The effect of risk elements in soil to nitric oxide metabolism in tobacco plants

D. Procházková[1], D. Haisel[1], D. Pavlíková[2], R. Schnablová[1], J. Száková[2], R. Vytášek[3], N. Wilhelmová[1]

[1]*Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Prague, Czech Republic*
[2]*Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Prague, Czech Republic*
[3]*Department of Medical Chemistry and Biochemistry, Charles University, Prague, Czech Republic*

ABSTRACT

We studied changes of endogenous nitric oxide content (NO) and of reactive nitrogen species metabolism in transgenic tobacco with prolonged life span (SAG) and in wild tobacco (WT) cultivated in the control and in the polluted soil. There was no difference in the metal accumulation between WT and SAG plants however SAG ones showed better ability to cope with risk elements, as they retained higher membrane stability index and chlorophyll content together with better photochemical efficiency and lower deepoxidation status. Risk elements induced higher NO production in the youngest leaves of both plant types. Low and middle leaves of both WT and SAG plants showed similar activities of nitrate reductase and nitrosoglutathione reductase. Increase of nitrotyrosine content in leaf soluble proteins suggests that risk elements induced nitrosative stress in both plant types.

Keywords: nitrate reductase; nitrosoglutathione reductase; nitrotyrosine; *Nicotiana tabacum* L.

Nitric oxide (NO) plays a definitive role in regulating a number of fundamental biological processes in plants (Neill et al. 2003). Number of articles reported the effects of exogenous NO in alleviating risk element toxicity (e.g. Xiong et al. 2010). All of these reports reveal the importance of exogenous NO in the protection against deleterious effects of risk elements, and suggest the mechanisms by which NO helps plants to resist risk element stress. First, by indirectly scavenging risk element-induced reactive oxygen species, NO might be involved in increasing the antioxidant content and antioxidant enzyme activity. Second, by affecting root cell wall components NO might increase risk element accumulation in root cell walls and decrease risk element accumulation in the soluble cell fraction of leaves. Finally, NO could function as a signalling molecule in the cascade of events leading to changes in gene expression under risk element stress (Xiong et al. 2010). However, the reports regarding the effects of risk elements on endogenous NO content are scarce and the results in different plant species and tissues are often contradictory (Xiong et al. 2010).

Plants with introduced *SAG*12*:ipt* gene construct, which increases cytokinin biosynthesis in response to senescence and thus these plants have longer life span, showed better tolerance against abiotic stresses compared to nontransformed plants (Huynh et al. 2005, Xu et al. 2009, Merewitz et al. 2010). Their resistance was associated with the maintenance of greater antioxidant enzyme activities (Merewitz et al. 2011). As the metal specific regulation of pro-oxidative and antioxidative genes was described (Opdenakker et al. 2012), we suppose that these plants are also more resistant against premature senescence induced by altered levels of risk elements. Our hypothesis is that they

Supported by the Czech Science Foundation, Grant No. P501/11/1239.

will also differ in their reactive nitrogen species (RNS) metabolism compared to wild plants similarly as they differ in their reactive oxygen species (ROS) metabolism during both natural and stress induced senescence. To our knowledge, it is the first study of the effect of risk elements on RNS metabolism in transgenic plants with increased cytokinin content.

MATERIAL AND METHODS

We used tobacco (*Nicotiana tabacum* L., cv. Wisconsin 38) transformed with a construct consisting of *SAG*12 promoter fused with *ipt* gene for cytokinin synthesis (SAG plants). As a control we used its wild type (WT plants). After *in vitro* precultivation, plants were cultivated for 60 days in the polluted soil (Gleyic Cambisol – pH = 6.1, CEC = 134 $mmol_{(+)}$/kg, C_{org} = 2.1%) from the site Příbram (highly polluted mainly by atmospheric emissions from the smelter), marked as PS and in nonpolluted soil (Chernozem – pH = 7.2, CEC = 258 $mmol_{(+)}$/kg, C_{org} = 1.8%) from the site Prague-Suchdol, marked as CS, both in Central Bohemia, Czech Republic. Plants were grown in greenhouse as described in Procházková et al. (2008). We employed two individual groups of samples; each was comprised of the mixture of six leaves per each group. Leaves were divided into three groups: low (1st and 2nd leaf), middle (3rd and 4th leaf) and upper (5th and 6th leaf).

Membrane stability index (MSI) was measured as leaf electrolyte leakage (Sairam et al. 1997). Pigment contents were established by HPLC (ECOM, Prague, Czech Republic) from acetone extracts using a reversed phase column Sepharon SGX C18 (Tessek, Praha, Czech Republic), data were captured and calculated by PC-software Clarity (DataApex, Prague, Czech Republic) (Procházková et al. 2008). Chlorophyll fluorescence parameters from slow kinetics were measured after a 15 min dark period with the PAM Chl fluorometer (Walz, Effeltrich, Germany) on adaxial side of fresh leaves, using the DA 100 data acquisition system (Walz, Effeltrich, Germany) for sampling and calculation (Wilhelmová et al. 2005).

Contents of 'pseudo-total' and mobile portions of As, Cd, Pb, and Zn in soils and in leaf digests were determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Varian, VistaPro, Mulgrave, Australia) (Žalud et al. 2012).

Assay of total N content, NO and relative RNS. For determination of total nitrogen content, the leaves were analyzed by the Kjeldahl method (Kjeltec Auto 1030, Neuberg et al. 2010). For leaf NO detection *in situ* by confocal laser scanning microscopy (CLSM), leaf segments were incubated with 4-aminomethyl-20,70-difluorofluoresceein diacetate (DAF-2) and then inspected with a CLSM system (Zeiss 5 Duo, Jena, Germany), using standard filters and collection modalities for DAF-2 green fluorescence (excitation at 495 nm; emission at 515 nm) (Corpas et al. 2008). The protein nitrotyrosine content in soluble fraction was assayed by a competitive ELISA with the aid of monoclonal antibodies (Wilhelmová et al. 2006). Nitrate reductase (NR) and nitrosoglutathione reductase (GSNOR) activities were determined spectrophotometrically (Hitachi U 3300, Tokyo, Japan) at 540 nm (Gaudinová 1990) and 340 nm (Corpas et al. 2008), respectively. The protein content for nitrotyrosine and GSNOR activity assays were estimated according to Lowry et al. (1951) and Bradford (1976), respectively.

Statistical treatment. Each measurement was assayed in triplicate. Shapiro-Wilk test has been used for the normality test. For the analysis of difference between both genotypes, the data were subjected to two-way ANOVA (NCSS, Kaysville, USA).

RESULTS AND DISCUSSION

The contents of As, Cd, Pb and Zn in both soils are shown in Table 1. Compared to the plants from CS, the contents of As, Cd, Pb and Zn increased in leaves of plants grown in PS without statistical difference between these contents in WT and SAG plants (Table 2). Zn is an essential element for plant cell physiological processes, but it can be toxic when present in excess (Broadley et al. 2007). Other risk elements are reported to have adverse effect on photosynthetic apparatus (Mobin and Khan 2007). Contrary to the other metal accumulation, Zn content in leaves of both plant types increased in upper leaves; only As content was the highest in the middle leaves of both tobacco types. Compared to the same leaves of SAG plants, leaves

Table 1. Contents of As, Cd, Pb, and Zn in the polluted soil (% of the control soil)

	As	Cd	Pb	Zn
Pseudototal portion	538	707	3 562	226
CH_3COOH	182	4 000	21 214	726

Table 2. Contents of selected metals in wild type (WT) and cytokinin synthesis (SAG) plants in low, middle and upper leaves (mg/kg dry weight)

Parameter	WT – control			WT – polluted		
	low	middle	upper	low	middle	upper
As	1.23 ± 0.55	0.77 ± 0.24	0.37 ± 0.05	2.67 ± 0.35	3.94 ± 0.09	1.18 ± 0.61
Cd	1.96 ± 1.59	0.94 ± 0.75	0.82 ± 0.49	23.5 ± 5.52	13.65 ± 3.04	11.26 ± 1.89
Pb	1.26 ± 0.13	0.66 ± 0.03	0.39 ± 0.07	20.69 ± 12.32	9.62 ± 3.09	11.45 ± 10.39
Zn	19.45 ± 5.02	15.9 ± 2.97	20.45 ± 4.31	28.25 ± 0.35	38.85 ± 6.26	36.50 ± 6.65
	SAG – control			SAG – polluted		
	low	middle	upper	low	middle	upper
As	1.48 ± 0.06	0.74 ± 0.16	0.35 ± 0.25	2.43 ± 0.93	5.37 ± 0.08	1.18 ± 0.29
Cd	1.24 ± 1.00	0.77 ± 0.56	0.89 ± 0.85	28.75 ± 10.39	15.45 ± 6.01	9.21 ± 2.53
Pb	0.14 ± 0.04	0.45 ± 0.39	0.96 ± 1.12	28.95 ± 25.10	18.10 ± 3.82	12.80 ± 6.91
Zn	21.8 ± 7.49	12.00 ± 1.27	18.75 ± 3.61	27.75 ± 10.11	28.05 ± 2.47	29.10 ± 6.08

Table 3. Contents of total chlorophyll (mg/g dry weight), maximal efficiency of photosystem II, membrane stability index (%), de-epoxidation status (% of total nitrogen [%]), nitrate reductase activity (nmol NO_2^-/g fresh weight/min), S-nitrosoglutathione reductase activity (ΔA_{340} mg protein/min), protein nitrotyrosine concentration in soluble fraction activity (pmol/mg protein) in low, middle and upper leaves of wild type (WT) and cytokinin synthesis (SAG) plants grown in control and polluted soil

Parameter	WT – control			WT – polluted		
	low	middle	upper	low	middle	upper
Chlorophyll content	454 ± 29	522 ± 19	495 ± 33	349 ± 52	462 ± 40	443 ± 52
F_v/F_m	0.73 ± 0.04	0.79 ± 0.02	0.82 ± 0.01	0.62 ± 0.14	0.76 ± 0.03	0.79 ± 0.03
MSI	87 ± 6	93 ± 3	87 ± 5	79 ± 6	87 ± 2	85 ± 4
DEPS	0.16 ± 0.01	0.16 ± 0.01	0.18 ± 0.02	0.22 ± 0.01	0.19 ± 0.01	0.19 ± 0.02
N (%)	1.42 ± 0.50	2.05 ± 0.74	3.65 ± 0.04	1.87 ± 0.14	2.91 ± 0.34	4.81 ± 0.40
NR	12.6 ± 0.5	20.5 ± 0.9	24.6 ± 2.5	7.8 ± 2.7	17.8 ± 2.8	23.4 ± 4
GSNOR	1.14 ± 0.17	1.01± 0.11	1.07 ± 0.19	1.66 ± 0.34	1.27 ± 0.23	0.92 ± 0.04
Nitrotyrosine	447 ± 37	454 ± 140	264 ± 29	1593 ± 186	1538 ± 159	580 ± 98
	SAG – control			SAG – polluted		
	low	middle	upper	low	middle	upper
Chlorophyll content	509 ± 35	603 ± 28	566 ± 57	472 ± 36*	603 ± 40*	579 ± 40*
F_v/F_m	0.77 ± 0.04	0.80 ± 0.02	0.81 ± 0.01	0.76 ± 0.02*	0.78 ± 0.03*	0.80 ± 0.03
MSI	91 ± 5	93 ± 2	91 ± 4	90 ± 7*	87 ± 6	86 ± 4
DEPS	0.12 ± 0.01*	0.14 ± 0.02	0.15 ± 0.01	0.15 ± 0.01*	0.14 ± 0.01*	0.14 ± 0.01*
N (%)	1.56 ± 0.18	2.04 ± 0.30	3.42 ± 0.16	1.98 ± 0.21	2.84 ± 0.11	4.91 ± 0.42
NR	12.1 ± 1.9	24.0 ± 0.58	26.7 ± 2.7	7.6 ± 1.7	16.5 ± 3.2	26.1 ± 1.3
GSNOR	0.94 ± 0.16	0.94 ± 0.13	0.74 ± 0.16	1.20 ± 0.04	1.23 ± 0.11	0.64 ± 0.09
Nitrotyrosine	354 ± 106	303 ± 14	206 ± 18	1243 ± 180	969 ± 41*	320 ± 13*

Asterisks indicate significance of differences between corresponding leaves of WT and SAG plants ($P < 0.05$). MSI – membrane stability index; DEPS – de-epoxidation state; NR – nitrate reductase; GSNOR – nitrosoglutathione reductase activity

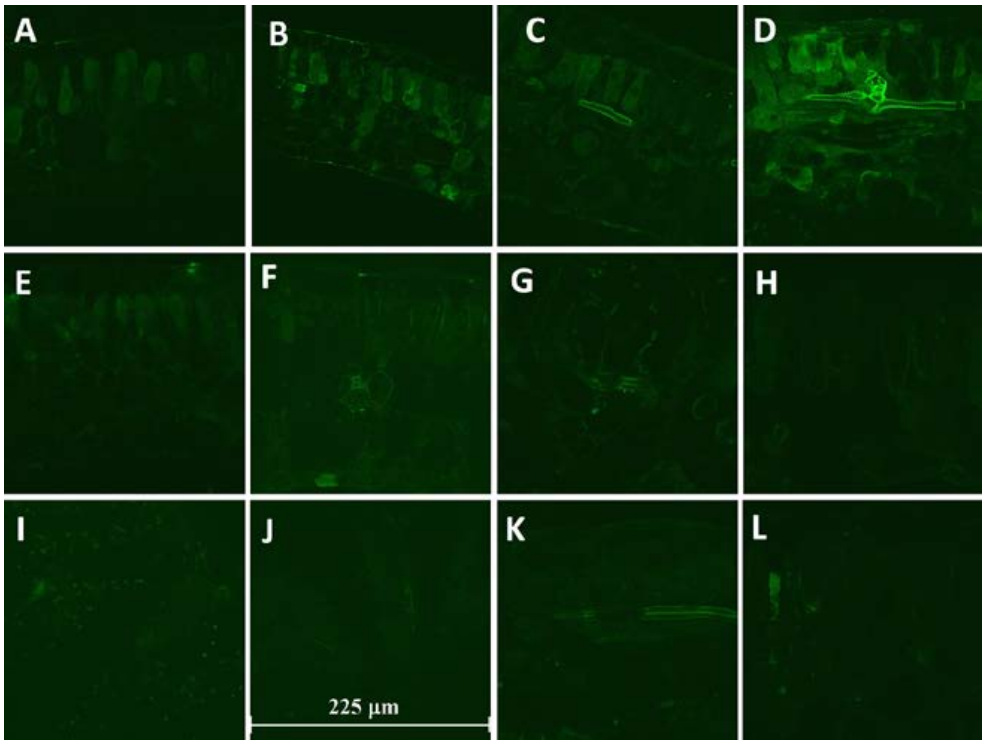

Figure 1. Representative images illustrating the confocal laser scanning microscopy (CLMS) detection and visualization of endogenous NO in tobacco leaves. First row: cross section of the upper leaf of wild type (WT) plant in control soil (A), plants with prolonged life spain (SAG) in control soil (B), WT plant in polluted soil (C), SAG plant in polluted soil (D). Second row: cross section of the middle leaf of WT plant in control soil (E), SAG plant in control soil (F), WT plant in polluted soil (G), SAG plant in polluted soil (H). Third row: cross section of the low leaf of WT plant in control soil (I), SAG plant in control soil (J), WT plant in polluted soil (K), SAG plant in polluted soil (L). Scale bar = 0.22 µm

of WT plants grown in the PS showed lower total chlorophyll content and also lower photosystem II efficiency, which is considered to be a sensitive parameter reflecting stress induced damage (Humbeck et al. 1996) (Table 3). We also suppose that photosynthetic electron transport was declined in WT plants from PS, as is demonstrated by their higher deepoxidation state of the xanthophyll cycle pigments. WT plants from PS had also lower MSI compared to SAG plants. Hence, we could presume that SAG plants might be better equipped to cope with risk element pollution.

NO and related molecules such as S-nitrosoglutathione (GSNO) and nitrotyrosine, among others, are involved in the mechanisms of response to stress conditions (Chaki et al. 2011). The analysis of NO production by CLMS showed an increase of NO content in the upper leaves of plants from PS, which was more prominent in SAG plants (Figure 1). NR, except for being a key enzyme of nitrate assimilation (Lea 1999), has also the capacity to generate NO (Kaiser et al. 2002). A decrease in NR activity was described with increasing metal concentration (e.g. Gautam et al. 2008). In our experiment, NR activity decreased with leaf age (from the top to the lower leaves) but it is evident that risk elements promoted this decrease both in WT and in SAG plants without any difference in values in both plant types (Table 3). % of total N increased in PS in both genotypes.

Although nitrosoglutathione reductase (GSNOR) was proposed to protect cells from nitrosative stress (Marozkina et al. 2012) response of its activity to various risk elements is ambiguous. For example, GSNOR activity decreased in pea plants grown in the presence of 0.05 mmol Cd/kg (Barroso et al. 2006) but significantly increased *Arabidopsis* seedlings grown in the presence of 0.5 mmol As/kg (Leterrier et al. 2011). In our experiment, GSNOR activity increased in low and middle leaves but slightly decreased in upper leaves (Table 3). These results clearly show the influence of leaf age on metabolism changes and confirm the above mentioned proposition of Groppa et al. (2008), who suggested that the use of different risk element concentrations, the age of the plants and the duration of treatment used are decisive in endogenous NO content in plants treated with risk element loads (Xiong et al. 2010).

The tyrosine nitration of proteins is considered as an indicator of the peroxynitrite action (Radi 2004) and 3-nitrotyrosine is often used as a marker of nitrosative stress (e.g. Valderrama et al. 2007). Although SAG plants had lower nitrotyrosine content both in CS and in PS, there was no difference in relative increase between WT and SAG plants grown in PS (Table 3). Hence, it is clear that risk element treatment induced nitrosative stress both in WT and SAG plants.

In summary, the results reported in this work show that although SAG plants seem to be better equipped to cope with risk element stress they did not show better protection against nitrosative stress.

Acknowledgements

The seeds were the kind gift of Prof. R. Amasino, University of Wisconsin, USA.

REFERENCES

Barroso J.B., Corpas F.J., Carreras A., Rodríguez-Serrano M., Esteban F.J., Fernández-Ocaña A., Chaki M., Romero-Puertas M.C., Valderrama R., Sandalio L.M., del Río L.A. (2006): Localization of S-nitrosoglutathione and expression of S-nitrosoglutathione reductase in pea plants under cadmium stress. Journal of Experimental Botany, *57*: 1785–1793.

Bradford M.M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, *72*: 248–254.

Broadley M.R., White P.J., Hammond J.P., Zelko I., Lux A. (2007): Zinc in plants. New Phytologist, *173*: 677–702.

Chaki M., Valderrama R., Fernández-Ocaña A.M., Carreras A., Gómez-Rodríguez M.V., Pedrajas J.R., Begara-Morales J.C., Sánchez-Calvo B., Luque F., Leterrier M., Corpas F.J., Barroso J.B. (2011): Mechanical wounding induces a nitrosative stress by down-regulation of GSNO reductase and an increase in S-nitrosothiols in sunflower (*Helianthus annuus*) seedlings. Journal of Experimental Botany, *62*: 1803–1813.

Corpas F.J., Mounira C., Fernández-Ocaña A., Valderrama R., Palma J.M., Carreras A., Begara-Morales J.C., Airaki M., del Río L.A., Barroso J.B. (2008): Metabolism of reactive nitrogen species in pea plants under abiotic stress conditions. Plant and Cell Physiology, *49*: 1711–1722.

Gaudinová A. (1990): The effect of cytokinins on nitrate reductase activity. Biologia Plantarum, *32*: 89–96.

Gautam M., Sengar R.S., Garg S.K., Sengar K., Chaudhary R. (2008): Effect of lead on seed germination, seedling growth, chlorophyll content and nitrate reductase activity in mung bean (*Vigna radiata*). Research Journal of Phytochemistry, *2*: 61–68.

Groppa M.D., Rosales E.P., Iannone M.F., Benavides M.P. (2008): Nitric oxide, polyamines and Cd-induced phytotoxicity in wheat roots. Phytochemistry, *69*: 2609–2615.

Humbeck K., Quast S., Krupinska K. (1996): Functional and molecular changes in the photosynthetic apparatus during senescence of flag leaves from field-grown barley plants. Plant, Cell and Environment, *19*: 337–344.

Huynh le N., Vantoai T., Streeter J., Banowetz G. (2005): Regulation of flooding tolerance of *SAG12:ipt Arabidopsis* plants by cytokinin. Journal of Experimental Botany, *56*: 1397–1407.

Kaiser W.M., Weiner H., Kandlbinder A., Tsai C.-B., Rockel P., Sonoda M., Planchet E. (2002): Modulation of nitrate reductase: some new insights, an unusual case and a potentially important side reaction. Journal of Experimental Botany, *53*: 875–882.

Lea P.J. (1999): Nitrate Assimilation. Wiley, London.

Leterrier M., Chaki M., Airaki M., Valderrama R., Palma J.M., Barroso J.B., Corpas F.J. (2011): Function of S-nitrosoglutathione reductase (GSNOR) in plant development and under biotic/abiotic stress. Plant Signalling and Behaviour, *6*: 789–793.

Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. (1951): Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, *193*: 265–275.

Merewitz E.B., Gianfagna T., Huang B. (2010): Effects of *SAG12-ipt* and *HSP18.2-ipt* expression on cytokinin production, root growth, and leaf senescence in creeping bentgrass exposed to drought stress. Journal of the American Society for Horticultural Science, *135*: 230–239.

Merewitz E.B., Gianfagna T., Huang B. (2011): Protein accumulation in leaves and roots associated with improved drought tolerance in creeping bentgrass expressing an *ipt* gene for cytokinin synthesis. Journal of Experimental Botany, *9*: 1–23.

Mobin M., Khan N.A. (2007): Photosynthetic activity, pigment composition and antioxidative response of two mustard (*Brassica juncea*) cultivars differing in photosynthetic capacity subjected to cadmium stress. Journal of Plant Physiology, *164*: 601–610.

Marozkina N.V., Wei C., Yemen S., Wallrabe H., Nagji A.S., Liu L., Morozkina T., Jones D.R., Gaston B. (2012): S-nitrosoglutathione reductase in human lung cancer. American Journal of Respiratory Cell and Molecular Biology, *46*: 63–70.

Neill S.J., Desikan R., Hancock J.T. (2003): Nitric oxide signalling in plants. New Phytologist, *159*: 11–35.

Neuberg M., Pavlíková D., Pavlík M., Balík J. (2010): The effect of different nitrogen nutrition on proline and asparagine content in plant. Plant, Soil and Environment, *56*: 305–311.

Opdenakker K., Remans T., Keunen E., Vangronsveld J., Cuypers A. (2012): Exposure of *Arabidopsis thaliana* to Cd or Cu excess leads to oxidative stress mediated alterations in MAPKinase transcript levels. Environmental and Experimental Botany, *83*: 53–61.

Procházková D., Haisel D., Wilhelmová N. (2008): Antioxidant protection during ageing and senescence in chloroplasts of tobacco with modulated life span. Cell Biochemistry and Function, *26*: 582–590.

Radi R. (2004): Nitric oxide, oxidants, and protein tyrosine nitration. Proceedings of the National Academy of Sciences of the United States of America, *23*: 4003–4008.

Sairam R.K., Deshmukh P.S., Shukla D.S. (1997): Tolerance to drought and temperature stress in relation to increased antioxidant enzyme activity in wheat. Journal of Agronomy and Crop Science, *178*: 171–177.

Valderrama R., Corpas F.J., Carreras A., Fernández-Ocaña A., Chaki M., Luque F., Gómez-Rodríguez M.V., Colmenero-Varea P., del Río L.A., Barroso J.B. (2007): Nitrosative stress in plants. FEBS Letters, *581*: 453–461.

Wilhelmová N., Fuksová H., Srbová M., Miková D., Mýtinová Z., Procházková D., Vytásek R., Wilhelm J. (2006): The effect of plant cytokinin hormones on the production of ethylene, nitric oxide, and protein nitrotyrosine in ageing tobacco leaves. BioFactors, *27*: 203–211.

Wilhelmová N., Procházková D., Šindelářová M., Šindelář L. (2005): Photosynthesis in leaves of *Nicotiana tabacum* L. infected with tobacco mosaic virus. Photosynthetica, *43*: 597–602.

Wilson I.D., Neill S.J., Hancock J.T. (2008): Nitric oxide synthesis and signalling in plants. Plant, Cell and Environment, *31*: 622–631.

Xiong J., Fu G., Tao L., Zhu C. (2010): Roles of nitric oxide in alleviating heavy metal toxicity in plants. Archives of Biochemistry and Biophysics, *497*: 13–20.

Xu Y., Tian J., Gianfagna T., Huang B. (2009): Effects of *SAG12-ipt* expression on cytokinin production, growth and senescence of creeping bentgrass (*Agrostis stolonifer* L.) under heat stress. Plant Growth Regulation, *57*: 281–291.

Žalud P., Száková J., Sysalová J., Tlustoš P. (2012): The effect of contaminated urban particulate matter on risk element contents in leafy vegetables. Central European Journal of Biology, *7*: 519–530.

Received on April 23, 2012

Corresponding author:

Ing. Dagmar Procházková, Ph.D., Ústav experimentální botaniky, Akademie věd České republiky, v.v.i., Rozvojová 263, Praha 6, Česká republika
phone: + 420 224 310 109, fax: + 420 224 310 113, e-mail: prochazkovad@ueb.cas.cz