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REGULAR ARTICLE

Metals and selenium induce medicarpin accumulation and excretion from the roots of fenugreek seedlings: a potential detoxification mechanism

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Abstract Medicarpin (M), an isoflavonoid phytoalexin, accumulates in plants of the Fabaceae family as a response to biotic and abiotic stresses. In an attempt to investigate the potential participation of M in metal detoxification, we studied the effect of three metals (copper, cadmium, and aluminum) and selenium on M synthesis and excretion from the roots of fenugreek (*Trigonella foenum-graecum* L.) seedlings. Medicarpin content and gene expressions were determined by RP-HPLC and RT-PCR, respectively. All treatments significantly induced increase in the expression of M biosynthetic genes and concomitant increase of M content in the roots and the culture medium. The metal and Se-induced M excretion inhibited by either orthovanadate or KCN, an ATPase and an ATP synthesis inhibitor respectively, and the elicitor-induced increase of *GST* transcript levels may imply the involvement of an ABC-type transport system in which GST is involved. Interestingly, a parallel increase of citrate exudation, a common metal detoxification agent, was measured in response to the elicitors used. The above results, along with the moderate effects of these elicitors on root growth and the plasma membrane integrity, imply that M as well as citrate exudation may participate in metal and Se detoxification, as part of a non element-specific resistance mechanism.

Keywords Fenugreek · Medicarpin excretion · Medicarpin synthesis · Metals · Selenium · *Trigonella foenum-graecum* L

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Introduction

As a consequence of industrial development, the environment is increasingly polluted by heavy metals (Michalak 2006). The uptake of these elements by plants and their accumulation at toxic levels interrupts various plant processes at the physiological, biochemical and molecular level, leading to growth reduction and ultimately to cell death. However, there are plant species, which may tolerate toxic amounts of these elements. The identification of metal stress-resistance mechanisms is a fundamental step for understanding the biochemical and molecular mechanisms of stress resistance, towards developing tolerant genotypes by either conventional breeding or by genetic modification.

Several approaches have been employed towards understanding trace element stress resistance mecha-

nisms. These include the identification of metal-induced genes or proteins in metal-tolerant species or varieties (Ahsan et al. 2009 and references therein; Chandran et al. 2008; Hoekenga et al. 2006; Zhao et al. 2009), or mechanisms of metal detoxification, which is achieved by the synthesis and/or exudation of metal chelating agents. Chelating agents, excreted by root apexes to the rhizosphere, chelate with the metal excluding it from the root apex or the ligand-metal complex detoxify metals internally (Ma et al. 2001; Salt et al. 1998). Organic acids and phenolics are included among these ligands (Barceló and Poschenrieder 2002; Michalak 2006). It is hypothesized that metal chelating ability of some organic acids, such as citrate, malate or oxalate, confers tolerance through the formation of stable complexes with metals like Al, Cu, Cd or Zn (Ma et al. 2001; Murphy et al. 1999; Nian et al. 2002; Pellet et al. 1997; Pinto et al. 2008; Qin et al. 2007; Rangel et al. 2010). However, a number of studies have indicated that organic acid chelation may not be the only mechanism responsible for Al resistance (Kochian et al. 2002; Nian et al. 2002; Pellet et al. 1997; Piñeros et al. 2005). Other ligands with potential for metal detoxification in the rhizosphere have received less attention. Varietal differences in the exudation rate of flavonoid-type phenolics by root tips of maize have been related to differences in Al resistance (Kidd et al. 2001). In *Medicago truncatula* Al induced the expression of genes encoding enzymes of the isoflavonoid biosynthetic pathway and glutathione-S-transferase (GST, EC. 2.5.1.18) (Chandran et al. 2008), which apart from other functions, is involved in the transport of secondary plant products (Dixon et al. 2010; Zhao and Dixon 2010).

Isoflavonoids, which are mainly restricted to the Papilionoideae subfamily of the Fabaceae, function as phytoalexins (Dixon et al. 2002) and antioxidants (Dixon and Steele 1999; Rice-Evans et al. 1997). The isoflavonoids genistein (Jung et al. 2003) or daidzein (Toda and Shirataki 2001) have Cu-chelating ability. Production and excretion of genistein by the roots of the legume *Lupinus albus* seedling in response to Cu has been proposed as the plant mechanism to alleviate Cu-mediated toxicity (Jung et al. 2003). Another isoflavonoid, the phytoalexin medicarpin (M), in the form of its malonyl glucoside (MGM), is a constitutive constituent of several leguminous plants (Dixon 1999; Tsiri et al. 2009). However, biotic or abiotic stress induced M accumulation (Cachinero et al. 2002; Carlsen et al. 2008; Dewick and Martin 1979; Dixon 1999; Farag et al. 2008; Jasiński et al. 2009 and references therein; Lopez-Meyer and Paiva 2002; Naoumkina et al. 2007; Parry et al. 1994; Saunders and O'Neill 2004; Tsiri et al. 2009). Medicarpin accumulation in response to Cu originated either from *de novo* synthesis or it was synthesized at the expense of its malonyl glucoside (Parry et al. 1994; Tsiri et al. 2009) and its Cu-induced exudation by the roots of fenugreek seedlings has been attributed to the operation of an ABC-type transport system (Tsiri et al. 2009).

Induction of isoflavonoid synthesis and/or exudation by biotic stress is a general response of plant species of *Papillionoidae* subfamily (Jasiński et al. 2009 and references therein). However, to the best of our knowledge, such a general response to metal or Se stress has not been considered yet to this class of phenolics. In this report, we investigated M synthesis and exudation by fenugreek seedlings in response to toxic amounts of two heavy metals (Cu and Cd), one light metal (Al) and a non metal (Se) in an attempt to evaluate the potential of M to play a metal and Se detoxification role. Thus, we have studied M content in fenugreek roots and in the culture medium of the seedlings, and the expression of the M biosynthetic pathway genes, chalcone synthase (*CHS*; EC 2.3.1.74), which catalyses the first committed step to the flavonoid biosynthetic pathway, isoflavone reductase (*IFR*; EC 1.3.1.45), a key enzyme involved in the latter steps of M biosynthetic pathway, and vestitone reductase (*VR*), which catalyzes the penultimate step of M biosynthesis (Guo et al. 1994; Paiva et al. 1991). The mechanism of elicitor-induced M exudation was studied using pharmacological approach, concomitantly with the determination of the transcript levels of *GST*, since M is transported into vacuoles *via* an ABC-type transport mechanism as S-glutathionylated conjugate, the formation of which is mediated by GST (Li et al. 1997; Zhao and Dixon 2010).

Material and methods

Plant material

Trigonella foenum-graecum L. (fenugreek) seeds were purchased from a local market. Surface sterilized seeds were sawn on moistened filter paper under sterile conditions and maintained at 25±1°C with a 16/8 h photoperiod (5.3 $W cm^{-2}$) in a growth chamber.

Six-day old seedlings were treated hydroponically with varying concentrations (0, 1, 5, 10 and 50 μM) of $AlCl_3$, $CuCl_2$, $CdCl_2$ or Na_2SeO_3. Seedlings (12 per vial) were placed into sterile vials containing 80 ml of sterile de-ionized water (control) or the Al, Cu, Cd or Se solutions, so that only part of their roots were immersed in the growth medium. At the start of the experiment the medium pH was 4.6. Six or 24 h post-treatment, the roots from the fenugreek seedlings were excised, blow dried, weighed and stored at −80°C, while culture media from 24-h-treated seedlings were collected and kept at −20°C. All experiments were performed at the same temperature and light conditions. For the inhibition experiments 5-d-old fenugreek seedlings were transferred into vials containing 80 ml sterile de-ionized water (control), 100 μM sodium orthovanadate (OV), or 100 μM KCN. After 24 h of incubation $AlCl_3$ $CuCl_2$, $CdCl_2$ or Na_2SeO_3 were added to the media to a final concentration of 10 μM and seedlings were incubated for another 24 h. All experiments were performed at least in triplicates.

Isoflavonoid extraction and analysis

Fenugreek roots were extracted with 80% methanol and the samples were prepared for HPLC analysis as described by Tsiri et al. (2009). Seedling culture media were adjusted to pH 2.5, extracted and prepared for HPLC analysis according to Tsiri et al. (2009). HPLC analysis was performed using a quarternary gradient pump (PU-2089, Jasco, Japan) connected to a multi-wavelength detector (MD-2015, Jasco, Japan) under the following chromatographic conditions: 20 μl of sample were injected onto a Lichrosorb RP 18, 5.0 mm, 250×4 mm column; elution profile: 0–25 min, 55% A in B, 25–30 min 100% A, 30–35 min 100% A, 35–40 min step return to 100% B, 40–45 min re-equilibration with 100% B. [solvents: (A) acetonitrile and (B) water with 0.1% acetic acid]; flow rate: 1.5 ml min^{-1}; detection: at 283 nm. Isoflavonoid quantification was based on a calibration curve, plotting peak area as monitored at 283 nm against known concentrations of medicarpin, which has been purified in our laboratory (Tsiri et al. 2009).

Citrate quantification

After a 24-h treatment with metals or Se roots from fenugreek seedlings were blow dried, weighed and extracted with 80% ethanol according to Silva et al. (2001). The supernatants of the centrifuged samples (3,800 rpm) were evaporated to dryness and the dried residues were taken up with distilled water (1 ml per mg root tissue). Seedling culture media were lyophilized and the residues were taken up with 2 ml distilled water. Samples were tested for citrate concentration using the enzyme assay method (Li et al. 2002). Preliminary assays indicated that Al, Cu, Cd or Se in the samples did not interfere with the assay for citrate quantification because the amount of citric acid detected after addition of known amounts of citric acid on the mixture was approx. the same regardless of the presence of these elements.

Semi-quantitative RT-PCR and DNA sequence analysis

Total RNA from fenugreek tissues was extracted with the NucleoSpin RNA Plant Kit (Macherey-Nagel, Germany) and treated with 10 U DNase I (Takara Bio) for 10 min at 37°C. First strand cDNA was synthesized from 0,5–1 μg total RNA with Prime-Script Reverse Transcriptase (200 U/mL, Takara Bio) according to the manufacturer's protocol. Fenugreek *CHS* and *VR* specific fragments were amplified with degenerated primers, designed using sequence information from known *CHS* and *VR* genes. PCR was performed in a mixture of 50 μL that contained 1 μL of first-strand cDNA, 0.4 mM dNTP's, 1X PCR buffer, 2.5 U Expand High Fidelity DNA Polymerase (Roche) and 10 pmole each of the gene-specific degenerated primer. Primers were as follows:

for *CHS*, 5′-AA(G/A)GGTGCTCGTGTGCT(G/T)GTTG-3′ and 5′-AGTCCAGGTCCAAA(G/C)CCAAA(C/T)-3′ (amplifying a 542 bp fragment), and for *VR*, 5′-CAGG(T/A)TT(T/C)CTTGGTTCATGG-3′ and 5′-ATCACTCCAA(T/A)CA(C/G)TCTCATCC-3′ (amplifying a 420 bp fragment). *IFR* (accession number X58078) and *GST* (accession number AB040439) specific fragments were amplified using primer pair 5′-CTGCTAATCCTGAAACCAAG-3′, 5′-GCTCCTTTCACATTTCCATC-3′ (amplifying a 425 bp fragment), and 5′-CAATAAAAGTGCACGGAAGCCC-3′, 5′-GCAAATCCACCAAGGTGAAACA-3′ (amplifying a 498 bp fragment), respectively. The amplified PCR products were subsequently cloned and sequenced, in order

to verify by BLAST analysis (National Centre for Biotechnology Information) the homology to the known *CHS*, *VR*, *IFR* and *GST* sequences deposited in the databases. The 18 S rRNA gene was used as an internal control for RNA calibration (5′-TTGTGTTGGCTTCGGGATCGGAGTAAT-3′ and 5′-GCACCACCACCCATAGAATCAAGAA-3′). To verify the exponential phase of RT-PCR amplification, 15, 25 and 35 cycles were tested for each gene, and data was collected at 16 cycles for 18 S rRNA and at 25–30 cycles for *CHS*, *VR*, *IFR* and *GST*. All experiments were performed in triplicate. DNA was sequenced by the dideoxy chain termination method with the use of an automated sequencer model 377 (Applied Biosystems).

Assessment of the loss of integrity of the plasma membrane

Fenugreek roots from 6-d-old seedlings were treated for 24 h with several concentrations of Al, Cu, Cd or Se. Histochemical detection of the loss of plasma membrane integrity in roots was performed with Evans blue according to Yamamoto et al. (2001). All stained roots with Evans blue were washed extensively, and observed under a Zeiss Axioplan light microscope equipped with a Zeiss Axiocam MRcs digital camera.

Results

Root growth and integrity of plasma membrane of the root cells are suitable phenotypic characteristics to assess metal resistance (Magnavaca et al. 1987; Cançado et al. 1999). Thus, we used these parameters to rank metal and Se resistance of fenugreek seedlings. Six-d-old seedlings were treated with several concentrations (1–50 μM) of $AlCl_3$, $CuCl_2$ $CdCl_2$ or Na_2SeO_3 for 24 h. As expected, these trace elements caused reduction of root growth. Reduction of root fresh weight and root elongation in response to these elements was dose-dependent and in the order Cu> Cd> Al> Se and Cd> Cu> Al> Se for the root fresh weight and the root elongation, respectively (Fig. 1a and b). The highest reduction of root fresh weight was caused by 50 μM Cu (38%), while that of root elongation by 50 μM Cd (88%).

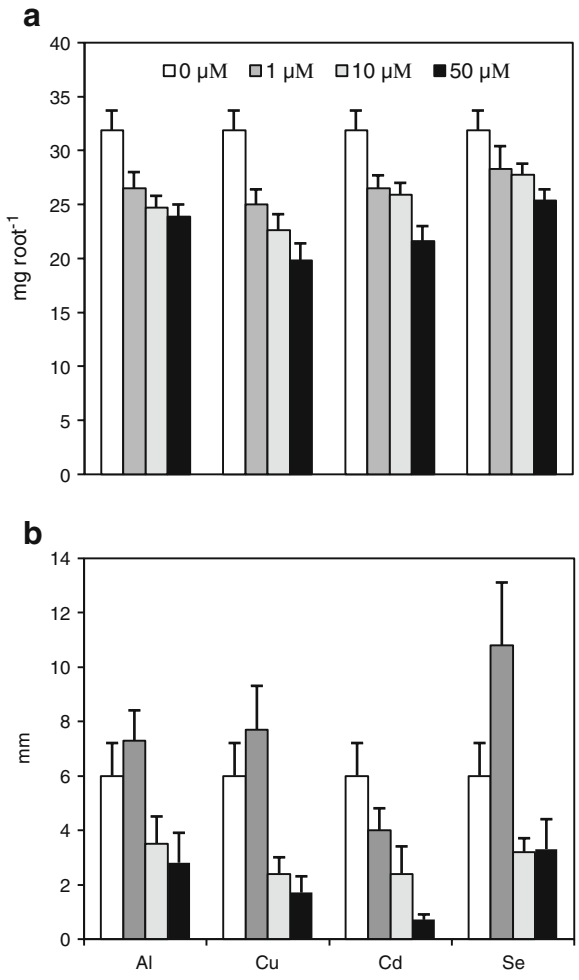


Fig. 1 Root fresh weights (**a**) and root elongation (**b**) of 6-d-old fenugreek seedlings treated with (1, 5, 10 and 50 μM) Al, Cu, Cd and Se for 24 h. Values are the mean±SE of at least 24 roots

The assessment of the effect of the above mentioned concentrations of Al, Cu, Cd or Se on plasma membrane integrity of roots from treated seedlings was performed by histochemical staining using Evans blue (Fig. 2). The extent of root staining was trace element concentration-dependent; staining intensity increased with increasing concentration. The roots which were treated with 50 μM concentrations of Al, Cu or Cd were stained extensively, while those treated with Se were slightly affected. Stain intensity of roots from 50 μM metal-treated seedlings was in the order Se< Cd< Cu< Al.

Next, we determined the expression of M biosynthetic pathway genes in the roots and the content of M in both the roots of treated seedlings and in the culture medium. In the presence of trace elements the

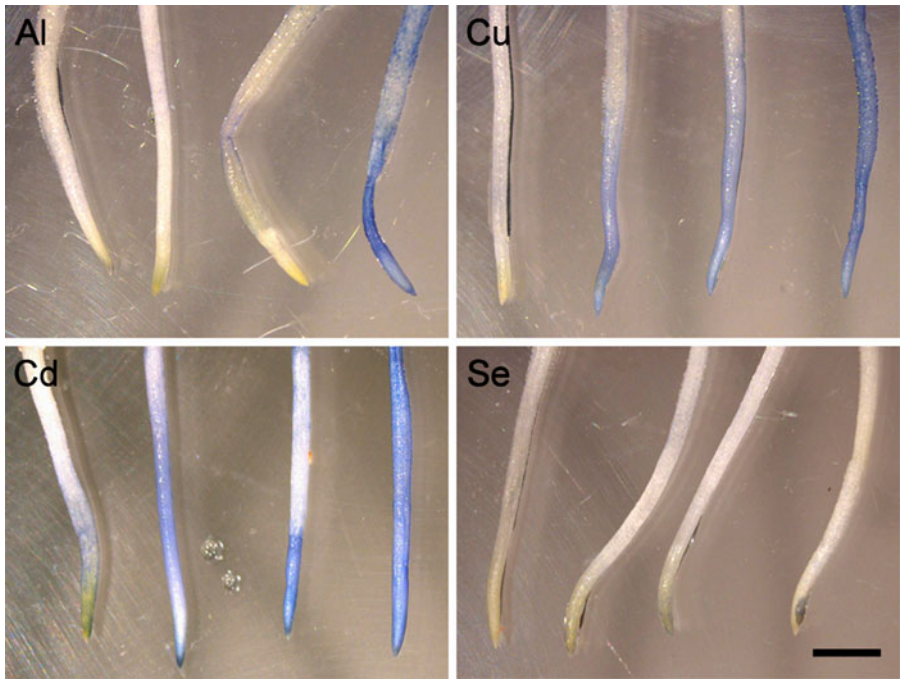


Fig. 2 Effect of Al, Cu Cd, or Se treatment on the loss of the root plasma membrane integrity. Six-d-old fenugreek seedlings were treated for 24 h with Al, Cu, Cd or Se (1, 5, 10 and 50 μM), and roots were stained with Evans blue (see Material and Methods). Concentrations used for all treatments were, from left to right 1, 5, 10 and 50 μM. Bar, 5 mm

abundance of transcript levels of the root *CHS*, *IFR*, and *VR* was increased (Fig. 3). In agreement with the molecular data, a significant increase in M accumulation in both roots and the seedling culture medium was measured (Fig. 4). The highest total M production (M in roots + M in the seedling culture medium) was observed at 1 μM Cd treatment and the lowest in response to Al treatment. However, the amount of synthesized M decreased with increasing Cd. In contrast, in Al, Cu or Se-treated seedlings M synthesis peaked at the concentration of 10 μM whereas at 50 μM it decreased significantly. At this concentration of Cu or Se MGM was reduced by approx. 45%.

An appreciable amount of M, which was synthesized in response to the studied elements, was excreted in the seedling culture medium, and the amount was dependent on the element and on its concentration. However, in any of these treatments, the amount of excreted M was considerably higher to that accumulated in the roots. The pattern of M exudation *vs.* M accumulation in the roots was depended on the element. The total amount of M accumulated in both the roots and in the seedling culture medium was 25-fold higher in seedlings treated with 1 μM Cd, 88% of which was excreted. However, M synthesis decreased with increasing Cd concentration and so did the percentage of excreted

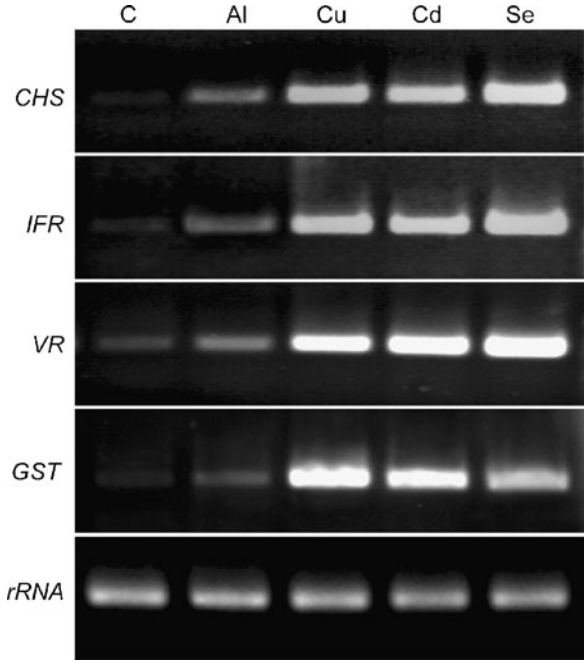
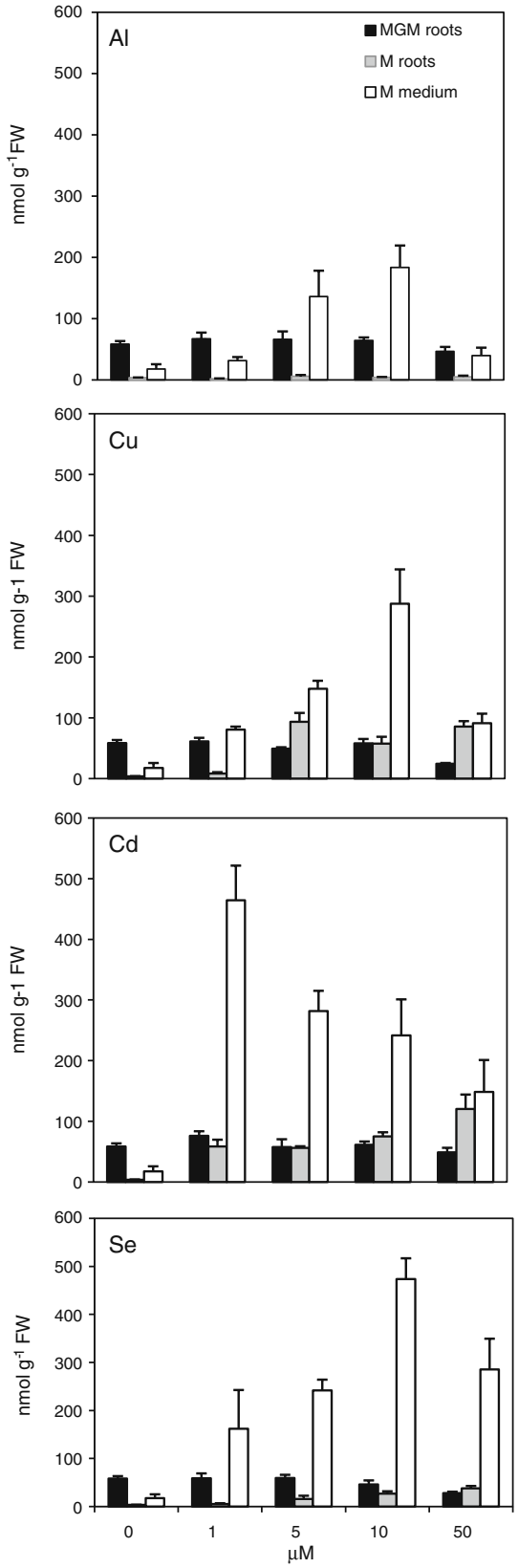


Fig. 3 RT-PCR analysis of *CHS*, *IFR*, *VR* and *GST* gene expression. Semi-quantitative RT-PCR of steady state *CHS*, *IFR*, *VR*, and *GST* mRNA in roots of 6-day-old fenugreek seedlings after 6 h elicitation with 10 μM Al, Cu, Cd, or Se



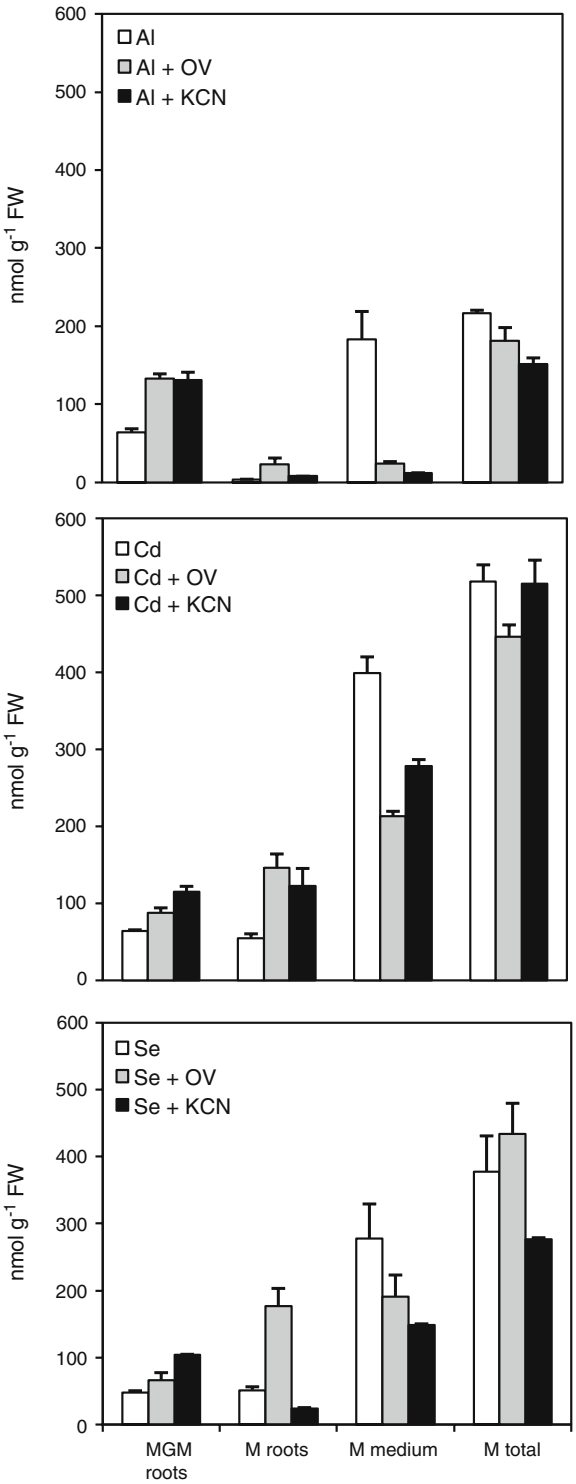
◀ **Fig. 4** MGM and M concentrations in the roots and the seedling culture media in response to Al, Cu, Cd, or Se treatment (1, 5, 10 and 50 µM). Values are the mean of at least three experiments±SE

M; reduction of M excretion was followed by increased M in the roots. In contrast, the pattern of M excretion *vs.* total M in response to Al, Cu or Se differed. The highest amount of synthesized M as well as the highest percentage of its excretion was detected when fenugreek seedlings were treated with 10 µM Cu, Al or Se (Fig. 4). M excretion in response to the tested elements does not seem to be due to non-specific leakage, since further increase of metal or Se concentration resulted in less M excretion.

Treatment of fenugreek seedlings with 10 µM Al, Cd or Se in the presence of the ATPase or the ATP synthesis inhibitors, OV (100 µM) or KCN reduced the metal or Se-induced M excretion, and the percentage of excreted M. In Cd or Se-treated seedlings, a great part of M which was not excreted, due to the inhibition by OV, accumulated in the roots mostly in the form of free M. In contrast, in Al-treated seedlings in the presence of OV or KCN and in Se-treated seedlings in the presence of KCN, most of the non-excreted M accumulated in the roots in its malonyl glucoside form (Fig. 5). The increase of total "M" (root MGM and M plus M of the seedling medium) which was effected in the presence of these elicitors was not affected significantly in the presence of OV or KCN, with the exception of Se-treated seedlings in the presence of KCN (Fig. 5). The amount of M excreted and the percentage of its excretion were further reduced in the presence of higher OV or KCN concentrations (data not shown).

Medicarpin is sequestered into vacuoles *via* an ABC-type transport mechanism in the form of S-glutathionylated conjugate, the formation of which is through the action of GST (Li et al. 1997). In order to test a possible implication of GST in M excretion, we studied the induction of a Phi *GST* gene (AB040439) in the presence of 10 µM Al, Cu, Cd and Se. Semi-quantitative RT-PCR showed that these elements induced an increase of the *GST* transcript levels in the order Cu> Cd> Se> Al (Fig. 3).

Twenty four-h treatment of fenugreek seedlings increased the total amount of citrate (citrate in the roots plus seedling culture medium), but most of it was excreted and the amount was being dependent on the element and its concentration. The highest amount



◀ **Fig. 5** MGM and M concentrations in the roots, M concentration in the seedling culture media and total "M" (MGM + M in roots + M in the seedling culture medium) as affected by the presence of an ATPase (OV) and an ATP synthesis (KCN) inhibitor. Five-day old seedlings were incubated in the absence or presence of 100 μM OV or 50 mM KCN. After 24 h of incubation $AlCl_3$, $CuCl_2$, $CdCl_2$, or Na_2SeO_3 were added to the media to a final concentration of 10 μM and seedlings were incubated for another 24 h. Values are the mean of at least three experiments±SE

of excreted citrate was induced by Cu and, like in the presence of Al, increased with increasing metal concentration, although citrate excretion in the presence of 50 μM Al was only slightly higher compared to that of control. The opposite occurred when seedlings were treated with Cd or Se (Fig. 6).

Discussion

Among the common effects of metal stress is inhibition of root growth and loss of the root plasma membrane integrity, the extent of which depends on the species, developmental stage and the kind and metal concentration (Peralta-Videa et al. 2004). The trace element-induced reduction of the root fresh weight of fenugreek seedlings was significantly lower compared to that of root elongation. The highest reduction of root fresh weight was caused by 50 μM Cu (38%), but the corresponding reduction of root elongation was considerably higher (72%). Yet, in other plant species the induced inhibition of root elongation by certain metals, like Cd or Al, was found to be higher compared to that of fenugreek seedlings. One-day treatment of *Arabidopsis* seedlings with

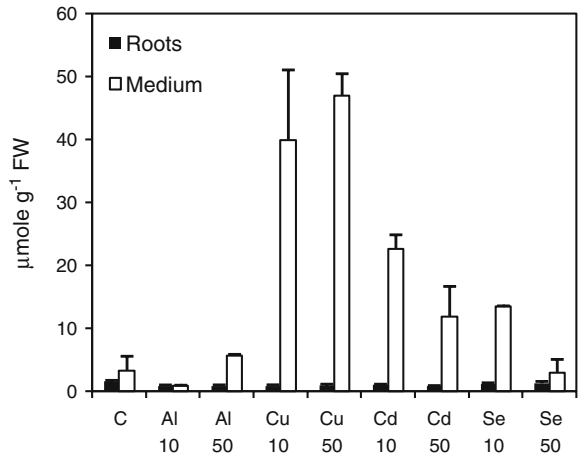


Fig. 6 Citrate concentration in the roots and culture media of fenugreek seedlings treated with 10 or 50 μM Al, Cu, Cd or Se for 24 h. Values are the mean of at least three experiments±SE

10 μM Cd caused a 75% reduction of root elongation (Hou et al. 2010) against a 60% inhibition in fenugreek seedlings,, while treatment of Al-tolerant *Medicago truncatula* seedlings with 5 μM Al caused a 50% reduction (Chandran et al. 2008) against a 42% reduction of fenugreek root elongation caused by 10 μM Al.

Loss of the root plasma membrane integrity caused by metal treatment may be the result of metal-induced plasma membrane peroxidation (De Vos et al. 1993). Moderate to severe metal toxicity effects, which were deduced from the intensity of Evans blue root staining, appeared only when fenugreek seedlings were treated with 50 μM Al, Cu or Cd, while roots from 50 μM Se-treated seedlings were hardly stained. This is substantiated further by the operation of an M-specific transport system, possibly an ABC-type transporter, by which M, in the presence of these trace elements, is excreted in the seedling growth medium. These results suggest that fenugreek seedlings are resistant at a different extent to 10 μM Cd, Cu, Al and Se.

Synthesis and excretion mainly of organic acids, but also of phenolics, has been proposed as a potential mechanism of amelioration of metal toxicity in plants (Ma et al. 2001; Michalak 2006). In contrast, no such mechanisms have been reported for Se resistance. On the other hand, induction of isoflavonoid synthesis in response to heavy metals is restricted to only certain plant species of the *Papillionoidae* subfamily. Cd treatment of endocarps from immature pea pods induced phenylalanine ammonia lyase (PAL) activity and the synthesis of the isoflavonoid phytoalexin pisatin (Hadwiger et al. 1973), while Al treatment of *Medicago truncatula* seedlings induced the expression of genes encoding enzymes of the isoflavonoid biosynthetic pathway (Chandran et al. 2008). In our previous work we showed that roots of 6-day old fenugreek seedlings synthesized M, which accumulated mainly in the form of MGM. Moderate Cu concentration (10 μM) induced *de novo* biosynthesis of M in the roots of these seedlings, while in seedlings treated with higher Cu concentrations (from 50 to 1,000 μM) the accumulated M was mainly or totally formed at the expense of its constitutive malonyl conjugate (Tsiri et al. 2009). Our results showed that fenugreek seedlings responded to both metals and Se by inducing *de novo* synthesis of M in the roots, most of which was exuded in the seedling culture medium, although at 50 μM concentration of Cu and Se, part of the accumulated M seems to be formed at the expense of constitutive MGM, since the concentration of the later was reduced by approx. 45%.

For certain plant species metal-induced citrate excretion plays a central role in mechanisms of metal and especially of Al resistance (Murphy et al. 1999; Ma et al. 2001; Nian et al. 2002; Pinto et al. 2008; Poschenrieder et al. 2008; Rangel et al. 2010). In contrast, to date, no such a role has been attributed for metal-induced isoflavonoid excretion, apart from the Cu-induced excretion of genistein from the roots of white lupin (Jung et al. 2003) and flavonoid exudation by root tips of maize seedlings (Kidd et al. 2001), which have been related to Cu or Al resistance, respectively. Fenugreek seedlings responded to the studied metals and Se by excreting citrate and M, two potential metal and Se chelators, which may contribute to metal detoxification mechanisms. Despite the significant increase in the amount of excreted M in the presence of these elicitors, the contribution of citrate to Cu, Cd, or Se detoxification seems to be considerably higher to that of M, since roots, depending on the elicitor concentration, excreted about 10 to100-folds more citrate than M. Interestingly, in Al treated seedlings, the possible detoxification mechanism seems to involve M rather than citrate. Similarly, in maize seedlings flavonoids, and not organic acids, are involved in Si-induced amelioration of Al toxicity (Kidd et al. 2001).

Although flavonoid transport mechanisms are far from being clarified there is increasing body of evidence which implicates ABC transporters in their transport (Zhao and Dixon 2010 and references therein). In the rhizobium-induced excretion of the isoflavonoid genistein is involved an ABC-type transporter, which is specific for isoflavones, like daidzein and formononetin (Sugiyama et al. 2007). If elicitor-induced M excretion by the roots of fenugreek seedlings is mediated by ABC-type transporters, ATP must play a central role and inhibition of ATP production must modify the excretion process. Potassium cyanide inhibits ATP synthesis (Lew and Spanswick 1984) while OV, a typical inhibitor of ATP-binding cassette transporters, inhibits the membrane ATPases by acting as a phosphate analogue (Sugiyama et al. 2007). The mechanism of M excretion induced by Al, Cd, or Se, like that by Cu (Tsiri et al. 2009) is likely to involve an ATP-dependent transport system, as indicated by the

inhibition of metal and Se-induced M excretion in the presence of OV or KCN.

Medicarpin excretion induced by these trace elements may also involve GST. GSTs have been implicated in the transport of secondary metabolites and are regarded as important cellular detoxifiers of toxic endogenous metabolites or xenobiotics (Dixon et al. 2010; Zhao and Dixon 2010). The plant-specific Phi GSTs are involved in cellular detoxification by catalyzing the conjugation of glutathione (GSH) with a wide range of endogenous and xenobiotic agents. Some Phi GSTs have other functions including transport of flavonoid pigments into the vacuole and GSH peroxidase activity (Alfenito et al. 1998; Dixon et al. 2010; Zhao and Dixon 2010). Also, GST activity is induced in response to a variety of environmental stresses, including heavy metals (Dixit et al. 2001; Ezaki et al. 2004; Edwards and Dixon 2004; Gajewska and SkŁodowska 2008). In *Medicago sativa*, for example, carbon monoxide alleviates Cd-induced oxidative damage by modulating GSH metabolism in the roots (Han et al. 2008). The hypothesis that metal or Se-induced expression of *GST* in the roots of fenugreek seedlings might be related to the M excretion mechanism is based on a proposed mechanism of flavonoid transport across various membranes; flavonoid, transport likely takes place in the form of GST-flavonoid *via* an ABC-type transporter (Zhao and Dixon 2010). By this mechanism anthocyanins are sequestered into plant vacuoles (Alfenito et al. 1998). Moreover, GST is used for M sequestration in vacuoles. It has been demonstrated that M, when reacting with GST-containing cellular extracts from maize, forms a GS conjugate that is transported into mung bean tonoplast vesicles by a similar to ATP-dependent mechanism and in a four-fold higher velocity when compared to un-conjugated M (Li et al. 1997). Based on these findings and on our results, it may be speculated that M is transported through the root plasma membrane to the rhizosphere as GS-M conjugate, which is formed through the action of GST. The relatively lower *GST* gene transcript levels in the roots of Al-treated seedlings correlate well with the significantly smaller amount of Al-induced M excretion.

The induction of *de novo* synthesis and excretion of M and of an ABC-type transport system for M, together with citrate excretion, by four different in their nature elements, two heavy metals, one light metal and a non metal, may be part of a non-trace element specific resistance mechanism mediated by common signal(s). In *Medicago sativa* cell cultures the induction of PAL activity and M production by an isolate of *Verticillium albo-atrum* implicated reactive oxygen species (ROS) in the signal pathway that activated these responses (Tang and Smith 2001). The generation of ROS is a common event in both biotic and abiotic stresses, including metal and Se stress (Gomes-Junior et al. 2007; Michalak 2006; Mithöfer et al. 2004; Tamaoki et al. 2008). Oveproduction of ROS signals the induction of the antioxidant genes necessary for cell survival/adaptation like genes of ROS scavenging enzymes and secondary defence metabolites (Moschou et al. 2008a and literature therein; Tang and Smith 2001; Zhao et al. 2009). Thus, in metal and Se-treated fenugreek seedlings, endogenous ROS may be candidate signals which induced M and citrate synthesis and excretion, as it was the case with salinity-induced ROS generation and induction of protection mechanisms (Moschou et al. 2008b).

In this report we have shown for first time that apart from heavy metals, Al and Se induced *de novo* biosynthesis and excretion from the roots of fenugreek seedlings, of an isoflavonoid phytoalexin, M, which may be involved in metal and Se detoxyfication. Furthermore, we have shown that all elicitors induced the operation of an ABC-type transport system of M probably involving GST. In parallel to M excretion fenugreek seedlings responded to the same elicitors by citrate exudation, which may be part of metal and Se detoxification mechanism. These responses seem to be part of a non-element specific resistance mechanism of fenugreek seedlings, induced by common signal(s). The identification of these signals as well as the role of GST is currently being investigated.

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References

Ahsan N, Renaut J, Komatsu S (2009) Recent developments in the application of proteomics to the analysis of plant responses to heavy metals. Proteomics 9(10):2602–2621

Alfenito M, Souer E, Goodman CD, Buell R, Mol J, Koes R, Walbot V (1998) Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione-*S*-transferases. Plant Cell 10:1135–1149

Barceló J, Poschenrieder C (2002) Fast root growth responses, root exudates and internal detoxification as clues to the mechanisms of aluminum toxicity and resistance: a review. Environ Exp Bot 48:75–82

Cachinero JM, Hervás A, Jiménez-Díaz RM, Tena B (2002) Plant defence reactions against fusarium wilt in chickpea induced by incompatible race of *Fusarium oxysporum* f. sp. ciceris and nonhost isolates of *F. oxysporum*. Plant Pathol 51:765–776

Cançado GMA, Loguercio LL, Martins PR, Parentoni SN, Paiva E, Borém A, Lopes MA (1999) Hematoxylin staining as a phenotypic index for aluminum tolerance selection in tropical maize (*Zea mays* L.). Theor Appl Genet 99:747–754

Carlsen SCK, Understrup A, Fomsgaard IS, Mortensen AG, Ravnskov S (2008) Flavonoids in roots of white clover: interaction of arbuscular mycorrhizal fungi and a pathogenic fungus. Plant Soil 302:33–43

Chandran D, Sharopova N, Vandenbosch KA, Garvin DF, Samac DA (2008) Physiological and molecular characterization of aluminium resistance in *Medicago truncatula*. BMC Plant Biol 8:89

De Vos CHR, Ten Bookum WM, Vooijs R, Schat H, De Kok LJ (1993) Effect of copper on fatty acid composition and peroxidation of lipids in the roots of copper tolerant and sensitive *Silene cucubalus*. Plant Physiol Biochem 31:151–158

Dewick PM, Martin M (1979) Biosynthesis of pterocarpan and coumestan metabolites of *Medicago sativa*: chalcone, isoflavone and isoflavanone precursors. Phytochemistry 18:597–602

Dixit V, Pandey V, Shyam R (2001) Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). J Exp Bot 52:1101–1109

Dixon RA (1999) Isoflavonoids: biochemistry, molecular biology and biological function. In: Sankawa U (ed) Comprehensive natural products chemistry, vol. 1. Elsevier, Oxford, pp 773–823

Dixon RA, Steele CL (1999) Flavonoids and isoflavonoids—a gold mine for metabolic engineering. Trends Plant Sci 4:394–400

Dixon RA, Achnine L, Kota P, Liu C, Reddy MSS, Wang L (2002) The phenylpropanoid pathway and plant defence—a genomics perspective. Mol Plant Pathol 3:371–390

Dixon DP, Skipsey M, Edward R (2010) Roles for glutathione transferases in plant secondary metabolism. Phytochemistry 71:338–350

Edwards R, Dixon DD (2004) Metabolism of natural and xenobiotic substrates by the plant glutathione S-transferase superfamily. In: Sandermann H (ed) Molecular ecotoxicology of plants. Ecological studies, vol 170. Springer-Verlag Berlin, Heidelberg, pp 17–50

Ezaki B, Suzuki M, Motoda H, Kawamura M, Nakashima S, Matsumoto H (2004) Mechanism of gene expression of Arabidopsis glutathione S-transferase, *AtGST1* and *AtGST11* in response to aluminium stress. Plant Physiol 134:1672–1682

Farag MA, Huhman DV, Lei Z, Sumner LW (2008) Metabolic profiling and systematic identification of flavonoids and isoflavonoids in roots and cell suspension cultures of *Medicago truncatula* using HPLC-V-ESI-MS and GC–MS. Phytochemistry 68:342–354

Gajewska E, SkŁodowska M (2008) Differential biochemical responses of wheat shoots and roots to nickel stress: antioxidative reactions and proline accumulation. Plant Growth Regul 54:179–188

Gomes-Junior RA, Gratao PL, Gaziola SA, Mazzafera P, Lea PJ, Azevedo RA (2007) Selenium-induced oxidative stress in coffee cell suspension cultures. Funct Plant Biol 34:449–456

Guo L, Dixon RA, Paiva NL (1994) Conversion of vestitone to medicarpin in alfalfa (*Medicago sativa* L.) is catalyzed by two independent enzymes. J Biol Chem 269:22372–22378

Hadwiger LA, von Broembsen S, Eddy R Jr (1973) Increased template activity in chromatin from cadmium chloride treated pea tissues. Biochem Biophys Res Commun 50:1120–1128

Han Y, Zhang J, Chen X, Gao Z, Xuan W, Xu S, Ding X, Shen W (2008) Carbon monoxide alleviates cadmium-induced oxidative damage by modulating glutathione metabolism in the roots of Medicago sativa. New Phytol 177:155–166

Hoekenga OA, Maron LG, Piñeros MA, Cançado GMA, Shaff J, Kobayashi Y, Ryan PR, Dong B, Delhaize E, Sasaki T, Matsumoto H, Yamamoto Y, Koyama H, Kochian LV (2006) *AtALMT1*, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in Arabidopsis. PNAS 103:9738–9743

Hou L, Shi W, Wei W, Hong Shen H (2010) Cadmium uptake, translocation, and tolerance in AHA1OX *Arabidopsis thaliana*. Biol Trace Elem Res. doi:10.1007/s12011-010-8657-6

Jasiński M, Kachlicki P, Rodziewicz P, Figlerowicz M, Stobiecki M (2009) Changes in the profile of flavonoid accumulation in Medicago truncatula leaves during infection with fungal pathogen Phoma medicaginis. Plant Physiol Biochem 47:847–853

Jung C, Maeder V, Funk F, Frey B, Sticher H, Frossard E (2003) Release of phenols from Lupinus albus L. roots exposed to Cu and their possible role in Cu detoxification. Plant Soil 252:301–312

Kidd PS, Llugany M, Poschenrieder C, Gunsé B, Barceló J (2001) The role of root exudates in aluminum resistance and silicon-induced amelioration of aluminum toxicity in three varieties of maize (Zea mays L.). J Exp Bot 52:1339–1352

Kochian LV, Pence NS, Letham DLD, Piñeros MA, Magalhaes JV, Hoekenga OA, Garvin DF (2002) Mechanisms of metal resistance in plants: aluminum and heavy metals. Plant Soil 247:109–119

Lew MM, Spanswick RM (1984) Characterization of electrogenicity of soybean (Glycine max) roots. ATP dependence and effect of ATPase inhibitors. Plant Physiol 75:1–6

Li ZS, Alfenito M, Rea PA, Walbot V, Dixon RA (1997) Vacuolar uptake of the phytoalexin medicarpin by the glutathione conjugate pump. Phytochemistry 45:689–693

Li XF, Ma JF, Matsumoto H (2002) Aluminum-induced secretion of both citrate and malate in rye. Plant Soil 242:235–243

Lopez-Meyer M, Paiva NL (2002) Immunolocalization of vestitone reductase and isoflavone reductase, two enzymes

involved in the biosynthesis of the phytoalexin medicarpin. Physiol Mol Plant Pathol 61:15–30

Ma JF, Ryan PR, Delhaize E (2001) Aluminium tolerance in plants and the complexing role of organic acids. Trends Plant Sci 6:273–278

Magnavaca R, Gardner CO, Clark RB (1987) Evaluation of inbred maize lines for aluminum tolerance in nutrient solution. In: Loughman BC, Gabelman HW (eds) Genetic aspects of plant mineral nutrition. Martinus Nijhoff, Dordrecht, pp 255–265

Michalak A (2006) Pnenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Pol J Environ Stud 15:523–530

Mithöfer A, Schulze B, Boland W (2004) Biotic and heavy metal stress response in plants: evidence for common signals. FEBS Lett 566:1–5

Moschou PN, Delis ID, Paschalidis KA, Roubelakis-Angelakis KA (2008a) Transgenic tobacco plants over-expressing polyamine oxidase are not able to cope with oxidative burst generated by abiotic factors. Physiol Plant 133(2):140–156

Moschou PN, Paschalidis KA, Delis ID, Andriopoulou AH, Lagiotis GD, Yakoumakis DI, Roubelakis-Angelakis KA (2008b) Spermidine exodus and oxidation in the apoplast induced by abiotic stress is responsible for H_2O_2 signatures that direct tolerance responses in tobacco. Plant Cell 20:1708–1724

Murphy AS, Eisinger WR, Shaff JE, Kochian LV, Taiz L (1999) Early copper-induced leakage of K^+ from *Arabidopsis* seedlings is mediated by ion channels and coupled to citrate efflux. Plant Physiol 121:1375–1382

Naoumkina M, Farag MA, Sumner LW, Tang Y, Liu C-J, Dixon RA (2007) Different mechanisms for phytoalexin induction by pathogen and wound signals in *Medicago truncatula*. PNAS 104:17909–17915

Nian H, Yang ZM, Ahn SJ, Cheng ZJ, Matsumoto H (2002) A comparative study on the aluminium- and copper-induced organic acid exudation from wheat roots. Physiol Plant 116:328–335

Paiva NL, Edwards R, Sun Y, Hrazdina G, Dixon RA (1991) Stress responses in alfalfa (*Medicago sativa* L.). 11. Molecular cloning and expression of alfalfa isoflavone reductase a key enzyme of isoflavonoid phytoalexin biosynthesis. Plant Mol Biol 17:653–667

Parry AD, Tiller SA, Edwards RE (1994) The effects of heavy metals and root immersion on isoflavonoid metabolism in alfalfa (*Medicago sativa* L.). Plant Physiol 106:195–202

Pellet DM, Papernik LA, Jones DL, Darrah PR, Grunes DL, Kochian LV (1997) Involvement of multiple aluminium exclusion mechanisms in aluminium tolerance in wheat. Plant Soil 192:63–68

Peralta-Videa JR, de la Rossa G, Gonzalez JH, Gardea-Torresdey JL (2004) Effects of the growth stage on the heavy metal tolerance of alfalfa plants. Adv Environ Res 8:679–685

Piñeros MA, Staff JE, Manslank HS, Alvez VMC, Kochian LV (2005) Aluminum resistance in maize cannot be solely explained by root organic acid exudation. A comparative physiological study. Plant Physiol 137:231–241

Pinto AP, Simões I, Mota AM (2008) Cadmium impact on root exudates of sorghum and maize plants: a speciation study. J Plant Nutr 31:1746–1755

Poschenrieder C, Gunsé B, Corrales I, Barceló J (2008) A glance into aluminium toxicity in plants. Sci Total Environ 400:356–368

Qin RJ, Hirano Y, Brunner I (2007) Exudation of organic acid anions from poplar roots after exposure to Al, Cu and Zn. Tree Physiol 27:313–320

Rangel FA, Rao IM, Braun HP, Horst WJ (2010) Aluminum resistance in common bean (*Phaseolus vulgaris*) involves induction and maintenance of citrate exudation from root apices. Physiol Plant 138:176–190

Rice-Evans CA, Miller NJ, Paganga G (1997) Antioxidant properties of phenolic compounds. Trends Plant Sci 2:152–159

Salt DE, Smith RD, Raskin I (1998) Phytoremedation. Annu Rev Plant Physiol Mol Biol 49:643–668

Saunders JA, O'Neill NR (2004) The characterization of defence responses to fungal infection in alfalfa. Biocontrol 49:715–728

Silva IR, Smytha TJ, Rapera CD, Carterb TE, Rufty TW (2001) Differential aluminum tolerance in soybean: an evaluation of the role of organic acids. Physiol Plant 112:200–210

Sugiyama A, Shitan N, Yazaki K (2007) Involvement of a soybean ATP-binding cassette-type transporter in the secretion of genistein, a signal flavonoid in legume-*Rhizobium* symbiosis. Plant Physiol 144:2000–2008

Tamaoki M, Freeman JL, Pilon-Smits EAH (2008) Cooperative ethylene and jasmonic acid signaling regulates selenate resistance in *Arabidopsis*. Plant Physiol 146:1219–1230

Tang M, Smith CJ (2001) Elicitor induced defence responses in *Medicago sativa*. New Phytol 149:401–418

Toda S, Shirataki Y (2001) Comparison of antioxidative and chelating effects of daidzein and daidzin on protein oxidative modification by copper in vitro. Biol Trace Elem Res 79:83–89

Tsiri D, Chinou I, Halabalaki M, Haralampidis K, Ganis-Spyropoulos C (2009) The origin of copper-induced medicarpin accumulation and its secretion from roots of young fenugreek seedlings are regulated by copper concentration. Plant Sci 176:367–374

Yamamoto Y, Kobayashi Y, Matsumoto H (2001) Lipid peroxidation is an early symptom triggered by aluminium, but not the primary cause of elongation inhibition in pea roots. Plant Physiol 125:199–208

Zhao J, Dixon RA (2010) The 'ins' and 'outs' of flavonoid transport. Trends Plant Sci 15:72–80

Zhao CR, Ikka T, Sawaki Y, Kobayashi Y, Suzuki Y, Hibino T, Sato S, Sakurai N, Shibata D, Koyama H (2009) Comparative transcriptomic characterization of aluminium, sodium chloride, cadmium and copper rhizotoxicities in *Arabidopsis thaliana*. BMC Plant Biol 9:32