

1 **ACCEPTED MANUSCRIPT**

2 **Article type: REVIEW**

3

4 **Title: Implication of nitric oxide (NO) in excess element-induced morphogenic responses**  
5 **of the root system**

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7 Zsuzsanna Kolbert<sup>1</sup>

8 <sup>1</sup>Department of Plant Biology, Faculty of Science and Informatics, University of Szeged,  
9 HUNGARY

10

11 Postal address: Department of Plant Biology  
12 University of Szeged  
13 Közép fasor 52.  
14 H-6726 HUNGARY

15

16 Corresponding Author: Zsuzsanna Kolbert  
17 e-mail: [kolzsu@bio.u-szeged.hu](mailto:kolzsu@bio.u-szeged.hu)  
18 telephone/fax: +36-62-544-307

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22 **Running title:** NO in excess element-induced SIMR of the root system

23

## 1 **Abstract**

2           Extremes of metal and non-metal elements in the soils create a stressful environment  
3 and plants exposed to sub-lethal abiotic stress conditions show a broad range of morphogenic  
4 responses designated as stress-induced morphogenic response (SIMR). Being the first plant  
5 organ directly contacting with elevated doses of elements, the root system shows remarkable  
6 symptoms and deserves special attention. In the signalling of root SIMR, the involvement of  
7 phytohormones (especially auxin) and reactive oxygen species (ROS) has been earlier  
8 suggested. Emerging evidence supports that nitric oxide (NO) and related molecules (reactive  
9 nitrogen species, RNS) are integral signals of root system development, and they are active  
10 components of heavy metal-induced stress responses as well. Based on these, the main scope  
11 of this review is to demonstrate the contribution of NO/RNS to the emergence of excess  
12 element-induced root morphogenic responses. The SIMR-like root system of lead-treated  
13 *Arabidopsis thaliana* contained elevated NO levels compared to the root not showing SIMR.  
14 In NO-deficient *nia1nia2* plants, the degree of selenium-induced root SIMR was, in some  
15 characteristics altered compared to the wild-type. Moreover, among the molecular elements of  
16 SIMR several potential candidates of NO-dependent S-nitrosylation or tyrosine nitration have  
17 been found using computational prediction. The demonstrated literature data together with  
18 own experimental results strongly outline that NO/RNS are regulating signals in the  
19 development of root SIMR in case of excess metal and non-metal elements. This also reveals  
20 a new role of NO in acclimation emphasizing its importance in defence mechanisms against  
21 abiotic stresses.

22

23 **Keywords:** excess element, nitric oxide, stress-induced morphogenic response, root system

24

## 25 **Abbreviations:**

26 CK cytokinin; cPTIO 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1-imidazolyl-1-  
27 oxy-3-oxide; ET ethylene; H<sub>2</sub>O<sub>2</sub> hydrogen peroxide; LR lateral root; NO nitric oxide; PR  
28 primary root; RNS reactive nitrogen species; ROS reactive oxygen species; SIMR stress-  
29 induced morphogenic response; SNP sodium nitroprusside.

## 1. Excess element-induced morphogenic responses of the root system: common features induced by different conditions

Due to their cumulative effects and long-term interactions, the inordinate accumulation of different metal (e.g. heavy metals like copper, Cu; cadmium, Cd; lead, Pb) and non-metal (e.g. selenium, Se; bromine, Br) elements in the soils can be a challenge for living organisms, especially for plants. Being sessile organisms, the reorientation of growth is the only option for plants to survive e.g. in an environment exposed to excess doses of elements. The common morphological symptoms of this developmental adaptation were determined and their manifestation was named as stress-induced morphogenic responses (SIMR, Potters et al., 2007). After a literature survey, it's evident that during excess element-triggered SIMR the main target is the root system, which is not surprising giving the fact that it is the first organ growing in the soil. Therefore this organ is in direct contact with the high doses of elements. Furthermore, in case of excessive external supply, the uptake of elements is often accompanied by their disproportionate accumulation in root cells. For the above reasons, roots show alterations in their growth and morphology as a part of their SIMR. At cellular level the main symptoms are the blocked cell division in the primary meristem, the inhibited cell elongation, the induced pericycle cell division and the altered cell differentiation (Potters et al., 2007). Blocked cell division and intensified cell differentiation could be supported by molecular data in the primary root (PR) meristem of *Arabidopsis* treated with the metal element chromium (in the form of dichromate salt). In these roots, the expression of the mitotic marker *CycB1;1* gene was decreased and the expression of cell differentiation marker (*Exp7:uidA*) appeared closer to the meristem (Castro et al., 2007). As a result of the counteracting growth inhibition and activation mechanisms, the root system showing SIMR phenotype is generally shorter but contains more lateral root (LR) compared with control roots. These main symptoms of SIMR were observed in case of several elements and numerous plant species (Table 1) indicating that the disturbance of element homeostasis is an effective inducer of root growth alterations. Among essential microelements, the effect of copper is well documented. For example, in *Arabidopsis* grown and treated with Cu in agar, the PR shortening was accompanied by the reduction of the mitotic index and the intensification of meristem cell death (Lequeux et al., 2010). Moreover, the copper-triggered SIMR phenotype appeared also in *Brassica juncea*, *Brassica napus*, *Triticum aestivum* and *Origanum vulgare* grown in nutrient solution or soil (Feigl et al., 2013; Singh et al., 2007; Mahmood et al., 2007; Panou-Filotheou and Bosabalidis, 2004). Similarly, in case of excess zinc (Zn), SIMR phenotype appeared in several mono- and dicot species grown in various

1 media. Among them, the Zn hyperaccumulator and tolerant *Thlaspi caerulescens* developed  
2 more lateral roots as a response to localized Zn enrichment; while in case of the non-  
3 accumulator *T. arvense* excess Zn had a negative effect on PR elongation and LR formation  
4 (Whiting et al., 2000). In case of localized selenium supply, the non-hyperaccumulator  
5 *Brassica juncea* showed no SIMR phenotype, while the Colorado ecotype of the  
6 hyperaccumulator *Stanleya pinnata* was able to reorient its root growth (Goodson et al.,  
7 2003). These results reveal a correlation between the hyperaccumulating capacity and the  
8 ability of growth reprogramming. However, question arises about the adaptive advantages (if  
9 any) of the appearance of SIMR phenotype. The possible contribution of root SIMR to the  
10 direct evasion of metal contaminated sites has been raised by Potters et al. (2007). According  
11 to this idea, the function of excess element-induced changes in root morphology may be to  
12 redirect root development away from a local source of xenobiotics. The SIMR-type root  
13 system contains more lateral roots, which provide a lateral expansion at the same time a better  
14 fixation for the root system. Moreover, the enhanced number of LRs can contribute to  
15 improved water and nutrient uptake promoting endurance of the plant. The possible  
16 involvement of SIMR in tolerance supports the hypothesis that SIMR is not the inevitable  
17 consequence of stress, but a joint of active acclimation processes. This is also suggested by  
18 the fact that SIMR is induced by mild doses of excess elements while under severe stress the  
19 growth responses are inhibited. For instance, in *Arabidopsis* 10  $\mu\text{M}$  selenite or 5  $\mu\text{M}$  copper  
20 increased but 40  $\mu\text{M}$  selenite or 50  $\mu\text{M}$  copper reduced the number of LR primordia (Lehotai  
21 et al., 2012; Kolbert et al., 2012). Furthermore, non-essential elements are also able to trigger  
22 the formation of the SIMR phenotype. As shown in Fig 1A, exposure of *Arabidopsis thaliana*  
23 to 25  $\mu\text{M}$  lead nitrate ( $\text{PbNO}_3$ ) resulted in shorter PR (by 28%) and enhanced number of  
24 lateral roots (by 60%). Similarly, *Brassica juncea* roots grown and treated in nutrient solution  
25 also developed SIMR in response to lead (Fig 1B).

26 Based on the above detailed literature (summarized in Table 1) and experimental data  
27 (presented in Fig 1), the emergence of root growth responses seems to be independent from  
28 the type (e.g. essential, non-essential) and the property (e.g. redox-active, redox-inactive  
29 metal, non-metal) of the element. The fact that SIMR appears in various monocot and dicot  
30 plant species grown in different conditions (soil, agar, solution) supports the species-  
31 independence thus the general nature of this stress response. Although, both the concentration  
32 of the element and the duration of the exposure determines the emergence of SIMR; generally  
33 low doses (corresponding to mild, sublethal stress) result in growth reprogramming in a  
34 relative long duration. As it was mentioned above, the emergence of SIMR phenotype can be

1 connected to tolerance, which emphasizes its ecological relevance in contaminated areas.  
2 Although, it has to be mentioned, that there is no direct experimental result demonstrating  
3 how does SIMR lead to tolerance so far. Therefore, an important task for future research is to  
4 answer this exciting question.

5

## 6 **2. Components of the signal transduction of excess element-induced SIMR**

7 With respect that during mild stress-induced growth responses, the inhibition of PR  
8 elongation is accompanied by LR initiation, the cell division of pericycle considered to be  
9 more tolerant compared to cell elongation possibly due to the endodermal barrier and the  
10 characteristic structure of central cylinder. By all means, these morphological alterations  
11 induced by environmental signals (e.g. excess element) are needed to be tightly coordinated  
12 by endogenous signal networks.

### 13 *2.1 Hormonal components of SIMR induced by excess elements*

14 The architecture of the root system is highly determined by the distribution of growth  
15 hormones such as auxin, cytokinin (CK) and ethylene (ET).

#### 16 *2.1.1 Auxin as integral growth signal during SIMR*

17 Auxin is a major player in altering PR growth and in promoting root hair and LR  
18 formation. All of these parameters are altered during SIMR suggesting the involvement of  
19 auxin. The phenotype of *Arabidopsis* seedlings exposed to e.g. copper sulphate resembles  
20 those of plants altered in auxin metabolism (Pasternak et al., 2005; Kolbert et al., 2012).  
21 The excess of different elements (e.g. copper, cadmium) results in the redistribution of  
22 auxin within the root system, which has been revealed mainly by the *in situ* detection of the  
23 auxin responsive DR5 promoter activity. E.g. Potters et al. (2007) found that the  
24 DR5::*GUS* activity decreased in the root tips, but intensified in the upper root parts in Cd-  
25 exposed *Arabidopsis*. Copper induced similar alterations in auxin distribution (Lequeux et  
26 al., 2010). In a detailed study it was shown that the auxin signal disappeared from  
27 columella, but increased in the meristematic and elongation zones of the PR (Yuan et al.,  
28 2013). In the tips of Cu- treated *Arabidopsis* roots, DR5 promoter activity was strong and  
29 expanded in a short term (7 days), but was reduced in a longer term (17 days), which  
30 demonstrated the temporal evolution of auxin redistribution induced by excess metal (Pető  
31 et al., 2011; Kolbert et al., 2012). The spatiotemporal distribution of auxin is controlled via  
32 the regulation of its *de novo* biosynthesis, transport, degradation and conjugation reactions.

1           Theoretically all of these processes can be modulated by stress; although the stress-  
2 induced alterations in auxin transport are principally studied. The directional, intercellular  
3 transport of auxin is achieved by precisely localized and regulated influx and efflux carriers.  
4 AUX1 is an influx protein being responsible for indole-acetic-acid (IAA) uptake into the cell.  
5 The main group of carriers involved in auxin export is formed by the PIN-FORMED (PIN)  
6 proteins. Under Cu exposure, PIN1, but not PIN2 or AUX1 seems to be needed for auxin  
7 redistribution in the root tips of *Arabidopsis* (Yuan et al., 2013). In the case of localized iron  
8 supply, the density of lateral roots increased in the wild-type, but not in the *aux1-3* mutant,  
9 which suggests the necessity of AUX1-mediated auxin redistribution for iron-triggered LR  
10 development (Giehl et al., 2012). According to the work of Hu et al. (2013), application of the  
11 auxin transport inhibitor naphthylthalamic acid (NPA) as well as mutations in the *aux1-7* and  
12 the *pin2* genes mitigated cadmium-induced LR formation. These observations reflect that LR  
13 number increase under Cd exposure requires auxin redistribution and the activity of the  
14 AUX1-7 and PIN2 transport proteins. In case of arsenite, SIMR phenotype was more intense  
15 in the *aux1-7* mutant compared to the wild-type. Due to the mutation, both the acro- and the  
16 basipetal auxin transport were reduced. Moreover, inhibitors of auxin transport intensified the  
17 arsenite sensitivity and exogenous IAA improved tolerance in the *aux1-7* mutant  
18 (Krishnamurthy and Rathinasabapathi, 2013).

19           Besides the transport, the regulation of auxin biosynthesis, catabolism and conjugation  
20 also appear to be involved in the evolution of SIMR phenotype. In Cd-exposed *Arabidopsis*  
21 showing SIMR phenotype, the auxin biosynthetic nitrilase (*AtNIT*) gene was upregulated and  
22 consequently the IAA content was elevated (Vitti et al. 2013). Similarly, *Arabidopsis* exposed  
23 to combined treatment of copper, cadmium and zinc showed increased *AtNIT* expression and  
24 enhanced IAA concentration in the root system (Sofa et al., 2013). Also, cadmium induced  
25 the expression of *NIT1*, *NIT2* and the cytochrome P450 monooxygenase *CYP79B3* genes  
26 leading to the intensification of *DR5::GUS* activity in the root system (Wang et al., 2015). In  
27 the same study, lead treatment of *Arabidopsis* seedlings resulted in the up-regulation of the  
28 Gretchen Hagen genes (*GH3.4*, *GH3.1*, *GH3.3*), which are IAA-amido synthases and thought  
29 to be important in controlling free IAA levels. Excess Cd triggered the expression of the auxin  
30 biosynthetic *NIT* and *YUCCA* (*NIT1*, *NIT2*, *YUCCA1*) and the *GH3.9* genes in *Arabidopsis*  
31 seedlings (Li et al., 2015). In these plants, the activity of the *DR5* promoter decreased and that  
32 of the IAA oxidase enzyme increased (Li et al., 2015). Likewise in cadmium-treated  
33 *Medicago* and *Arabidopsis*, the activity of auxin catabolic enzyme IAA oxidase increased and  
34 the IAA content consequently decreased (Xu J et al., 2010b; Hu et al., 2013).

1 Based on the above, the redistribution of the active auxin pool of the root system plays a  
2 fundamental role in excess element-induced root growth responses. However auxin transport,  
3 metabolism, and conjugation processes leading to SIMR, respond differentially to various  
4 treatments suggesting the existence of element-specific background mechanisms.

### 6 2.1.2. Other hormonal components of SIMR-related signalling induced by excess element

7 Besides auxin, cytokinins and ethylene are also notable regulators in the shaping of the  
8 root system architecture; therefore their involvement is also presumable in the evolution of the  
9 SIMR phenotype.

10 Cytokinins are present in high quantities in the root cap and compensate the effect of  
11 auxin in LR initiation therefore help to maintain root apical dominance thus the suitable root  
12 system architecture. In the PR of selenium-exposed *Arabidopsis*, the activity of the cytokinin-  
13 inducible *ARR5::GUS* promoter notably increased. Additionally, the CK overproducer *ipt-161*  
14 mutant was completely insensitive to Se-induced growth inhibition further supporting the role  
15 of CKs in selenium tolerance (Lehotai et al., 2012). In response to Cu exposure, the  
16 *ARR5::GUS* activity was enhanced in the root tips indicating CK accumulation (Lequeux et  
17 al., 2010). However in case of cadmium treatment, *Arabidopsis* plants showed cytokinin  
18 oxidase (CKX) up-regulation in the roots, which could be associated with the observed  
19 increase in LR number (Vitti et al., 2013). In a comprehensive study, Sofu et al. (2013) found  
20 that the excess of copper, cadmium, or zinc as well as the combination of them increased the  
21 *trans*-zeatin riboside and dihydrozeatin riboside content in *Arabidopsis* roots.

22 Ethylene levels are known to be positively regulated by heavy metals like e.g. copper and  
23 cadmium. In case of selenite treatment, the activity of *ACC synthase8::GUS* promoter was  
24 enhanced in the root tips of *Arabidopsis* reflecting ethylene generation. Furthermore, in ET-  
25 deficient (*hls1-1*) and -perception mutant (*etr1-1*), the selenite-induced root growth inhibition  
26 proved to be slighter compared to the WT suggesting the requirement of a normal ET content  
27 and signalling for root growth responses to selenium (Lehotai et al., 2012). In *Lotus*  
28 *japonicus*, aluminium exposure led to the generation of ethylene which was associated with  
29 PR shortening (Sun et al., 2007). In *Arabidopsis*, aluminium treatment triggered ethylene  
30 production that acted as a signal to modify auxin distribution in the roots by disrupting  
31 AUX1- and PIN2-dependent auxin transport (Sun et al., 2010). This was considered to lead to  
32 the observed inhibition of root elongation (Sun et al., 2010). In contrast, ethylene does not  
33 seem to participate in the long-term remodelling of *Arabidopsis* root growth under excess Cu

1 (Lequeux et al., 2010; Yuan et al., 2013). The above literature data imply the involvement of  
2 cytokinin and ethylene in the regulation of root system architecture under stress induced by  
3 excess elements; although the related mechanisms need further elucidation. For instance,  
4 endogenous hormone levels are needed to be measured in the SIMR root systems in order to  
5 reveal the possible changes in hormone distribution as main background mechanisms of the  
6 morphological response. The appearance of SIMR in the root system of transgenic and mutant  
7 plants with altered hormone contents or signalling may also serve interesting findings in the  
8 future. Furthermore, e.g. abscisic acid, brassinosteroids and gibberellic acid participate in the  
9 regulation of root system development (De Smet et al. 2015), but the determination of their  
10 possible action in excess element-triggered SIMR has to be a future research objective.

## 11 2.2. ROS-dependent signals in SIMR induced by excess element

12 Since most stress situations are accompanied by the increased production of reactive  
13 oxygen species (ROS), Potters et al. (2007; 2009) have raised the idea that they are involved  
14 in SIMR. Although the overproduction of ROS can cause oxidative damages which  
15 consequently lead to cell death, this group of molecules acts also as signal components during  
16 plant growth and development. Regarding root cell elongation, moderate levels of hydrogen  
17 peroxide (H<sub>2</sub>O<sub>2</sub>) -the most studied ROS acting as a developmental signal- promote PR  
18 growth, while excessive amount of it inhibits this process. The H<sub>2</sub>O<sub>2</sub>-induced root growth  
19 inhibition in *Arabidopsis* is mediated by the mitogen activated protein kinase (MPK6) and a  
20 calcium influx across the plasma membrane (Han et al., 2015). Additionally, among genes  
21 required for lateral root emergence peroxidase genes are highly represented, and peroxidase  
22 activity and ROS signalling is specifically required for LR formation but not for primordium  
23 specification. The ROS signalling of the later phases of LR development proved to be  
24 independent from auxin signal transduction (Manzano et al., 2014). During Cd exposure,  
25 H<sub>2</sub>O<sub>2</sub> accumulated in the root tips of rice, which influenced auxin distribution and the  
26 expression of cell cycle regulatory genes (e.g. *CDKs* and *CYC*s) within the root system (Zhao  
27 et al., 2012). Unlike the *aux1-7* mutant, wild-type *Arabidopsis* showed decreased expression  
28 of catalase genes and consequently enhanced formation of H<sub>2</sub>O<sub>2</sub> in response to arsenate. This  
29 indicates the role of AUX1-mediated auxin transport in H<sub>2</sub>O<sub>2</sub> formation during arsenic stress  
30 (Krishnamurthy and Rathinasabapathi, 2013). The ascorbic acid-deficient *vtc2-1* mutant with  
31 slightly elevated ROS content in its roots (Pető et al., 2013) maintains better root growth  
32 under selenite stress compared to the WT. Therefore, ROS might be involved in the control of  
33 root elongation in the presence of excess selenium (Lehotai et al., 2012). In the regulation of



1 boron-induced root growth inhibition, auxin- and cytokinin-related processes seem to be not  
2 involved, since the expressions of ARR5 and DR5 reporters in the roots were unaffected  
3 (Aquea et al., 2012). Instead the participation of ROS is assumable; although abscisic acid  
4 (ABA) may also play a role since boron up-regulated several ABA- and water stress-induced  
5 genes (Aquea et al., 2012). A direct link between ROS and the SIMR phenotype was  
6 demonstrated in *Arabidopsis* treated with the ROS-generating compound paraquat or a H<sub>2</sub>O<sub>2</sub>  
7 derivative (*tert*-butyl-hydroperoxide) (Pasternak et al., 2005). These ROS-exposed plants  
8 showed SIMR-like root system, namely enhanced number of lateral roots and shorter primary  
9 roots compared to the untreated plants. According to the results of Olmos et al. (2006) the  
10 ascorbic acid-deficient *vtc1* mutant contains enhanced number of lateral roots, which further  
11 supports the possible involvement of ROS in the appearance of SIMR. The role of ascorbic  
12 acid in SIMR response may derive not only from the control of ROS levels, but from its effect  
13 on cell wall structure and on cell cycle progression (reviewed by Gallie 2013). Although, the  
14 molecular mechanisms of ROS action during the development of SIMR remain to be  
15 elucidate. An important task for the future is to determine the excess-element induced  
16 modifications in the levels of different ROS within the SIMR root system. Also, the ROS-  
17 dependent post-translational modifications and gene expressions are needed to be compared in  
18 control and SIMR roots of the wild-type and ROS homeostasis mutants.

19

### 20 **3. Reactive nitrogen species, as signals in root system growth and development**

21 Reactive nitrogen species are nitric oxide (NO)-derived radicals (e.g. nitrogen dioxide  
22 radical, NO<sub>2</sub><sup>•</sup>) and non-radical molecules (e.g. peroxyxynitrite, ONOO<sup>-</sup>, S-nitrosoglutathione,  
23 GSNO) generated by both algae and higher plants. The central molecule is the redox active  
24 gas signal, nitric oxide, having a fundamental role in coordinating, *inter alia*, plant growth  
25 and development. Emerging evidence suggests that NO regulates all three stages of the life  
26 cycle of seed-bearing plants. Nitric oxide proved to be involved in embryogenesis and seed  
27 germination just like in the determination of flower development, flowering time or pollen  
28 tube growth (reviewed by Yu et al., 2014). In the vegetative growth phase of numerous plant  
29 species, the effect of NO proved to be concentration-dependent, since low levels of it caused  
30 an increase in the biomass (e.g. fresh weight, hypocotyl elongation), while higher NO  
31 contents reduced growth (reviewed by Hebelstrup et al., 2013). In plants, the intracellular  
32 concentration of NO is controlled by several biosynthetic and removal routes. The classic  
33 enzyme of nitrogen metabolism, nitrate reductase (NR) has been widely accepted as a

1 candidate for NO source especially in the root tissues. In *Arabidopsis*, NR is encoded by *NIA1*  
2 and *NIA2* genes, and the *nia1nia2* double mutant possesses less than 1% of the NR activity of  
3 the wild-type (Wilkinson and Crawford, 1993). In the roots of these plants, also the NO levels  
4 were reduced by 60% (Pető et al., 2013), supporting the contribution of NR activity to NO  
5 production in the root system. Another enzyme playing direct or indirect role in NO  
6 production of plant cells is the nitric oxide associated1/resistant to inhibition by fosfidomycin  
7 1 (AtNOA1/RIF1) protein. More recently, the *nia1nia2noa1-2* triple mutant has been  
8 generated, which is impaired in nitrate reductase- and nitric oxide associated1- mediated NO  
9 biosynthetic pathways and it contains extremely low NO level in their roots (Lozano-Juste  
10 and León, 2010). In contrast, the *nox1/cue1* mutant being deficient in the chlorophyll a/b  
11 binding protein under expressed 1 (*CUE1*) gene contains elevated L-arginine, L-citrulline and  
12 NO contents compared to the wild-type (He et al., 2004). Indeed, the mutation resulted in 2-  
13 fold NO accumulation in the root system (Pető et al., 2013); although the molecular  
14 mechanism of NO overproduction has not been revealed yet. The S-nitrosoglutathione  
15 reductase (GSNOR) plays a role in the conversion of S-nitrosoglutathione (GSNO) into  
16 oxidized glutathione and ammonia thus contributing to the reduction of active RNS pool. In  
17 *gsnor1-3* plants, the GSNOR activity is reduced by 80% and the total S-nitrosothiol, nitrate  
18 and NO contents are enhanced compared to the WT (Feechan et al., 2005; Pető et al., 2013).  
19 The above mentioned nitric oxide overproducer mutants (*nox1* and *gsnor1-3*) as well as plants  
20 containing reduced NO levels (*nia1nia2*, *nia1nia2noa1-2*) possess lower fresh weight and  
21 smaller leaf area than the WT (Frungillo et al., 2014; Kolbert et al., 2015), which supports the  
22 requirement of an optimal NO level for normal growth.

23         Among the organ development processes, shaping of the root system as a NO-  
24 coordinated mechanism received the most attention. The key processes determining root  
25 system architecture such as adventitious and lateral root formation, root hair differentiation  
26 and primary root elongation take place with the participation of NO (Yu et al., 2014).  
27 Regarding lateral root emergence, nitric oxide was proved to be a downstream element of  
28 auxin signalling promoting the process (Correa-Aragunde et al., 2004). Indeed, exogenous  
29 auxin treatment induces NO generation in LR primordia of wild-type *Arabidopsis*, but the  
30 nitrate reductase-deficient *nia1nia2* mutant failed to produce NO reflecting the fundamental  
31 role of nitrate reductase activity in auxin-triggered NO synthesis during LR formation  
32 (Kolbert et al., 2008). The mechanism of NO action in LR development materializes to be the  
33 modulation of the auxin-induced expression of cell cycle regulatory genes. In case of NO  
34 scavenging; IAA was not able to increase the expression of the genes coding for cyclin-

1 dependent kinase and cyclins (e.g. *CDKA1*, *CYCA2;1*, *CYCD3;1*) (Correa-Aragunde et al.,  
2 2006). In contrast, Shi et al. (2015) indicated that auxin-dependent lateral root induction was  
3 impaired in the *gsnor1-3* mutant having elevated S-nitrosothiol content, which indicated the  
4 negative effect of NO/SNO overproduction on auxin signalling leading to LR formation.  
5 Similar results were published in a recent paper of Correa-Aragunde and co-workers (2015),  
6 where the pharmacological inhibition of the NADPH-dependent thioredoxin reductase (NTR)  
7 in auxin-treated roots led to the accumulation of S-nitrosothiol compounds, to the  
8 intensification of protein S-nitrosylation and to the inhibition of LR formation. These suggest  
9 the involvement of NTR in protein denitrosylation during auxin-mediated root development.  
10 Moreover, high NO concentration induced NTR activity, which implies the possibility that  
11 NO controls the level of protein S-nitrosylation through a negative feedback mechanism in  
12 plant cells (Correa-Aragunde et al., 2015). Using either mutant *Arabidopsis* lines deficient in  
13 NO homeostasis or pharmacological treatments, the necessity of NO for normal root  
14 elongation, and for the maintenance of the root apical meristem has been demonstrated (Sanz  
15 et al., 2014). Moreover, high NO levels have been reported to reduce PIN1-mediated polar  
16 auxin transport and negatively affect the activity of the primary root meristem thus inhibiting  
17 root elongation (Fernández-Marcos et al., 2011). These results were supported by that of Shi  
18 et al. (2015), where significantly reduced basipetal auxin transport and lower protein levels of  
19 PIN1 and PIN2 were measured in the NO/SNO overproducing *gsnor1-3* mutant. In addition  
20 to the regulation of auxin transport, nitric oxide also modulates auxin signalling and  
21 sensitivity. Auxin perception and signal transduction can be altered as a consequence of NO-  
22 dependent S-nitrosylation of the auxin receptor TIR1 (TRANSPORT INHIBITOR  
23 RESPONSE 1) promoting its interaction with AUX/IAA (AUXIN/INDOL-3-ACETIC ACID)  
24 transcriptional co-repressor proteins. The NO-related modulation of signalling results in the  
25 subsequent promotion of auxin-dependent gene expression (Terrile et al., 2012). In  
26 accordance with this, reduced NO levels in *noa1*, *nia1nia2*, *nia1nia2noa1-2* mutants as well  
27 as the pharmacological inhibition of nitric oxide synthase in wild-type plants resulted in  
28 decreased activity of the *DR5::GUS* auxin response marker (Sanz et al., 2014). Furthermore,  
29 in a root elongation inhibition test, the mutants showed decreased auxin sensitivity compared  
30 to the wild-type revealing the fundamental role of NO in the maintenance of auxin sensitivity  
31 of the primary root (Sanz et al., 2014). More recently, the degradation of AXR3NT-GUS  
32 (reporter for auxin-mediated degradation of AUX/IAA by TIR1) was found to be delayed in  
33 *gsnor1-3* plants compared with the WT showing that the TIR1-mediated auxin signalling  
34 pathway was compromised in this mutant (Shi et al., 2015). These inconsistent results indicate

1 the necessity for further experiments (experimental setups) in order to reveal the exact  
2 molecular mechanism of NO action in the regulation of auxin signalling.

3 In several physiological processes, such as root system development, nitric oxide  
4 seems to be in complex interactions with cytokinins, as well. The synergistic action of  
5 cytokinin and NO was demonstrated by Shen et al. (2013), where NO-deficient *nos1/nao1*  
6 plants showed impaired cytokinin-triggered activation of the cell cycle gene *CYCD3;1*  
7 supposing that NO might be a downstream element of cytokinin signalling directed to  
8 *CYCD3*. However, antagonism between cytokinins and NO has also been evidenced. The  
9 NO-derivative peroxynitrite is able to participate in the regulation of the bioactivity of at least  
10 certain types of cytokinins, like zeatin, via chemical interaction (Liu W-Z et al., 2013). On the  
11 other hand, zeatin may also modulate the homeostasis of RNS due to these reactions.  
12 Moreover, the NO-triggered S-nitrosylation directly inhibits the activity of HISTIDINE  
13 PHOSPHOTRANSFER PROTEIN (AHP1) which reveals and supports an antagonistic  
14 interference between cytokinin and NO signalling at the molecular level (Feng et al., 2013).  
15 Regarding root development, the interaction between cytokinins and NO presently seems to  
16 be synergistic. The NO-deficient *nos1/nao1* mutant possesses significantly smaller root apical  
17 meristem, which could be complemented with the overexpression of *CYCD3;1*. The  
18 expression of *CYCD3;1* is regulated, among others, by cytokinin. Therefore the positive  
19 regulatory role of NO in the maintenance of root apical meristem (RAM) activity was  
20 hypothesized (Shen et al., 2013).

21 In physiological processes, like fruit ripening, the relationship between ethylene and  
22 NO is antagonistic, while during e.g. biotic stress responses their interaction proved to be  
23 rather synergistic (Freschi, 2013). However, during growth processes of the root system, only  
24 a few literature data are available regarding the putative crosstalk between them. Interestingly,  
25 mutant *hls1-1* and *etr1-1* plants deficient in ethylene content or signal transduction contain  
26 extremely high NO levels in their primary root tips, which indicate a relevant antagonism  
27 between these signalling molecules during *Arabidopsis* root growth (Lehotai et al., 2012). In  
28 contrast, *Arabidopsis* and cucumber plants supplemented with 1-aminocyclo-propane-1-  
29 carboxylic acid (ACC) showed increased NO levels in their roots (Garcia et al., 2011).

30 Although the nature of their crosstalk depends on the physiological process and condition,  
31 the signal transduction of nitric oxide and ROS (especially H<sub>2</sub>O<sub>2</sub>) is connected at several  
32 points and they coordinate developmental processes in a tight cooperation. As for the root  
33 system, NO-deficient *nia1nia2* and *nia1nia2noal-2* mutants showed two-fold accumulation  
34 of the highly reactive superoxide anion as well as total ROS in their RAM (Petó et al., 2013;

1 Kolbert et al., 2015). In *gsnor1-3* plants with two-fold increased NO content, the levels of  
2 superoxide and H<sub>2</sub>O<sub>2</sub> were 50% lower relative to the wild-type (Pető et al., 2013) clearly  
3 indicating a negative relationship between ROS and NO in the root system. Similarly, the root  
4 meristems of *noal*, *nia1nia2* and *nia1nia2noal-2* and wild-type plants treated with L-NMMA  
5 contained elevated ROS levels, which were supposed to contribute to the reduced root growth  
6 properties of these plants (Sanz et al., 2014).

7

#### 8 **4. Reactive nitrogen species, as possible signals in SIMR induced by excess element**

9 It is increasingly obvious that like ROS, nitric oxide and related species are formed in  
10 response to almost all biotic and abiotic stressors. In addition, they regulate several aspects of  
11 growth and development which makes the assumption apparent that NO/RNS are integrating  
12 elements of SIMR signalling as it has recently been proposed by Leung (2015). If the growth  
13 responses are taken in a wider sense and considered to appear in case of the presence of a  
14 single symptom (e.g. an element in excess only inhibits primary and lateral root elongation  
15 without inducing lateral root formation), several literature data can be found about the  
16 involvement of NO. For instance, in the roots of *Lupinus luteus*, rice or *Medicago truncatula*  
17 treated with lead, cadmium or arsenic, the NO donors (S-Nitroso-N-acetyl-DL-penicillamine,  
18 SNAP and sodium nitroprusside, SNP) ameliorated growth inhibition possibly through the  
19 reduction of the accumulation of different ROS forms such as superoxide anion (Kopyra and  
20 Gwóźdź, 2003) or hydrogen peroxide (Xu J et al., 2010b) and the level of oxidative damage  
21 (Singh et al., 2009). The crosstalk between NO and auxin during excess element-induced  
22 growth inhibition has also been revealed in recent years. The NO donor (SNP) had a positive  
23 effect on the IAA content of Cd-exposed *Medicago* roots, which was supposed to be the result  
24 of the inhibition of IAA oxidase activity (Xu J et al., 2010b). In pretty different experimental  
25 systems, copper, selenium or cadmium oppositely influenced the levels of nitric oxide and  
26 auxin in the root meristem of *Arabidopsis* (Pető et al., 2011; Lehotai et al., 2012; Yuan and  
27 Huang, 2016). Genetic and biochemical experiments confirmed their antagonistic  
28 relationship in these experimental systems (Pető et al., 2011; Lehotai et al., 2012; Yuan and  
29 Huang, 2016). The application of the NO scavenger 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-  
30 tetramethyl-1-imidazolyl-1-oxy-3-oxide (cPTIO), prevented the reduction of root meristem  
31 growth of cadmium-treated *Arabidopsis*. The cPTIO treatment also prevented the Cd-induced  
32 decrease in the auxin content and in the level of the PIN protein and inhibited the stabilization  
33 of the IAA17 protein, one of the transcriptional repressors of auxin-responsive gene

1 expression. These observations support the involvement of NO in the changes induced by Cd  
2 regarding auxin metabolism and signalling and consequently PR growth (Yuan and Huang,  
3 2016).

4 Taken rigorously, stress-induced morphogenic response is a totality of both growth  
5 inhibition and induction processes (Potters et al., 2007). In case of roots it means that the  
6 shortening of the PR is associated with LR induction as a SIMR. Regarding this more  
7 complex developmental response, there is only few supporting evidence for the involvement  
8 of NO as a signal.

9 In one of their early works, Correa-Aragunde and co-workers (2004) demonstrated that  
10 the supplementation of tomato seedlings with the NO donor SNP inhibited PR elongation and  
11 concomitantly induced LR formation resulting in a SIMR-like root system. Contrary, the  
12 reduction of endogenous NO content by cPTIO led to the formation of a root system  
13 containing elongated PR and almost no LRs. Also in *Arabidopsis*, SNP was able to induce the  
14 appearance of SIMR phenotype in the root system (Méndez-Bravo et al., 2010) and this effect  
15 could be reversed by NO scavenging. Moreover, the NO overproducing *cue1* and *argah1-1*,  
16 *argah2-1* arginase-negative mutants showed enhanced LR density/number compared with the  
17 WT supporting the positive effect of NO on LR development (Lira-Ruan et al., 2013; Flores  
18 et al., 2008). However, in control situation, the root system of *nialnia2* mutant contains a  
19 similar number of LR than the WT (Fig 3 and Kolbert et al., 2010) but the *gsnor1-3* mutant  
20 has seriously reduced LR number (Shi et al., 2015). When the root length of the NO  
21 homeostasis mutants is compared, all the mutants (*nox1/cue1*, *gsnor1-3*, *noal*, *nialnia2*,  
22 *nialnia2noal-2*) can be characterized by reduced capability of elongation independently from  
23 the up- or down-regulation of the NO content (Fig 3, Fernández-Marcos et al., 2011; Espunya  
24 et al., 2006; Lehotai et al., 2012; Sanz et al., 2014; Xu W et al., 2015; Yuan and Huang, 2016;  
25 Pető et al., 2011; Kolbert et al., 2015). This shows that there is no correlation between the NO  
26 level and root elongation but there is a necessity of tight NO-level regulation to allow normal  
27 root elongation to take place.

28 In certain cases, the excess element-induced SIMR phenotype correlates with elevated NO  
29 levels in the root tissues referring to the participation of the NO signal in the growth response.  
30 For instance, within the SIMR-type root system of *Arabidopsis* induced by Pb (shown in Fig  
31 1) NO levels were intensified not only in the PR tips (Fig 2 AB) but also in the upper root  
32 parts and in LRs (Fig 2 CD). The NO formation was more intense in the primary root tips,  
33 where three-fold increase was detected, while root tissues far from the tip showed only two-  
34 fold NO accumulation as the effect of lead (Fig 2). Moreover, in case of both *Arabidopsis* and

1 *Brassica*, the SIMR-type roots that were formed due to excess copper or selenium had higher  
2 NO-related fluorescence in the PR tip than control roots (Kolbert et al., 2012; Lehotai et al.,  
3 2012; Feigl et al., 2013).

4 In order to provide direct evidence for the regulatory role of nitric oxide in excess  
5 element-triggered SIMR, the selenite-induced root system architecture changes of the WT and  
6 the NO-deficient *nia1nia2* double mutant were compared. The *nia1nia2* double mutant has  
7 approximately 40% NO content in its root as compared to the WT (Pető et al., 2013). Ten  
8 days of Se treatment (10  $\mu$ M sodium selenite) decreased root elongation (Fig 3A) and  
9 enhanced the number of emerged LRs (Fig 3B) resulting in the SIMR phenotype of both plant  
10 lines (Fig 3C); although the extent of growth alterations were different. In case of the NO  
11 deficient line, selenite caused slighter LR induction, which means that NO promotes or is  
12 even required for the effect of selenium on this process. In contrary, selenite inhibited PR  
13 elongation more intensively in the *nia1nia2* mutant than in the wild-type suggesting the  
14 negative influence of NO on selenite-induced PR shortening. More interestingly, during Se-  
15 triggered SIMR, the approximately 1:1 ratio of LR primordia and emerged LRs shifted, since  
16 the number of older LRs increased, while that of primordia diminished (Fig 3B). This is more  
17 pronounced in the *nia1nia2* mutant indicating that reduced NO content has negative influence  
18 on the initiation of LR primordia during selenium-induced SIMR. Although, the appearance  
19 of SIMR itself was not affected, the extent of the response proved to be influenced by NO  
20 deficiency suggesting the regulatory role of NO in SIMR. The results also evidence that the  
21 developmental processes of SIMR (PR and LR growth) are differentially modulated by NO.

22 The next question to be answered, concerns the possible molecular mechanisms of  
23 NO-mediated signalling during SIMR. The bioactivity of NO is manifested through specific,  
24 chemical modifications of target proteins. These biologically relevant NO-dependent  
25 posttranslational modifications (PTMs) influence protein activity or cellular function (Freschi,  
26 2013). Among them, S-nitrosylation, the reversible formation of S-nitrosothiol (-SNO) from  
27 the thiol (-SH) groups of certain cysteine residues is a well-known modification related to NO  
28 signalling. Besides, the NO-associated nitration of certain tyrosine amino acids known as  
29 tyrosine nitration is also a specific, potentially reversible PTM, which triggers changes in  
30 protein activity and function. This process is catalysed by peroxynitrite yielding from the in  
31 vivo reaction between NO and superoxide reflecting the tight crosstalk between NO and ROS  
32 in cellular signalling. The specificity of these NO-dependent PTMs is based on the molecular  
33 environment (particular amino acids) which surrounds the target amino acid (cysteine or

1 tyrosine) of the affected protein (Chaki et al., 2014). With the help of certain software tools  
2 e.g. Group-based Prediction Systems (GPS-SNO 1.0, Xue et al., 2010; GPS-YNO2 1.0 Liu Z  
3 et al., 2011), the occurrence of potential NO-dependent PTMs can be predicted in particular  
4 proteins with a good probability (Chaki et al., 2014). Therefore, among the hypothesized  
5 molecular elements of SIMR (Potters et al., 2009), we searched for potential candidates of  
6 NO-dependent S-nitrosylation or tyrosine nitration. As shown in Table 2, NO-related PTMs  
7 presumably influence proteins involved in three major molecular processes of SIMR: in cell  
8 cycle progression, microtubule organization and the development or modification of cell wall  
9 structure (Potters et al., 2009). In case of nitration, larger number of possible sites of  
10 modification could be predicted compared to S-nitrosylation. The significance of this  
11 potential regulation should be addressed in more details in the near future. For example, the  
12 comparison of nitration pattern in control and SIMR roots could provide interesting results. In  
13 addition, the effect of the biochemical influence (inhibition or promotion) of nitration or S-  
14 nitrosylation on the emergence of SIMR phenotype is also an important issue to be addressed.  
15 Although, the tyrosine nitration of alpha-tubulin and the effect of this modification in plant  
16 cell division have already been evidenced (Blume et al., 2013), laccase or callose synthase,  
17 which are implicated in heavy metal-induced cell wall alterations, can be interesting  
18 candidates of NO-dependent modifications for future research.

19

## 20 **5. Conclusions and Perspectives**

21 In this review, literature data have been collected and direct experimental evidence has  
22 been provided to support the hypothesis regarding the contribution of NO and related  
23 molecules to the emergence of excess element-induced root morphogenic responses. Thus, a  
24 new role of NO in acclimation has been revealed emphasizing its importance in defence  
25 mechanisms against abiotic stresses. Hypothetically, at least three pathways of NO/RNS  
26 action are conceivable in the complex signalling network of SIMR. Previously, Potters and  
27 co-workers (2007; 2009) suspected the involvement of hormones and ROS in SIMR  
28 signalling. NO has been proven to synergistically or antagonistically interact with several  
29 phytohormones such as auxin, cytokinin and ethylene (reviewed by Freschi, 2013) modulating  
30 root growth and development. Therefore, the phytohormone-associated involvement of NO in  
31 SIMR is an attractive hypothesis. The ROS-dependent participation of NO in SIMR is also  
32 probable. This can be realized by the formation of peroxynitrite and consequently by the  
33 tyrosine nitration of SIMR-related proteins like tubulin (Table 2). Thirdly, the NO-dependent



1 S-nitrosylation may also regulate SIMR via the modification of involved proteins (Table 2).  
2 This pathway might be independent from both phytohormones and ROS, but can be part of  
3 them as well.

4 Despite of the increasing number of results in plant NO biology in the last two  
5 decades, there are still questions to be clarified. Regarding the excess element-triggered root  
6 growth responses, an important issue to be addressed is to elucidate the possible role of SIMR  
7 in stress tolerance. Moreover, signalling mechanisms linked to hormonal changes (such as  
8 cytokinin, ethylene, abscisic acid etc.) in SIMR roots have to be future research objective. As  
9 to nitric oxide, the characterisation of the molecular mechanisms of its action during SIMR  
10 has to be a task of future research. Among them, processes that regulate morphogenesis and  
11 are simultaneously affected by excess elements have to be in the focus. A system-based  
12 approach including morphophysiology of the root system, ionomics, transcriptomics of NO-  
13 dependent gene expressions, metabolomics of hormone-NO-ROS metabolism and proteomics  
14 of NO-dependent posttranslational modifications is proposed to be applied in order to clarify  
15 the molecular mechanism of NO action during SIMR, which can bring us closer to the better  
16 understanding of its complex role during stress acclimation.

17

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- 17



1 **Table 1 Overview of root morphogenic responses induced by the excess of different elements.** Only publications reporting decreased root  
 2 elongation accompanied by concomitant increase in lateral/seminal root number are indicated.

3

4	<b>Element</b>	<b>Concentration</b>	<b>Duration</b>	<b>Growth medium</b>	<b>Species</b>	<b>References</b>
5	<b>Essential microelements</b>					
6	Cu	30-50-100 $\mu\text{M}$ $\text{CuSO}_4$	7 days	agar	<i>Arabidopsis thaliana</i>	Pasternak et al., 2005
7	Cu	10 $\mu\text{M}$ $\text{CuSO}_4$	17 days	agar	<i>Arabidopsis thaliana</i>	Kolbert et al., 2012
8	Cu	10 $\mu\text{M}$ $\text{CuSO}_4$	7 days	solution	<i>Brassica juncea</i> , <i>Brassica napus</i>	Feigl et al., 2013
9	Cu	1 $\mu\text{M}$ $\text{CuSO}_4$	4 weeks	solution	<i>Pinus pinaster</i>	Arduini et al., 1995
10	Cu	5 or 25 $\text{mg L}^{-1}$ $\text{CuSO}_4$	14 days	solution	<i>Triticum aestivum</i>	Singh et al., 2007
11	Cu	50 $\mu\text{M}$ $\text{CuSO}_4$	8 days	agar	<i>Arabidopsis thaliana</i>	Lequeux et al., 2010
12	Cu	0.66 $\mu\text{M}$ , 1.17 $\mu\text{M}$ Cu	4 weeks	solution	<i>Chloris gayana</i> Knuth.	Sheldon and Menzies, 2005
13	Cu	13 $\mu\text{M g}^{-1}$ soil	2 months	soil	<i>Origanum vulgare</i>	Panou-Filothou and Bosabalidis, 2004
14	Cu	10 $\mu\text{M}$ $\text{CuSO}_4$	3 days	solution	<i>Triticum aestivum</i>	Mahmood et al., 2007
15	Cu	5 $\mu\text{M}$ $\text{CuSO}_4$	12 days	solution	<i>Arabidopsis thaliana</i>	Sofo et al., 2013
16						
17	Fe	200 $\mu\text{M}$ Fe-EDTA	15 days	agar	<i>Arabidopsis thaliana</i>	Giehl et al., 2012
18						
19	Zn	10 $\mu\text{M}$ $\text{ZnSO}_4$	3 days	solution	<i>Triticum aestivum</i>	Mahmood et al., 2007
20	Zn	50 $\mu\text{M}$ $\text{ZnSO}_4$	7 days	solution	<i>Brassica juncea</i> , <i>Brassica napus</i>	Feigl et al., 2015
21	Zn	1000 $\text{mg kg}^{-1}$ ZnO	42 days	soil in rhizobox	<i>Thlaspi caerulescens</i>	Whiting et al., 2000

22

1	<b>Element</b>	<b>Concentration</b>	<b>Duration</b>	<b>Growth medium</b>	<b>Species</b>	<b>References</b>
2	Zn	400 µM ZnCl <sub>2</sub>	4 or 10 days	solution	<i>Solanum nigrum</i>	Xu J et al., 2010a
3	Zn	150 µM ZnSO <sub>4</sub>	12 days	solution	<i>Arabidopsis thaliana</i>	Sofo et al., 2013
4						
5	Ni	75 µM NiCl <sub>2</sub>	12 days	agar	<i>Arabidopsis thaliana</i>	Wang et al., 2015
6						
7	<b>Ocasionally essential elements</b>					
8	Se	25 mg kg <sup>-1</sup> Na <sub>2</sub> SeO <sub>3</sub>	79 days	soil in rhizobox	<i>Stanleya pinnata</i>	Goodson et al., 2003
9	Se	10 µM Na <sub>2</sub> SeO <sub>3</sub>	14 days	agar	<i>Arabidopsis thaliana</i>	Lehotai et al., 2012
10						
11	Co	50 µM CoCl <sub>2</sub>	4 days	solution	<i>Lycopersicon esculentum</i>	Xu S et al., 2010
12	Co	50 or 70 µM CoCl <sub>2</sub>	12 days	agar	<i>Arabidopsis thaliana</i>	Wang et al., 2015
13	Co	10 or 20 µM CoCl <sub>2</sub>	3 days	solution	<i>Oryza sativa</i>	Hsu et al., 2013
14						
15	Al	50 µM AlCl <sub>3</sub>	5 days	solution	<i>Zea mays</i>	Doncheva et al., 2005
16	Al	100 µM, 200 µM AlCl <sub>3</sub>	4 days	agar	<i>Arabidopsis thaliana</i>	Illés et al., 2006
17						
18	<b>Other essential elements</b>					
19	Cr	500 µg ml <sup>-1</sup> CrCl <sub>3</sub>	-	solution	<i>Triticum aestivum</i>	Hasnain and Sabri, 1997
20	Cr (VI)	200 µM K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	5 days	agar	<i>Arabidopsis thaliana</i>	Castro et al., 2007
21						
22						

1	<b>Element</b>	<b>Concentration</b>	<b>Duration</b>	<b>Growth medium</b>	<b>Species</b>	<b>References</b>
2	Va	20-40-80 mg L <sup>-1</sup> NH <sub>4</sub> VO <sub>3</sub>	7 days	solution	<i>Brassica campestris</i>	Vachirapatama et al., 2011
3						
4	<b>Non-essential elements</b>					
5	Pb	10 μM PbCl <sub>2</sub>	3 days	solution	<i>Oryza sativa</i>	Mahmood et al., 2007
6	Pb	10 <sup>-3</sup> M PbNO <sub>3</sub>	3 days	solution	<i>Zea mays</i>	Obroucheva et al., 1998
7	Pb	1200 μM PbNO <sub>3</sub>	12 days	agar	<i>Arabidopsis thaliana</i>	Wang et al., 2015
8						
9	Cd	50 μM Cd	48 hours	agar	<i>Arabidopsis thaliana</i>	Potters et al., 2007
10	Cd	50 μM CdSO <sub>4</sub>	5 days	agar	<i>Arabidopsis thaliana</i>	Hu et al., 2013
11	Cd	10 μM CdSO <sub>4</sub>	12 days	solution	<i>Arabidopsis thaliana</i>	Vitti et al., 2013;
12						Sofo et al., 2013
13	Cd	25, 50, 75, 100 μM CdCl <sub>2</sub>	5 days	agar	<i>Arabidopsis thaliana</i>	Li et al., 2015
14						
15	As	25 μM As(III)	3 days	agar	<i>A. thaliana</i>	Krishnamurthy and Rathinasabapathi, 2013
16						
17	<b>Combination of elements</b>					
18	Cd+Cu+Zn	10 μM CdSO <sub>4</sub> +				
19		5 μM CuSO <sub>4</sub> +				
20		150 μM ZnSO <sub>4</sub>	12 days	solution	<i>Arabidopsis thaliana</i>	Sofo et al., 2013
21						

1 **Table 2** S-nitrosylation and tyrosine nitration sites in examples of SIMR-related proteins  
2 predicted by GPS-SNO 1.0 (Xue et al., 2010) and GPS-YNO2 1.0 (Liu Z et al., 2011)  
3 software, respectively. The medium threshold condition and the batch prediction tool were  
4 applied. - no site was predicted, + one or two sites were predicted, ++ more than two sites  
5 were predicted

6	<b>Accession number</b>	<b>Protein name</b>	<b>S-nitrosylation</b>	<b>tyrosine nitration</b>
7	<b><u>Cell cycle</u></b>			
8	At1g44110	cyclinA1-1	+	++
9	At1g77390	cyclinA1-2	++	++
10	At5g25380	cyclinA2-1	+	++
11	At5g11300	cyclinA2-2	++	++
12	At1g15570	cyclinA2-3	+	+
13	At1g80370	cyclinA2-4	+	+
14	At5g43080	cyclinA3-1	+	++
15	At1g47210	cyclinA3-2	+	++
16	At1g47220	cyclinA3-3	+	++
17	At1g47230	cyclinA3-4	+	++
18	At4g37490	cyclinB1-1	+	++
19	At5g06150	cyclinB1-2	+	++
20	At3g11520	cyclinB1-3	+	++
21	At2g2676	cyclinB1-4	+	++
22	At1g34460	cyclinB1-5	+	++
23	At2g17620	cyclinB2-1	++	-
24	At4g35620	cyclinB2-2	+	+
25	At1g20610	cyclinB2-3	+	-
26	At1g76310	cyclinB2-4	+	+
27	At1g20590	cyclinB2-5	+	+
28	At1g16330	cyclinB3-1	+	++
29	At5g48640	cyclinC1-1	-	+
30	At5g48630	cyclinC1-2	-	+
31	At1g70210	cyclinD1-1	-	++
32	At2g22490	cyclinD2-1	+	-
33	At4g34160	cyclinD3-1	+	+
34	At5g67260	cyclinD3-2	-	+

1	<b>Accession number</b>	<b>Protein name</b>	<b>S-nitrosylation</b>	<b>tyrosine nitration</b>
2	At3g50070	cyclinD3-3	++	-
3	At5g65420	cyclinD4-1	+	+
4	At5g10440	cyclinD4-2	-	+
5	At4g37630	cyclinD5-1	+	++
6	At4g03270	cyclinD6-1	+	+
7	At5g02110	cyclinD7-1	++	++
8	At5g27620	cyclinH1-1	-	++
9	At3g21870	cyclinU1-1	-	+
10	At2g45080	cyclinU2-1	+	++
11	At3g60550	cyclinU2-2	+	++
12	At3g63120	cyclinU3-1	-	-
13	At2g44740	cyclinU4-1	-	+
14	At5g07450	cyclinU4-2	-	++
15	At5g61650	cyclinU4-3	-	+
16	At1g35440	cyclinT1-1	-	+
17	At4g19560	cyclinT1-2	++	++
18	At1g27630	cyclinT1-3	++	+
19	At4g19600	cyclinT1-4	+	++
20	At5g45190	cyclinT1-5	+	++
21				
22	At3g48750	cyclin-dependent kinase A-1	-	++
23	At3g54180	cyclin-dependent kinase B1-1	-	++
24	At2g38620	cyclin-dependent kinase B1-2	+	++
25	At1g76540	cyclin-dependent kinase B2-1	-	++
26	At1g20930	cyclin-dependent kinase B2-2	-	++
27	At5g10270	cyclin-dependent kinase C-1	-	+
28	At5g64960	cyclin-dependent kinase C-2	+	++
29	At1g73690	cyclin-dependent kinase D-1	-	+
30	At1g66750	cyclin-dependent kinase D-2	-	++
31	At1g18040	cyclin-dependent kinase D-3	-	+
32	At5g63610	cyclin-dependent kinase E-1	-	+
33	At4g28980	cyclin-dependent kinase F-1	-	+
34				

1	<b>Accession number</b>	<b>Protein name</b>	<b>S-nitrosylation</b>	<b>tyrosine nitration</b>
2	At3g12280	Retinoblastoma-related 1	+	-
3				
4	At2g36010	Transcription factor E2FA	-	-
5	At5g22220	Transcription factor E2FB	-	++
6	At1g47870	Transcription factor E2FC	-	++
7	At5g14960	E2F Transcription factor-like E2FD	-	++
8	At3g48160	E2F Transcription factor-like E2FE	+	+
9	At3g01330	E2F Transcription factor-like E2FF	-	++
10				
11	At5g02470	Transcription factor-like protein DPA	-	+
12	At5g03415	Transcription factor-like protein DPB	+	++
13				
14	At1g02970	WEE1-like protein kinase	+	++
15				
16	<b><u>Microtubuli</u></b>			
17	At1g64740	$\alpha$ -tubulin-1 chain	++	++
18	At1g50010	$\alpha$ -tubulin-2 chain	++	++
19	At5g19770	$\alpha$ -tubulin-3 chain	++	++
20	At1g04820	$\alpha$ -tubulin-4 chain	++	++
21	At5g19780	$\alpha$ -tubulin-5 chain	++	++
22	At4g14960	$\alpha$ -tubulin-6 chain	++	++
23	At1g75780	$\beta$ -tubulin-1 chain	++	++
24	At5g62690	$\beta$ -tubulin-2 chain	++	++
25	At5g62700	$\beta$ -tubulin-3 chain	++	++
26	At5g44340	$\beta$ -tubulin-4 chain	++	++
27	At1g2001	$\beta$ -tubulin-5 chain	++	++
28	At5g1225	$\beta$ -tubulin-6 chain	++	++
29	At2g29550	$\beta$ -tubulin-7 chain	++	++
30	At5g23860	$\beta$ -tubulin-8 chain	++	++
31	At4g20890	$\beta$ -tubulin-9 chain	++	++
32	At3g61650	$\gamma$ -tubulin-1 chain	++	++
33	At5g05620	$\gamma$ -tubulin-2 chain	++	++
34				

1	Accession number	Protein name	S-nitrosylation	tyrosine nitration
2	<b><u>Cell wall elongation</u></b>			
3	At1g69530	Expansin-A1	-	-
4	At5g05290	Expansin-A2	-	-
5	At2g37640	Expansin-A3	-	-
6	At2g39700	Expansin-A4	-	-
7	At3g29030	Expansin-A5	-	-
8	At2g28950	Expansin-A6	-	-
9	At1g12560	Expansin-A7	-	-
10	At2g40610	Expansin-A8	-	++
11	At5g02260	Expansin-A9	-	-
12	At1g26770	Expansin-A10	-	-
13	At1g20190	Expansin-A11	-	-
14	At3g15370	Expansin-A12	-	+
15	At3g03220	Expansin-A13	-	-
16	At5g56320	Expansin-A14	-	-
17	At2g03090	Expansin-A15	+	-
18	At3g55500	Expansin-A16	-	-
19	At4g01630	Putative expansin-A17	-	-
20	At1g62980	Expansin-A18	-	-
21	At4g38210	Expansin-A20	-	+
22	At5g39260	Expansin-A21	-	-
23	At5g39270	Expansin-A22	-	-
24	At5g3928	Expansin-A23	-	-
25	At5g39310	Expansin-A24	-	-
26	At5g3930	Expansin-A25	-	+
27	At5g39290	Putative expansin-A26	-	-
28	At2g20750	Expansin-B1	++	-
29	At1g65680	Putative expansin-B2	++	++
30	At4g28250	Expansin-B3	++	-
31	At2g45110	Expansin-B4	-	-
32	At3g60570	Expansin-B5	++	-
33				
34				

1	Accession number	Protein name	S-nitrosylation	tyrosine nitration
2	At4g32410	Cellulose synthase A catalytic subunit 1	+	++
3	At4g39350	Cellulose synthase A catalytic subunit 2	++	++
4	At5g05170	Cellulose synthase A catalytic subunit 3	++	++
5	At5g44030	Cellulose synthase A catalytic subunit 4	+	++
6	At5g09870	Cellulose synthase A catalytic subunit 5	++	++
7	At5g64740	Cellulose synthase A catalytic subunit 6	++	++
8	At5g17420	Cellulose synthase A catalytic subunit 7	++	++
9	At4g18780	Cellulose synthase A catalytic subunit 8	++	++
10	At2g21770	Cellulose synthase A catalytic subunit 9	++	++
11	At2g25540	Cellulose synthase A catalytic subunit 10	++	++
12				
13	At1g05570	Callose synthase 1	+	++
14	At2g31960	Callose synthase 2	++	++
15	At5g13000	Callose synthase 3	-	++
16	At5g36870	Callose synthase 4	++	++
17	At2g13680	Callose synthase 5	+	++
18	At3g59100	Callose synthase 6	++	++
19	At1g06490	Callose synthase 7	++	++
20	At3g14570	Callose synthase 8	++	++
21	At3g07160	Callose synthase 9	-	++
22	At2g36850	Callose synthase 10	++	++
23	At4g04970	Callose synthase 11	+	++
24	At4g03550	Callose synthase 12	+	++
25				
26	At1g18140	Laccase-1	+	+
27	At2g29130	Laccase-2	+	++
28	At2g30210	Laccase-3	+	+
29	At2g38080	Laccase-4	+	++
30	At2g40370	Laccase-5	+	++
31	At2g46570	Laccase-6	+	+
32	At3g09220	Laccase-7	+	+
33	At5g01040	Laccase-8	+	++
34	At5g01050	Laccase-9	+	++



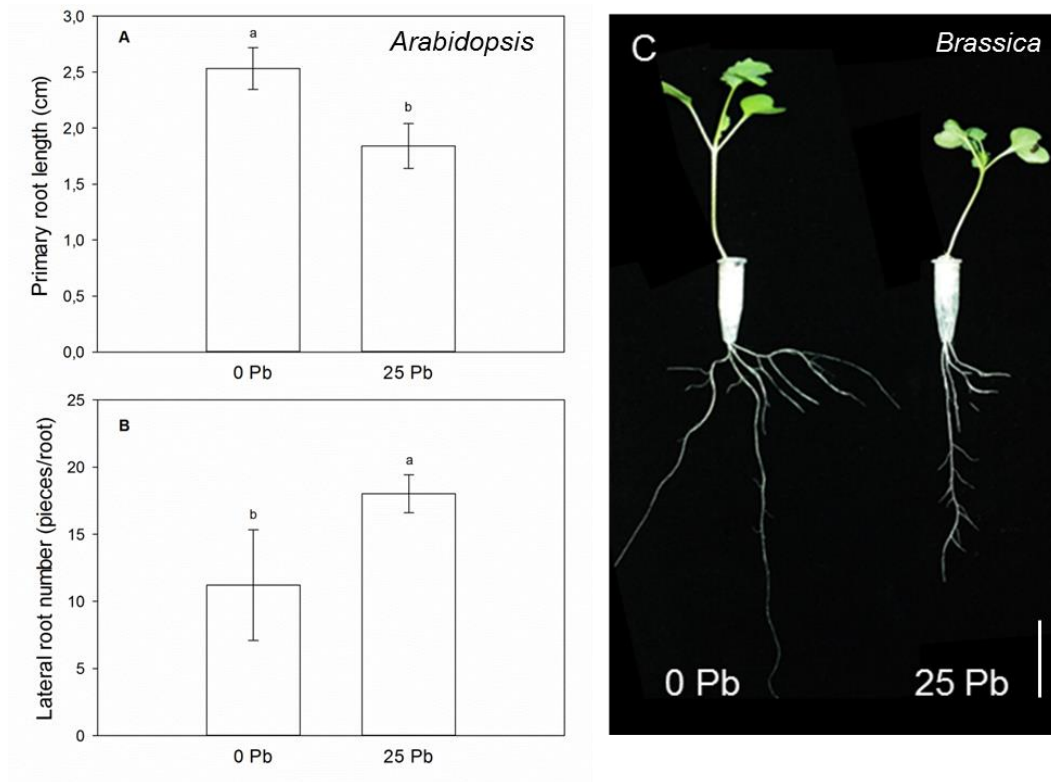
1	2	3	4	5
	<b>Accession number</b>	<b>Protein name</b>	<b>S-nitrosylation</b>	<b>tyrosine nitration</b>
3	At5g01190	Laccase-10	+	++
4	At5g03260	Laccase-11	+	++
5	At5g05390	Laccase-12	+	++
6	At5g07130	Laccase-13	+	++
7	At5g09360	Laccase-14	+	+
8	At5g48100	Laccase-15	++	++
9	At5g58910	Laccase-16	+	++
10	At5g60020	Laccase-17	+	++
11				
12	At4g13080	Putative xyloglucan endotransglucosylase/hydrolase protein 1	-	++
13	At4g13090	Xyloglucan endotransglucosylase/hydrolase protein 2	+	++
14	At3g25050	Xyloglucan endotransglucosylase/hydrolase protein 3	-	+
15	At2g06850	Xyloglucan endotransglucosylase/hydrolase protein 4	+	+
16	At5g13870	Probable xyloglucan endotransglucosylase/hydrolase protein 5	++	+
17	At5g65730	Probable xyloglucan endotransglucosylase/hydrolase protein 6	-	++
18	At4g37800	Probable xyloglucan endotransglucosylase/hydrolase protein 7	+	-
19	At1g11545	Probable xyloglucan endotransglucosylase/hydrolase protein 8	+	+
20	At4g03210	Xyloglucan endotransglucosylase/hydrolase protein 9	-	+
21	At2g14620	Probable xyloglucan endotransglucosylase/hydrolase protein 10	-	-
22	At3g48580	Probable xyloglucan endotransglucosylase/hydrolase protein 11	-	-
23	At5g57530	Probable xyloglucan endotransglucosylase/hydrolase protein 12	-	+
24	At5g57540	Putative xyloglucan endotransglucosylase/hydrolase protein 13	-	+
25	At4g25820	Xyloglucan endotransglucosylase/hydrolase protein 14	++	+
26	At4g14130	Xyloglucan endotransglucosylase/hydrolase protein 15	-	+
27	At3g23730	Probable xyloglucan endotransglucosylase/hydrolase protein 16	-	++
28	At1g65310	Probable xyloglucan endotransglucosylase/hydrolase protein 17	+	-
29	At4g30280	Probable xyloglucan endotransglucosylase/hydrolase protein 18	+	-
30	At4g30290	Probable xyloglucan endotransglucosylase/hydrolase protein 19	-	+
31	At5g48070	Xyloglucan endotransglucosylase/hydrolase protein 20	+	-
32	At2g18800	Probable xyloglucan endotransglucosylase/hydrolase protein 21	-	++
33	At5g57560	Xyloglucan endotransglucosylase/hydrolase protein 22	-	+
34	At4g25810	Probable xyloglucan endotransglucosylase/hydrolase protein 23	-	++

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	<b>Accession number</b>	<b>Protein name</b>	<b>S-nitrosylation</b>	<b>tyrosine nitration</b>										
	At4g30270	Xyloglucan endotransglucosylase/hydrolase protein 24		-	+									
	At5g57550	Probable xyloglucan endotransglucosylase/hydrolase protein 25		-	-									
	At4g28850	Probable xyloglucan endotransglucosylase/hydrolase protein 26		-	++									
	At2g01850	Probable xyloglucan endotransglucosylase/hydrolase protein 27		-	+									
	At1g14720	Probable xyloglucan endotransglucosylase/hydrolase protein 28		-	++									
	At4g18990	Probable xyloglucan endotransglucosylase/hydrolase protein 29		-	+									
	At1g32170	Probable xyloglucan endotransglucosylase/hydrolase protein 30		-	++									
	At3g44990	Xyloglucan endotransglucosylase/hydrolase protein 31		+	-									
	At2g36870	Probable xyloglucan endotransglucosylase/hydrolase protein 32		-	-									
	At1g10550	Probable xyloglucan endotransglucosylase/hydrolase protein 33		++	-									

1 **Figures**

2 **Figure 1**

3



4

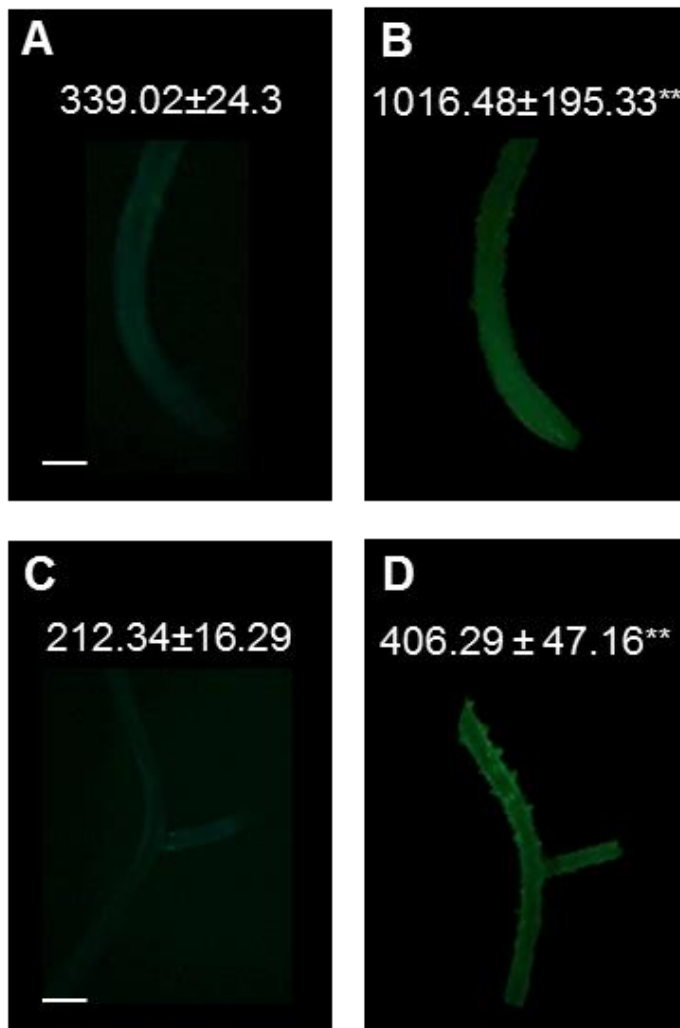
5

6 **Fig 1** Lead-induced SIMR in *Brassicaceae*. Primary root length (cm, A) and lateral root  
7 number (pieces/root, B) of wild-type *Arabidopsis thaliana* grown in agar medium  
8 supplemented with 0 or 25  $\mu\text{M}$   $\text{PbNO}_3$  for two weeks. Different letters indicate statistically  
9 significant differences according to Duncan-test ( $n=20$ ,  $P\leq 0.05$ ). (C) Representative images  
10 showing SIMR-phenotype (short primary root and enhanced number of lateral roots) in the  
11 root system of 15-days-old *Brassica juncea* seedlings treated with 0 or 25  $\mu\text{M}$   $\text{PbNO}_3$  in  
12 nutrient solution for one week. Bar=5 cm.

13

14

1 **Figure 2**



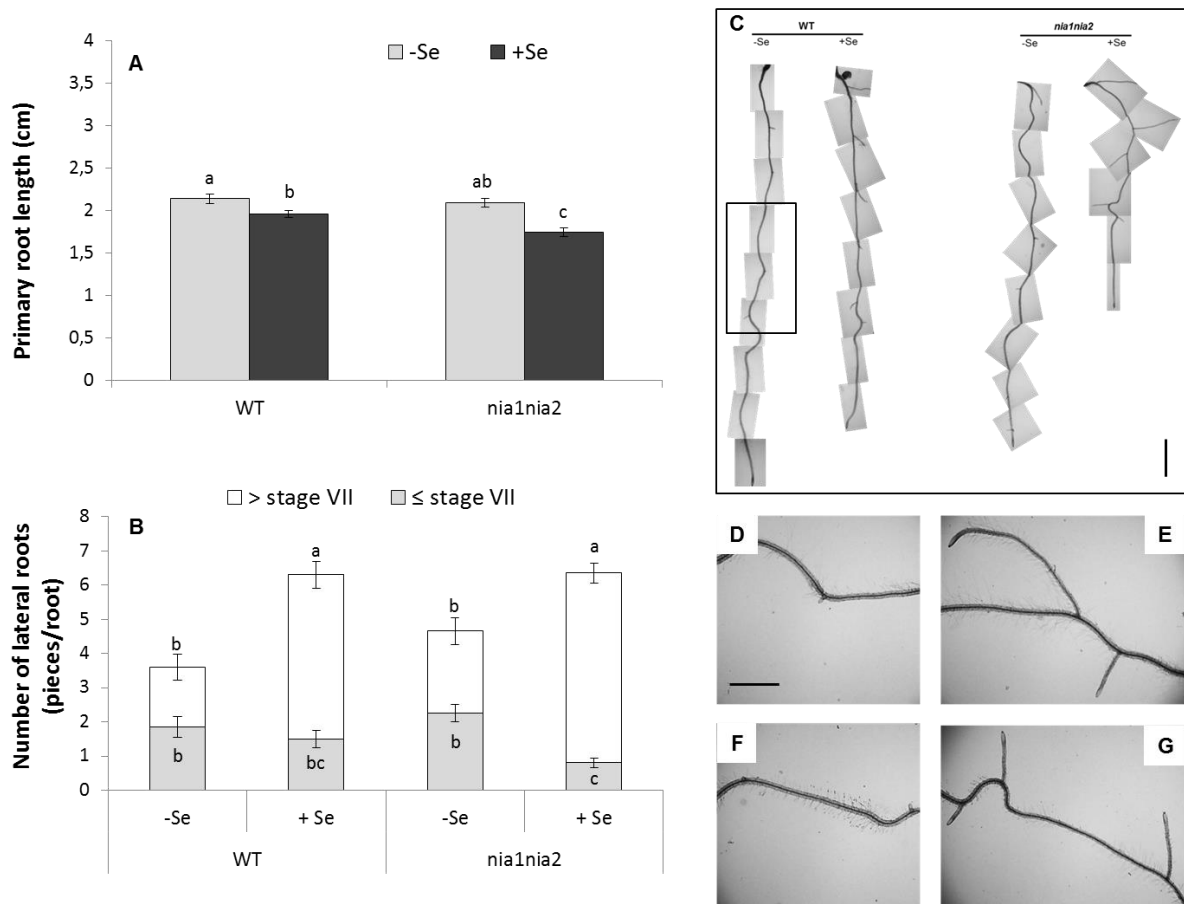
2

3

4 **Fig 2** Lead induces nitric oxide formation in SIMR root system. Representative microscopic  
5 images showing 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA)  
6 stained primary root tips (A and B) and lateral roots (C and D) of wild-type *Arabidopsis* (*Col-*  
7 *0*) treated with 0 (A and C) or 25 μM (B and D) PbNO<sub>3</sub> for two weeks. Bars=100 μm. Mean  
8 and standard error values of NO-associated pixel intensities are also indicated. Statistically  
9 significant differences were determined by using Microsoft Office software and Student's t-  
10 test (n=10, \*\*P≤0.01). The staining procedure, the microscopic detection (Zeiss Axiovert  
11 200M, 10x magnification) and the measurement of fluorescence intensity were carried out as  
12 described by Kolbert et al. 2015.

13

1 **Figure 3**



2

3 **Fig 3** The effect of NO-deficiency on the extent of SIMR. Primary root length (cm, A) and  
 4 lateral root number (pieces/root, smaller and larger than stage VII) of the wild-type and the  
 5 NO-deficient *nia1nia2* mutant grown in nutrient agar medium supplemented with 0 (-Se) or  
 6 10  $\mu$ M  $\text{Na}_2\text{SeO}_3$  (+Se) for 10 days. The developmental stages of LRs were determined after  
 7 Malamy and Benfey (1997) under Zeiss Axiovert 200M microscope (Carl Zeiss, Jena,  
 8 Germany). Different letters indicate significant differences according to Duncan-test (n = 20,  
 9  $P \leq 0.05$ ). (C) Representative photographs showing the whole root system of control (-Se) and  
 10 Se-treated (+Se) wild-type and *nia1nia2* plants. Bar=2 mm. (D-G) The middle part of the root  
 11 system (indicated by a square in C) was examined using 2.5x magnification. D=wild-type -Se;  
 12 E=wild-type +Se; F=*nia1nia2* -Se; G=*nia1nia2* +Se. Bar=1 mm.  
 13