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Nitric oxide enhances the adaptive responses of lupine plants against heavy-metal stress

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Abstract

The present work investigates the possible protective role of nitric oxide (NO) against heavy metals stress in *Lupinus termis* L. plants. A greenhouse experiment was conducted as a 2 x 2 factorial design with five replications. Lupine seedlings were treated with two concentrations (0.4 mM and 0.6 mM) of sodium nitroprusside (SNP as an NO donor) and subjected to high levels of nickel (Ni) sulfate (100 and 150 mM). The toxic effects of Ni on *L. termis* plants were evaluated by measuring the oxidative stress markers such as lipid peroxidation, hydrogen peroxide and electrolyte leakage. In addition, growth parameters, endogenous phytohormones, photosynthetic pigment, calcium, magnesium and Ni accumulation in lupin plants grown under Ni toxicity in the presence or absence of NO were also determined. Carbohydrate, organic acid and proline contents were also measured to determine the possible NO-mediated defense strategies in lupine plants to resist Ni stress. The obtained results proved that, Ni toxicity resulted in significantly elevated levels of oxidative stress markers, as well as abscisic acid, jasmonic acid and proline contents, which are associated with significant reduction in growth parameters, photosynthetic pigments, carbohydrate, indole acetic acid and gibberellic acid contents compared with control plants. SNP treatments significantly alleviated the toxic effect of Ni on *Lupinus termis* L. and increased the amounts of proline, soluble sugars and polysaccharides in shoots and roots, which could be an induced defensive mechanism against heavy-metal stress. Treatment with 0.4 mM SNP was more effective in increasing lupine plants tolerance to Ni toxicity than the 0.6 mM SNP.

Keywords: *Lupinus termis*; nickel, oxidative markers; phytohormones; sodium nitroprusside.
Abbreviations: ABA_ Abscisic acid; DW_ dry weight; FW _fresh weight; GA_3_Gibberellic acid; IAA_indole acetic acid; JA_jasmonic acid; MDA_malondialdehyde; NED_N-naphthyl-ethylenediamine; NO_nitric oxide; NOS_nitric oxide synthase; ROS_reactive oxygen species; SNP_sodium nitroprusside; TBA_thiobarbituric acid ; TCA_trichloroacetic.

Introduction

Nitrogen monoxide or nitric oxide (NO) is a unique ubiquitous signaling molecule in animals and plants (Wendehenne et al., 2001). NO is produced in plants through different pathways: enzymatically by nitrate reductase, peroxidases and/or xanthine oxidoreductases and putative nitric oxide synthases; and non-enzymatically through, for example, nitrite reduction by ascorbic acid at a low pH or by carotenoids in light (Wendehenne et al., 2001; Belligni et al., 2001; Wendehenne et al., 2004; Desikan et al., 2004; Zemojtel et al., 2006). NO also plays vital roles in diverse physiological functions in plants, such as the induction of seed germination and reduction of seed dormancy (Beligni and Lamattina 2000; Bethke et al., 2006, 2007; Libourel et al., 2006; Zheng et al., 2009), regulation of plant metabolism and senescence (Guo and Crawford, 2005), induction of cell death (Pedroso and Durzan, 2000), regulation of stomatal movement (García-Mata and Lamattina, 2001; Guo et al., 2003; Neill et al., 2003; Sakihama et al., 2003; Bright et al., 2006; García-Mata and Lamattina, 2007), regulation of photosynthesis (Takahashi and Yamasaki 2002) and the regulation of flowering (He et al., 2004), as well as in mitochondrial functionality (Zottini et al. 2002) and gravitropism (Hu et al., 2005). It is believed that NO can tightly interact with the plant hormonal system and may serve as a secondary messenger (Lamattina et al., 2003).

In addition to its regulatory roles in plant development, NO has been implicated in plant responses to a variety of abiotic and biotic stresses, such as drought (García-Mata and Lamattina, 2002), salt (Zhang et al., 2006a, b; 2007), heat (Uchida et al. 2002) and infection (Modolo et al. 2005; Floryszak-Wieczorek et al., 2007). Both biotic and abiotic stresses alter (promote or suppress) NO production, while externally applied NO donors enhance plant tolerance to specific stresses (García-Mata and Lamattina, 2002; Uchida et al., 2002; Zhao et al., 2007).

Although heavy metals are normal constituents of soils and occur naturally in the environment, the contamination of soils by toxic metals and metalloids is of major concern worldwide (Rascio and Navari-Izzo, 2011; Villiers et al., 2011). Their toxic influence on plants is largely a strong and fast inhibition of the

growth processes of the above- and underground parts, together with a decreased activity level of the photosynthetic apparatus, which is often correlated with progressing senescence-related processes (Molas, 2002; Sobkowiak and Dekert, 2003; Alaoui-Sossé et al., 2004; Lin et al., 2005). Growth inhibition and senescence stimulation, resulting from an excess of heavy metals are important effects (Maksymiec, 2007). In higher plants, heavy metals induce oxidative stress by the generation of superoxide radicals, hydrogen peroxide (H_2O_2), hydroxyl radicals and singlet oxygen, collectively termed as reactive oxygen species (ROS). ROS can rapidly attack all types of bio-molecules, such as nucleic acids, proteins and amino acids, which lead to irreparable metabolic dysfunction and cell death. ROS produced under stress are detrimental to growth because these molecules cause gradual lipid peroxidation and the inactivation of antioxidant enzymes (Teisseire and Guy, 2000). NO is a highly reactive molecule and, as a free radical, it scavenges other reactive intermediates. It can alleviate the oxidative stress in plants generated by abiotic stresses (Kopyra and Gwozdz, 2003; Neill et al. 2003; Guo and Crawford, 2005).

The present work investigated the capability of NO to protect *Lupinus termis* plants against nickel (Ni) stress and highlighted the defense mechanism(s) underlying the NO-induced tolerance.

Results

Endogenous NO content

In general, lupine plants accumulate higher amounts of NO in their roots than shoots. An increase in endogenous NO content was observed in both shoots and roots of *L. termis* when treated with 0.4 mM and 0.6 mM SNP, compared with water as the control. The amount of NO was directly proportional to the applied concentration of SNP.

Treatment with Ni sulfate (100 and 150 mM) caused significant increases in the NO content. The maximum increase was detected in response to 150 mM Ni sulfate and was calculated at 142.7% and 106.67% above the control value in shoots and roots, respectively (Table 1). Plants treated with both 150 mM Ni sulfate + 0.6 mM SNP produced the highest amounts of NO in both shoots and roots, compared with other treated plants. The increase was evaluated as 24.78% and 4.03% in shoot and 10.51% and 40.31% in root over those treated with 150 mM Ni sulfate or 0.6 mM SNP alone, respectively.

Growth parameters

Data illustrated in Table 2 indicated that the effect of exogenous NO applications on plant growth depended on the SNP concentration applied. The 0.4-mM SNP treatment caused a significant increase in all of the measured growth parameters, while the 0.6-mM SNP treatment decreased almost all of the measured growth parameters, including a significant reduction ($P < 0.05$) in shoot and root lengths calculated at 10.31% and 18.08%, respectively, less than control plants.

Ni toxicity (100 and 150 mM) dramatically reduced lupine growth. The detected toxic effect on growth was concentration dependent. The reduction in the measured growth parameters ranged from significant ($P < 0.05$) to extremely significant ($P < 0.001$) compared with the control values. Root growth in lupine was more affected than shoot growth by Ni stress, as shown by the 37.5% and 46.9% reductions in root length and root FW, respectively, in plants that received 150 mM Ni sulfate compared with control values. The growth of lupine plants treated with both SNP and Ni sulfate were less than those treated with the same concentration of SNP alone and greater than those treated with the corresponding concentration of Ni sulfate (Table 2).

An exogenous application of 0.4 mM SNP completely alleviated the inhibitory effect of 100 mM Ni sulfate on lupine growth.

Phytohormonal contents

The effects of SNP and Ni sulfate on the endogenous phytohormonal contents of lupine shoots and roots are illustrated in Table 3. Treatments with 0.4 mM SNP caused extremely significant increases in the endogenous pool of phytohormones (including IAA, GA_3, ABA and JA) in lupine plants. Such increases ranged from 11.76% for IAA to 18.62% for JA contents of shoots treated with 0.4 mM SNP, compared with control untreated plants. This stimulatory effect was pronounced in both shoots and roots. In contrast, the higher SNP concentration (0.6 mM) and both applied Ni sulfate concentrations (100 and 150 mM) had inhibitory effects on plant hormone levels. The maximum inhibition of endogenous phytohormones was detected in plants receiving 150 mM Ni sulfate and was evaluated in lupine shoots as 32.35%, 62.88%, 69.23% and 58.62% and in roots as 62.96%, 57.14%, 54.54% and 77.78% of IAA, GA_3, ABA and JA, respectively, compared with control values.

Photosynthetic pigment contents

Data presented in Table 4 show that SNP treatments (0.4 and 0.6 mM) have positive impacts on photosynthetic pigment accumulation in lupine plants. Chlorophyll a underwent a highly significant increase compared with in untreated control plants. In contrast, Ni stress (100 mM and 150 mM) caused the significant to extremely significant inhibitions of chlorophyll a and b, carotenoids and total pigment content when compared with those of the control. These inhibition levels were directly proportional to the applied concentration of Ni sulfate and were calculated as 79.47%, 62.04%, 79.27% and 75.82% lower in plants receiving 150 mM Ni sulfate for chlorophyll a, chlorophyll b, carotenoids and total pigment content, respectively, than in the untreated control plants. Treatment with 0.4 mM SNP partially alleviated the inhibitory effect of Ni toxicity on the lupine pigment content.

Table 1. Effect of exogenous application of SNP (as NO donor) on nitric oxide content of *Lupinus termis* L. under Ni stress (0, 100 and 150 mM).Results are expressed in μg g^{-1} fresh weight. Data are mean ±SE (Standard Error) of five replicates.

Treatment		Shoot			Root		
Control	H_2O	155.22	±	0.87	242.55	±	0.83
	SNP 0.4mM	302.15	±	2.19^{c}	380.79	±	0.82^{c}
	SNP 0.6mM	451.91	±	0.08^{c}	394.79	±	0.08^{c}
Nickel sulphate	100 mM	330.16	±	11.57^{b}	372.35	±	12.99^{a}
Nickel sulphate 100mM + SNP0.4mM		359.53	±	1.70^{***}	399.96	±	5.48
Nickel sulphate 100mM + SNP0.6mM		379.77	±	10.22^{*}	418.29	±	6.29
Nickel sulphate	150 mM	376.75	±	12.67^{b}	501.28	±	1.15^{c}
Nickel sulphate 150mM + SNP0.4mM		427.07	±	13.77^{*}	510.70	±	2.08^{***}
Nickel sulphate 150mM + SNP0.6mM		470.11	±	64.60	553.94	±	4.40^{***}

Values with a superscript are significantly different from the control (tap water). Letter "a" at P<0.05 (significant), "b" at P<0.01 (highly significant), "c" at P<0.001 (extremely significant), and the absence of letter = non-significant. Values with a superscript are significantly different from the control (SNP 0.4 mM, SNP 0.6mM). Asterisk * at P<0.05 (significant), ** at P<0.01 (highly significant), *** at P<0.001 (extremely significant), and the absence of letter = non-significant.

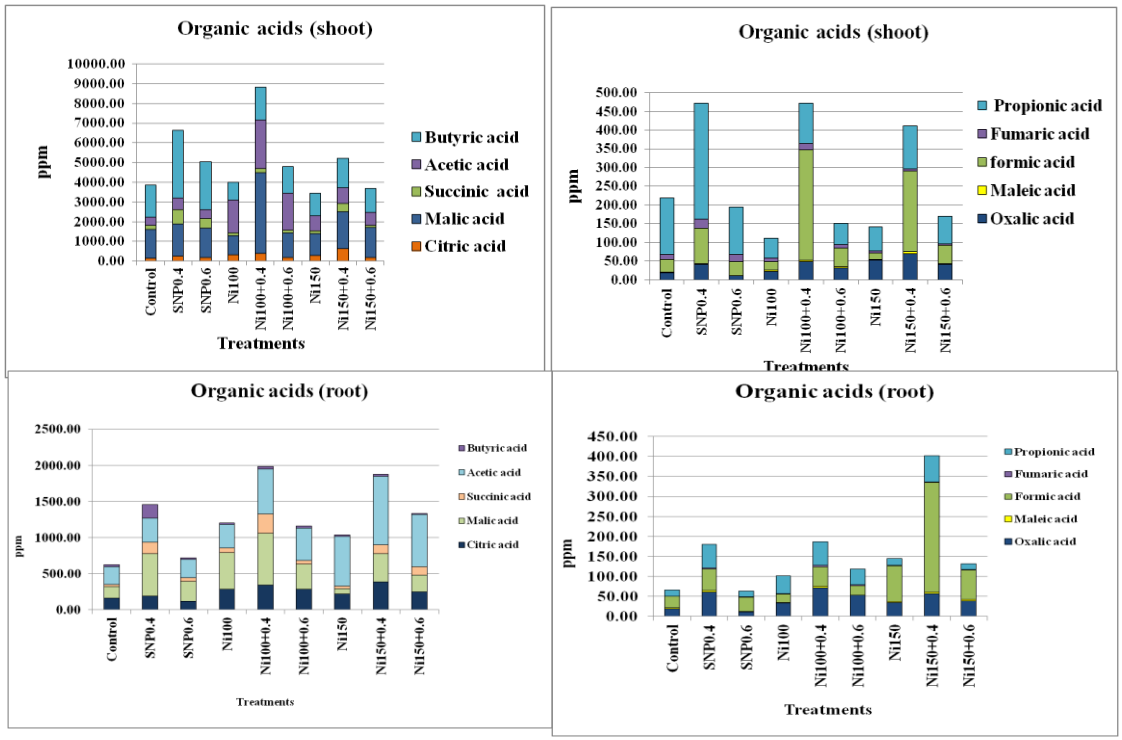

Fig 1. Effect of exogenous application of SNP (as NO donor),on organic acids of shoots and roots of *Lupinus termis* L. under Ni stress (0, 100 and 150 mM).

Table 2. Effect of exogenous application of SNP (as NO donor) on growth characteristics of *Lupinus termis* L. under Ni stress (0, 100 and 150 mM). Data are mean ±SE (Standard Error) of five replicates.

Treatment	Growth parameters	Shoot Length (cm)	Root Length (cm)	no. of leaves/plant	area of leaves (cm^2)	fresh wt. of shoot (g)	dry wt. of shoot (g)	fresh wt. of root (g)	dry wt. of root (g)
Control	H_2O	13.97 ± 0.03	12.00 ± 0.50	13.33 ± 0.33	66.99 ± 6.41	4.36 ± 0.00	0.49 ± 0.00	1.94 ± 0.06	0.33 ± 0.02
	SNP 0.4mM	14.53 ± 0.03^{c}	13.67 ± 0.44	16.00 ± 0.00^{a}	73.42 ± 2.40	4.65 ± 0.07^{a}	0.61 ± 0.01^{a}	2.23 ± 0.11	0.42 ± 0.01^{a}
	SNP 0.6mM	12.53 ± 0.03^{c}	9.83 ± 0.17^{a}	13.00 ± 0.00	67.06 ± 3.22	4.24 ± 0.15	0.54 ± 0.01	1.88 ± 0.02	0.31 ± 0.03
Nickel sulphate	100 mM	12.53 ± 0.03^{c}	9.17 ± 0.33^{a}	12.00 ± 0.00	56.10 ± 3.08	3.91 ± 0.06^{a}	0.42 ± 0.00^{c}	1.34 ± 0.02^{b}	0.29 ± 0.00
Nickel sulphate 100mM +SNP0.4mM		14.17 ± 0.60	11.17 ± 0.33^{*}	13.33 ± 0.33^{*}	68.61 ± 3.68	4.33 ± 0.13	0.50 ± 0.04	1.87 ± 0.40	0.41 ± 0.01
Nickel sulphate 100mM +SNP0.6mM		11.50 ± 0.00^{***}	8.83 ± 0.17^{*}	11.67 ± 0.33	65.05 ± 4.29	3.86 ± 0.19	0.49 ± 0.03	1.44 ± 0.02^{***}	0.35 ± 0.01
Nickel sulphate	150 mM	9.83 ± 0.67^{a}	7.50 ± 0.29^{b}	10.33 ± 0.33^{b}	44.85 ± 2.89	2.91 ± 0.05^{c}	0.32 ± 0.01^{b}	1.03 ± 0.08^{c}	0.23 ± 0.01^{b}
Nickel sulphate 150mM +SNP0.4mM		11.33 ± 0.16^{**}	10.33 ± 0.16^{*}	12.67 ± 0.33^{*}	51.86 ± 7.19^{*}	3.82 ± 0.28	0.50 ± 0.03	1.67 ± 0.04^{*}	0.39 ± 0.01
Nickel sulphate 150mM +SNP0.6mM		10.00 ± 0.50^{*}	8.17 ± 0.16^{**}	11.67 ± 0.67	49.12 ± 0.88^{*}	3.59 ± 0.21	0.46 ± 0.01^{*}	1.27 ± 0.01^{***}	0.33 ± 0.02

Values with a superscript are significantly different from the control (tap water). Letter "a" at P<0.05 (significant), "b" at P<0.01 (highly significant), "c" at P<0.001 (extremely significant), and the absence of letter = non-significant. Values with a superscript are significantly different from the control (SNP0.4mM, SNP0.6mM). Asterisk * at P<0.05 (significant), ** at P<0.01 (highly significant), *** at P<0.001 (extremely significant), and the absence of letter = non-significant.

Table 3. Effect of exogenous application of SNP (as NO donor) on acidic hormones (IAA, GA_3 and ABA) and JA content of *Lupinus termis* L. under Ni stress (0, 100 and 150 mM). Results are expressed in ppm fresh weight. Data are mean ±SE (Standard Error) of five replicates.

Treatment	Conc.	IAA		GA_3		ABA		JA	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Control	(H_2O)	0.34 ± 0.01	0.27 ± 0.01	1.32 ± 0.01	0.21 ± 0.01	0.13 ± 0.01	0.011 ± 0.01	1.45 ± 0.00	0.18 ± 0.01
	SNP 0.4mM	0.38 ± 0.12^{c}	0.30 ± 0.00^{c}	1.51 ± 0.05^{c}	0.23 ± 0.01^{c}	0.14 ± 0.01^{c}	0.011 ± 0.01^{c}	1.72 ± 0.01^{c}	0.21 ± 0.05^{c}
	SNP 0.6mM	0.30 ± 0.01^{c}	0.20 ± 0.01^{c}	1.05 ± 0.32^{c}	0.18 ± 0.01^{c}	0.10 ± 0.01^{c}	0.006 ± 0.00^{c}	1.68 ± 0.01^{c}	0.18 ± 0.01^{c}
Nickel sulphate	100 mM	2.61 ± 0.01^{c}	0.14 ± 0.01^{c}	0.70 ± 0.04^{c}	0.14 ± 0.01^{c}	0.06 ± 0.00^{c}	0.006 ± 0.00^{c}	0.60 ± 0.00^{c}	0.10 ± 0.01^{c}
Nickel sulphate 100mM +SNP0.4mM		0.35 ± 0.01^{***}	0.21 ± 0.01^{***}	1.05 ± 0.00^{***}	0.16 ± 0.01^{***}	0.10 ± 0.01^{***}	0.009 ± 0.00^{***}	1.59 ± 0.01^{***}	0.19 ± 0.02^{***}
Nickel sulphate 100mM +SNP0.6mM		0.29 ± 0.00^{***}	0.18 ± 0.00^{***}	0.74 ± 0.01^{***}	0.14 ± 0.03^{***}	0.09 ± 0.00^{***}	0.008 ± 0.00^{***}	1.27 ± 0.00^{***}	0.16 ± 0.02^{***}
Nickel sulphate	150 mM	0.23 ± 0.01^{c}	0.10 ± 0.01^{c}	0.49 ± 0.01^{c}	0.09 ± 0.01^{c}	0.04 ± 0.01^{c}	0.005 ± 0.01^{c}	0.60 ± 0.00^{c}	0.04 ± 0.01^{c}
Nickel sulphate 150mM +SNP0.4mM		0.34 ± 0.01^{***}	0.19 ± 0.01^{***}	0.90 ± 0.03^{***}	0.14 ± 0.01^{***}	0.05 ± 0.00^{***}	0.009 ± 0.01^{***}	1.48 ± 0.01^{***}	0.15 ± 0.01^{***}
Nickel sulphate 150mM +SNP0.6mM		0.27 ± 0.01^{***}	0.17 ± 0.01^{***}	0.69 ± 0.01^{***}	0.09 ± 0.00^{***}	0.03 ± 0.00^{***}	0.005 ± 0.01^{***}	0.79 ± 0.00^{***}	0.13 ± 0.03^{***}

Values with a superscript are significantly different from the control (tap water). Letter "a" at P<0.05 (significant), "b" at P<0.01 (highly significant), "c" at P<0.001 (extremely significant), and the absence of letter = non-significant. Values with a superscript are significantly different from the control (SNP0.4mM, SNP0.6mM). Asterisk * at P<0.05 (significant), ** at P<0.01 (highly significant), *** at P<0.001 (extremely significant), and the absence of letter = non-significant.

Table 4. Effect of exogenous application of SNP (as NO donor) on photosynthetic pigments content of *Lupinus termis* L. leaves under Ni stress (0, 100 and 150 mM). Results are expressed as μg g^{-1} fresh weight. Data are mean ±SE (Standard Error) of five replicates.

Treatment		Chlorophyll a			Chlorophyll b			Carotenoids			Total pigments		
	H_2O	3.41	±	0.01	1.37	±	0.05	1.93	±	0.02	6.70	±	0.06
Control	SNP 0.4mM	4.53	±	0.06^{b}	2.37	±	0.10^{b}	3.49	±	0.08^{b}	10.39	±	0.22^{b}
	SNP 0.6mM	3.98	±	0.04^{b}	2.16	±	0.21	3.16	±	0.09^{b}	9.29	±	0.28^{b}
Nickel sulphate	100 mM	0.86	±	0.00^{c}	0.69	±	0.01^{b}	0.41	±	0.03^{c}	1.96	±	0.03^{c}
Nickel sulphate 100mM +SNP0.4mM		1.98	±	0.34^{*}	1.46	±	0.04^{**}	1.49	±	0.13^{***}	4.93	±	0.47^{**}
Nickel sulphate 100mM +SNP0.6mM		0.96	±	0.00^{***}	0.69	±	$^{*}0.01$	0.38	±	0.00^{***}	2.03	±	0.01^{***}
Nickel sulphate	150 mM	0.70	±	0.01^{c}	0.52	±	0.00^{b}	0.40	±	0.01^{c}	1.62	±	0.01^{c}
Nickel sulphate 150mM +SNP0.4mM		1.00	±	0.00^{***}	0.79	±	0.01^{**}	0.88	±	0.10^{***}	2.67	±	0.09^{***}
Nickel sulphate 150mM +SNP0.6mM		0.35	±	0.00^{***}	0.28	±	0.00^{*}	0.21	±	0.01^{***}	0.84	±	0.01^{***}

Values with a superscript are significantly different from the control (tap water). Letter "a" at P<0.05 (significant), "b" at P<0.01 (highly significant), "c" at P<0.001 (extremely significant), and the absence of letter = non-significant. Values with a superscript are significantly different from the control (SNP0.4mM, SNP0.6mM). Asterisk * at P<0.05 (significant), ** at P<0.01 (highly significant), *** at P<0.001 (extremely significant), and the absence of letter = non-significant.

Table 5. Effect of exogenous application of SNP (as NO donor) on soluble sugar, polysaccharide and total carbohydrates content of *Lupinus termis* L. under Ni stress (0, 100 and 150 mM). Results are expressed in mg 100g^{-1} dry weight. Data are mean ±SE (Standard Error) of five replicates.

Treatment		Soluble Sugar						Polysaccharide						Total carbohydrates					
		Shoot			Root			Shoot			Root			Shoot			Root		
	H_2O	894.44	±	11.56	1035.18	±	1.85	2899.63	±	1.77	2253.70	±	12.96	3794.07	±	13.30	3290.74	±	14.46
Control	SNP 0.4mM	1148.15	±	9.80^{c}	1092.59	±	18.51	3504.81	±	28.26^{b}	2401.67	±	3.69^{b}	4652.97	±	28.98^{c}	3506.42	±	7.11^{c}
	SNP 0.6mM	794.45	±	5.55^{b}	855.55	±	38.88^{a}	3016.67	±	5.56^{c}	1935.18	±	1.85^{c}	3811.11	±	5.55	2778.39	±	35.82^{b}
Nickel sulphate	100 mM	635.18	±	1.85^{b}	588.89	±	5.55^{c}	1633.33	±	202.78^{a}	1755.56	±	0.96^{c}	2268.52	±	201.88^{a}	2343.15	±	4.38^{c}
Nickel sulphate 100mM +SNP0.4mM		801.85	±	1.85^{***}	1033.33	±	0.00	2512.96	±	4.89^{***}	1926.18	±	9.13^{***}	3314.81	±	3.70^{***}	2953.43	±	3.05^{***}
Nickel sulphate 100mM +SNP0.6mM		377.78	±	39.02^{**}	968.52	±	1.85	2392.59	±	3.70^{***}	1616.67	±	5.55^{***}	2770.37	±	40.74^{***}	2585.80	±	4.82^{*}
Nickel sulphate	150 mM	229.63	±	18.52^{c}	568.52	±	12.96^{c}	1485.74	±	4.42^{c}	1428.15	±	0.18^{c}	1715.37	±	22.88^{c}	1988.15	±	4.26^{c}
Nickel sulphate 150mM +SNP0.4mM		416.66	±	16.03^{***}	590.74	±	1.85^{***}	1582.03	±	1.29^{***}	1465.93	±	0.18^{***}	1998.70	±	14.92^{***}	2057.22	±	1.47^{***}
Nickel sulphate 150mM +SNP0.6mM		244.44	±	0.00^{***}	246.29	±	1.85^{**}	1419.44	±	0.32^{***}	1279.63	±	0.37^{***}	1663.89	±	0.32^{***}	1524.94	±	0.49^{***}

Values with a superscript are significantly different from the control (tap water). Letter "a" at P<0.05 (significant), "b" at P<0.01 (highly significant), "c" at P<0.001 (extremely significant), and the absence of letter = non-significant. Values with a superscript are significantly different from the control (SNP0.4mM, SNP0.6mM). Asterisk * at P<0.05 (significant), ** at P<0.01 (highly significant), *** at P<0.001 (extremely significant), and the absence of letter = non-significant

Table 6. Effect of exogenous application of SNP (as NO donor) on proline content of *Lupinus termis* L. under Ni stress (0, 100 and 150 mM). Results are expressed in mg $100g^{-1}$ dry weight. Data are mean ±SE (Standard Error) of five replicates.

Treatment		Shoot			Root		
Control	H_2O	2.29	±	0.19	1.05	±	0.10
SNP	0.4mM	2.15	±	0.01	0.76	±	0.00
SNP	0.6mM	2.27	±	0.05	0.90	±	0.07
Nickel sulphate	100 mM	3.19	±	0.07^{a}	1.12	±	0.00
Nickel sulphate 100mM +&SNP0.4mM		2.58	±	0.22	1.01	±	0.01^{***}
Nickel sulphate 100mM + SNP0.6mM		2.66	±	0.14	1.02	±	0.01
Nickel sulphate	150 mM	3.95	±	1.24^{**}	1.53	±	0.05^{a}
Nickel sulphate 150mM + SNP0.4mM		2.85	±	0.04^{**}	1.09	±	0.05^{*}
Nickel sulphate 150mM + SNP0.6mM		3.29	±	0.08^{***}	1.20	±	0.00^{*}

Values with a superscript are significantly different from the control (tap water). Letter "a" at P<0.05 (significant), "b" at P<0.01 (highly significant), "c" at P<0.001 (extremely significant), and the absence of letter = non-significant. Values with a superscript are significantly different from the control (SNP0.4mM, SNP0.6mM). Asterisk * at P<0.05 (significant), ** at P<0.01 (highly significant), *** at P<0.001 (extremely significant), and the absence of letter = non-significant.

Table 7. Effect of exogenous application of SNP (as NO donor) on mineral ions (Ca^{2+} and Mg^{2+} content) and Ni^{2+} content of *Lupinus termis* L. under Ni stress (0, 100 and 150 mM).Results are expressed in mg g^{-1} dry weight. Data are mean ±SE (Standard Error) of five replicates.

Treatment	Mineral Ions	Ca^{2+}						Mg^{2+}						Ni^{2+}					
		Shoot			Root			Shoot			Root			Shoot			Root		
Control	H_2O	2.90	±	0.06	2.93	±	0.06	14.02	±	0.04	12.27	±	0.18	27.97	±	0.32	38.27	±	0.38
	SNP 0.4mM	3.54	±	0.03^{b}	3.31	±	0.21	14.60	±	0.06^{b}	17.51	±	0.06^{c}	7.69	±	0.25^{c}	26.33	±	0.88^{b}
	SNP 0.6mM	2.94	±	0.03	3.17	±	0.04^{b}	14.03	±	0.04	12.21	±	0.01	48.10	±	1.06^{c}	67.01	±	1.72^{b}
Nickel sulphate	100 mM	6.60	±	0.06^{c}	6.33	±	0.17^{b}	3.62	±	0.40^{c}	9.03	±	0.02^{b}	350.08	±	1.09^{c}	367.20	±	1.56^{c}
Nickel sulphate 100mM+SNP0.4mM		7.15	±	0.08^{***}	7.03	±	0.03^{**}	12.50	±	0.06^{***}	10.70	±	0.01^{***}	140.05	±	2.27^{***}	215.50	±	2.47^{***}
Nickel sulphate 100mM +SNP0.6mM		6.17	±	0.17^{**}	6.17	±	0.17^{**}	10.39	±	0.01^{***}	5.56	±	0.02^{***}	368.09	±	1.07^{***}	319.38	±	1.72^{***}
Nickel sulphate	150 mM	4.63	±	0.09^{c}	4.50	±	0.00^{c}	4.12	±	0.04^{c}	1.90	±	0.06^{c}	690.32	±	10.69^{c}	1254.92	±	0.65^{c}
Nickel sulphate 150mM +SNP0.4mM		6.20	±	0.11^{***}	6.03	±	0.03^{**}	8.19	±	0.01^{***}	4.19	±	0.00^{***}	249.85	±	0.71^{***}	377.42	±	1.39^{***}
Nickel sulphate 150mM +SNP0.6mM		5.37	±	0.18^{**}	5.57	±	$`0.07^{***}$	4.05	±	0.01^{***}	4.16	±	0.01^{***}	633.99	±	0.58^{***}	690.09	±	10.93^{**}

Values with a superscript are significantly different from the control (tap water). Letter "a" at P<0.05 (significant), "b" at P<0.01 (highly significant), "c" at P<0.001 (extremely significant), and the absence of letter = non-significant. Values with a superscript are significantly different from the control (SNP0.4mM, SNP0.6mM). Asterisk * at P<0.05 (significant), ** at P<0.01 (highly significant), *** at P<0.001 (extremely significant), and the absence of letter = non-significant.

Table 8. Effect of exogenous application of SNP (as NO donor) on some oxidative markers (Hydrogen Peroxide, electrolyte leakage and lipid peroxidation content) of *Lupinus termis* L. under Ni stress (0, 100 and 150 mM). Data are mean ±SE (Standard Error) of five replicates.

Treatment		Hydrogen Peroxide (mM g^{-1})						Electrolyte Leakage (%)			Lipid Peroxidation (MDA) (mM g^{-1})					
		Shoot			Root			Shoot			Shoot			Root		
Control	H_2O	16.92	±	0.23	1.47	±	0.01	19.29	±	0.00	0.00059	±	0.0000	0.00038	±	0.0000
	SNP 0.4mM	14.02	±	0.43^{a}	1.04	±	0.02^{c}	15.39	±	0.00^{c}	0.00034	±	0.0000^{c}	0.00022	±	0.0000^{b}
	SNP 0.6mM	18.12	±	0.32^{a}	1.22	±	0.01^{c}	19.02	±	0.17	0.00047	±	0.0000^{c}	0.00028	±	0.0000^{a}
Nickel sulphate	100 mM	55.24	±	0.17^{c}	17.74	±	0.13^{c}	61.88	±	0.00^{c}	0.00132	±	0.0000^{c}	0.00046	±	0.0000^{b}
Nickel sulphate 100mM +SNP0.4mM		38.32	±	0.13^{***}	6.44	±	0.11^{***}	21.64	±	3.32	0.00064	±	0.0000	0.00022	±	0.0000
Nickel sulphate 100mM +SNP0.6mM		51.27	±	0.06^{***}	8.59	±	0.73^{*}	27.89	±	0.00^{***}	0.00070	±	0.0001	0.00024	±	0.0000
Nickel sulphate	150 mM	82.89	±	0.50^{c}	19.44	±	1.12^{b}	74.49	±	0.00^{c}	0.00196	±	0.0000^{c}	0.00056	±	0.0001
Nickel sulphate 150mM +SNP0.4mM		40.97	±	0.92^{***}	7.39	±	0.11^{***}	28.21	±	0.00^{***}	0.00068	±	0.0000^{***}	0.00032	±	0.0000^{**}
Nickel sulphate 150mM +SNP0.6mM		80.81	±	0.06^{***}	9.79	±	0.17^{***}	31.61	±	0.00^{***}	0.00075	±	0.0000	0.00036	±	0.0000^{*}

Values with a superscript are significantly different from the control (tap water). Letter "a" at P<0.05 (significant), "b" at P<0.01 (highly significant), "c" at P<0.001 (extremely significant), and the absence of letter = non-significant. Values with a superscript are significantly different from the control (SNP0.4mM, SNP0.6mM). Asterisk * at P<0.05 (significant), ** at P<0.01 (highly significant), *** at P<0.001 (extremely significant), and the absence of letter = non-significant.

Carbohydrate contents

The effects of SNP treatments on the carbohydrate (soluble sugars, polysaccharides and total carbohydrates) contents of lupine shoots and roots were concentration-dependent (Table 5). The lower SNP concentration (0.4 mM) had a stimulatory effect, while the higher concentration (0.6 mM) had an inhibitory effect, when compared with water-treated control plants. The stimulatory effect of the 0.4-mM SNP treatment was much more pronounced in shoots than roots and was evaluated as 28.37%, 20.87% and 22.64% increases in soluble sugars, polysaccharides and total carbohydrates, respectively, in 0.4-mM SNP-treated shoots over the control values.

Treating Ni-stressed plants with 0.4 mM SNP partially alleviated the inhibitory effect of Ni toxicity on the soluble sugar content of lupine and increased the amounts of polysaccharides accumulated in both shoots and roots. Meanwhile, treating with Ni sulfate together with 0.6 mM SNP resulted in a continuous decrease in the soluble sugar contents of shoots to values lower than those of plants treated with 0.6 mM SNP alone. The extremely significant decrease was evaluated as 52.45% and 69.23% in plants treated with 0.6 mM SNP and subjected to 100 and 150 mM Ni sulfate stress, respectively, compared with the corresponding control plants treated with 0.6 mM SNP alone.

The maximum total carbohydrate content in lupine shoots in treated plants followed the order of: 0.4 mM SNP > control plants (tap water) > 100 mM Ni sulfate + 0.4 mM SNP > 0.6 mM SNP > 150 mM Ni + 0.6 mM SNP.

Proline content

The changes in proline content in response to treatment with SNP and/or Ni sulfate are illustrated in Table 6. The SNP treatment decreased the proline content, while Ni sulfate applications increased it in both shoots and roots of lupine plants. The maximum proline accumulation was detected in response to 150 mM Ni sulfate and was evaluated as 72.49% greater in shoots compared with unstressed control plants. The lowest proline value was detected in plants treated with 0.4 mM SNP and evaluated as 27.62% less in roots compared with the untreated control. Plants treated with both SNP and Ni sulfate recorded intermediate proline content, greater than those treated with SNP alone and less than those exposed to Ni sulfate alone.

Organic acids profile

The data in Figure 1 represent the changes in organic acid profiles in shoots and roots of *L. termis* L. in response to treatments with SNP and/or Ni sulfate. Generally, the shoot organic acid contents were much greater than in roots. Among the organic acids, butyric and malic acids were the most abundant compounds in shoots, while citric and malic acids were the major compounds in roots. Applications of SNP and/or Ni sulfate caused marked changes in the organic acid profiles of lupine plants, compared with the control. Exogenous applications of NO (in the form of SNP at 0.4 and 0.6 mM) significantly increased citric, malic, succinic, formic, acetic, fumaric and butyric acid levels. The increase was much more pronounced in plants treated with 0.4 mM SNP and was evaluated as 73.78%, 13.09%, 249.12%, 173.53%, 31.24%, 85.714% and 114.80% in shoots, and 19.713%, 266.8%, 453.4%, 86.206%, 34.75%, 233.00% and 633.3% in roots, respectively, over the untreated control values. However, oxalic, maleic and propionic acids were increased only in response to the low concentration of SNP (0.4 mM) and significantly decreased in response to the higher concentration (0.6 mM SNP).

Ni toxicity had a significant effect on the organic acid composition of lupine plants. Oxalic, citric, maleic and acetic acids were increased in both shoots and roots in response to both applied concentrations of Ni sulfate, while malic, succinic and propionic acids were increased only in roots and decreased in shoots compared with the control. In most cases, Fumaric and formic acids were significantly inhibited in both shoots and roots in response to Ni treatments. As a percentage, acetic acid was most affected by Ni toxicity. The accumulated acetic acid increased by 274.38% and 64.79% in shoots and 31.03% and 175.64% in roots in response to 100- and 150-mM Ni sulfate treatments, respectively.

Treating lupine plants with a combination of Ni sulfate and SNP significantly increased the accumulated amounts of oxalic, citric and maleic acids over those treated with SNP or Ni alone. Maximum values were detected in response to 150 mM Ni + 0.4 mM SNP. NO production (using 0.4 and 0.6 mM SNP) completely overcame the inhibitory effect of Ni on the malic acid content, and the maximum values were detected in plants treated with 100 mM Ni sulfate + 0.4 mM SNP, at 151.70% and 23.25% in shoots and roots, respectively, over 0.4 mM SNP as the control value. However, only the 0.4-mM SNP treatment could alleviate the inhibitory effect of Ni toxicity on the production of succinate and butyric acid.

A highly significant increase in formic acid and acetic acid contents was occurred, when different concentrations of Ni sulfate together with SNP applied. The highest values in shoot were detected by applying 100 mM Ni sulfate + 0.4 mM SNP (8.69- and 5.54-fold in case of formic acid and acetic acid, respectively), whereas the maximum values in root were detected in response to 150 mM Ni + 0.4 mM SNP (9.43 and 3.79-fold in formic acid and acetic acid, respectively) of untreated control value.

All of the treatments caused an increase in the propionic acid content of roots of *Lupinus termis* L., when different concentrations of Ni sulfate were applied together with 0.4 mM SNP, compared with the 0.4 mM SNP control value. The highest extremely significant increase ($P < 0.001$) was detected when 150 mM Ni sulfate + 0.4 mM SNP was applied and was calculated as 13.79% greater than the 0.4 mM SNP control value.

Mineral ion contents and Ni^{2+} accumulation

The effects of SNP treatments and Ni toxicity on the Mg^{2+} and Ca^{2+} contents and Ni accumulation in lupine plants are presented in Table 7.

The effect of SNP on divalent cations is concentration dependent. The 0.4-mM SNP treatment highly significantly increased the Mg^{2+} and Ca^{2+} concentrations compared with the

untreated control, while the 0.6-mM SNP treatment had no significant effects on their contents.

Ni toxicity caused extremely significant increases in Ca^{2+} and highly significant decreases in Mg^{2+} contents in both shoots and roots as compared with the untreated control. The reductions in Mg^{2+} were calculated as 74.18% and 70.61% in shoots and 26.41% and 84.52% in roots of plants treated with 100 and 150 mM Ni sulfate, respectively. The treatment of Ni-stressed plants with 0.4 mM SNP partially alleviated the inhibitory effect of Ni on the Mg^{2+} level and led to the accumulation of additional amounts of Ca^{2+}.

The endogenous Ni^{2+} ion content showed extremely significant increases ($P < 0.001$) in both shoots and roots of *L. termis* L. in response to Ni sulfate treatments, as compared with the untreated control. The detected increase was much greater in roots than shoots and was directly proportional to the concentration of Ni applied. The highest accumulation of Ni ions was found in *L. termis* plants receiving 150 mM Ni sulfate and was evaluated as 24.68- and 32.76-fold greater than the control values in shoots and roots, respectively. The treatment of Ni-stressed plants with 0.4 and 0.6 mM SNP extremely and significantly reduced the amounts of Ni accumulated in the plants. The lower dose of SNP (0.4 mM) was more effective in reducing Ni accumulation than the higher dose (0.6 mM). The Ni levels were reduced by 63.81% and 69.92% in the shoots and roots of plants, respectively, treated with 150 mM Ni sulfate + 0.4 mM SNP compared with those treated with 150 mM Ni sulfate alone.

Oxidative markers

Oxidative markers, including H_2O_2, electrolyte leakage and lipid peroxidation, increased in response to heavy-metal stress with Ni sulfate (100 mM and 150 mM) as shown in Table 8, while treatments with both Ni sulfate (100 mM and 150 mM) + SNP (0.4 mM), as a stress inhibitor, decreased H_2O_2, electrolyte leakage and lipid peroxidation when compared to Ni-stress treatments alone. The lower SNP concentration (0.4 mM) was more effective than the higher concentration (0.6 mM) in alleviating the oxidative stress caused by different Ni sulfate concentrations.

Discussion

The current results indicated that exogenous applications of SNP (0.4 and 0.6 mM) caused a significant increase in the endogenous NO content in both shoots and roots of lupine compared with the non-treated control. Grossi and D'Angelo (2005) hypothesized that the release mechanism of the NO molecule from SNP involves the sulfhydryl-containing compounds glutathione and cysteine, which help to form the corresponding disulfides and S-nitrosothiols, NO and cyanide ions.

Several past studies on NO functions in plant responses to heavy metals were based on the use of an exogenous NO donor (Hsu and Kao, 2004; Gallego et al., 2005; Laspina et al., 2005). However, stress-induced NO might be endogenously produced and; therefore, play specific roles in responses to heavy metals (Besson-Bard et al., 2009). According to the present results, Ni sulfate treatments (100 and 150 mM) caused significant increases in the NO content, and the increases were directly related to the Ni concentration applied. NO can modulate the expression of metal transporters, such as iron regulated transporter 1 and; therefore, affect the accumulation of these metals in plants (Parani et al., 2004; Besson-Bard et al., 2009). An increase in the endogenous NO content upon exposure to heavy metal (Cd) has also been reported for different crop plants (Groppa et al., 2008; Xiong et al., 2009; Valentovičová et al., 2010). Treatments with Ni + SNP had an accumulating NO effect in lupine plants. The highest endogenous NO content was detected in plants treated with 0.6 mM SNP + 150 mM Ni sulfate.

Plant growth is highly influenced by NO. Here, the effect of NO on lupine growth was concentration-dependent, with 0.4 mM SNP causing a significant increase and 0.6 mM SNP causing a significant decrease in all of the measured growth parameters (Table 2). A similar dual behavior of the NO donor, SNP, was also noted by several authors. For example, treating wheat seedlings with a lower concentration of SNP promoted root growth, while a higher concentration was inhibitory (Tian and Lei, 2006). Seedlings of canola, raised from the seeds treated with a lower concentration of SNP had longer roots and greater dry masses, while those treated with a higher concentration had reduced values for these parameters (Zanardo et al., 2005).

The most common response of plants to stress conditions, such as exposure to heavy metals, is growth reduction. A marked decrease was detected in all of the growth parameters in response to 100 and 150 mM Ni compared with unstressed control plants. Mihailovic and Drazic (2011) illustrated that Ni induced a significant inhibition of leaf and root growth in bean plants after a 4-d exposure, which confirmed previous results (Gajewska and Sklodowska 2005). Interestingly, NO alleviates this effect, similar to the findings of Kopyra and Gwóźdź (2003) and Laspina et al. (2005) that were obtained for lead- and cadmium (Cd)-stressed plants, respectively.

Changes in NO levels are triggered by exogenous plant hormones (Freschi, 2013). In the current study, we detected the changes in endogenous hormones in response to exogenous applications of NO. Endogenous phytohormones, including IAA, GA_3, ABA and JA, were significantly increased in response to the 0.4 mM SNP treatment. NO appeared to modulate elements controlling either the plant hormone levels (e.g., biosynthesis, degradation and conjugation enzymes), distribution (e.g., transport proteins) or signaling (e.g., receptors and signal transduction proteins). This modulation occurs either at the transcriptional (Xu et al., 2010; Leon and Lozano-Juste, 2011) or post-translational level (Terrile et al., 2012; Feng et al., 2013).

In contrast, all of the applied Ni sulfate concentrations significantly decreased the hormonal contents in both shoots and roots of lupine plants. Heavy-metal stress leads to a decrease in endogenous levels of auxins. For example, arsenic is able to alter levels of three auxins (IAA, naphthalene-1-acetic acid and indole-3-butyric acid) in *Brassica juncea* (Srivastava et al. 2013). In another case, a short-term Cd treatment disturbed IAA homeostasis in barley root tips (Zelinova et al., 2015). Besson-Brad et al. (2009) also indicated that Cd suppresses primary root elongation in *Arabidopsis*. In contrast to our results, increases in the ABA content have been detected in

germinating chickpea (*Cicer arietinum*) seeds under lead toxicity conditions (Atici et al., 2005) and in crowberries (*Empetrum nigrum*) exposed to copper (Cu) and Ni (Monni et al. 2001). Further studies are needed to reveal the changes in the ABA biosynthesis level caused by applied Ni.

The detected increases in growth-stimulating hormones (such as IAA and GA_3) could explain the observed growth stimulation in response to NO treatments. The increase in the ABA level in response to NO could explain the protective effect of NO against Ni toxicity that is connected with ABA-induced stomatal closure. Preventing a decline in the water potential by closing stomata contributes to the plant's adaptability to unfavorable conditions (Pantin et al., 2013). Exposure to toxic metal concentrations impairs the plant's water balance (Mukhopadhyay and Mondal, 2015). Thus, SNP responds to Ni stress by elevating internal hormones.

The two applied SNP concentrations had positive impacts on photosynthetic pigments in lupine leaves. NO enhanced the chlorophyll content in potato, lettuce and *Arabidopsis* (Beligni and Lamattina, 2000). Additionally, SNP treatments delay yellowing and retard chlorophyll degradation in broccoli (Hyang et al., 2009). They also improve the rate of photosynthesis, chlorophyll content, transpiration rate and stomatal conductance in cucumber seedlings (Fan et al., 2007). In contrast, Ni stress caused a significant to extremely significant decrease in the photosynthetic pigment content. Decreases in chlorophyll contents under Cu stress were reported in *Atriplex halimus* (Brahim and Mohamed, 2011) and mangrove plant seedlings (Zhang et al., 2007). Exogenous applications of NO alleviate the inhibitory effects of Ni toxicity on the leaf pigment content. Similarly, Chen et al. (2010) detected that the NO-mediated stability and integrity of the subcellular structure under Cd stress contributed to its effective role in preventing Cd-induced leaf chlorosis and in the inhibition of photosynthesis in barley seedlings. The detected increase in the photosynthetic pigments under Ni toxicity could be attributed to the role of NO in protecting chloroplast membranes from ROS or its involvement in the chlorophyll metabolic pathway (Siddiqui et al., 2013).

Based on the obtained results, SNP (0.4 mM) has a stimulatory effect on the lupine carbohydrate content, including soluble sugars, polysaccharides and total carbohydrates. The soluble sugar content increases after an exogenous NO pre-treatment in wheat seedlings, which was in accordance with the increased amylase activity levels in the germinating seeds (Zheng et al., 2009). Ni stress significantly decreased the soluble sugar and total carbohydrate contents in lupine shoots and roots. Similarly, Latif (2010) demonstrated that a Ni sulfate treatment significantly decreases the total carbohydrate content of the white radish at moderate and higher concentrations (0.178, 0.36, 0.53 and 0.71 mM) compared with the untreated samples, but the red radish shows a significant decrease only at a higher concentration. Thus, the detected reduction in the photosynthetic pigment content had an indirect influence on the photosynthesis process and; therefore, the carbohydrate accumulation in plants. Heavy metals directly affect the photosynthetic machinery by binding to the various sensitive sites of the photosynthetic apparatus **(**Aggarwal et al., 2011). Interestingly, Ni-stressed plants treated with SNP (0.4 mM) significantly increased the soluble sugar and polysaccharide contents compared with plants treated with 100 or 150 mM Ni sulfate alone. The increased accumulation of soluble sugar, which has been assigned an osmo-regulatory role, provides evidence for the development of water stress in $NiSO_4$-treated plants (Najafi et al., 2011).

Based on the obtained results, SNP treatments decreased the proline contents in shoots and roots of lupine plants, while $NiSO_4$ increased the contents when compared with untreated control plants. Similar results were obtained by Theriappan et al. (2011) who found that the applied concentrations of $ZnCl_2$, $HgCl_2$ and $CdCl_2$ caused proline accumulation in cauliflower seedlings. Mourato et al. (2012) and Tripathi and Gaur (2004) proposed that ROS scavenging by proline, which is stimulated by heavy-metal stress, is primarily conducted by detoxifying hydroxyl radicals and quenching singlet oxygen. Proline can also act as a metal chelator and protein stabilizer (Mishra and Dubey, 2006). Proline accumulation in plant tissues has been attributed to the following: (1) an increase in proline biosynthesis; (2) a decrease in proline degradation; (3) a decrease in protein synthesis or proline use; and (4) the hydrolysis of proteins (Ashraf and Foolad, 2007). Further studies are needed to address the effects of NO and Ni stress on proline biosynthetic and degradative enzymes. The detected amounts of proline in plants treated with SNP + Ni sulfate were significantly lower than those treated with Ni alone. Similarly, Mihailovic and Drazic (2011) found that NO supplementation caused the proline accumulation stimulated by Ni to be completely suppressed in roots and partly suppressed in leaves.

Exogenous applications of NO significantly increased citric, malic, succinic, formic, acetic, fumaric and butyric acids in lupine plants, compared with in the control. Additionally, oxalic, citric, maleic, acetic and butyric acids were increased in both shoots and roots in response to all of the applied Ni sulfate concentrations, as shown in Figure 1. Organic acids not only act as intermediates in carbon metabolism but also as key components in coping mechanisms used by some plants in metal tolerance. The increased organic acid content may be a defense mechanism against Ni stress. At least two organic acids of the Krebs cycle, malic and citric, play important roles in metal-chelating processes by forming complexes with Ni (Brooks et al., 1991). Ni may be escorted to the vacuoles in the form of metal-organic acid complexes (Krämer et al., 2007).

Exogenous applications of an NO donor (0.4 mM SNP) highly significantly increased the Mg^{2+} and Ca^{2+} contents in lupine shoots and roots compared with untreated plants, while the Ni sulfate treatment highly significantly decreased the Mg^{2+} content compared with control plants. Exogenous NO alleviates the impact of Ni on the Mg^{2+} concentration, which may result from the activation of weakly selective Ca-uptake channels or increased requirements for Mg^{2+} by the NO-activated synthesis of enzymes and/or other proteins. Ni stress increased the accumulation of Ca^{2+} in lupine plants, and NO treatments caused additional Ca^{2+} accumulations compared with plants treated with Ni alone. Thus, NO responses to stresses in plants are mediated by a cytosolic Ca^{2+} increase. NO stimulates Ca^{2+} release from intracellular stores (Sokolowski and Blatt, 2007) but can also act as a strong stimulator of a Ca^{2+} influx across the plasma membrane (Lamotte et al., 2006). The mechanism involved is assumed to be a direct covalent

modification of ion channels by the S-nitrosylation of proteins (Vandelle et al., 2006). The high accumulation level of Ca^{2+} in Ni-treated plants is probably necessary by the increased mobilization of intracellular and extracellular Ca^{2+} as mediators in the NO-regulated protective responses of the plant cells. Ni stress significantly increased endogenous Ni accumulations in lupine shoots and roots, while the NO treatment decreased the accumulated Ni amounts compared with untreated Ni-stressed plants (Table 7). This might be attributed to the detected NO-stimulated organic acids, the formation of organic acid–metal complexes or to Ni accumulations in root cell wall, which subsequently decreased its levels in the aerial parts of the plant (Xiong et al., 2009).

NO treatment decreased the oxidative markers in lupine plants grown under normal or Ni-stress conditions (Table 8). SNP applications promoted ROS-scavenging enzymes, reduced H_2O_2 accumulation, increased the activity levels of H^+-ATPase and H^+-PPase in the plasma membrane or tonoplasts, and also significantly alleviated the growth inhibition induced by $CuCl_2$ in tomato plants (Cui et al., 2009). Heavy metals cause cell toxicity by overproducing ROS, which impairs antioxidant defense systems and causes oxidative stress (Rui et al., 2016).

NO is most likely involved in H_2O_2 detoxification and in the maintenance of the cellular redox status (Tewari et al., 2008). SNP-exposed wheat plants show decreases in H_2O_2 and MDA under Al^{3+} stress (Zhang et al., 2008). NO protects the plants from oxidative damage by regulating the general mechanisms for cellular redox homeostasis, promoting the transformation of O_2^- to H_2O_2 and O_2, and enhancing the activity levels of H_2O_2-scavenging enzymes (Lamattina et al., 2003; Shi et al., 2007; Zheng et al., 2009).

Materials and Methods

Plant materials

Seeds of lupine (*Lupinus termis* L. cv. Balady) were obtained from Agriculture Research Center, Ministry of Agriculture, Giza, Egypt.

Growth conditions

This experiment was performed in the greenhouse of the National Institute of Occupational Safety and Health (NIOSH) in 2014 / 2015. Uniform seeds of *Lupinus termis* L. were surface sterilized with 2.5% sodium hypochlorite for 15 min and washed thoroughly with distilled water. 10 seeds were sown per plastic pot (22-cm diameter and 20-cm length). Each pot was filled with 4 kg soil (clay: sand, 2:1 w/w). The plants were irrigated using tap water to 80% of their saturation capacity, and then 14 d after sowing; seedlings were thinned to 6 healthy seedlings per pot.

Three weeks after sowing, the pots were divided into three groups. The pots of the first group were sprayed with tap water to serve as a control; the second and third groups were sprayed two times (at 23 d and 30 d after sowing) with two concentrations of sodium nitroprusside (0.4 and 0.6 mM, respectively) as a nitric oxide (NO) donor. Each group was divided into three subgroups treated with one of the following concentrations of nickel sulfate: 0, 100 and 150 mM. Samples were taken from 2-month-old plants to measure lipid peroxidation, H_2O_2, electrolyte leakage, growth parameters, photosynthetic pigments (including chlorophyll a, b and carotenoids), endogenous phytohormones (IAA, GA_3, ABA and JA), soluble sugar and polysaccharide contents, proline, organic acids content and divalent cation Ca^{2+}, Mg^{2+} and Ni^{2+} accumulations. The endogenous NO content in each treatment was also determined. Five planted pots from each level of treatment were considered.

Estimation of endogenous NO

Nitric oxide (NO) was detected using Griess reagent (1% sulphanilamide, 0.1% naphthyethylene diamine dihydro-chloride in 2% H_3PO_3) as described by Sun et al. (2003). Results are expressed as $\mu g\ g^{-1}$ fresh weight (FW).

Extraction, separation and estimation of growth regulating substances using gas chromatography (GC)

The method of extraction was similar to that adopted by Shindy and Smith (1975) and described by Hashem (2006). To estimate the amounts of acidic hormones IAA, ABA and GA_3, the plant hormone fractions and standards were methylated according to Vogel (1975). JA was determined according to Kramell (1996) using a NUCLEODEX beta-PM, 200 mm × 4 mm ID column. The flow rate was adjusted to 1 ml/min and detected at UV 210 nm. Results are expressed as ppm.

Estimation of photosynthetic pigments

The photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) were determined in leaves of the investigated plants according to the spectrophotometric method described by Metzner et al. (1965). Pigment contents were calculated as $\mu g\ g^{-1}$ FW of leaves.

Estimation of carbohydrates

Soluble sugars and polysaccharides were determined in plant tissues using anthrone method described by Whistler et al. (1962). Soluble sugars and polysaccharides were expressed as mg $100\ g^{-1}$ DW.

Estimation of proline

Proline was determined using ninhydrin reagent (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid) according to Bates et al. (1973). Results expressed as mg proline $100\ g^{-1}$ DW. Griess Reaction assay was described by Bratton and Marshall in 1939.

Estimation of organic acids

An HP 1090 Hewlett-Packard liquid chromatograph equipped with a Cl8 guard column (25 × 4.6 mm I.D.), a column-switching valve, a Nucleosil ODS 100-5 analytical column (250 × 4.6 mm I.D.) and an auto sampler were used. UV-detection at 210 nm was accomplished with the HP 1040 diode-array detector.

Organic acids were analyzed as described by Wodecki et al (1991).

Estimation of mineral ions content and nickel accumulation

The contents of Ca^{2+}, Mg^{2+} and Ni^{2+} were analyzed using atomic absorption spectrometry (flame atomizer technique) (Marounek et al., 2006).

Oxidative markers

Hydrogen peroxide content

The hydrogen peroxide content was determined according to Shi et al. (2007) using 3% (w/v) trichloroacetic acid, and the absorbance was measured at 390 nm (Alexieva et al., 2001). Data was expressed as mM H_2O_2 g^{-1} FW of plant.

Lipid peroxidation

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content using the method of Hodges et al. (1999). The MDA content was calculated using its absorption coefficient of 155 mM^{-1} cm^{-1} and expressed as mM MDA g^{-1} FW.

Electrolyte leakage

Electrolyte leakage (EL) was determined according to Dionisio-Sese and Torbita (1998).

Statistical analysis

Experiments utilized a completely randomized design. Mean values were calculated from the measurements of five replicates, and standard deviations of the means were analyzed using an independent sample t-test (SPSS program 17.0). To determine significant difference between means, two-tailed P-values were obtained (Snedecor and Cochran, 1980).

Conclusion

The present study showed that Ni toxicity can be ameliorated by an exogenous NO donor, as indicated by increased growth parameters, an enhanced photosynthetic pigment content and decreased oxidative-stress markers in Ni-stressed plants treated with NO, compared with untreated plants subjected to the same Ni levels. Such effects could be attributed to the detected NO-enhanced hormonal levels, organic acid production and increased divalent cation (Ca^{2+} and Mg^{2+}) content. The extent of the impact depends on the NO concentration applied. A lower SNP concentration (0.4 mM) alleviated the deleterious effects of up to 150 mM Ni sulfate, while a higher SNP concentration (0.6 mM) alleviated the harmful effects of up to 100 mM Ni sulfate.

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