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Original Research

Effect of cobalt nanoparticles and cobalt ions on alkaloids production and expression of CrMPK3 gene in *Catharanthus roseus* suspension cultures

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Abstract: The capabilities of cobalt ions (Co^{2+}) and cobalt nanoparticles (CoNPs) in enhancing alkaloids accumulation in *Catharanthus roseus* suspension cultures were evaluated in relation to the expression of CrMPK3 gene. Four concentrations (5, 10, 15 and 20 mg/L) of each cobalt form were utilized in addition to control. Both forms induced oxidative stress that was more pronounced for Co^{2+} treatments. A positive correlation was observed between cobalt concentration and expression of CrMPK3 gene. However, a characteristic temporal expression profile was recorded for each cobalt form. Also, positive correlations were detected between both cobalt concentration and expression of CrMPK3 gene on one hand and the activities of antioxidant enzymes (SOD and APX) and alkaloids content on the other hand. Such correlations suggest CrMPK3 gene as a common player in cobalt-induced stress signaling; regardless of cobalt form. Results revealed the higher capability of Co^{2+}, compared with CoNPs, in enhancing alkaloids accumulation. However, results supported CoNPs as a novel tool in manipulating cobalt-induced alkaloids production in *C. roseus*.

Key words: *Catharanthus roseus*; Tissue culture; Cobalt; Nanoparticles; Alkaloids; Lipid peroxidation; Antioxidants; SOD; APX; Real-time PCR.

Introduction

Alkaloids are a group of heterocyclic nitrogen-containing low-molecular-weight compounds (1). Many of which are known for their antitumor activity eg: vinblastine and vincristine (2). Chemical synthesis of alkaloids is hindered by low productivity and high cost (3). As an alternative, alkaloids are now industrially extracted from higher plants (4). *Catharanthus roseus*, family *Apocynaceae*, is an alkaloid plant able to synthesis a huge number (>130) of precious monoterpenoid indole alkaloids (MIA) (5).

For production of plant secondary metabolites, *in vitro* techniques have several superiorities over traditional cultivation (6-8). Dealing with *C. roseus*, different types of *in vitro* cultures including callus, cell suspension, shoot, root and somatic embryos were employed to study and improve alkaloids production (5, 9-10).

Phytotoxic concentrations of heavy metals are known to induce oxidative stress associated with enhancement in reactive oxygen species (ROS) production (11) that provoke antioxidant defense mechanisms (12). Beside provocation of ascorbate-glutathione cycle enzymes and other antioxidant enzymes [eg: superoxide dismutase (SOD, EC 1.15.1.1), peroxidases (POD; EC 1.11.1.7) and catalase (CAT; EC 1.11.1.6)], metabolic processes are directed towards biosynthetic pathways that consume reduction equivalents. As a consequence, the biosynthesis of reduced compounds including isoprenoids, phenols or alkaloids, is enhanced (13). The potentialities of heavy metals in enhancing alkaloids production in *C. roseus* were recorded using Cd (14), Mn, Ni, and Pb (15), Hg (16) and Cr (17).

Though having an undefined role, cobalt is usually defined as beneficial microelement for plant growth (18). However, excess amount of this heavy metal is accompanied with oxidative stress symptoms (19-20). The ability of cobalt to enhance biosynthesis of secondary metabolites was documented by several research groups (21, 7, 22-23). However, few reports demonstrated the impact of cobalt on alkaloids accumulation (24-25).

Mitogen-activated protein kinases (MAPKs) are a class of protein kinases playing a key role linking perception of stress stimuli with changes in gene expression (26). MAPK cascade is essential for alkaloids biosynthesis under stress conditions (27). In response to copper treatments, induction of CrMPK3 expression was correlated with enhancements in expression of several genes involved in alkaloids biosynthesis in *C. roseus* (28).

Stress is a double edged sword; it enhances alkaloids biosynthesis with simultaneous decrease in biomass accumulation that may nullify enhancements in total amount of alkaloids produced per plant. Thus stress should be manipulated to make a compromise between these two opposite effects. Dealing with heavy metal-induced stress, such compromise can be reached through variations in metal concentration and duration of stress treatment. The unique properties of nanoparticles as compared with their large-particle counterparts (29), may introduce a new button for fine tuning stress treatment. Cobalt nanoparticles were employed to enhance artemisinin synthesis in *Artemisia annua* suspension cultures (30).

The aim of this investigation is to study the poten-

tialities of different concentrations of cobalt nanoparticles (CoNPs) and cobalt ions (Co^{2+}) in enhancement of alkaloids accumulation in *C. roseus* suspension cultures in relation to the expression of CrMPK3 gene. Being polyvinylpyrrolidone (PVP)-coated nanoparticles, the impacts of PVP was also evaluated.

Materials and Methods

CoNPs characterization and dispersion

CoNPs utilized in this investigation were provided by Nanotech, Cairo, Egypt, as suspension of polyvinylpyrrolidone (PVP)-coated particles in deionized water (DW). Figure 1 showed the transmission electron microscopy (TEM) image of CoNPs used in this study. Particles are roughly spherical with 20-50 nm average size. Preparation of experimental concentrations was carried out by diluting stock solution (1 g/L) in DW and sonication at 100 W and 30 kHz for 40 min. All dilutions were prepared just before use.

Plant material and explant preparation

C. roseus seeds utilized in this investigation were kindly provided from Horticulture Research Institute, Agricultural Research Centre, Giza, Egypt. Healthy seeds were sterilized in 70 % (v/v) ethanol for one min followed by 0.1% (m/v) $HgCl_2$ for 10 min. Sterilized seeds were thoroughly rinsed in sterile distilled water then plated on basal medium in 350 ml glass jars (10–15 seed per jar) and incubated at 25 ± 2 °C under light intensity of 30 µmol m^{-2} s^{-1} with 16/8 h photoperiod. Two weeks later, seedlings were obtained and used as a source of explants for callus initiation. Basal medium was MS medium (31) supplemented with sucrose (30 g/L) and gelled with agar (7 g/L). The pH was adjusted to 5.8 before autoclaving at 121 °C for 20 min.

Establishment of callus cultures

Callus cultures were initiated by placing hypocotyl explants (0.5-0.7 cm length) on basal medium fortified with 1 mg/L 2,4- dichlorophenoxyacetic acid (2,4-D), 1 mg/L indole acetic acid (IAA) and 0.5 mg/L kinetin. Cultures were incubated in dark at 25 ± 2 °C, the same growth conditions were applied all over this study unless otherwise indicated. After 4 weeks, developed calli were separated from explants then subcultured monthly on the same but fresh medium.

Establishment of cell suspension cultures and experimental treatments

Cell suspension culture was initiated by transferring 10 g callus to 500 ml flasks containing 200 ml callus induction medium (without agar); cultures were maintained on orbital shaker at 130 rpm. A subculture step was carried out at 10-day intervals by transferring 20 ml of old culture to 180 ml fresh medium. In the seventh day of third subculture, filter sterilized aqueous CoNPs solution was aseptically added to reach a final concentration of 5, 10, 15 and 20 mg/L. Likewise, cobalt chloride and PVP were used in two positive control experiments. All Results were compared with a negative control in which cobalt and PVP solutions were replaced with sterile DW.

Cultures were maintained on shaker for 0.5, 4, 8 and 72 h; thereafter cells were harvested by filtration.

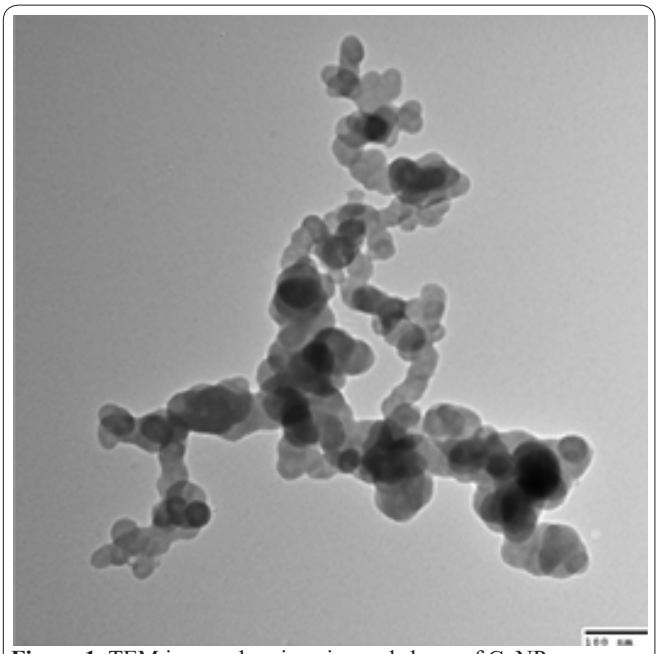

Figure 1. TEM image showing size and shape of CoNPs.

The 0.5, 4 and 8 h treatments were devoted for real-time quantitative PCR while 72 h treatment was used in all other measurements. Fresh weights were determined then cells were ground into fine powder in liquid nitrogen and kept at -80 °C till used. Dry weights were determined after drying at 50 °C till constant weight. Dried tissues and broth were kept for alkaloids quantifications.

Lipid peroxidation assay

Lipid peroxidation assay was carried out by measuring malondialdehyde (MDA) formation, using the thiobarbituric acid method (32). Liquid nitrogen-powdered cells of 72 h treatment were homogenized in 100 mM Tris–HCl buffer, pH 7.4, containing 1.5% (m/v) PVP. The homogenates were filtered and centrifuged for 20 min at 10 000 *g*. 1 ml clear supernatant was mixed with 4 ml of 0.5% (m/v) thiobarbituric acid in 20% (m/v) trichloroacetic acid and heated for 30 min at 90 °C. After cooling, samples were centrifuged for 20 minutes at 10 000 *g* then the absorbance of clear supernatant was read at 532 nm then nonspecific absorbance at 600 nm was subtracted. The MDA concentration was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1}.

Antioxidant enzymes and soluble proteins

Liquid nitrogen-powdered cells of 72 h treatment were homogenized in 50 mM phosphate buffer (pH 7.8) containing 1 % PVP (m/v) and 0.2 mM EDTA (33). The homogenate was centrifuged at 4 °C for 15 min at 15 000 *g*, the supernatant was used for measurements of enzymes activity and soluble proteins content.

SOD activity was measured by observing the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (34). Assay was carried out in 50 mM phosphate buffer (pH 7.8) containing 33 mM NBT, 3.3 mM riboflavin, 0.66 mM Na-EDTA and 10 mM methionine. Reactions were carried out at a light intensity of about 300 µmol m^{-2} s^{-1} for 10 min at 25 °C. One unit of enzyme activity was defined as the quantity of SOD required for 50% inhibition of NBT reduction (35).

Ascorpate peroxidase (APX) activity was measured in 50 mM phosphate buffer, pH 7.0, containing 0.5 mM ascorbate, 1.2 mM H_2O_2 and 0.1 mM EDTA (36). Rate of decrease in oxidised ascorbate absorbance was measured at 290 nm and activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} for ascorbate.

Soluble proteins were quantified as described by Lowry *et al.* (1951) using Folin-Ciocaltcau reagent at 750 nm with bovine serum albumin as a calibration standard.

Alkaloid extraction and determination

Alkaloid extraction and determination was carried out, for 72 h treatments, as described by Lee *et al.* (1981). Fine powdered dry tissue was extracted three times using methanol, the extracts were combined then methanol was vacuum-evaporated. Residues were partitioned between acid water (pH 3) and petroleum ether. Using 1 M NaOH, pH of the aqueous phase was raised to 8.5 then total alkaloids were extracted with chloroform. Extraction of alkaloids from broth was started with acidification to pH 3 then completed as previously mentioned for extraction from dry tissue. Based on a standard curve constructed with ajmalicine, total alkaloids were determined spectrophotometrically at 280 nm.

Real-time quantitative PCR

Fresh calli of 0.5, 4 and 8 h treatments were used for total RNA extraction using *Direct-zol™ RNA MiniPrep* (http:// www.zymoresearch.com). RNA was purified of contaminating residual genomic DNA using DNase (*Fermentas*, Waltham, MA, USA). RNA purity and concentration were checked employing *Nanodrop* spectrophotometer (ND-2000c, *Thermo Fisher Scientific*, Wilmington, DE, USA). For cDNA synthesis, one µg RNA was reverse transcribed with the aid of *SensiFAST™* cDNA synthesis kit (http://www.bioline.com). Quantitative amplifications of *CrMPK3* gene was performed using a *Mx3000P* (*Stratagene*, CA, USA) qPCR system with the specific primers 5'-ACGAAATGAGGATGCAAAAAGATAC-3' and 5'-TGCTAACTGCTGACGAGGGAAT-3' (28). Transcription levels were normalized to that of *actin* as endogenous control using the primers: 5'-GCTTCCCGATGGTCAAGTCA-3' and 5'-GGATTCCAGCTGCTTCCATTC-3'. The amplification protocol was 40 two-step cycles of amplification (95 °C for 15 s and 60 °C for 60 s) preceded by 95 °C for 10 min. Expression recorded in fresh cells collected just before treatments was considered as a quantification unit.

Statistical analysis

Results of each treatment were expressed as mean of three replicates ± standard deviation (SD). For each evaluated parameter, differences among means were calculated using the LSD (least significant difference) range test with a family error rate of 0.05 using the *SPSS v. 14* software. Correlations between parameters were calculated based on Pearson correlation at P values < 0.05 using *Minitab v. 10.0* software.

Results

Results of the present investigation reflected that PVP treatments had no significant effects, compared with the corresponding controls, on any of the considered parameters. Such results were confirmed with insignificant Pearson correlations (Table 1). Conversely, significant correlations were recorded between Co^{2+} concentration and all measured parameters except expression level of CrMAPK gene in 8 h treatments. On the other hand, CoNPs concentration was significantly correlated with all recorded parameters for all durations.

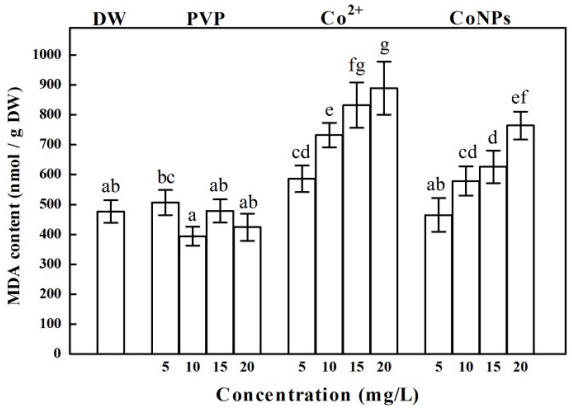

Figure 2. Effect of different concentrations of PVP, Co^{2+} and CoNPs on MDA content in C. roseus cell suspension cultures. One-week-old cultures were subjected to 72 h treatments. Bars with different letters are significantly different, according to the LSD test, at $P \leq 0.05$. Means + SDs, n = 3.

Lipid peroxidation

Compared with control, lipid peroxidation; indicated with increase in MDA content, increased in a dose dependent manner following exposure to Co^{2+} reaching 174.6% of control at 15 mg/L that remained without further significant increase at 20 mg/L (figure 2). Except at 5 mg/L, all CoNPs treatments were associated with significant increase in MDA content to reach a maximum of 160.3% of control at 20 mg/L. Compared with Co^{2+} treatments, significantly lower values for MDA content were recorded following CoNPs treatments at all used concentrations.

Growth parameters

Under control conditions, the average values recorded for fresh and dry weights were 59.49 and 3.38 g/L, respectively (figure 3). In response to Co^{2+} treatments, both growth parameters showed no significant changes at 5 mg/L. Increase in Co^{2+} concentration was associated with decrease in fresh and dry weights to reach 56.7 and 62.9% of corresponding control, respectively, at 20 mg/L.

Compared with control values, significant increases in both fresh and dry weights were distinguished upon exposure to CoNPs at 5 mg /L. The growth promoting effect of CoNPs was vanished at 10 mg/L. Further increases in concentration were accompanied with gradual decrease in fresh weight to reach 73% of corresponding control at 20 mg/L, while significant decrease in dry weight was only recorded at 20 mg/L. Replacing Co^{2+} with CoNPs was associated with significantly higher values for both fresh and dry weights at all used concentrations.

Table 1. Pearson correlation matrix for PVP, cobalt ion and CoNPs concentration and different evaluated parameters.

			Conc.	MDA content	Fresh Weight	Dry Weight	CrMPK3			SOD activity	APX activity	Alkaloids content
							0.5 h	4 h	8 h			Cells
PVP	MDA content		-0.360									
	Fresh Weight		0.266	0.264								
	Dry Weight		0.204	-0.408	-0.189							
	CrMPK3	0.5 h	-0.372	-0.088	0.012	-0.131						
		4.0 h	0.507	0.201	0.541	-0.110	-0.332					
		8.0 h	-0.349	-0.047	-0.460	-0.391	0.183	-0.116				
	SOD activity		-0.504	0.740*	0.026	-0.286	-0.251	0.040	-0.153			
	APX activity		-0.299	-0.407	-0.073	0.200	0.668*	-0.335	-0.168	-0.286		
	Alkaloids content	Cells	-0.254	0.607*	0.156	-0.534	-0.106	0.025	0.087	0.437	-0.200	
		Medium	-0.155	0.215	-0.055	0.145	0.196	-0.115	0.186	-0.094	0.019	0.423
Co^{2+}	MDA content		0.941*									
	Fresh Weight		-0.900*	-0.841*								
	Dry Weight		-0.861*	-0.825*	0.878*							
	CrMPK3	0.5 h	0.950*	0.950*	-0.930*	-0.917*						
		4.0 h	0.871*	0.786*	-0.798*	-0.822*	0.824*					
		8.0 h	-0.172	-0.133	-0.007	0.132	-0.040	-0.092				
	SOD activity		0.982*	0.931*	-0.925*	-0.900*	0.961*	0.850*	-0.193			
	APX activity		0.980*	0.933*	-0.912*	-0.865*	0.968*	0.826*	-0.106	0.974*		
	Alkaloids content	Cells	0.936*	0.902*	-0.929*	-0.887*	0.956*	0.848*	-0.035	0.961*	0.961*	
		Medium	0.955*	0.885*	-0.924*	-0.880*	0.941*	0.817*	-0.120	0.986*	0.966*	0.974*
CoNPs	MDA content		0.893*									
	Fresh Weight		-0.687*	-0.769*								
	Dry Weight		-0.619*	-0.742*	0.815*							
	CrMPK3	0.5 h	0.704*	0.840*	-0.690*	-0.785*						
		4.0 h	0.890*	0.851*	-0.745*	-0.753*	0.792*					
		8.0 h	0.906*	0.851*	-0.830*	-0.752*	0.725*	0.899*				
	SOD activity		0.969*	0.901*	-0.797*	-0.721*	0.730*	0.908*	0.919*			
	APX activity		0.956*	0.937*	-0.791*	-0.729*	0.765*	0.911*	0.925*	0.969*		
	Alkaloids content	Cells	0.880*	0.814*	-0.815*	-0.748*	0.725*	0.811*	0.890*	0.915*	0.900*	
		Medium	0.914*	0.874*	-0.701*	-0.608*	0.805*	0.879*	0.907*	0.874*	0.913*	0.869*

*Correlation is significant at the 0.05 level.

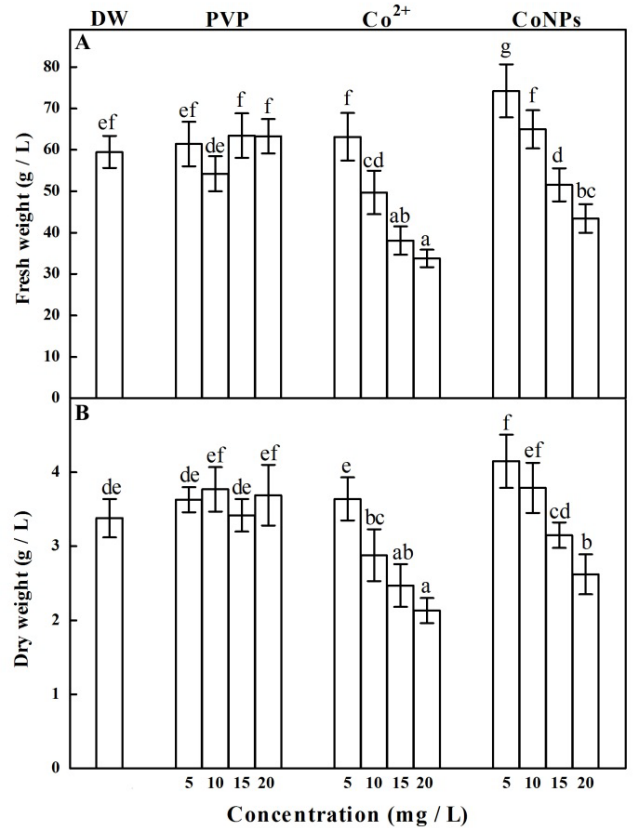

Figure 3. Effect of different concentrations of PVP, Co2+ and CoNPs on fresh (A) and dry (B) weights of cells in C. roseus cell suspension cultures. One-week-old cultures were subjected to 72 h treatments. Bars with different letters are significantly different, according to the LSD test, at $P \leq 0.05$. Means + SDs, n = 3.

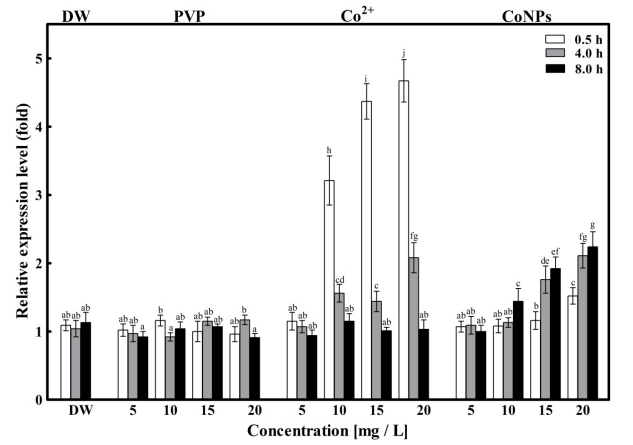

Figure 4. Effect of different concentrations of PVP, Co2+ and CoNPs on relative expression of CrMPK3 gene in C. roseus cell suspension cultures. One-week-old cultures were subjected to 0.5, 4 and 8 h treatments. Values are relative to average expression recorded in cultures just before treatments. Bars with different letters are significantly different, according to the LSD test, at $P \leq 0.05$. Means + SDs, n = 3.

Relative expression of CrMPK3 gene

Regarding the corresponding controls, Co^{2+} failed to significantly enhance expression of CrMPK3 gene at 5 mg/L regardless exposure time (figure 4). Increase in Co^{2+} concentration was accompanied with significant enhancement in transcript accumulation in 0.5 and 4 h treatments that was more significantly pronounced in 0.5 h treatments. Expression was down-regulated to control level upon extending treatment to 8 h.

Except at 20 mg/L where expression level reached about 1.4 fold of corresponding control, no significant changes in CrMPK3gene expression were quantified following 0.5 h exposure to CoNPs. Regarding 4 h treatments, gene expression remained unaffected at 5 and 10 mg/L while it increased significantly reaching 1.7 and 2 folds of corresponding control at 15 and 20 mg/L, respectively. Except significantly higher expression level recorded at 10 mg/L, the same results were recorded following doubling exposure time. Compared with each other, the effects of different cobalt forms on expression of CrMPK3 gene was complicated with exposure time. Generally, significantly greater impact for Co^{2+} was observed in 0.5 h treatments; the opposite was observed for the 8 h treatments.

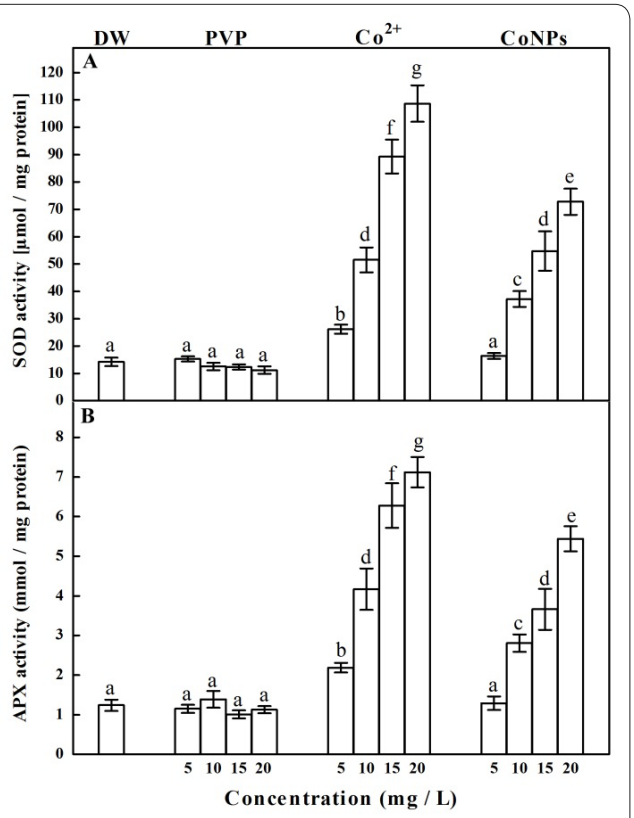

Figure 5. Effect of different concentrations of PVP, Co2+ and CoNPs on SOD (A) and APX (B) activities in C. roseus cell suspension cultures. One-week-old cultures were subjected to 72 h treatments. Bars with different letters are significantly different, according to the LSD test, at $P \leq 0.05$. Means + SDs, n = 3.

Antioxidant enzymes

With respect to corresponding control, the activity of both antioxidant enzymes showed a dose dependent increase in response to Co^{2+} treatments reaching 7.6 and 5.7 folds of control for SOD and APX, respectively, at 20 mg/L (figure 5). The enhancing effect of cobalt ions was significantly attenuated upon using cobalt nanoparticles at all used concentrations. Compared with control, no significant changes were recorded at 5 mg/L for both enzymes. Further increases in CoNPs concentration were accompanied with significant gradual increases in enzymatic activities to reach 5.1 and 4.4 folds of corresponding control for SOD and APX, respectively, at 20 mg/L.

Alkaloids content

Alkaloids content recorded in control cultures was

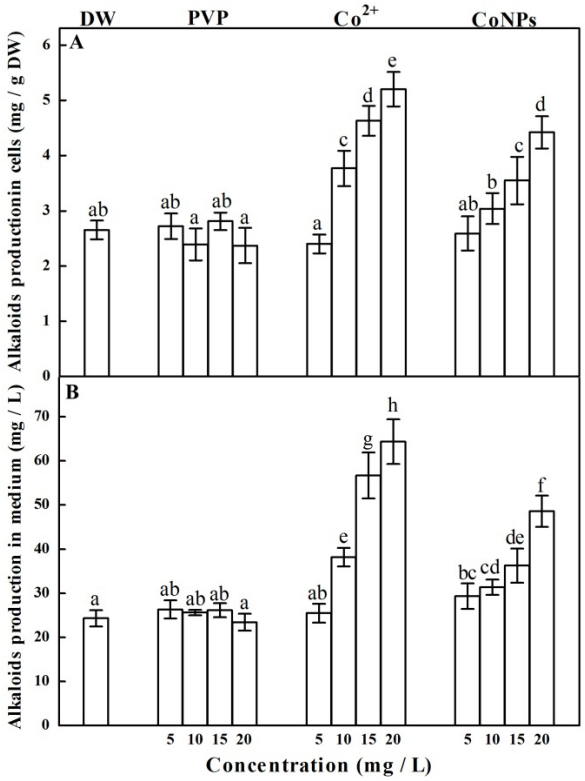

Figure 6. Effect of different concentrations of PVP, Co2+ and CoNPs on total alkaloids accumulation in C. roseus suspension cells (A) and medium (B). One-week-old cultures were subjected to 72 h treatments. Bars with different letters are significantly different, according to the LSD test, at $P \leq 0.05$. Means + SDs, n = 3.

2.7 and 24.3 in terms of mg/g dry weight and mg/L medium, respectively (figure 6). Compared with control, no significant change was observed upon using Co^{2+} at 5 mg/L. Increase in Co^{2+} concentration was associated with significant dose-dependent increase in alkaloids content to reach 196.1 and 265.5% of controls related to dry weight and medium, respectively, at 20 mg/L. Regarding CoNPs treatments, increase in alkaloids content in dry tissues was restricted to 15 and 20 mg/L. On the other hand, alkaloids accumulation in growth medium increased steeply in response to increasing concentrations of CoNPs reaching about 2 folds of control at 20 mg/L. Compared with CoNPs, cobalt ions were significantly more efficient in enhancing alkaloids content at all used concentrations except at 5 mg/L where insignificantly different results were recorded following application of any cobalt form.

Discussion

Cobalt is a trace element in most plant tissue culture media (39); low level of cobalt ion enhances plant growth through different mechanisms (40-41). However, exceeding a threshold concentration of such element results in oxidative stress as indicated by appearance of oxidative stress markers (eg: MDA) (19). Enhancement in ROS formation, associated with stress conditions, results in decrease in biomass accumulation attributed to the damaging role played by ROS on biomolecules (eg: nucleic acids, proteins and lipids). This damage leads to inhibition of cell proliferation and protein synthesis, loss of enzymatic activities, alteration in membrane characteristics, and finally cell death (42). As a defense response, MAPKs genes are up regulated (43-44) which in turn stimulates expression and activities of antioxidant enzymes (45) as well as genes involved in alkaloids biosynthesis (27-28) resulting in accumulation of such secondary metabolites in tissues and growth medium.

The previous scenario is easily recognized in results of this investigation following cobalt treatments with some differences, characteristic for each cobalt form. For Co^{2+} treatments, the positive correlation between cobalt concentration and MDA content in addition to the negative correlations between both cobalt concentration and MDA content on one hand and fresh and dry weights on the other hand describe growth retardation accompanied with the onset of oxidative stress following Co^{2+} treatments.

Similar to our results, Karuppanapandian and Kim (2013) recorded significant enhancements in MDA content associated with growth retardation in *Brassica juncea* seedlings following exposure to $CoCl_2$ at 100 µM. Increase in MDA content following Co^{2+} treatment was also recorded in *Ocimum basilicum* plants (20). In harmony with our results, Amarasinghe (2009) recorded decrease in rate of callus proliferation in four rice cultivars growing on media containing 10 mg/L of cobalt chloride. In the same context, Javed and Anis (2015) demonstrated decrease in shoot length of *Erythrina variegate in vitro* plants at 100 and 150 µM of cobalt chloride.

Results of this investigation reflected positive correlations between Co^{2+} concentration and MDA content on one hand and expression of CrMPK3 gene (in 0.5 and 4 h treatments) on the other hand. Such correlations may reflect a role played by CrMPK3 gene in cobalt-induced stress signaling. This role is supported by the positive correlations between gene expression and antioxidant defense mechanisms represented by SOD and APX in addition to alkaloids accumulation.

Association between MAPK induction and enhancements in activities of antioxidant enzymes was recorded following cobalt treatment in *Brassica juncea* (19) as well as ABA and H_2O_2 treatments in maize (45) and rice (47). SOD and APX are known for their role in scavenging ROS generated in the course of oxidative stress (48). Enhancements in these enzymes were recorded following cobalt treatments in mung bean (49), Spinach (50), groundnut (51), *Brassica juncea* (52).

Increase in alkaloids accumulation underlined with enhancements in expression of CrMPK3 gene was demonstrated following wounding and UV as well as methyl jasmonate treatment in two-months-old *in vitro* grown *C. roseus* plants (27). The authors observed induction of gene expression within 30 min post treatment followed with decline in expression level to reach basal level 90 min later. Not far from our results, Pan *et al.* (2015) observed enhancement in alkaloids accumulation in roots of 3-month-old *C. roseus* plants following exposure to 300 µM of copper. Enhancements were preceded with significant increase in MPK3 expression level that detected 4 h following treatment.

Alkaloids accumulation recorded following stressful Co^{2+} treatments is a defense response against oxidative stress (13). It was recorded in *C. roseous* following exposure to different types of stress including heavy

metals (14-17), drought (53) and fungal attack (54).

Based on results of this study, attenuated oxidative stress; indicated with lower values for MDA content, and consequently better growth was observed following replacement of Co^{2+} with CoNPs at all used concentrations. In contrast, Faisal *et al.* (2016) recorded significant higher growth retarding effects for Co_3O_4 nanoparticles (250-1000 mg/L), compared with bulk cobalt oxide, on eggplant seedlings derived from treated seeds. The disagreement with our results may be attributed to difference in concentration range used and nature of plant material.

The observed attenuation can be ascribed to PVP coating that may reduce Co^{2+} leaching from CoNPs. Similar effects for PVP were recorded in silver nanoparticles (56-57). This hypothesis is strengthened by the mitigated slow long-acting effect of CoNPs, compared with Co^{2+}, on induction of CrMPK3 expression. The exhaustion in gene expression was accompanied with decrease in defense stimulation, in terms of activity of antioxidant enzymes and alkaloids accumulation.

Statistical analysis revealed positive correlations between expression of CrMPK3 (in 0.5, 4 and 8 h treatments) and both of CoNPs concentration and MDA content. Also, positive correlations were recorded between gene expression and both of activity of antioxidant enzymes and alkaloids accumulation. Combining these correlations with corresponding ones recorded for Co^{2+} treatments suggests that CrMPK3, through different temporal expression profiles, is a common player in cobalt-induced stress signaling regardless cobalt form.

In conclusion, both Co^{2+} and PVP-coated CoNPs are able to induce oxidative stress provoking alkaloids accumulation in *C. roseus* suspension cultures with higher capabilities recorded for Co^{2+}. However, results revealed CoNPs as a novel tool in manipulating cobalt-induced alkaloids production in *C. roseus*. Enhancements in alkaloids biosynthesis employing different cobalt forms were underlined with different characteristic temporal expression profiles for CrMPK3 gene. Fast, short-lasting induction was accompanied with Co^{2+} treatments. On the other hand, exposure to CoNPs was associated with slow extended induction for CrMPK3 gene.

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