrMAPK3*, the TF *ORCA3*, the biosynthetic enzymes *CrPRX1*, *STR*, *DAT*, *GS;* and finally, the relationship between alterations in gene expression and the production of TIA alkaloids.

2 MATERIAL AND METHODS

2.1 Plant growth conditions and elicitor preparation

Seeds of *C. roseus* var. *pacifica* 'XP Cherry Red Halo' procured by the Pan American Seed Company (U.S.A.) were germinated in MS medium in a growth chamber with a temperature of 25 ± 2 °C and a 16-h 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 Mj (Sigma–Aldrich) separately and in combination with 50 and 100 µM concentrations of Cr^{+6} as $K_2Cr_20_7$ (Merck). For the control group, explants were cultured in basal MS medium. Treated and control samples were then harvested after 0.5, 4, 8, 24 hours (h) and one week elicitation, chilled in liquid N_2, and then kept at −80 °C until analysis.

2.2 Lipid peroxidation

The malondialdehyde (MDA) content represents lipid peroxidation in plant tissue, and it was measured by thiobarbituric acid reaction using the method of Heath and Packer (1968).

2.3 Total phenolic and flavonoid contents

To evaluate total phenolic content, the Folin–Cio-calteu method was used (Dewanto et al., 2015). 0.5 ml of deionised water and 125 µl of the Folin–Cio-calteu reagent were added to 125 µl of the diluted sample extract. After standing for 6 min and then adding 1.25 ml of a 7 % aqueous Na_2CO_3 solution, the ultimate

volume was arranged to 3 ml with water. Consideration was performed after 90 min in 760 nm. The results were expressed as mg gallic acid equivalents per g fresh mass(mg GAE g^{-1} FM). Flavonoid content was determined using a colorimetric method that was explained by Dewanto et al. (2015). 0.05 ml of a 33 % aqueous acetic acid solution and 0.1 ml of a newly made 10 % $AlCl_3$ solution were added to 0.5 ml of the suitably diluted sample. By using ethanol, the ultimate volume was reached to 2.5 ml and after 30 min, absorption of samples was read at 414 nm. The outcomes were displayed as mg quercetin equivalents per g fresh mass(mg QE g^{-1} FM).

2.4 Alkaloid extraction and analysis

Alkaloids were extracted according to Miranda-Ham et al. (2007). To determine the content of vincristine, vinblastine, catharanthine, vindoline and ajmalicine, a quantitative HPLC by a Knauer GmbH HPLC system was used. A5 µm C18 vertex column (125 mm × 4 mm ID) was applied for the separation of samples. A volume of 20 µl was injected, and the column temperature was 25 °C. The mobile phase was made up 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^{-1}. The UV detector of the HPLC system was adjusted at 258 nm. Alkaloids were computed as µg g^{-1} DW. Total alkaloid content was counted at 280 nm using a UV-VIS spectrophotometer (Vario 2600).

2.5 Protein content and assays of antioxidant enzyme activity

Bradford's method was used to consider the protein content. Catalase (CAT; EC 1. 11.1.6), peroxidase (POD; EC 1. 11.1.7), and superoxide dismutase (SOD; EC 1. 15.1.1) activities were determined by standard methods as previously described in Sanchez-Rojo et al. (2015).

2.6 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). The qualities and concentrations of the extracted RNA were checked with agarose gel electrophoresis and spectrophotometer, respectively. After DNaseI 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) 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 genes transcripts was normalized to *rbcl* mRNA and was determined by the standard $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen (2001).

3 RESULTS AND DISCUSSION

3.1 Alkaloid contents

As it was considered before, the low levels of dimeric anticancer drugs, their costliness, and their difficult chemical biosynthesis have attracted the attention of many researchers and prompted them to find ways to optimize the production of these TIAs. Using Cr + Mj as an inducer and studying the responses of the plant to this abiotic stress and examining individual gene expression in the biosynthetic pathway and the relationship between genes and the construction of TIAs could enhance the comprehension of the whole interplay. Previous researches explained that heavy metals, when applied in low concentrations, in most cases causes positive effects and increased metabolite production (Wójciak-Kosior et al., 2016). Also Mj has been proved to be able to elicit the production of several compounds (alkaloids, terpenoids and phenolic phytoalexines) in many plant species (Van der Fits & Memelink, 2000). The present investigation found that after 0.5 and 8 h treatment there wasn't any significant difference between groups. After 4 h-treatment, only 100 µM Mj + 100 µM Cr and 100 µM Mj + 50 µM Cr caused a significant increase in ajmalicine and vinblastine respectively. After 24 h-treatment, Mj separately and in combination with two concentrations of Cr significantly elevated vincristine content compared to control but the content of vinblastine, ajmalicine and catharanthine significantly increased only in joint treatment. About vindoline, only 100 µM

Mj + 50 µM Cr significantly increased it compared to control. The maximum total yield of vincristine was 1.52-fold more than control in response to 100 µM Mj. This increase was 2.16, 4.01, 2.39 and 1.97-fold for ajmalicine, vinblastine, vindoline and catharanthine respectively, in response to 100 µM Mj + 50 µM Cr (Figures 1a, 1b, 1c, 2a, 2b). Mj alone and combined with 50 and 100 µM Cr significantly increased total alkaloids after 4, 8, 24 and one week of treatment (Figure 2c). So, this result confirmed earlier reporters on TIA biosynthesis under Copper treatment (Pan et al., 2015) or Mj application (Peebles et al., 2009). Also it can be deduced that the highest values for all alkaloids were observed after application of two treatments simultaneously that shows the additive effects of combined treatments on increasing alkaloid. Reduction in 100 µM Cr treatment is probably due to the effects of high concentration of metal and gradual degredation of the plant. This improvement in indole alkaloid production may also be explained by the activation of the transcription of their biosynthetic genes.

3.2 Gene expression analysis

In the current study, qRT-PCR was used to study *PRX1*, *DAT*, *STR*, *GS*, *ORCA3*, and *MAPK3* transcripts in response to Mj + Cr. As seen in Figure 3, when exposed to100 µM of Mj combined with 50 and 100 µM of Cr, the expression of *PRX1*, *DAT*, *STR*, and *GS* increased significantly compared to control. The maximum expression level of *PRX1* and *GS* were obtained after 24 h treatment and was 6.25 and 4.87-fold respectively in response to100 µM of Mj + 50 µM Cr. *DAT* expression levels didn't show any significant difference in response to100 µM of Mj + 50 and 100 µM Cr after 24 h. The maximum expression level of *STR* was obtained after 24 h of treatment and was 5.92-fold in response to to100 µM of Mj + 100 µM Cr. This result is in agreement with previous studies that showed vincristine and vinblastine were accumulated significantly in plants with PRX1, DAT, and STR overexpression, indicating that *PRX1*, *DAT*, and *STR* are actively involved in the biosynthesis of these alkaloids (Pan et al., 2016). GS expression under stress has never been investigated, but from these results, it seems to have a pattern similar to *PRX1*, *DAT*, and *STR*. Some investigations about the effects of exogenous Mj on the expression of the biosynthetic genes of TIA pathway has been done and all of them have shown that Mj cause an up regulation of many genes like G10H, TDC, STR, D4H, etc in this pathway (Zhang et al., 2011). Also, many abiotic stresses (like heavy metal Cr^{+6}) not only increased the TIA biosynthetic genes but also induced the genes related to biosynthesis and signaling of JA (Raina et al., 2012). From these studies, we infer that both of these two exogenous treatments, Mj and Cr are participating in signal transduction pathways that cause the accumulation of TIAs in stress conditions in *C. roseus*.

The molecular and signal transduction mechanisms involved in the plant's defense against Cr stress was partially known, but recent findings suggest that transcriptional regulation of TIA pathway is under a complex control containing many TFs, known as ORCAs, which are regulating the primary and secondary metabolism of *C. roseus* in response to jasmonate. The TFs directly regulate the expression of many downstream stress-related genes by making a connection with the cis-elements located in the promoter region and thus leading to abiotic stress tolerance (Trinh et al., 2014). There are many reports about the role of ORCAs against various abiotic stresses in recent researches (Singh et al., 2002). Also in *C. roseus*, the elevated expression of several genes from the biosynthetic pathway of TIAs, like *As*, *Cpr*, *Str*, *Sgd*, *Tdc*, *D4h*, and *Dxs*, due to overexpression of *ORCA3* can enhance the accumulation of TIAs. Some previous studies on DAT have shown that this gene is regulated by another TF named ORCA2 (Liu et al., 2007).

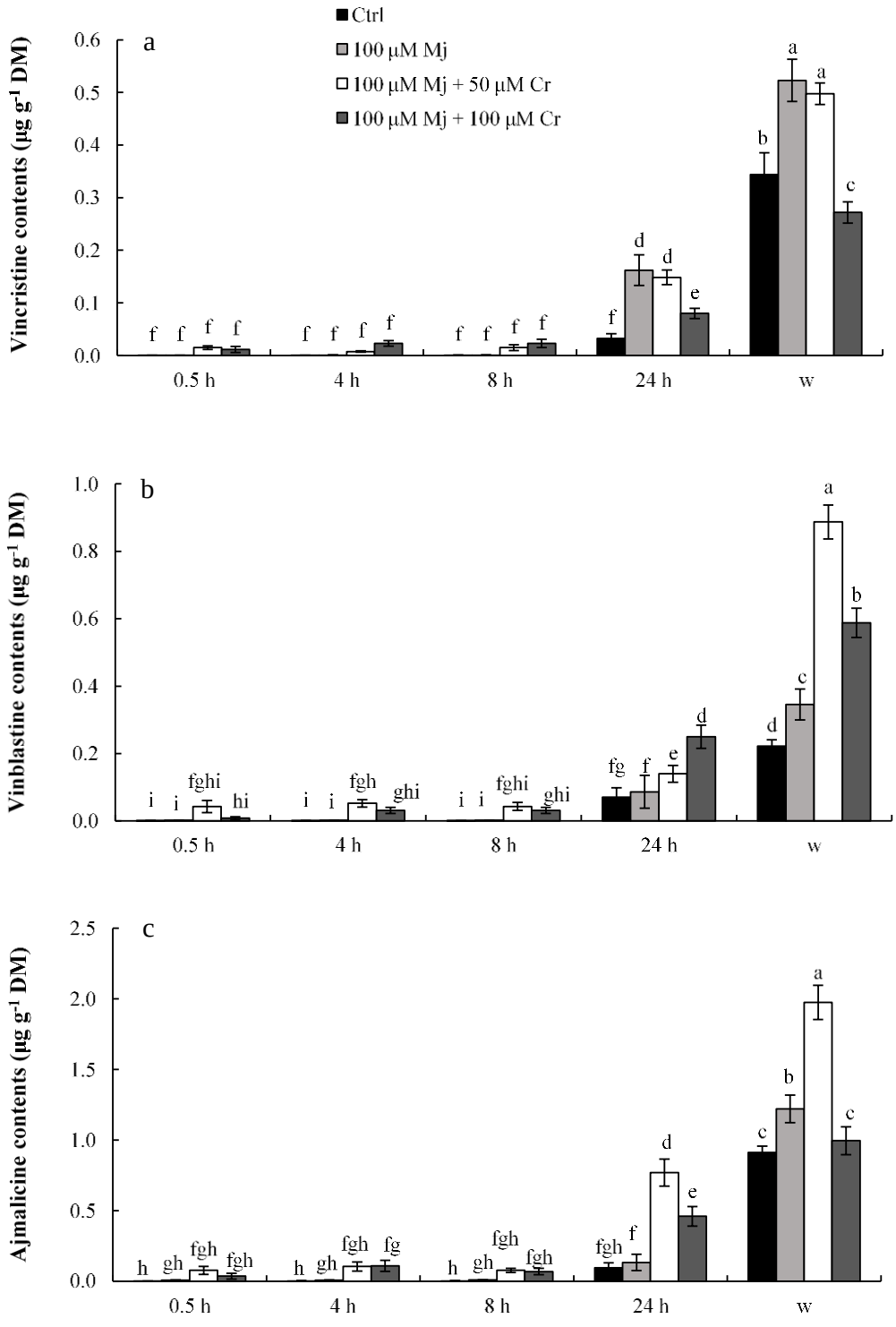

Figure 1: Effects of Cr + Mj treatments on vincristine (a), vinblastine (b), ajmalicine (c) contents on in vitro propagated *C. roseus* shoots after 0.5, 4, 8, 24 h and one week (w) treatment. The experiments were performed in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at $P < 0.05$ according to Duncan test.

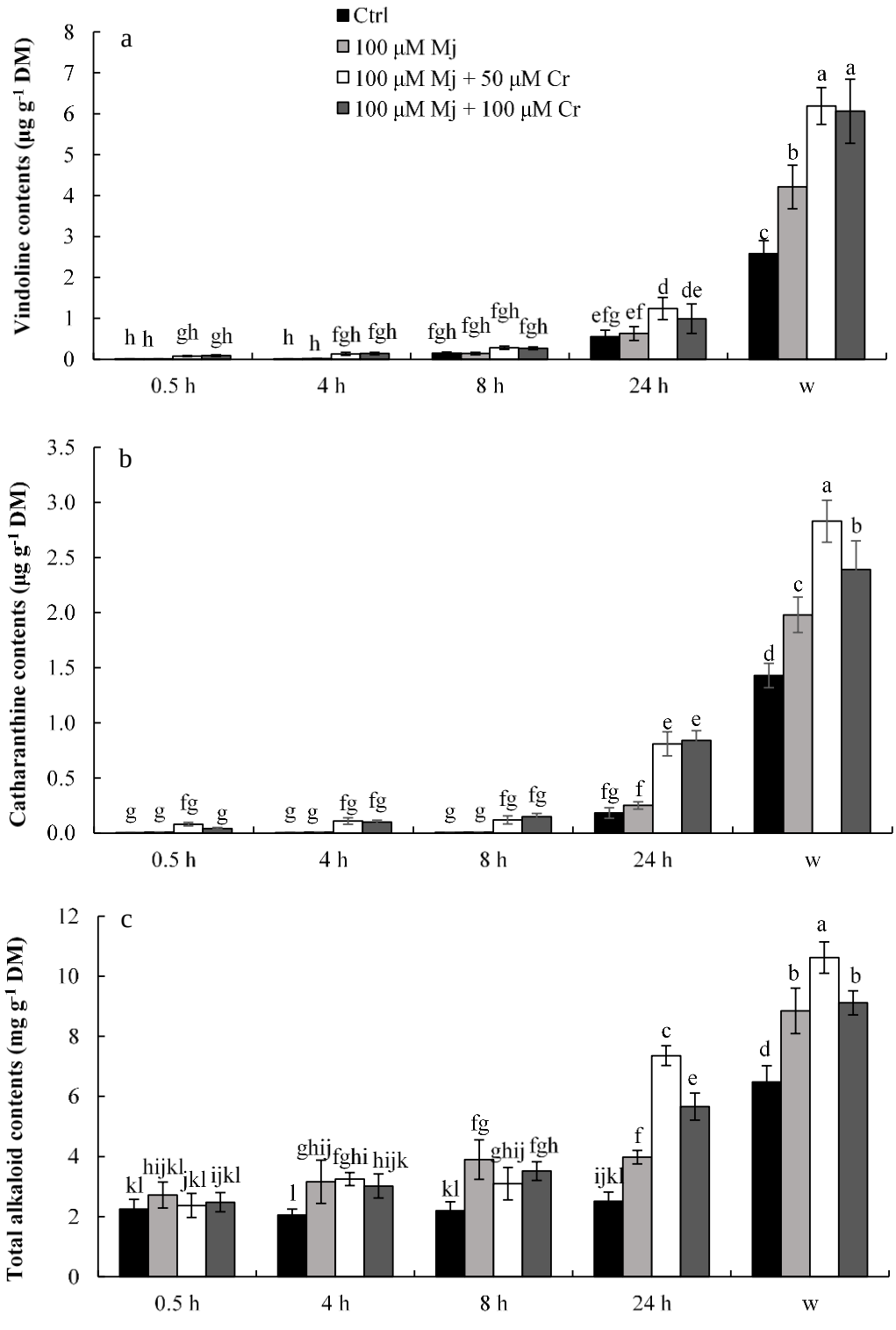

Figure 2: Effects of Cr + Mj treatments on vindoline (a), catharanthine (b), total alkaloid (c) contents on in vitro propagated *C. roseus* shoots after 0.5, 4, 8, 24 h and one week (w) treatment. The experiments were performed in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at $P < 0.05$ according to Duncan test.

Internal or external signals regulate the TFs and cause controlled responses (Pan et al., 2016). Protein kinases and protein phosphatases are two main parts of signal transduction and response to stresses in plants that work through the phosphorylation and de-phosphorylation of proteins. MAPK cascade is one of these protein kinases that have several critical tasks, such as activation in defense responses to many stresses jasmonates biosynthesis, expression of jasmonate-inducible genes, responding to hormones that include ROS signaling (Dubey et al., 2010, Gao et al., 2010). In all eukaryotic cells, the activation of MAPK cascade results in the activation of TFs which convert the extracellular stimulus to intracellular responses. This cascade includes three major kinases, one of which is the MAPK that is the terminal component of this signaling cascade. *MAPK3* is the most well characterized of these MAPKs. The current results showed that there was an upregulation of *ORCA3* and *MAPK3* in response to Mj alone and in combination with Cr treatments, in consistent with Gao et al. (2010) reporting that transcription of *ORCA3* and orthologs of *MAPK3* in other plants were upregulated by Mj treatment and different stresses like high salinity, cold, heat, wounding, chitin, UV, osmotic, and oxidative stresses. The highest level of *MAPK3* and *ORCA3* expression was 7.67 and 5.38-fold higher than control appear 8 h after 100 μM of Mj + 50 μM Cr treatment.

Based on our results, 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. Their expression is increased, but over time, they may influence the other defense responses and biosynthetic genes of secondary metabolites. One of the most important points that previous studies have detected 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). In the current work, *ORCA3* and *MAPK3* reacted positively to the signals, signifying peculiar cognition and exhibited their maximal activity. They also promoted each other in the expression of TIA pathway genes to combat Mj and Cr stress. According to recent studies, abiotic stresses, for example dehydration, cold, H_2O_2 and salicylic acid (SA), initiate the signal transduction pathway which is similar for heavy metal responsive TFs (DalCorso et al., 2008). Therefore, following these findings, the mechanism of Mj and Cr may be done through two distinct ways and cross-talk across these two separate ways or stimulation of a third way by the joint attendance of Mj and Cr." This could explain the increasable effect perceived for the accumulation of vinblastine and vincristine.

3.3 Lipid peroxidation

Cr as a toxic heavy metal has several effects and mechanisms to induce ROS. This is the initiation of oxidative stress, because these free radicals might mutilate the membrane architecture, cause oxidative damage, and motivate lipid peroxidation as reported in other higher plants. Here, the results (Figure 4a) suggest that combined treatment elevated the amount of lipid peroxidation after 4 h and one week but more precisely, 100 μM Mj + 100 μM Cr causes significant increase in all time courses, indicates that extensive oxidative damages could have occurred to the cells under Cr stress especially in its higher concentrations. It was showed that Cr, like other metals recently studied such as aluminum, lead, and arsenic, has promoted production of ROS leading to a rise in lipid peroxidation and have similar toxic effects (Sharmin et al., 2012). Also, Kupper et al. (2009) demonstrated that Mj has a strong potential to stimulate ROS production and oxidative stress with the strongest response at 100 mM according to Kumari et al. (2015). In proceed to the previous studies, application of joint treatment in our work increased ROS production, mutilate the membrane architecture, create an oxidative damage and motivate lipid peroxidation.

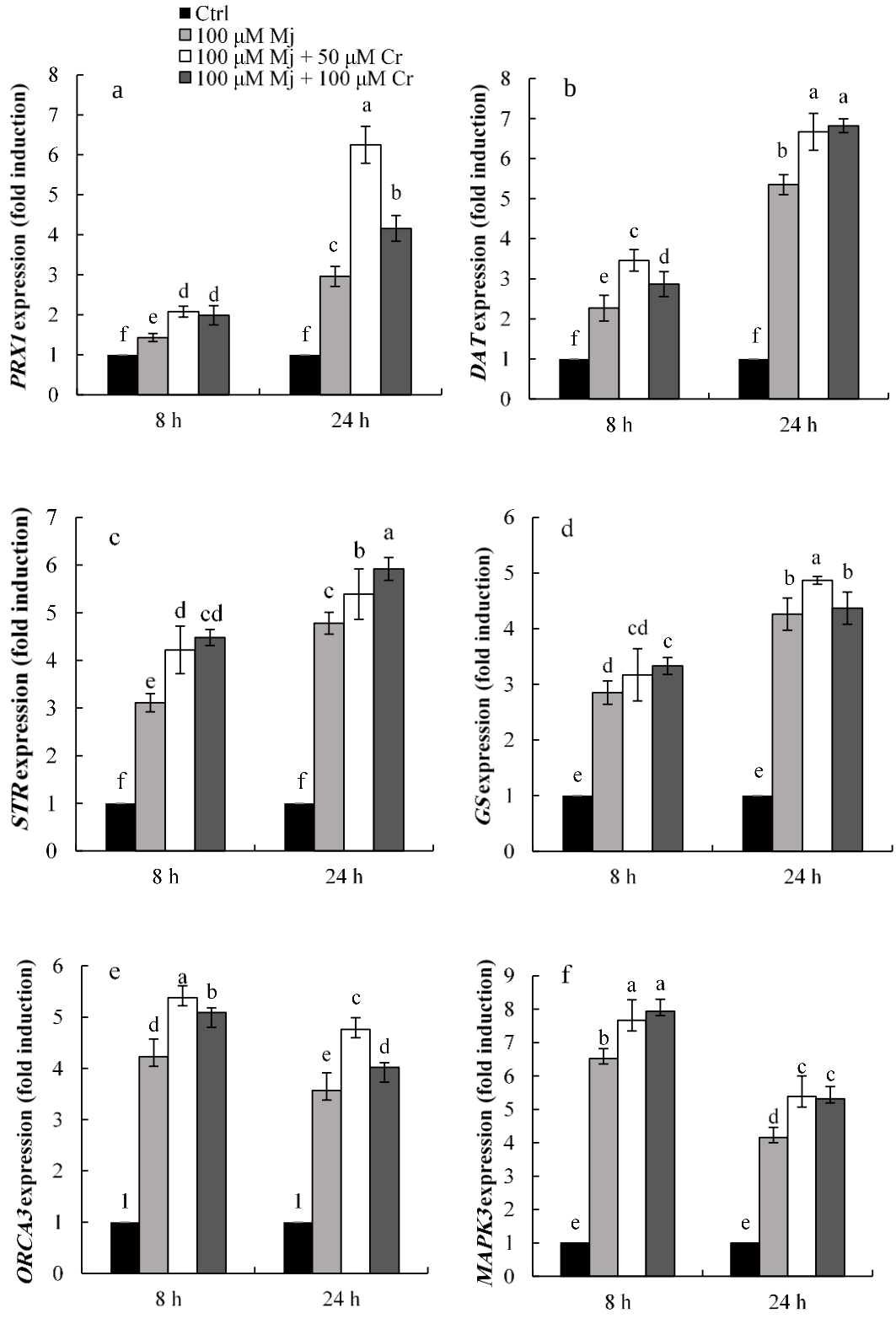

Figure 3: Effects of Cr + Mj treatments on expression patterns of *PRX1* (a), *DAT* (b), *STR* (c), *GS* (d), *ORCA3* (e), *MAPK3* (f) for 8 h and 24 h. The experiments were performed in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at $P < 0.05$ according to Duncan test.

3.4 Phenol, flavonoid and carotenoid contents

In addition to producing alkaloids as nonenzymatic antioxidants, carotenoids and phenolic compounds may also play a role during stress by preserving unstable macromolecules and inducing stability against metals. After 0.5 h treatment, there wasn't any significant difference in phenol and flavonoid contents compared to control but slowly over time, variations happened and Mj alone and combined with two concentration of Cr caused significant increases in total phenol and flavonoid contents after 8, 24 h and one week. The highest contents of total phenol and flavonoid (1.310 and 1.042 mg g^{-1} FM, respectively) were observed in the 100 µM Mj + 100 µM Cr treatments after one week (Figures 4b and 4c).

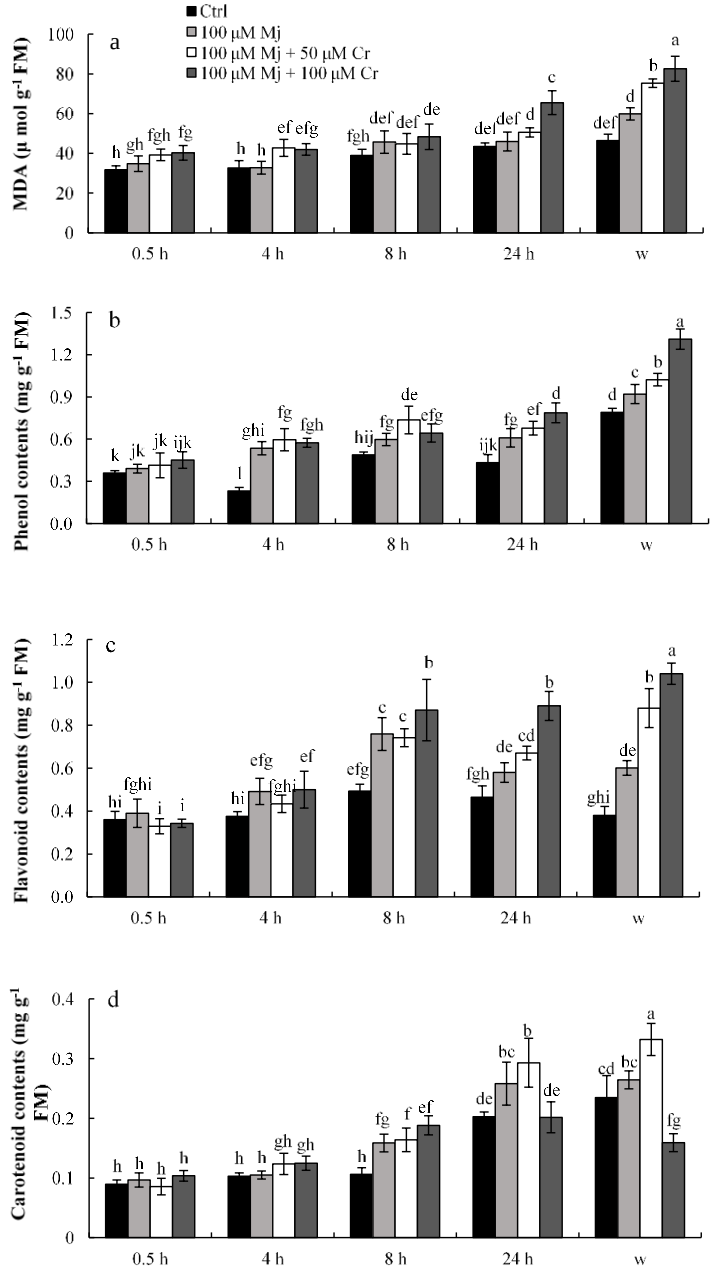

Figure 4: Effects of Cr + Mj treatments on MDA (a) total phenol (b), flavonoid (c), carotenoid (d) contents on in vitro propagated *C. roseus* shoots after 0.5, 4, 8, 24 h and one week (w) treatment. The experiments were performed in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at $P < 0.05$ according to Duncan test.

This increase offered elevated defense system because the hydroxyl and carboxyl groups of these metabolites have the ability to attach metal ions like Cr, chelate them, and thereby prevent Fenton's reaction, which is the main origin of ROS production. Flavonoids also regulate the polar transportation of auxin which controls the stomatal opening and manages the allocation of resources to help the plant overcome weak growth conditions under stress (Singh et al., 2016). The increase in total phenol and flavonoid contents in a separately heavy metal and Mj stress was proved in previous studies like Emamverdian et al. (2015) reviewed heavy metal effects on plants, and also the study of Ozturket al. (2015) worked on effects of pre-harvest Mj and reported matching results. İncreased contents of total phenolic and flavonoid compounds in the current work are in consonance with those studies, it also proves that the simultaneous effect of these two treatments has strengthened this antioxidant property.

C. roseus showed increased carotenoid contents after 8 h as a defense strategy when encountering metal stress, because these pigments protect the chlorophyll pigments and avoid the excited singlet oxygen biosynthesis. Furthermore, they suppress the photodynamic reactions and replace peroxidation. Meanwhile, it should be noted that 100 µM Cr significantly decreased the carotenoid contents after one week which may be again due to gradual degredation of the plant. The highest content was 0.33 mg g^{-1} FM and observed in the 100 µM Mj + 50 µM Cr treatments after one week (Figure 4d).

3.5 Enzymatic analysis

Plants possess another kind of defense known as enzymatic antioxidants (SOD, CAT, and POD) that act as the scavengers of free radicals. Antioxidative enzymes may behave variably in response to oxidative stresses. Antioxidative enzymes work in a contributive or synergistic manner to safeguard against oxidative stress. The current results showed after 0.5 h, Mj combined with 50 µM Cr increased CAT and combined with 100 µM Cr increased SOD activity. Combined treatment also increased POD activities after 4, 8 and 24 h. After one week, all treatments increased POD activity but for the two enzymes CAT and SOD, this happened only at the treatments of Mj alone and combined with 50 µM Cr and a decline at 100 µM compared to 50 µM Cr (Figure 5a, 5b and 5c) was observed. This decrease may be attributed to the high affinity of Cr ions to thiol compounds that interrupts protein synthesis and enzyme activity (Mourato et al., 2012). As can be seen in some conditions, lower concentrations of a metal may cause an increase in enzyme activities, but using higher concentrations breaks the defense system and decreases the activities. The activation of an enzyme itself or the upregulation of its gene expression may be reasons for the increase in amounts of an enzyme. Furthermore, metals can change enzyme structures, and therefore the enzymes activities are decreased. On the other hand, it was found that Mj (50 and 100 µM) elevated production of numerous antioxidative enzymes and their storage (Giri & Zaheer, 2016) so it can alleviate the oxidation by improving the ROS scavenging system stress (Jung, 2004, Aftab et al., 2011). Therefore, it is inferred that combined use of Mj + Cr induces a stronger activation of enzyme activities and these defense responses could act separately or be joined into one strategy to reduce membrane destruction and elevate cell growth or preserve cell maintenance in response to stress.

Our findings demonstrated that after 0.5 h, combined treatments increased the protein contents. After 4, 8 and 24 h, there wasn't any significant difference but after one week only Mj alone significantly increased it and a significantly decrease in protein contents occurred by 100 µM Cr compared to other treatment groups (Figure 5d). The protein rising in early hours is probably due to the plant's rapid response for launching defensive responses under stress conditions. The elevation by jasmonates may be because of induction of gene expression leading to biosynthesis of many proteins maybe proteins related to defense mechanisms, in agreement with Poonam et al. (2013) reported the accumulation of proteins induced by Mj in *Cajanus cajan* (L.) Millsp., but Cr induced decrease after one week might be a consequence of the elevation of catabolic enzymes like proteases, which were stimulated under Cr stress. Another reason is the protein denaturation and oxidation as a consequence of changes in thiol groups of proteins, which leads to increases in the production of carbonyl groups and in the rate of proteolysis that similar to our results was reported by Mourato et al. (2012).

The present study is a small-scale assay aimed at revealing the signal transduction mechanism of *C. roseus* and treatment with Cr and Mj to provide a way to produce significant values of these anticancer metabolites, the only precursors of anticancer drugs. Based on the findings, it is suggested that Mj responsive MAPK3 and ORCA3 are important components of the signal transduction pathway. However, full recognition of the regulatory mechanisms of this biosynthetic pathway requires further studies in this regard.

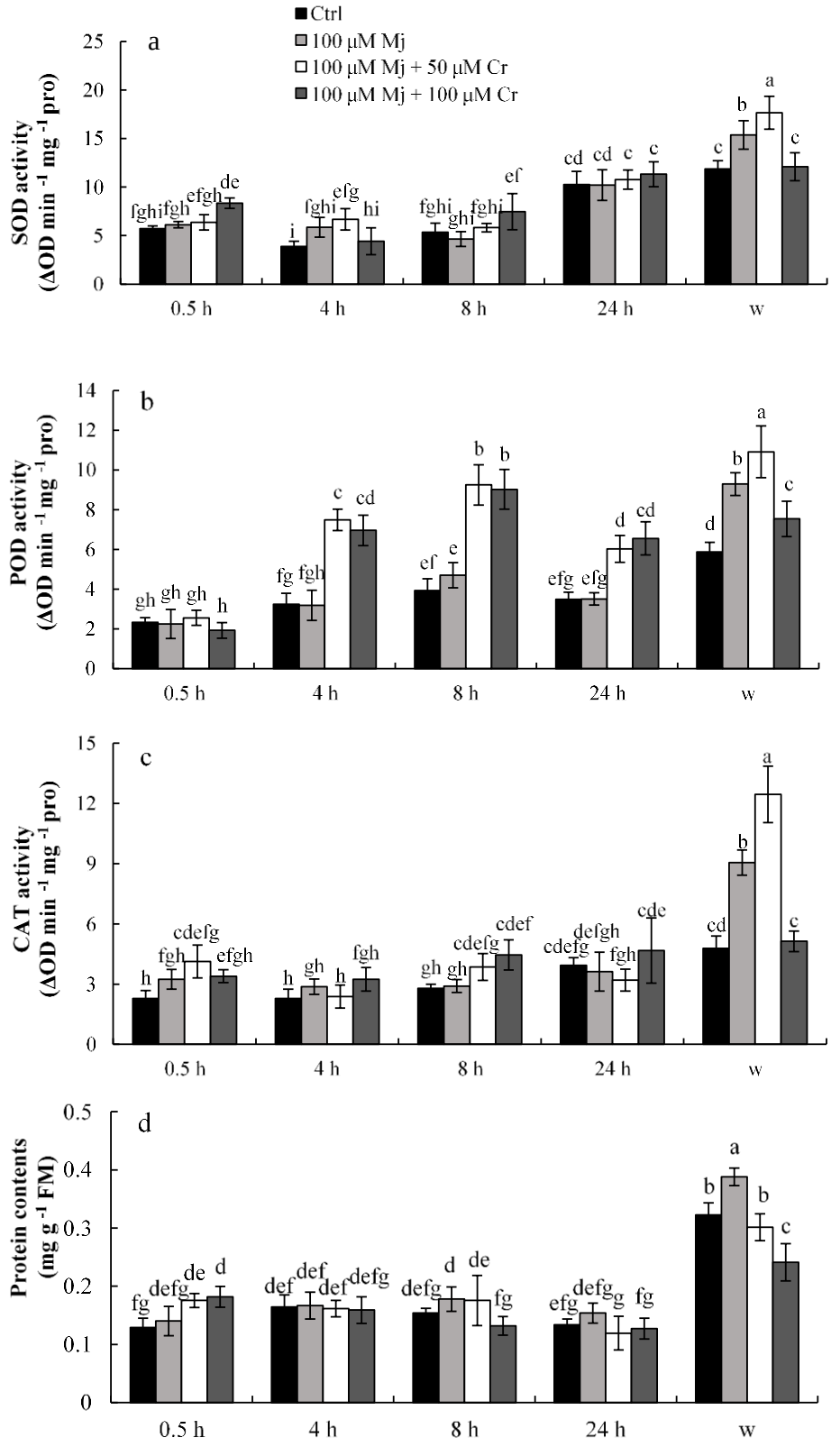

Figure 5: Effects of Cr + Mj treatments on activities of SOD (a), POD (b), CAT(c) and protein contents (d) on in vitro propagated *C. roseus* shoots after 0.5, 4, 8, 24 h and one week (w) treatment. The experiments were performed in biological triplicates. Error bars indicate the standard deviations. Different letters indicate significant differences at $P < 0.05$ according to Duncan test.

4 CONCLUSIONS

The results of the current study have demonstrated that combined abiotic treatments such as Mj + Cr can influence the production of secondary metabolites and can be a commercial way to enhance the potential to overproduce medicinal valuable chemicals (here, alkaloids like vindoline, catharanthine, vincristine, vinblastine, and ajmalicine) with high pharmaceutical values. Both Mj and Cr are oxidizing agents and induce the formation of free radicals and hyperactivates the antioxidant defense system, like phenolics, flavonoids, carotenoids, and enzymes, as a part of the general stress response. However, 100 µM Mj + 100 µM Cr showed a toxic effect on samples and reduced the alkaloids. The results of the current study agreed with a recent model named "elicitor-based signaling model" for appended stimulation of gene expression in this plant, which explained the connection of elicitor to receptor turns on the signal transduction pathway of the MAPK cascade, leading to endogenous JA biosynthesis. Increase in endogenous in addition to exogenous JA as a signal messenger activates the synthesis of nuclear proteins ORCA3. These proteins cooperate with the promoter of the biosynthetic genes and motivate the biosynthesis of TIA alkaloids.

5 ACKNOWLEDGEMENT

The authors would like to express thanks to Shahed University of Tehran for providing the facilities necessary to carry out the work. This research is financed in part by a grant from Medicinal Plant Research Centre of Shahed University, Tehran, Iran.

6 REFERENCES

Aftab, T., Khan, M. M. A., Idrees, M., Naeem, M., & Hashmi, N. (2011). Methyl jasmonate counteracts boron toxicity by preventing oxidative stress and regulating antioxidant enzyme activities and artemisinin biosynthesis in *Artemisia annua* L. *Protoplasma*, *248*(3), 601-612. https://doi.org/10.1007/s00709-010-0218-5

DalCorso, G., Farinati, S., Maistri, S., & Furini, A. (2008). How plants cope with cadmium: staking all on metabolism and gene expression. *Journal of integrative plant biology*, *50*(10), 1268-1280. https://doi.org/10.1111/j.1744-7909.2008.00737.x

Dewanto, V., Wu, X., Adom, K. K., & Liu, R. H. (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *Journal of agricultural and food chemistry*, *50*(10), 3010-3014. https://doi.org/10.1021/jf0115589

Dubey, S., Misra, P., Dwivedi, S., Chatterjee, S., Bag, S. K., Mantri, S. & Tripathi, P. (2010). Transcriptomic and metabolomic shifts in rice roots in response to Cr (VI) stress. *BMC genomics*, *11*(1), 648. https://doi.org/10.1186/1471-2164-11-648

Eleftheriou, E. P., Adamakis, I. D. S., Panteris, E., & Fatsiou, M. (2015). Chromium-induced ultrastructural changes and oxidative stress in roots of *Arabidopsis thaliana*. *International journal of molecular sciences*, *16*(7), 15852-15871. https://doi.org/10.3390/ijms160715852

Emamverdian, A., Ding, Y., Mokhberdoran, F., & Xie, Y. (2015). Heavy metal stress and some mechanisms of plant defense response. *The Scientific World Journal*, *2015*. http://dx.doi.org/10.1155/2015/756120

Gao, F., Su, Q., Fan, Y., & Wang, L. (2010). Expression pattern and core region analysis of AtMPK3 promoter in response to environmental stresses. *Science China Life Sciences*, *53*(11), 1315-1321. https://doi.org/10.1007/s11427-010-4079-0

Giri, C. C., & Zaheer, M. (2016). Chemical elicitors versus secondary metabolite production in vitro using plant cell, tissue and organ cultures: recent trends and a sky eye view appraisal. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *126*(1), 1-18. https://doi.org/10.1007/s11240-016-0985-6

Heath, R. L., & Packer, L. (1968). Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of biochemistry and biophysics*, *125*(1), 189-198. https://doi.org/10.1016/0003-9861(68)90654-1

Jung, S. (2004). Effect of chlorophyll reduction in Arabidopsis thaliana by methyl jasmonate or norflurazon on antioxidant systems. *Plant Physiology and Biochemistry*, *42*(3), 225-231. https://doi.org/10.1016/j.plaphy.2004.01.001

Kumari, P., Reddy, C. R. K., & Jha, B. (2015). Methyl jasmonate-induced lipidomic and biochemical

alterations in the intertidal macroalga *Gracilaria dura* (Gracilariaceae, Rhodophyta). *Plant and Cell Physiology*, *56*(10), 1877-1889. https://doi.org/10.1093/pcp/pcv115

Kupper, F. C., Gaquerel, E., Cosse, A., Adas, F., Peters, A. F., Müller, D. G. & Potin, P. (2009). Free fatty acids and methyl jasmonate trigger defense reactions in *Laminaria digitata*. *Plant and Cell Physiology*, *50*(4), 789-800. https://doi.org/10.1093/pcp/pcp023

Liu, D. H., Jin, H. B., Chen, Y. H., Cui, L. J., Ren, W. W., Gong, Y. F., & Tang, K. X. (2007). Terpenoid indole alkaloids biosynthesis and metabolic engineering in *Catharanthus roseus*. *Journal of integrative plant biology*, *49*(7), 961-974. https://doi.org/10.1111/j.1672-9072.2007.00457.x

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, *25*(4), 402-408. https://doi.org/10.1006/meth.2001.1262

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. *Biochemistry and Molecular Biology Education*, *35*(3), 206-210. https://doi.org/10.1002/bmb.60

Mourato, M., Reis, R., & Martins, L. L. (2012). Characterization of plant antioxidative system in response to abiotic stresses: a focus on heavy metal toxicity. In *Advances in selected plant physiology aspects*. InTech. https://doi.org/10.5772/34557

Ncube, B., & Van Staden, J. (2015). Tilting plant metabolism for improved metabolite biosynthesis and enhanced human benefit. *Molecules*, *20*(7), 12698-12731. https://doi.org/10.5772/34557

Ozturk, B., Yıldız, K., & Ozkan, Y. (2015). Effects of pre-harvest methyl jasmonate treatments on bioactive compounds and peel color development of 'Fuji' apples. *International journal of food properties*, *18*(5), 954-962. https://doi.org/10.1080/10942912.2014.911312

Pan, Y. J., Liu, J., Guo, X. R., Zu, Y. G., & Tang, Z. H. (2015). Gene transcript profiles of the TIA biosynthetic pathway in response to ethylene and copper reveal their interactive role in modulating TIA biosynthesis in *Catharanthus roseus*. *Protoplasma*, *252*(3), 813-824. https://doi.org/10.1007/s00709-014-0718-9

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. *Phytochemistry reviews*, *15*(2), 221-250. https://doi.org/10.1007/s11101-015-9406-4

Peebles, C. A., Hughes, E. H., Shanks, J. V., & San, K. Y. (2009). Transcriptional response of the terpenoid indole alkaloid pathway to the overexpression of ORCA3 along with jasmonic acid elicitation of *Catharanthus roseus* hairy roots over time. *Metabolic engineering*, *11*(2), 76-86. https://doi.org/10.1016/j.ymben.2008.09.002

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. *American Journal of Plant Sciences*, *4*(04), 817. https://doi.org/10.4236/ajps.2013.44100

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. *Proceedings of the National Academy of Sciences*, *115*(12), 3180-3185. https://doi.org/10.1073/pnas.1719979115

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 biology*, *12*(1), 134. https://doi.org/10.1186/1471-2229-12-134

Sanchez-Rojo, S., Cerda-García-Rojas, C. M., Esparza-García, F., Plasencia, J., Poggi-Varaldo, H. M., Ponce-Noyola, T., & Ramos-Valdivia, A. C. (2015). Long-term response on growth, antioxidant enzymes, and secondary metabolites in salicylic acid pre-treated *Uncaria tomentosa* microplants. *Biotechnology letters*, *37*(12), 2489-2496. https://doi.org/10.1007/s10529-015-1931-0

Sharmin, S. A., Alam, I., Kim, K. H., Kim, Y. G., Kim, P. J., Bahk, J. D., & Lee, B. H. (2012). Chromium-induced physiological and proteomic alterations in roots of *Miscanthus sinensis*. *Plant science*, *187*, 113-126. https://doi.org/10.1016/j.plantsci.2012.02.002

Singh, K. B., Foley, R. C., & Oñate-Sánchez, L. (2002). Transcription factors in plant defense and stress responses. *Current opinion in plant biology*, *5*(5), 430-436. https://doi.org/10.1016/S1369-5266(02)00289-3

Singh, S., Parihar, P., Singh, R., Singh, V. P., & Prasad, S. M. (2016). Heavy metal tolerance in plants: role

of transcriptomics, proteomics, metabolomics, and ionomics. *Frontiers in plant science*, *6*, 1143. https://doi.org/10.3389/fpls.2015.01143

Trinh, N. N., Huang, T. L., Chi, W. C., Fu, S. F., Chen, C. C., & Huang, H. J. (2014). Chromium stress response effect on signal transduction and expression of signaling genes in rice. *Physiologia plantarum*, *150*(2), 205-224. https://doi.org/10.1111/ppl.12088

Van der Fits, L., & Memelink, J. (2000). ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. *Science*, *289*(5477), 295-297. https://doi.org/10.1126/science.289.5477.295

Van Moerkercke, A., Steensma, P., Schweizer, F., Pollier, J., Gariboldi, I., Payne, R. & Kellner, F. (2015). The bHLH transcription factor BIS1 controls the iridoid branch of the monoterpenoid indole alkaloid pathway in *Catharanthus roseus*. *Proceedings of the National Academy of Sciences*, 201504951. https://doi.org/10.1073/pnas.1504951112

Wójciak-Kosior, M., Sowa, I., Blicharski, T., Strzemski, M., Dresler, S., Szymczak, G. & Świeboda, R. (2016). The stimulatory effect of strontium ions on phytoestrogens content in *Glycine m*ax (L.) Merr. *Molecules*, *21*(1), 90. https://doi.org/10.3390/molecules21010090

Zhang, H., Hedhili, S., Montiel, G., Zhang, Y., Chatel, G., Pré, M. & 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*. *The Plant Journal*, *67*(1), 61-71. https://doi.org/10.1111/j.1365-313X.2011.04575.x