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Effects of exogenous nitric oxide on cadmium toxicity in black poplar (*Populus nigra*): physiological approaches

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Abstract – Cadmium (Cd) is a highly toxic metallic contaminant that negatively affects plant metabolism and causes reductions in productivity. Nitric oxide (NO) is a signaling molecule that regulates various physiological processes and is involved in response to biotic/abiotic stresses. This work investigated the effects of exogenous sodium nitroprusside (SNP), a nitric oxide (NO) donor, application on Cd toxicity in black poplar (*Populus nigra*). Black poplars were exposed to individual/combined $CdCl_2$ and SNP treatments for 21 days by complete randomized design with three replications. Cd concentrations increased in leaves, bark, and roots at Cd treatments, whereas Cd + SNP applications had alleviative effects on Cd exposures, except for leaves. Photosynthetic pigments (chlorophyll *a*, *b*, *a* + *b* and carotenoids) reduced with Cd treatments in leaves, while they increased in Cd + SNP applications. Similarly, plant biomass was reduced with Cd treatments, but Cd + SNP application prevented these reductions. SNP also alleviated malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) accumulation in leaves under Cd treatments. Catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11) activities were also affected by Cd and Cd + SNP applications. Cd exposure also decreased Zn^{2+}, Fe^{2+} and Mn^{2+} levels in leaves, bark and roots, while it increased Cu^{2+} level in leaves and roots. This study concludes that Cd toxicity caused a reduction of plant growth and mineral nutrition parameters. However, SNP indicates great potentials for improving the growth under Cd toxicity in *P. nigra*.

Keywords: antioxidant enzyme, growth inhibition, heavy metal stress, mineral nutrition, sodium nitroprusside

Introduction

Cadmium (Cd) is a non-essential trace metal and it is also one of the most toxic elements adversely affecting plant growth and development. It is accumulated in soil as a result of anthropogenic activities such as traffic emissions, mining, and agricultural practices like waste water irrigation, excessive fertilizer use and pesticide application to sewage sludge (White and Brown 2010, Tran and Popova 2013). Cd is toxic even at very low concentrations; it is taken up by roots with similar charged metal (Fe, Zn, Mn) ion transporters and loaded into xylem for transport to leaves (Schützendübel and Polle 2002, Verbruggen et al. 2009). Three major mechanisms are involved in plant response to Cd toxicity, such as the production of free radicals and reactive oxygen species (ROS), thiol groups contained in cysteine residues of proteins, and the competition among Cd and some mineral nutrients containing similar chemical properties (Schützendübel and Polle 2002, Paradiso et al. 2008). Its toxicity causes damage to root structure, limits nutrient absorption and translocation, decreases chlorophyll content and plant biomass, leads to leaf chlorosis and withering, and negatively affects stomatal opening, conductance and root water conductivity (Vandecasteele et al. 2003, Benavides et al. 2005, Wang et al. 2008). On the other hand, to reduce the toxic effects of Cd, plants employ two major mechanisms: (i) limiting Cd uptake by binding and sequestrating it to biomolecules, and (ii) increasing antioxidant enzyme activities such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11),

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glutathione peroxidase (GPX, EC 1.11.1.9), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and glutathione reductase (GR, EC 1.6.4.2) (Dixit et al. 2001, Gill and Tuteja 2010). In plants, thiol-containing chelating ligands such as glutathione (GSH) and phytochelatins (PCs) are used for transport of Cd-complexes into the vacuole or in the apoplast (Wolf et al. 1996, Cobbett 2000). Other low-molecular-weight cysteine (Cys) rich peptides, such as metallothioneins (MTs), also play important roles in the binding of metal ions like Cd and thus contribute to Cd detoxification from the cytosolic environment (Cobbett 2000).

Nitric oxide (NO) is a hydrophobic gaseous free radical and plays important roles in cell signaling, various physiological processes and response to biotic/abiotic factors (Arasimowicz and Floryszak-Wieczorek 2007). Plants have four types of enzymes involved in NO production (Gill et al. 2013), such as cytosolic nitrate reductase (NR, EC 1.6.6.1), plasma-membrane (PM)-nitrite: NO reductase (Ni:NOR), nitric oxide synthase (NOS, EC 1.14.13.39) and xanthine dehydrogenase (XDH, EC 1.1.1.204). NO regulates antioxidant enzyme machinery at gene expression and activity levels, resulting in an increase or decrease of the cellular redox status of cells under stress conditions (Groß et al. 2013). NO also interacts with other signaling molecules and plant hormones, including jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), the ethylene signaling pathway and hydrogen peroxide (H_2O_2; Neill 2007, Gill et al. 2013).

The genus *Populus* includes five sections with about 30–40 species distributed all around the worldwide, but mainly in the Northern Hemisphere (Polle and Douglas 2010). Previous studies projected that different clones of the same poplar species may show different heavy metal tolerance due to genetic variations (Castiglione et al. 2009). For example, He et al. (2013) reported various Cd tolerance levels and physiological changes among six poplar species. Gaudet et al. (2011) reported intraspecific variations of physiological and molecular response under Cd-stressed *P. nigra* trees. In another paper, Lomaglio et al. (2015) reported physiological variations at photosystem II (PSII) quantum yield, H_2O_2 generation and hormone levels from short-term Cd-stressed *P. nigra* trees. Accordingly, in this work an endeavour was made to understand the effects of exogenous sodium nitroprusside (SNP) that provides nitric oxide (NO) at physiological activities and mineral nutrition of black poplar (*P. nigra*) under different Cd treatment levels.

Materials and methods

Plant materials and treatments

Cuttings (~30 cm length × 1 cm diameter) from black poplar (*P. nigra* genotype Gazi) were obtained from the Poplar and Fast Growing Forest Trees Research Institute, Izmit, Turkey. At least 18 cuttings with sprouts were planted and rooted in perlite medium. After 10 weeks, these cuttings were transplanted at a rate of one plant per pot filled with 3 liters of perlite. To enhance acclimation in root zone, modified Hoagland solution was used for watering the plants. For this purpose, the poplar cuttings were grown two weeks by using one-quarter strength (1/4 rate dilution of full-strength modified Hoagland solution), one week by one-half strength (dilution of 1/2 rate of full-strength modified Hoagland solution) and one week full-strength modified Hoagland solutions each day. The full-strength modified Hoagland solution consisted of 5 mM $Ca(NO)_2{\times}4H_2O$, 5 mM KNO_3, 2 mM $MgSO_4{\times}7H_2O$, 1 mM KH_2PO_4, 45.5 μM H_3BO_3, 44.7 μM $FeSO_4{\times}7H_2O$, 30.0 μM NaCl, 9.1 μM $MnSO_4{\times}H_2O$, 0.77 μM $ZnSO_4{\times}7H_2O$, 0.32 μM $CuSO_4{\times}5H_2O$, 0.10 μM $(NH_4)_2Mo_7O_{24}4H_2O$ and 54.8 μM $Na_2EDTA{\times}2H_2O$ adjusted to pH 6.0. For Cd treatment, 18 acclimatized poplar cuttings with similar heights were selected and divided into two groups, including with and without sodium nitroprusside (SNP) (Sigma Aldrich St. Lois, MO, USA) treatments. Poplar cuttings were exposed to six treatments during 21 days, including (i) control (full-strength modified Hoagland solution), (ii) addition of 100 μM $CdCl_2$, (iii) addition of 500 μM $CdCl_2$, (iv) addition of 100 μM SNP, (v) addition of 100 μM $CdCl_2$ and 100 μM SNP, and (vi) addition of 500 μM $CdCl_2$ and 100 μM SNP dissolved in full-strength modified Hoagland solution. The experiment was designed as a complete randomized factorial design with three replications.

Three weeks after treatments, leaves in cuttings were carefully harvested and washed in tap water, after which samples were rinsed three times with de-ionized water. Similarly, root and bark in cuttings were removed, cleaned and washed. All leaf, bark and root tissues were oven-dried at 70 °C for at least three days, and dry weight (DW) was immediately determined.

Photosynthetic pigment analysis

Fresh leaf samples were taken from the youngest fully expanded leaves before harvest. Then, they (500 mg) were homogenized using a homogenizer (Heidolph, Diax 900) with added 10 mL of acetone (90% v v^{-1}). The absorbance of extracts was measured at 663, 645 and 470 nm wavelengths using a spectrophotometer (Shimadzu UV-1201; Tokyo). Chlorophyll *a, b, a + b,* and carotenoid amounts and the chlorophyll *a/b* ratio were calculated according to the Lichtenthaler (1987) formula.

Cd and nutrient ion analysis

All leaf, bark and root tissues were digested using the dry-ashing method in a muffle furnace at 500 °C for 6 hours (Miller 1998). Then, the concentrations of Cd and metal ions, iron (Fe), zinc (Zn), manganese (Mn), and copper (Cu) in leaf, bark and root tissues were measured by using inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer Optima 2100 DV, Waltham, MA).

H_2O_2 and lipid peroxidation estimation

H_2O_2 levels in fresh leaf samples were calorimetrically estimated as described by Mukherjee and Choudhuri (1983). Firstly, fresh leaf samples were extracted with cold acetone. An aliquot (3 mL) of extracted solution was mixed with 1 mL

0.1% titanium dioxide in 20% (v:v) H_2SO_4. Subsequently, the mixture was centrifuged at 6000 x *g* for 15 min. The intensity of yellow supernatant was measured at 415 nm. The concentration of H_2O_2 was calculated from a standard curve plotted with the range of 100–1000 nmol H_2O_2 and expressed as μmol g^{-1}, fresh weight. The amount of lipid peroxidation in fresh leaves was identified by measuring malondialdehyde (MDA), a major thiobarbituric acid reactive species (TBARS) and product of lipid peroxidation (Hodges et al. 1999). MDA concentration was calculated by means of an extinction coefficient of (ε = 155 mM^{-1} cm^{-1}).

Extraction and assaying enzyme activities

All enzymatic measurements were performed at 0–4 °C. Fresh leaf samples were homogenized with a homogenizer (Heidolph, Diax 900) in 5 mL of 100 mM potassium phosphate buffer (pH 7.5), including 1 mM EDTA-Na_2 and 0.5 mM ascorbate. Then, homogenate was centrifuged at 10,000 × *g* for 5 min. Supernatant was used as crude enzyme extract to analyze the activities of catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11). All colorimetric measurements with enzyme activities were made at 25 °C using a spectrophotometer (Shimadzu UV/VIS1201). Enzyme activities were expressed as units per gram fresh weight of tissue.

APX activity was determined by measuring the decrease in absorbance at 290 nm for 1 min in 2 mL reaction mixture, including 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA–Na_2, 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 50 mL crude enzyme extract (Nakano and Asada 1981). APX activity was calculated using extinction coefficient of (2.8 mM^{-1} cm^{-1}).

CAT activity was determined by measuring the decrease in absorbance at 240 nm for 1 min following decomposition of H_2O_2 (Cakmak et al. 1993). The reaction mixture (3 mL) consisted of 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2 and 50 m L crude enzyme extract. CAT activity was calculated using extinction coefficient (40 mM^{-1} cm^{-1}) for H_2O_2.

Statistical analyses

Statistical analysis was done with ANOVA using the MINItab package program (Minitab Corp., State College, PA). Multiple means comparisons between Cd treatments were analyzed using Duncan's Multiple Range Test at significance level (α = 0.05). The levels of significance are represented as * at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$, and ns – non-significant.

Results

Biomass and Cd accumulations

In this study, Cd exposure mostly negatively affected leaf and bark dry weights (DW) in black poplar trees, while 100 μM Cd + SNP and 500 μM Cd + SNP applications caused an increase in leaf biomass compared to Cd treatments as well as 500 μM Cd + SNP application in bark biomass (Tab. 1). SNP treatment increased DWs in plant leaves by 40.5% at 100 μM Cd and by 36.4% at 500 μM Cd following 100 μM Cd + SNP and 500 μM Cd + SNP treatments, respectively. The reduction in root DWs under Cd exposure was not found statistically significant, except at 500 μM Cd + SNP treatment.

In all plant tissues, Cd levels were significantly increased depending on applied concentrations (Tab. 1). Their increases in leaves, bark and roots respectively were about 35-, 16- and 215-fold at 100 μM Cd, and 39-, 15- and 242-fold at 500 μM Cd treatments in comparison to controls. However, the levels in bark and roots decreased by 29.0% and 5.3% respectively at 100 μM Cd + SNP compared to 100 μM Cd treatment, and by 51.7% and 21.6% at 500 μM Cd + SNP treatments compared to 500 μM Cd treatment. On the other hand, leaf Cd content increased by 77.8% at 100 μM Cd + SNP treatment in comparison to 100 μM Cd treatment, and by 86.3% at 500 μM Cd + SNP treatment in comparison to 500 μM Cd treatment. Although SNP application alleviated Cd content in *P. nigra* barks and roots, it was not effective in the plant leaves.

Tab. 1. Changes in biomass accumulation and total Cd content in leaf, bark and roots of black poplar plants exposed to individual Cd, sodium nitroprusside (SNP) and combined Cd + SNP treatments for 21 days. Values are the mean of three replicates (means ± SE, n= 3). Different letters in the same column denote significantly different means according to Duncan's multiple range test (α = 0.05). For F-test the levels of significance are represented as * at $P < 0.05$, ** at $P < 0.01$, *** at $P < 0.001$ DW – dry weight.

SNP (μM)	Cd (μM)	Dry weight (g $plant^{-1}$)			Cd content (μg g^{-1} DW)		
		Leaf	Bark	Root	Leaf	Bark	Root
0	0	3.37±0.16 ab	0.98±0.03 a	0.91±0.05 a	1.2±0.1 d	2.9±0.2 d	3.35±0.20 c
	100	2.58±0.15 cd	0.90±0.07 ab	0.69±0.06 ab	42.7±0.6 c	47.3±2.3 a	721.7±18.9 ab
	500	2.14±0.07 d	0.70±0.03 b	0.70±0.02 ab	46.8±0.9 c	43.5±3.2 a	811.0±59.5 a
100	0	3.40±0.11 ab	1.03±0.07 a	0.88±0.12 a	2.7±0.2 d	3.0±0.1 d	5.27±0.42 c
	100	3.62±0.28 a	1.11±0.11 a	0.75±0.07 ab	75.9±3.0 b	33.6±4.3 b	683.5±39.9 b
	500	2.92±0.17 bc	0.99±0.07 a	0.57±0.05 b	87.2±2.4 a	21.0±2.3 c	635.9±47.7 b
F-test significance		10.8***	4.1*	3.2*	477.7***	58.1***	105.9***

Photosynthetic pigments

Chlorosis is a common symptom of Cd toxicity. In this work, content of photosynthetic pigments was notably decreased upon Cd exposure (Tab. 2). The reductions in chlorophyll *a*, *b*, *a* + *b*, and carotenoid were 53.4%, 61.8%, 55.1% and 53.9% in 100 μM Cd-treated plants, and 65.7%, 72.9%, 67.2% and 63.3% in 500 μM Cd-treated plants, respectively. However, with 500 μM Cd + SNP application, chlorophyll *a*, *b*, *a* + *b* and carotenoid contents significantly increased as 2.50-, 2.87-, 2.56- and 2.35-fold at 500 μM Cd exposure, respectively. Also, chlorophyll *a*, *b*, *a* + *b* and carotenoid contents were decreased with 100 μM SNP exposure in comparison to the controls as a result of being toxic to plants depending on the level in growth media. Increasing the level of Cd toxicity remarkably enhanced the chlorophyll *a*/*b* ratio (Tab. 2). Moreover, chlorophyll *a*/*b* was decreased with 500 μM Cd + SNP application in comparison to 500 μM Cd treatments. In this study, SNP exposures were noted to have ameliorative effects on the photosynthetic pigments of Cd-exposed *P. nigra*.

MDA and H_2O_2 content

In this work, MDA contents were increased at all levels of Cd exposures (Fig. 1A). Lipid peroxidation significantly elevated by 17.3% and 34.3% at 100 and 500 μM Cd exposures respectively. However, its levels also decreased by 13.1% and 22.8% at 100 and 500 μM Cd + SNP treatments in comparison to 100 and 500 μM Cd, respectively. So, it appeared that SNP treatment attenuates the deleterious effect of Cd exposure in poplar plants. In parallel to MDA, H_2O_2 levels also increased under Cd exposure in black poplars (Fig. 1B). However, H_2O_2 content decreased by 14% with 500 μM Cd + SNP exposure in comparison to 500 μM Cd treatment.

Antioxidative enzyme activities

In this study, CAT activity notably increased at 500 μM Cd exposure; however, the changes in APX activity with all levels of Cd exposure were not found significant statistically (Fig. 2). Besides, CAT activity in black poplar was significantly decreased by 100 and 500 μM Cd + SNP applications compared to 100 and 500 μM Cd treatments (Fig. 2A). However, the APX activity was notably enhanced in leaves, 2.34- and 1.91-fold at 100 and 500 μM Cd + SNP treatments in comparison to 100 and 500 μM Cd applications, respectively (Fig. 2B).

Mineral nutrition

The concentrations of mineral nutrients Fe, Zn, Mn and Cu in poplar leaves, bark, and roots were influenced by Cd

Tab. 2. Changes in contents of photosynthetic pigments in leaves of black poplar plants exposed to individual Cd, sodium nitroprusside (SNP) and combined Cd + SNP treatments for 21 days. Values are the mean of three replicates (means ± SE, n= 3). Different letters in the same column denote significantly different means according to Duncan's multiple range test (α = 0.05). For F-test the levels of significance are represented as * at P < 0.05, ** at P < 0.01, *** at P < 0.001 FW – fresh weight

SNP (μM)	Cd (μM)	Chlorophyll *a*	Chlorophyll *b*	Chlorophyll *a*+*b*	Carotenoid	Chlorophyll *a*/*b* ratio
		(mg g^{-1} FW)				
0	0	0.959±0.024 a	0.251±0.009 a	1.211±0.033 a	0.603±0.016 a	3.82±0.08 d
	100	0.447±0.023 d	0.096±0.003 d	0.544±0.024 d	0.278±0.007 d	4.64±0.27 ab
	500	0.329±0.003 e	0.068±0.001 e	0.397±0.002 e	0.221±0.004 e	4.79±0.07 a
100	0	0.886±0.003 b	0.211±0.004 b	1.097±0.007 b	0.561±0.008 b	4.19±0.07 cd
	100	0.792±0.012 c	0.175±0.001 c	0.968±0.011 c	0.488±0.002 c	4.52±0.09 abc
	500	0.823±0.026 c	0.195±0.013 bc	1.018±0.040 c	0.519±0.002 c	4.25±0.15 bcd
F-test significance		200.3***	103.1***	189.4***	189.9***	6.2**

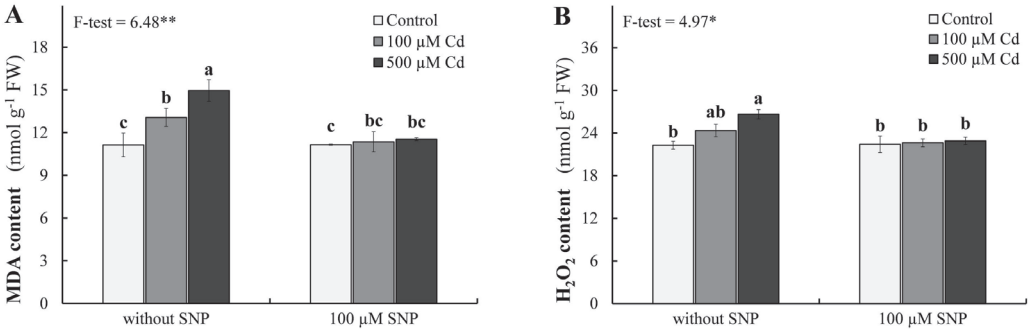

Fig. 1. Malondialdehyde (MDA; A) and hydrogen peroxide (H_2O_2; B) contents in leaves of black poplar exposed to Cd (0, 100, and 500 μM), sodium nitroprusside (SNP) and combined Cd + SNP (100 μM +100 μM or 500 μM +100 μM) for 21 days. Bars indicate means of three replicates ± SE. Different letters on the bars indicates significant differences according to Duncan's multiple range test (α = 0.05).

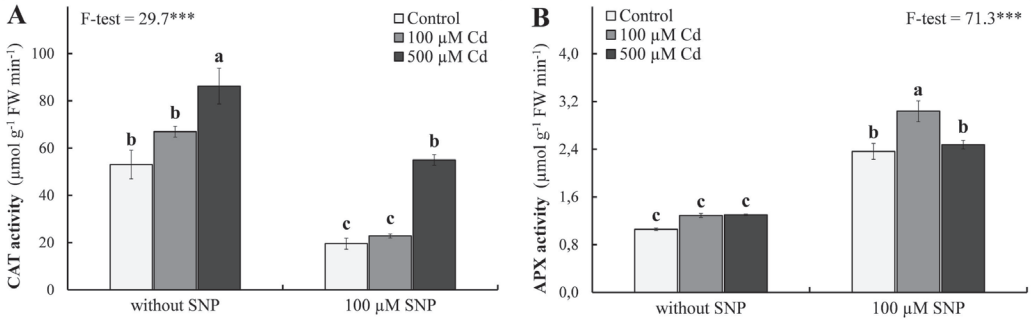

Fig. 2. Catalase (CAT; A) and ascorbate peroxidase (APX; B) activities in leaves of black poplar exposed to Cd (0, 100, and 500 µM), sodium nitroprusside (SNP) and combined Cd + SNP (100 µM + 100 µM or 500 µM + 100 µM) for 21 days. Bars indicate means of three replicates ± SE. Different letters on the bars indicates significant differences according to Duncan's multiple range test (α = 0.05).

exposure and SNP treatments, except for Cu concentrations in bark (Tab. 3). Cd treatments significantly reduced Fe, Zn and Mn levels in leaves, bark and roots but increased Cu levels in leaves and roots. However, Fe concentrations in leaves, bark, and roots, Mn concentrations in bark and roots were remarkably enhanced with Cd + SNP applications in comparison to Cd treatments. Cd exposure notably decreased Zn concentrations in all plant parts, and also Zn concentrations in leaves, bark, and roots were significantly reduced with Cd + SNP treatments in comparison to Cd treatments. In comparison with control, Cu concentrations in root were increased 100 and 500 µM Cd treatments while the increments in leaf Cu concentration were found significantly with only 500 µM Cd treatments. Otherwise, Cu concentration in leaves decreased with 500 µM Cd + SNP treatment compared to 500 µM Cd treatment, but Cu concentration in

Tab. 3. Changes in concentrations of Fe, Zn, Mn and Cu in leaf, bark and roots of black poplar plants exposed to individual Cd, sodium nitroprusside (SNP) and combined Cd + SNP treatments for 21 days. Values are the mean of three replicates (means ± SE, n= 3). Different letters in the same column denote significantly different results according to Duncan's multiple range test (α = 0.05). For F-test the levels of significance are represented as * at P < 0.05, ** at P < 0.01, *** at P < 0.001, and ns – non-significant.

SNP (µM)	Cd (µM)	Concentrations (µg g^{-1})			
		Fe	Zn	Mn	Cu
		Leaves			
0	0	79.09±3.11 d	30.65±0.73 a	42.59±1.29 ab	8.08±0.84 c
	100	56.79±1.57 e	21.08±0.18 c	36.41±1.41 c	7.02±1.29 c
	500	49.32±0.24 f	27.12±0.32 b	37.60±0.86 bc	24.61±2.16 a
100	0	116.96±1.55 a	28.07±0.69 b	47.33±2.70 a	9.05±0.15 c
	100	106.00±0.09 b	19.12±0.35 d	41.93±1.25 abc	4.00±0.22 c
	500	99.88±0.48 c	18.85±0.90 d	40.96±2.91 bc	17.69±3.37 b
F-test significance		324.7***	73.8***	4.2*	19.7***
		Bark			
0	0	29.32±1.80 c	30.49±1.03 a	18.33±0.47 a	5.14±0.37
	100	20.51±0.64 d	21.86±0.70 b	12.21±0.05 c	4.81±0.26
	500	25.08±0.47 cd	19.20±0.05 c	12.13±0.48 c	5.53±0.30
100	0	53.39±0.30 a	21.98±0.22 b	14.38±0.34 b	5.24±0.11
	100	54.22±0.77 a	15.24±0.32 d	15.34±0.49 b	5.51±0.47
	500	44.31±4.16 b	14.02±0.40 d	9.64±0.17 d	4.63±0.24
F-test significance		59.6***	111.9***	65.3***	ns
		Root			
0	0	447.4±12.2 c	44.64±2.25 a	49.10±1.52 c	10.35±0.19 c
	100	461.0±18.3 c	27.36±1.17 c	36.93±0.35 d	13.38±0.57 b
	500	302.3±3.5 d	35.70±1.11 b	33.90±2.35 d	12.74±0.79 b
100	0	1011.4±7.1 a	28.88±0.84 c	64.21±0.96 a	8.57±0.11 d
	100	942.3±27.8 a	17.65±0.19 d	60.23±5.32 ab	11.02±0.86 c
	500	736.1±103.0 b	29.55±0.69 c	53.18±1.15 bc	15.09±0.18 a
F-test significance		42.7***	54.7***	23.3***	18.4***

roots increased while changes in Cu concentrations in bark were not significant.

Discussion

In the current study, it was aimed to reveal alleviated effects of SNP exposure on Cd toxicity in black poplar. For this purpose, the black poplar was exposed to only Cd, only SNP, and combined Cd + SNP treatments. In this context, biomass accumulation was adversely affected by Cd exposure; in contrast, SNP treatment with Cd increased the biomass. In a similar study, total root DW in *P. nigra* was markedly lower at 200 μM Cd exposure (He et al. 2013). Dai et al. (2013) showed that Cd treatments decreased leaf, bark and root DWs at four Cd levels such as 10, 30, 50 and 70 μM. In two *P. nigra* genotypes, Cd treatment reduced leaf DW for genotype 58–861 whereas genotype Poli was not affected (Gaudet et al. 2011). NO plays crucial roles in ROS and hormone regulation (Lamattina et al. 2003). It also regulates gene expression patterns in signal transduction, transport, defense, cell death and ROS production/degradation (Palmieri et al. 2008). Besides, SNP, as NO donor, stimulates the electron transport through photosystem (PS) II (Ederli et al. 2009). Leaf biomass increments under SNP exposure may thus prove the crucial roles of NO in response to Cd toxicity. It is thought that increased biomass is closely correlated with the increase of chlorophyll content which promotes the photosynthesis.

Cd contamination is considered a major environmental problem due to chemical fertilizers, sewage wastewater irrigation, and rapid industrialization (Sanità di Toppi and Gabbrielli 1999). In this study, Cd exposure increased the Cd amounts in all plant tissues while SNP application generally decreased cadmium amounts. In *Populus* × *canescens* plantlets, Cd levels in roots, bark, wood, and leaves increased with different Cd exposures were found as roots > wood > bark > leaves (Dai et al. 2013). In another similar work, Cd amounts in *Populus* × *canescens* roots, wood, bark and leaves exposed to 50 μM $CdSO_4$ were increased for 1, 10 or 20 days (He et al. 2013). Gaudet et al. (2011) reported 22.5 and 33.9 mg kg^{-1} Cd levels at 50 μM treatment in leaves of the *P. nigra* Poli and 58–861 genotypes respectively. Those reported Cd concentrations were not significantly different than the findings of the present study. In *P. nigra* and *Salix alba* (willow), comparisons of Cd content at 50 μM revealed that black poplar roots contained ~2.4 fold higher Cd levels than those of willow, while by contrast, *S. alba* leaves had ~16 fold higher Cd concentrations than those of poplar (Zacchini et al. 2011). In six poplar species, mean Cd concentrations at 200 μM Cd exposure were found increased by 43-, 9-, 23-, and 25-fold in roots, wood, bark and leaves respectively (He et al. 2013). In this paper, Cd concentrations were also found significantly increased in root, leaves and bark without SNP applications, suggesting that these variations in different poplar species may be attributed to genomic background. Many studies also showed that endogenous NO plays a key role in regulation of Cd toxicity (Besson-Bard et al. 2009, De Michele et al. 2009, Gill et al. 2013). In this study, Cd + SNP exposures significantly decreased Cd accumulations in bark and roots compared to Cd treated plants. So, this reduction in Cd concentrations may be due to the positive signalling effects of NO in cellular metabolism.

Cd is considered one of the heavy metals most toxic to plant metabolic pathways. When plants are exposed to Cd toxicity, growth inhibition, necrosis, chlorosis, leaf rolling or drying symptoms are generally observed (Cosio et al. 2006, Amani 2008). Baszyński et al. (1980) showed that Cd disturbs the biosynthesis of chlorophyll and carotenoids. It negatively affects chloroplast metabolism, including chloroplast replication and cell division (Prasad, 1995, Baryla et al. 2001). In *Populus* × *canescens* plantlets, chlorophyll *a*, *b*, *a* + *b* and carotenoid were reported to be decreased at 50 μM Cd exposure (He et al. 2013). The same authors reported that upon 200 μM Cd exposure, chlorophyll *a* and *b* decreased in three poplar species but also found no statistical significance. These reports were also in parallel with the present findings. In *Arabidopsis*, SNP treatments induced the expression of 614 genes out of which 579 genes were upregulated, while 35 genes were down-regulated. Most of the over expressed genes play important roles in biotic and abiotic stress responses (Ahlfors et al. 2008). In our study, the recovery of photosynthetic pigments could be affected by gene expression related with abiotic stress responses by NO induced signaling in black poplar.

MDA, which is an indicator for membrane lipid oxidation, is used to evaluate plant tolerance to abiotic stresses. He et al. (2013) reported that MDA concentrations are not significantly affected by Cd exposure but some species-specific differences were present in six poplar species. In the same study, the highest MDA concentrations were also reported in *P. nigra* bark and leaves. In another study, MDA was increased by Cd treatments but no significant difference was detected in plants treated for four days, whereas eight and 12 day *P. yunnanensis* plants showed high MDA levels (Yang et al. 2015). It can be proposed that genotypic variations and the ameliorative effect of NO may cause a reduction of MDA amounts in response to oxidative stress.

Hydrogen peroxide (H_2O_2), a form of reactive oxygen species, is considered a common cellular metabolite. It is continually generated in enzyme and non-enzyme pathways in plants. Also, it plays important roles in cell signaling, including various physiological and biochemical processes in plants (Barba-Espín et al. 2011). He at al. (2013) reported that H_2O_2 concentrations in leaves were elevated by 31% in *P. cathayana* and by 41% in *P. nigra* under Cd treatment. In *P. yunnanensis*, H_2O_2 levels were elevated with an increase of exposure days (Yang et al. 2015). In plantlets of *Populus* × *canescens* (*P. tremula* × *P. alba*), H_2O_2 and MDA levels were significantly increased in root, followed by wood, bark and leaf (Dai et al. 2012). These results were corroborated by the findings of this work that H_2O_2 and MDA levels were increased under Cd exposure. Kopyra and Gwóźdź (2003) reported that exogenous NO reduces the devastating effect of heavy metals, ethylene and herbicides on plants. So, ROS

productions are suppressed and oxidative damage is limited during stress conditions. Also, NO may directly prevent the production of the superoxide anion ($O_2^{\cdot -}$) which results in an abrogation of toxic effects by the conversion of $O_2^{\cdot -}$ into peroxonitrite ($ONOO^-$; Neill et al. 2003). In this study, the reductions in MDA and H_2O_2 levels by SNP treatment in comparison to control may prove the efficacy of the physiological functions of NO in black poplar.

ROS as toxic by-products are considered to cause oxidative damage in cells (Suzuki and Mittler 2006). ROS can be generated by different pathways such as imbalance of the electron transport chains in both chloroplasts and mitochondria (Hernandez et al. 1995). Anti-oxidative enzymes play crucial roles in ROS detoxification under various abiotic and biotic stress conditions (Pandhair and Sekhon 2006). He et al. (2013) reported that APX activity was markedly enhanced, by 59%, in *P. deltoides* wood but APX was significantly inhibited by 43–49% in leaves of four poplar species (*Populus* × *euramericana*, *P. alba* × *P. glandulosa*, *P. nigra* and *P. popularis*) under Cd exposure. CAT activities were found higher in poplar roots and bark but lower in leaves exposed to 200 μM Cd. In hybrid poplar (*P. nigra* × *maximowitzii* × *P. nigra* var. *italica*), CAT and SOD activities were significantly higher at a 10^{-5} M Cd treatment, whereas the increase was not significant at a 10^{-4} M Cd treatment (Nikolić et al. 2008). In *Populus* × *canescens*, CAT activities were increased after 1 day Cd exposure in roots and leaves, and then decreased more strongly for 10 and 20 days in leaves. APX activities declined during Cd exposure in roots and leaves for 1, 10 or 20 days (He at al. 2013). In hybrid poplar (*Populus* × *canescens*), CAT and APX activities in leaves were significantly enhanced at 10 μM Cd treatment but also declined significantly according to the level of Cd exposures (30, 50, or 70 μM Cd) at 28 days (Dai et al. 2012). Yang et al. (2015) reported significant increases in APX, CAT and SOD activities under increasing Cd stress durations (0, 4, 8, and 12 days) in *P. yunnanensis*. In *P. tremula,* APX activity increased slightly on days 7 and 14 with Cd exposure in leaves, but it decreased rapidly at day 28 and reached a low on day 56 (Kieffer et al. 2009). To alleviate/eliminate Cd toxicity, plants induce many regulatory networks, including modulation of transcription factors, activation of metal transporters and biosynthesis of chelating compounds (DalCorso et al. 2010). In the light of the present findings about antioxidative enzyme activities and other available reports, it could be suggested that NO as signaling molecule by SNP treatment may induce different scavengers, including protein or non-protein antioxidants for ROS balance under Cd toxicity in *P. nigra*.

In the soil-plant relationship, Cd may mainly affect physiological processes and biochemical mechanisms by changing the concentration and functions of mineral nutrients. The interaction of Cd with the nutrient elements has been shown (Khan et al. 2007). Sarwar et al. (2010) reported that chemical similarity between Zn and Cd is a major reason for Cd toxicity in higher plants due to their interactions with each other. In this study, the decline in the Zn level may be attributed to the interactions of these metals. Several plant transporters are known to be responsible for the metal transport into the cytosol, such as zinc regulated transporter (ZRT), iron-regulated transporter (IRT)-like protein (ZIP), natural resistance-associated macrophage protein (NRAMP) family and oligo peptide transporters (OPTs) family. ZRT and ZIP families also transport other divalent metals, including Zn^{2+}, Mn^{2+} and Cd^{2+} (Colangelo and Guerinot, 2006, Vatansever et al. 2015, Vatansever et al. 2016). Overall, decline in divalent metal ions under Cd exposure may result from competition between Cd and other divalent ions, where Cd could suppress the influx of available divalent metal ions into cells. In addition, it has been determined that Cd + SNP treatment generally improves mineral nutrition in black poplar, demonstrating the positive effects of SNP for mineral nutrition.

Conclusion

The present work has investigated the effects of exogenous SNP treatments on Cd toxicity in *P. nigra*. It was revealed that Cd exposure causes reductions in growth parameters and inhibits physiological activities. Leaf and bark dry weights and photosynthetic pigments (chlorophyll *a*, *b*, *a* + *b*, and carotenoids) were decreased by Cd exposure. In addition, levels of MDA and H_2O_2 content, as stress indicators, were increased under Cd treatments and antioxidative enzyme activities such as those of CAT and APX also increased. The treatment also causes a decline in the influx of divalent metal ions like Fe, Zn and Mn. However, SNP applications alleviated the symptoms of Cd toxicity on growth, mineral nutrition and physiological activities, and enhance plant stress tolerance. Exogenous SNP application, accordingly, could be an efficient tool to cope with Cd toxicity in black poplar species.

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