Incomplete alleviation of nickel toxicity in bean by nitric oxide supplementation

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ABSTRACT

The aim of the experiment was to test the capacity of NO to reverse harmfull effects of nickel on bean (*Phaseolus vulgaris* L.) seedlings. Bean seedlings were grown on culture medium and treated with NO-donor – sodium nitroprusside (0.3 mmol/L) and Ni (0.2 mmol/L $NiCl_2$). After 4 days, the parameters of antioxidative response were determined in roots and leaves, as well as the concentrations of essential cations and Ni. In the presence of Ni alone, soluble protein, proline and superoxide-dismutase activity were increased, while peroxidase and especially catalase activities were supressed. Also, Ni induced a depletion of K, Ca, Mg, Mn and Zn, while the contents of Cu and Fe in the roots were increased. In the presence of NO, Ni-induced stimulation of superoxide-dismutase activity, soluble protein and proline accumulations was decreased, while the inhibition of peroxidase and catalase activities was eliminated. Calcium and Zn concentrations were increased by Ni in NO-treated seedlings, suggesting specific activation of the uptake of these elements as part of the protective processes regulated by NO. However, NO had no effect on the impact of Ni on K, Cu, Fe, and Mn concentrations. In conclusion, exogenous NO efficiently attenuates oxidative stress in bean, but does not prevent Ni-induced ion leakage.

Keywords: antioxidative response; ion accumulation; *Phaseolus vulgaris* L.; root; shoot

Nickel (Ni) is a transient metal present in most soils utilized for crop cultivation. The main sources of anthropogenic contamination with Ni are sewage sludge, coal-mine spoils, metalliferious tailings, smelters, emissions and ash from coal-fired power plants.

As a component of urease Ni is present in plants as an essential element (Dixon et al. 1975) and at low concentrations it stimulates the growth of some plants.

Nevertheless, at higher concentrations Ni is toxic for plants. The most frequent effects are growth inhibition, chlorosis, mineral nutrition disturbance and metabolic disorders (Seregin and Kozhevnikova 2006). The exact mechanisms of Ni toxicity are still unclear.

Similar to other transient metals, Ni was found to induce oxidative stress in plants (Gajewska and Sklodowska 2005, Yan et al. 2008). Besides, the impact of this metal on ion uptake and transport was also established by several authors (El-Enany et al. 2000, Parida et al. 2003, Kopittke et al. 2007).

During the last ten years the role of nitric oxide (NO) in plant metabolism was elucidated from many aspects, although the exact action of NO as a signalling and regulating agent is still unclear. It was found that NO participated in the regulation of growth and photosynthesis, programmed cell death and the plant resistance to biotic and abiotic stresses. In the presence of toxic elements synthesis of endogenous NO is stimulated (Bartha et al. 2005). Also, several authors described alleviation of the symptoms of Pb and Cd stress in plants by exogenous NO (Kopyra and Gwóźdź 2003, Laspina et al. 2005).

In the present study we investigated the effect of exogenous NO on Ni-induced oxidative stress expecting to confirm the beneficial action of NO,

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similarly to the results obtained for other toxic elements, but we also wished to investigate possible role of NO in reverting harmful effects of Ni on the accumulation of essential ions in plant roots and leaves, because there are very few data on NO effects on heavy-metal induced impairment of ion transport.

MATERIAL AND METHODS

Bean (*Phaseolus vulgaris* L.) seeds were obtained from a local market. The seeds were first shortly sterilized with diluted 95% ethyl alcohol (1:3) and then with 2% sodium-hypochlorite for 5 min and thoroughly rinsed with tap and distilled water. The seeds were germinated for 3 days between two sheets of filter paper soaked with distilled water in plastic containers in thermostat at 25°C in the dark. After germination, the seedlings were transferred to hydroponic culture solution as described by Liu et al. (2008) and grown in phytotrone chamber under the following conditions: humidity 70%, day/night cycle 14/10 h and day/night temperature 25/18°C, light intensity 150 µmol (photon)/m^2/s. The solutions were aerated. After two days, uniformly growing seedlings were selected and four treatments were applied: control (0), 0.2 mmol/L $NiCl_2$ (Ni), 0.3 mmol/L sodium nitroprusside (SNP) (NO) and the two combined (Ni + NO). The plants were treated for 4 days. The concentration of 0.2 mmol/L Ni was selected on the basis of previous results with Fabaceae as one sufficient to induce oxidative stress response and also a disturbance of ion transport (El-Enany et al. 2000, Gajewska and Sklodowska 2005). Also, the treatment period of 4 days was selected as optimal for allowing the development of the effects of Ni and NO on both metabolic and growth parameters, as it was demonstrated by several authors (Baccouch et al. 1998, Ruan et al. 2004, Llamas et al. 2008, Gajewska and Sklodowska 2009). Sodium nitroprusside concentration of 0.3 mmol/L was previously demonstrated as appropriate to alleviate oxidative stress in wheat plants (Tan et al. 2008).

Total soluble protein was assayed by the Lowry's method (Lowry et al. 1956). Proline was quantitated according to Bates et al. (1973). Superoxide dismutase (EC 1.15.1.1.) (SOD) activity was measured according to Giannopolitis and Ries (1977). Peroxidase (EC 1.11.1.7.) (POD) activity was determined by the method of Lin and Kao (1999). Catalase (EC 1.11.1.6.) (CAT) activity was determined according to Pandey and Sharma (2002). Spectrophotometric measurements were performed by UV/visible spectrophotometer Ultrospec 2000, Pharmacia Biotech (ADD, UK).

The concentrations of K, Ca, Mg, Mn, Zn, Fe, Cu, and Ni were determined by atomic absorption spectrophotometry (Pye Unicam SP 192, ADD, UK) after wet digestion of dried and ground samples by HNO_3 and H_2O_2. Standard solutions were obtained from Carlo Erba, Italy. Standard reference plant material used was obtained from NIST (1515 Apple leaves).

The results were statistically analysed (ANOVA) using the Tukey's test to check for significant differences between means ($P \leq 0.05$).

RESULTS AND DISCUSSION

In the present study we demonstrated highly beneficial effects of NO on plant growth and indicators of stress in bean plants subjected to 0.2 mmol/L Ni. However, these effects were accompanied with incomplete and specific NO interference with the ion uptake disturbance caused by Ni.

Nickel induced significant inhibition of growth in leaves and especially in roots of bean plants after 4 days exposure (Table 1), which confirms previous results (Gajewska and Sklodowska 2005). Nitric oxide completely eliminated this effect, similarly to the findings of Kopyra and Gwóźdź (2003) and Laspina et al. (2005) obtained for Pb- and Cd-stressed plants.

The activity of antioxidative enzymes was changed by Ni. Thus, SOD activity was significantly increased and CAT activity was decreased in roots and leaves after 4 days treatment, but POD activity was almost unaffected (Table 2). Stimulation of SOD is a common finding for a number of toxic elements (Schützendübel and Polle 2002, Kopyra and Gwóźdź 2003, Laspina et al. 2005) and was confirmed by Yan et al. (2008) in Ni-treated *Jathropa curcas* L.

Table 1. Effect of Ni on root and leaves fresh weight (g/plant) in control (0) and NO-treated bean seedlings

Treatment	Root	Leaves
0	0.72[a]	0.81[a]
0 + Ni	0.36[b]	0.59[b]
NO	0.50[c]	0.92[a]
NO + Ni	0.55[c]	0.86[a]

Each result is the average of 20 repetitions. Different small letters within the same column indicate significance at 0.05 level

Table 2. Effect of Ni on the parameters of antioxidant response in control (0) and NO-treated bean seedlings

	Treatment	SOD (U/g FW)*	POD (μmol/min/g FW)	CAT (μmol/min/g FW)	Soluble proteins (mg/g FW)	Proline (μmol/g FW)
Root	0	81.2[a]	9.4[a]	0.5[a]	4.3[a]	127.6[a]
	Ni	229.3[b]	8.5[a]	0.1[b]	6.8[b]	1527.5[b]
	NO	122.5[c]	3.0[b]	0.6[a]	5.9[b]	857.0[c]
	NO + Ni	123.5[c]	5.3[c]	12.8[c]	4.2[a]	798.5[c]
Leaves	0	63.2[a]	13.2[a]	50.7[a]	14.1[a]	27.0[a]
	Ni	100.0[b]	11.5[a]	25.1[b]	25.4[b]	817.5[b]
	NO	30.5[c]	29.3[b]	89.4[c]	16.1[a]	264.1[c]
	NO + Ni	61.8[a]	43.5[c]	198.1[d]	15.5[a]	525.0[d]

Each result is the average of 3 repetitions. Different small letters within the same column indicate significance at 0.05 level. *SOD activity unit (U) is the quantity of the enzyme extract required to cause 50% inhibition of NBT reduction measured at 560 nm

cotyledons. However, Gajewska and Sklodowska (2005) found a decrease of SOD activity in roots and leaves and a decrease of CAT activity only in leaves of pea plants at a Ni concentration of 0.2 mmol/L. The activity of POD was reported to be both stimulated (Schützendübel and Polle 2002, Kopyra and Gwóźdź 2003, Laspina et al. 2005) and depressed (Schützendübel and Polle 2002, Martins and Mourato 2006, Yan et al. 2008) by toxic elements. The reason for these discrepancies probably lies in the dependence of stress severity on the individual plant species, the tissue type and the heavy metal applied (Schützendübel and Polle 2002).

The nickel-induced increase of soluble protein concentration and strong stimulation of proline accumulation in roots and leaves of the bean plants (Table 2) confirm the results of Gajewska and Sklodowska (2005).

In our experiment, these responses to Ni stress were significantly modified by NO. The increase of SOD activity was diminished and the POD and CAT activities in roots and especially in leaves were stimulated by NO (Table 2). Proline stimulation by Ni was completely suppressed in roots and partly in leaves. Nickel-induced soluble protein accumulation was also eliminated. These findings are in accordance with the role of NO as a potent mediator in stress-induced physiological responses of plants. Suppression of SOD activation may be explained by NO itself being a direct scavenger of superoxide radicals. The other effects, NO-induced strengthening of POD and CAT activities resulting in more efficient H_2O_2 destruction and the supression of Ni-induced proline and soluble protein accumulation are further evidence for the well documented but still unclear complex role of NO-activated and – inactivated processes that are part of plant protection against oxidative stress (Kopyra and Gwóźdź 2003, Bartha et al. 2005, Laspina et al. 2005).

However, the observed increase of Ni concentration in Ni- and NO-treated plants in comparison with those Ni-treated (Table 3) indicates that the protective role of NO involves some mechanisms other than either inhibition of Ni uptake or stimulation of Ni extrusion. A mechanism was suggested that NO alleviated metal toxicity by increasing the pectin and hemicellulose contents of the cell wall and increasing the metal accumulation in cell walls (Xiong et al. 2009), but, considering that in our experiment NO did not decrease Ni transport to the leaves, it is more likely that some ligands resposible for Ni neutralization and complexation were activated. This assumption may be supported by the report of Elviri et al. (2010) about identification of nitrosylated phytochelatins in Cd-stressed *Arabidopsis thaliana* plants suggesting that NO, as a recognized regulator of protein activation by S-nitrosylation, may increase the transport,

Table 3. Nickel accumulation (mg/kg DW) in control and NO-treated bean seedlings submitted to 0.2 mmol/L Ni

Treatment	Root	Leaves
0 + Ni	1138.9[a]	335.2[a]
NO + Ni	1344.4[b]	435.3[b]

Each result is the average of 3 repetitions. Different small letters within the same column indicate significance at 0.05 level

but also the inactivation of Ni by modification of phytochelatins or some other ligands containing SH-group.

The presence of NO alone, applied at the concentration of 0.3 mmol/L, led to a substantial decrease of K, Ca, Mg, Mn, and Zn concentrations in roots and of Ca and Mn concentrations in leaves, and to a significant increase of Fe concentration both in roots and leaves (Table 4). Negative NO effects on ion accumulation may be ascribed to NO being a free radical itself, capable at higher concentrations to induce lipid peroxidation and S-nitrosylation of proteins, thus damaging plasma membranes (Belligni and Lamattina 1999). Moreover, a role for NO in enzyme-mediated or direct interference with ion channel function was recently demonstrated in plant cells (Sokolovski and Blatt 2007).

The stimulative effect of NO on Fe uptake is probably the consequence of a close relation between NO and Fe metabolism. Nitric oxide had been found to enhance the accumulation of mRNA responsible for Fe-transporter production in Fe-deficient tomato plants (Graziano and Lamattina 2007). In addition, NO has high affinity towards the Fe-containing active sites of many proteins and thus participates in the regulation of Fe transport in plants (Ramirez et al. 2010). Increased Fe accumulation in roots may also result from enhanced hemoglobin synthesis in the presence of exogenous NO, considering that non-symbiotic hemoglobins are involved in a fine mechanism of NO concentration regulation (Dordas 2009).

Nickel applied at 0.2 mmol/L induced the depletion of K, Ca, Mg, Mn, and Zn (Table 4). The decreases were very significant in roots, while in leaves they were less marked or absent. On the other hand, Ni induced an increase of Cu and Fe concentrations in roots, accompanied with lowered Fe concentration in leaves. The observed decrease of K, Ca, Mg and Mn is in accordance with the results of El-Enany et al. (2000). Similarly to our findings, Fe content was observed to increase in shoots and roots of *Trigonella corniculata* L. under the influence of Ni (Parida et al. 2003). The authors also reported an Ni-induced decrease of Zn and, contrary to our results, of Cu. However, they detected significant correlations with Ni concentration only for Zn and Fe, but not for Cu. Stimulation of Fe accumulation by Ni was also observed by Narwal et al. (1991). Exogenous NO affected the impact of Ni on some of the ion concentrations. Nickel-induced changes of K, Cu, Mn, and Fe concentrations were unaffected by NO presence. However, the action of Ni on Ca, Mg, and Zn concentrations changed significantly under the influence of NO (Table 4). Contrary to the effect of Ni alone, in NO-treated plants, a very significant increase of Ca concentration occurred in roots and leaves, accompanied with elevated Zn and unchanged Mg concentrations in roots.

It has been postulated recently that the NO response to stresses in plants is mediated by cytosolic [Ca^{2+}] increase. Nitric oxide stimulates Ca^{2+} release from intracellular stores (Sokolowski and Blatt 2007) but can also act as a strong stimulator of 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 S-nitrosylation of proteins. It appears, however, that NO is also capable of promoting inhibition of Ca^{2+} entry into cells (Vandelle et al. 2006). Our results show that NO alone inhibited Ca accumulation in roots, but under Ni-stress this accumulation was activated. High accumulation of Ca in Ni-treated plants is probably necessitated by increased mobilisation of intracellular and

Table 4. Effect of Ni on ion accumulation in control (0) and NO-treated bean seedlings

	Treatment	K	Ca	Mg	Cu	Zn	Mn	Fe
		(mg/g DW)			(mg/kg DW)			
Root	0	90.6^{a}	5.32^{a}	8.02^{a}	27.4^{a}	351.4^{a}	119.6^{a}	293.6^{a}
	Ni	71.5^{b}	1.67^{b}	4.93^{b}	53.2^{b}	202.8^{b}	60.9^{b}	773.0^{b}
	NO	74.7^{b}	2.11^{b}	5.81^{bc}	22.0^{c}	75.0^{c}	98.4^{c}	638.0^{c}
	NO + Ni	57.8^{c}	4.54^{c}	6.56^{c}	39.4^{d}	126.4^{d}	22.7^{d}	826.7^{b}
Leaves	0	39.9^{a}	4.21^{a}	2.34^{a}	17.3^{a}	58.5^{a}	47.5^{a}	186.4^{a}
	Ni	37.9^{a}	3.37^{b}	2.02^{a}	16.5^{a}	55.8^{a}	34.3^{b}	132.0^{b}
	NO	37.8^{a}	1.96^{c}	2.66^{a}	12.7^{b}	71.6^{b}	34.7^{b}	318.8^{c}
	NO + Ni	35.8^{a}	3.52^{b}	2.46^{a}	12.0^{b}	50.7^{a}	31.3^{b}	227.8^{d}

Each result is the average of 3 repetitions. Different small letters within the same column indicate significance at 0.05 level

extracellular Ca^{2+} as a mediator in NO-regulated protective responses of the plant cells. Absence of Mg decrease in NO-treated bean roots in the presence of Ni may be either due to activation of weakly selective Ca-uptake channels or to increased requirements for Mg by NO-activated synthesis of enzymes and/or other proteins.

The Ni-induced increase of Zn accumulation in the roots of NO-treated bean plants resulted from both activated root Zn uptake and inhibition of Zn transport into the shoots. It is probably connected with an increase of Cu/Zn-SOD synthesis. Contrary to Mn-SOD, this isoform of SOD is susceptible to H_2O_2 and its activity could be suppressed by Ni-evoked oxidative stress accompanied with H_2O_2 accumulation. However, in NO-treated plants, H_2O_2 concentration is likely to be decreased by higher activities of POD and CAT, so enabling the synthesis of Cu/Zn-SOD. This is the only isoform found in cytosol and thus can be more efficient in eliminating superoxide radicals.

Our results demonstrated that NO provides the resistance towards Ni and has an ameliorating effect on Ni-stressed bean plants. However, considering that NO itself is a stress factor, its application should be carefully adjusted to the severity of the stress and to the characteristics of the plant species. Our results also demonstrated that NO did not prevent most changes in ion concentration caused by the presence of Ni, except that it specifically stimulated Ca and Zn accumulation and prevented Mg decrease in Ni-treated plants. This was probably due to NO-activation of only specific pathways participating in plant antioxidative responses to the stress. Such a finding would also suggest that Ni effects on ion uptake may not be mediated only by oxidative stress, but by some additional mechanicms unsusceptible to NO.

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