

Role of arbuscular mycorrhizal fungi (*Rhizophagus irregularis*) on mercury tolerance of *Medicago truncatula* in relation to mercury and zinc concentration

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Abstract

Mercury (Hg) pollution of soils is a critical environmental problem. To rehabilitate Hg contaminated soils, arbuscular mycorrhizal fungi (AMF)-based phytoremediation may be supportive, yet the functional potential of AMF in response to Hg exposure is unclear. In a greenhouse experiment, we assessed the response of *Medicago truncatula* (biomass, Hg tolerance index (TI), Hg partitioning) to different Hg concentrations [0 (Hg0), 25 (Hg25), 50 (Hg50) $\mu\text{g g}^{-1}$] in treatments with (AM) and without (NM) inoculation of the AMF *Rhizophagus irregularis*. Additionally, zinc (Zn) uptake and the expression of two Zn transporter genes (*MtZIP2*, *MtZIP6*) were examined, because Hg and Zn share the same outer electronic configuration, inferring a potential competition for the same transporters. Although AM plants revealed lower biomass than NM plants, they showed a higher Hg TI. Plant roots were identified as dominant Hg reservoirs. At Hg25, *R. irregularis* decreased the Hg translocation from roots to stems, while Hg translocation was increased at Hg50. Hg in leaves originated mainly from atmospheric uptake. A lower Hg concentration in leaves of AM than NM plants was found, indicating a regulatory effect of *R. irregularis* on stomata functioning. The negative relationship between Hg and Zn concentrations in the roots of AM and NM plants implied a potential competition for the same transporters, although the expression of Zn transporters was upregulated by AMF inoculation at all Hg levels. In conclusion, this baseline study demonstrated that *R. irregularis* contributed to Hg tolerance of *M. truncatula*, suggesting the potential of *R. irregularis* for Hg-contaminated phytoremediation.

Introduction

Mercury (Hg) is a very toxic heavy metal (HM), ranked as the 3rd most hazardous substance on earth (Atsdr 2019). Due to its high mobility and persistence in the environment, Hg has been recognized as a global pollutant and major threat to human health (Driscoll et al. 2013). Pollution with Hg occurs naturally by weathering and to a large extent through anthropogenic activities. These include artisanal gold mining (Wang et al. 2022), which is done in many regions around the world. These activities are often uncontrolled and aggravate the distribution of Hg in terrestrial ecosystems and affect growing plants, including food crops, thereby compromising food safety (Gworek et al. 2020).

Phytoextraction (facilitating HM translocation to plant stems) and phytostabilization (facilitating HM storage in plant roots, while preventing HM translocation to plant stems) are the two efficient plant-based technologies for remediating soil HM contaminations (Bhat et al. 2022). However, remediating plants may suffer from negative effects of HM, especially when HM are excessively present in soils. To counteract negative impacts on plant growth and performance under high HM concentration, the roots of remediating plants are commonly inoculated with competent microbes to enhance HM tolerance and with this, enhancing the efficiency of phytoremediation (Yang et al. 2020).

Among microbe-assisted phytoremediation strategies proposed to date, arbuscular mycorrhizal fungi (AMF) have been verified to support plants in resisting HM stress (Sahraoui et al. 2022; Tiwari et al. 2022). AMF are ubiquitous in terrestrial environments and form intimate relationships with the majority of vascular plants (Smith and Read 2008), reflecting the strategic importance of plant-AMF interactions for environmental adaptation (Wang and Qiu 2006). Several studies reported that certain AMF facilitate HM transport to aboveground plant organs (phytoextraction) (Bissonnette et al. 2010; Fiqri et al. 2016; Singh et al. 2019). Other studies, however, revealed that AMF inhibit HM translocation by various means. These include binding of HM in AMF hyphae and glomalin, or sequestering HM in root structures (arbuscular, vesicle, vacuole). With this, the transport of HM to the plant stem is prevented (phytostabilization) (Garg and Singh 2018; Motaharpoor et al. 2019). The extent of HM uptake and the subsequent translocation *in planta* further depends on the HM concentration in soils, even with presence of the same AMF species (Huang et al. 2017).

Hg exists in three forms, i.e. elemental Hg, inorganic Hg (Hg^{1+} , Hg^{2+}) and organic Hg (Beckers and Rinklebe 2017). In soils, the predominant form is Hg^{2+} (Beckers and Rinklebe 2017; Kumari et al. 2020). Uptake of Hg^{2+} via roots is facilitated by an active process (Esteban et al. 2008; Wang et al. 2014; Ma et al. 2021), yet no precise membrane transporters involved in root Hg^{2+} have been identified. It is generally accepted that HM enter the roots via nutrient transporters (Manoj et al. 2020), due to the structural similarity of HM with other essential nutrients. For example, cesium (Cs^+) uptake is closely related to potassium (K^+) transporters (Boulois et al. 2006); Arsenic (As^{3+} / As^{5+}) shares the same transporters with silicon (Si^{3+}) (Ma et al. 2008) and phosphate (P^{5+}) (Meharg and Hartley-Whitaker 2002). Cadmium (Cd^{2+}) uptake by plants is facilitated via zinc (Zn) protein carrier (Kaur and Garg 2018). However, such pertinent information is lacking for Hg, despite the fact that Hg^{2+} , like Cd^{2+} , shares the same outer electronic configuration with Zn^{2+} (Jensen 2003). Competition between Hg^{2+} and Zn^{2+} was observed in bacteria upon addition of Zn to the Hg containing growth solution. These findings imply that Hg and Zn share affinity for the same transporters (Schaefer et al. 2014; Szczuka et al. 2015). In plants, the effect of Zn nutrient addition under Hg exposure depends on plant species, soil properties and growth conditions (Calgaroto et al. 2016; Debeljak et al. 2018). More importantly, AMF are considered as a critical regulator of Zn nutrient uptake via regulating Zn transporters (Ruytinx et al. 2020). Thus, how Zn nutrient and Zn transporters are tuned by AMF exposed to Hg, is fundamental to understand the mechanisms of Hg uptake and accumulation in mycorrhizal plants. Such advanced knowledge would help to optimize AMF-supported plant performance in phytoremediation. Among the involved transporters of Zn into the cytoplasm, the Zinc-Iron-Regulated Transporter (*ZRT-IRT*), called *ZIP*, have been mostly studied. In *Medicago truncatula*, four *ZIP* transporters - *ZIP1*, *ZIP2*, *ZIP5* and *ZIP6* - facilitating the transport of Zn^{2+} have been verified in yeast complementation assays (Burleigh et al. 2003; Stephens et al. 2011). Recent studies showed that only two of them are influenced by mycorrhizal colonization, yet under contrasting Zn conditions. Namely, *MtZIP6* was up-regulated at deficient and sufficient soil Zn concentrations, while *MtZIP2* was up-regulated at toxic Zn concentrations (Watts-Williams et al. 2017).

In this study, we used *Medicago truncatula* inoculated with *Rhizophagus irregularis* to gain fundamental insights into the underlying mechanisms of Hg uptake and accumulation by plants, emphasizing the role of AMF. *M. truncatula* is a primary model for genomic and functional genomic research and is frequently used for AMF-related studies (Frank and Engel 2013; Dursun et al. 2019). *R. irregularis* is widely adopted as a model species for AMF research (Chaturvedi et al. 2021). The aims of this study were to (1) examine the effect of *R. irregularis* on biomass and Hg accumulation of *M. truncatula* under Hg exposure; (2) determine the translocation strategies of Hg in different organs of *M. truncatula* associated with *R. irregularis*; and (3) investigate the effect of *R. irregularis* on Zn nutrient uptake and Zn transporters (*MtZIP2*, *MtZIP6*) under Hg exposure.

Materials And Methods

Preparation of biological materials

Medicago truncatula cv. Jemalong A17 seeds were scarified in 90% sulfuric acid for 7.5 min. Seeds were washed eight times with cold water to remove the acid, followed by 90 s surface sterilization in 3% 5-6 times active chlorine solution (bleach). Bleach was removed by repeated rinsing of the seeds in sterile water. Sterilized seeds were soaked in sterile water overnight under darkness. Then, the seeds were stratified in water-agar plates (0.8% (w/v)) for 24 h at 4°C and germinated at 20°C for 2-3 days in the dark. After germination, seeds were exposed to light for 2 days to form seedlings. Pure AMF inoculum, *Rhizophagus irregularis* (QS81), was kindly provided by INOQ GmbH (Schnega, Germany). The inoculum was produced with the host plant white clover (*Trifolium repens* L.) and made of pure root fragment containing mycorrhizal propagules. Those root fragments containing mycorrhizal propagules were cut into small pieces and sieved with 425 µm mesh. Prior to use, the inoculum was stored in an air-dried, well-ventilated and dark environment.

Experimental design and conditions

Sand quartz was twice autoclaved (121°C / 2 h) with 2 days interval. Plastic pots were filled with 50 ml sterilized sand mixed with 160 mg AMF inoculum (treatment AM). A control without AMF inoculation (treatment NM) was included. Twenty-five mg Osmocote Exact Mini fertilizer (ICL Specialty Fertilizers, Nordhorn, Germany) was applied to each pot prior to planting one *Medicago* seedling per pot. Plants were grown in the greenhouse from July to August 2021. Plants were watered daily with 5 ml tap water. After 3 weeks, when plants showed vigorous growth, a Hg solution was applied as HgCl₂ at the rates 0, 25 and 50 µg HgCl₂ g⁻¹, assigned as Hg0, Hg25, and Hg50. The experiment was a 2 × 3 complete factorial design, comprising 5 replications per treatment arranged in a randomized block design. The experiment was performed for 5 weeks after Hg additions until destructive harvest.

Harvest and parameters measurement

At the end of experiment, the roots were quickly washed with tap water and separated into 3 parts. One sub-sample (100 mg) of fresh root was immediately flash frozen in liquid nitrogen and stored at -80°C for RNA isolation. The second sub-sample was also stored at -80°C for AMF root colonization observation. The remaining roots as well as stem and leaf biomass were dried at room temperature until weight stability to determine plant biomass weight.

The tolerance index (TI) was calculated (Eq 1) to reflect the ability of the host plant to grow in the presence of different Hg concentration (Huang et al. 2017). In which, *mt* is dry weight of the plants growing in Hg substrates; *mc* denotes dry weight of the plants growing in substrates without Hg.

Eq 1

Hg concentrations in each plant organ were analyzed via inductively coupled plasma mass spectrometry (ICP-MS), and Zn concentrations in plant tissues were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES). Fresh roots stored for AMF observation were cleared using 10% NaOH and stained in 2% PARKER QUINK blue ink (Yon et al. 2015). Mycorrhizal colonization was checked with 30 fragments per sample using a light microscope.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from 100 mg root sub-samples (RNeasy® plant Mini Kit, QIAGEN GmbH, Hilden, Germany). RNA integrity was checked by gel electrophoresis, following quantification of RNA by Nanodrop 2100 (Thermo Fisher Scientific). The cDNA was synthesized from 2 µl (~500 ng) DNase-treated RNA using QIAGEN QuantiTect® Reverse Transcription Kit (QIAGEN GmbH). For quantitative real-time (RT)-PCR, transcripts were amplified from 1 µl of 10× diluted cDNA in a 20 µl reaction using gene-specific primers and SYBR® green PCR Master Mix in the StepOnePlus™ Real-Time PCR system (Applied Biosystems, Waltham, Massachusetts, USA). The RT-PCR settings were 94°C for 5 min, then 94°C for 30 s, 60°C for 30 s and 72°C for 30 s for 40 cycles, followed by generation of a dissociation curve. *MtASPP* and *β-actin* were selected as housekeeping genes using NormFinder (Mestdagh et al. 2009). Gene expressions were normalized to the geometric mean of 2 selected housekeeping genes (Vandesompele et al. 2002). Table S1 displays the sequences of the forward and reverse primers used in this study.

Statistical analysis

All statistical analyses were performed in R version 4.0.3. Homogeneity and normality of data were checked with LeveneTest and Shapiro-wilk test, respectively. Data, which did not meet the criteria, were Box-Cox transformed (Box and Cox 1964). An independent *t* test (5%) was used to evaluate the difference between NM and AM treatments under the same Hg level in the substrate. One-way ANOVA was used to examine the effects of Hg in both NM and AM plants. In case of significance ($P < 0.05$), Tukey's HSD test was conducted. The correlation matrix was calculated using the *spearman* method.

Results

Plant biomass

Mycorrhizal plants (AM) produced lower biomass than corresponding non-mycorrhizal plants (NM), which was significant ($P < 0.05$) for Hg0 and Hg50 (Table 1). Despite this, AM plants had a higher tolerance index (TI) than NM plants under the same Hg concentration. Particularly, TI of AM plants was higher than that of NM plants under Hg25 (Table 1) ($P < 0.05$).

Concentration and accumulation of Hg

Fig. 1 shows the Hg concentration in different plant organs (leaf, stem, root). Hg concentration in leaves of AM plants was lower than in NM plants under all Hg treatments ($P < 0.05$). Besides, Hg concentrations in leaves at different Hg levels were similar to Hg0 in both AM and NM plants (Fig. 1a). Since there was no Hg contamination in control substrate (Hg0) (Fig. S1), Hg accumulation in leaves of untreated Hg plants resulted most likely from absorbance of atmospheric Hg, since all plants were grown in the same growth chamber. AMF inoculation reduced the translocation of Hg to stems under Hg25, while Hg translocation to stems was increased under Hg50 ($P < 0.05$). Hg concentration of roots increased with increasing Hg concentration in both AM and NM plants (Fig. 1c). Under Hg25, Hg concentration in AM roots was similar to that in NM roots. However, under Hg50, Hg concentration in AM roots was lower than in NM roots ($P < 0.05$). Accumulation of Hg in different plant organs depended on Hg concentration in the substrate (Fig. 2). Without Hg addition, Hg accumulated mainly in leaves and the corresponding pattern was leaves > roots > stems in both AM and NM plants. In Hg treatments, Hg was preferentially stored in roots and accumulation followed the pattern roots > leaves > stems.

Zn concentration and Zn transporters

In leaves, Zn concentrations of AM plants were higher than those of NM plants at all Hg levels (Fig. 3a) ($P < 0.05$). Similarly, Zn concentrations in stems of AM plants were higher than those of NM plants at all Hg levels (Fig. 3b) ($P < 0.05$). In contrast, Zn concentrations in roots of AM plants were lower than those of NM plants (Fig. 3c). The difference of Zn concentrations between AM and NM roots was significant under Hg0 as well as under Hg25 ($P < 0.05$), but not under Hg50. Besides, AMF inoculation increased the proportion of Zn in stems and further into leaves (Fig. 4), although AMF inoculation did not affect the pattern of accumulation, which was roots > stems > leaves in both AM and NM plants (Fig. 4). Notably, AMF inoculation upregulated the expression of Zn transporters *MtZIP2* and *MtZIP6* at all Hg levels (Fig. 5) ($P < 0.05$). *MtZIP2* was similarly expressed in NM plants, and AMF inoculation increased the expression of *ZIP2* up to 3 times compared to NM plants. Particularly, the *MtZIP6* was exclusively expressed in AM plants (Fig. 5b).

Correlations between Hg and Zn concentrations in roots

To elucidate the relationship among measured parameters, a correlation matrix was performed in NM and AM plants (Fig. 6). AMF inoculation changed the profile of the correlation matrix (Fig. 6). Hg concentration in stems was positively correlated with Hg concentration in roots in NM plants (Fig. 6a) ($P < 0.05$), while this was not found in AM plants (Fig. 6b). Zn concentration in roots was negatively correlated with Hg concentration in roots in both NM and AM plants ($P < 0.05$).

Discussion

Effects of *Rhizopagus irregularis* on Hg partitioning and plant biomass

Our results showed that Hg in leaves originated mainly from atmospheric uptake, a finding in line with previous studies (Ericksen et al. 2003; Assad et al. 2016; Wohlgemuth et al. 2020). We found a significant reduction of Hg concentration in leaves of AM plants compared to NM plants. This indicates that the used AMF strain was able to regulate the Hg uptake by leaves via their stomata (Laacouri et al. 2013; Wohlgemuth et al. 2022). Stomatal closure and decreasing CO₂ fixation is part of the protective strategy initiated by AMF under exposure to pollutants (Wang et al. 2017). With this, plants can maintain optimal physiological conditions by reducing the assimilation of pollutants (García-Sánchez et al. 2019). This protective strategy may also explain the lower biomass of AM compared to NM plants observed in our study, a finding in line with (Chitarra et al. 2016). Besides, the effect that AMF alleviated HM stress rather than promoting plant growth was earlier verified (Wang et al. 2017). This observation was approved by the higher tolerance index (TI) of AM than NM plants, and further supported by observed leaf necrosis under Hg50 only in NM plants.

Although numerous studies exist regarding the role of AMF in phytoremediation, their application in HM contaminated soils is still not practicable. This is mainly manifested in the controversy whether AMF facilitate or inhibit HM uptake and/or translocation in plants (Gong and Tian 2019). Research in maize showed that AMF inoculation reduced Hg accumulation in roots compared non-inoculated plants with a Hg concentration of 2 and 4 mg kg⁻¹ (Yu et al. 2010). Other studies demonstrated, however, that AMF inoculation facilitated Hg uptake and its translocation from roots to shoots of maize at a Hg concentration of 50 mg kg⁻¹ (Kodre et al. 2017; Debeljak et al. 2018). These results indicated that the role of AMF in Hg translocation of plants is likely dependent of the Hg concentration in the substrate. This was confirmed by our results revealing a repression of Hg translocation from roots to stems at Hg25 and a respective increase at Hg50.

The observed increase of Hg concentrations in roots with increasing Hg levels was coherent with other reports. For example, Hg concentration in lupin roots revealed a linear and hyperbolic pattern with increasing Hg additions in the growth solution (Esteban et al. 2008). This verifies that plant roots generally accumulate Hg, but the extent of Hg storage in roots depends largely on the plant species (Greger et al. 2005). In our study, Hg concentrations in the roots of *M. truncatula* were similar under Hg25 in both AM and NM plants, indicating the ability of *M. truncatula* to accumulate Hg under the given concentration. On the other hand, AMF inoculation reduced Hg accumulation at Hg50, indicating a positive role of AMF under high Hg concentration. It seems likely that there is a threshold between Hg25 and Hg50. Within this threshold, plants are able to detoxify Hg by themselves (Kumar et al. 2017). Beyond that, however, plants benefit from microbes (in this case AMF) to resist biotic and abiotic stress (Sinha et al. 2022).

Effects of *Rhizopagus irregularis* on Zn under Hg exposure

Generally, AMF inoculation exerts a positive effect on absorption of elemental nutrients (Smith and Read 2008). In our study, AMF inoculation increased Zn concentration in aboveground organs exposed to Hg, a finding in line with other studies (Debeljak et al. 2018; Saboor et al. 2021). Moreover, AMF inoculation upregulated the expression of Zn transporter genes (*MtZIP2*, *MtZIP6*), independent of Hg levels in the substrate. This indicates that Hg uptake might not be related to these two transporters in our study. However, acknowledging this fact, we used the identical Zn nutrient in the substrate across the experiment, which could not rule out the stronger effect of Zn over that of other factors. Although the amount of Zn in the substrate was expected to be sufficient, the Zn

requirement of the plant under Hg stress is not known. Zn acts not only as a nutrient, but is also involved in the reduction of the oxidative stress caused by Hg (Calgaroto et al. 2011). However, the correlation analysis showed a negative relationship between Hg and Zn concentrations in the root of both AM and NM plants, implying a potential competition between both elements for the same transporter membranes. This is consistent with findings in bacteria (Schaefer et al. 2014; Szczuka et al. 2015). In order to fully understand the relationship between both Zn and Hg, further experiments with different Zn concentrations in the substrate, and studies on other Zn-related transporters are needed.

Conclusions

We verified that the regulatory role of AMF on Hg accumulation and translocation in plants from roots to stems is dependent on the concentration of Hg in the substrate and is also influenced by other elements, as was revealed for Zn. Conclusively, we demonstrated that *R. irregularis* contributed to Hg tolerance of plants, suggesting the potential of *R. irregularis* for phytoremediation of Hg-contaminated sites.

Declarations

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Author contributions

All authors contributed to the study conception and design. Experiments and data collection were performed by Yaqin Guo and Nadine Sommer. Data analysis and the first draft were done by Yaqin Guo. All authors commented and approved the final manuscript.

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Table

Table 1 Biomass, tolerance index (TI) of non-mycorrhizal (NM) and mycorrhizal (AM) plants in the substrate exposed to three different mercury (Hg) levels (Hg0, Hg25, Hg50)

	Leaf		Stem		Root		Total		TI	
	NM	AM	NM	AM	NM	AM	NM	AM	NM	A
Hg0	0.67 ± 0.03a*	0.62 ± 0.01a	0.63 ± 0.02a*	0.60 ± 0.01a	0.65 ± 0.04a	0.61 ± 0.02a	1.95 ± 0.07a*	1.82 ± 0.02a	1.000a	1
Hg25	0.65 ± 0.04a	0.62 ± 0.01a	0.61 ± 0.01a	0.60 ± 0.02a	0.61 ± 0.04a	0.60 ± 0.01ab	1.83 ± 0.23b	1.81 ± 0.26a	0.938b	0
Hg50	0.65 ± 0.04a	0.62 ± 0.01a	0.62 ± 0.00a*	0.60 ± 0.01a	0.62 ± 0.01a**	0.58 ± 0.01b	1.91 ± 0.39ab***	1.80 ± 0.02a	0.978ab	0

The asterisk (*) denotes significant mean differences between NM and AM treatments by the *t* test (5%), with *, ** and *** representing the significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. One-way ANOVA was performed to determine the effects of Hg concentration in the substrate, with significant differences shown by different lower cases ($P < 0.05$) in each column

Figures

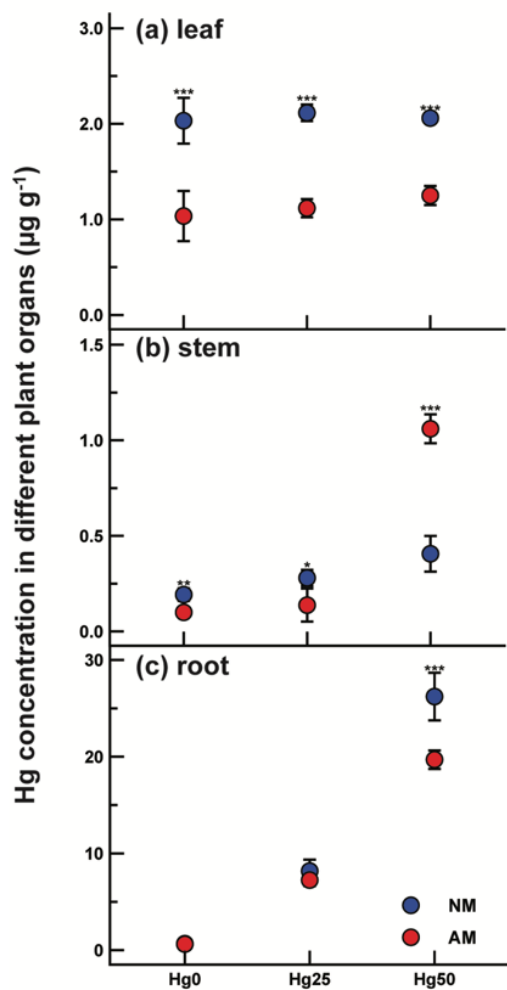


Figure 1

Mercury (Hg) concentration in leaves (a), stems (b) and roots (c) of non-mycorrhizal (NM) and mycorrhizal (AM) plants exposed to three different Hg levels (Hg0, Hg25, Hg50). Asterisks denote the significance level (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$)

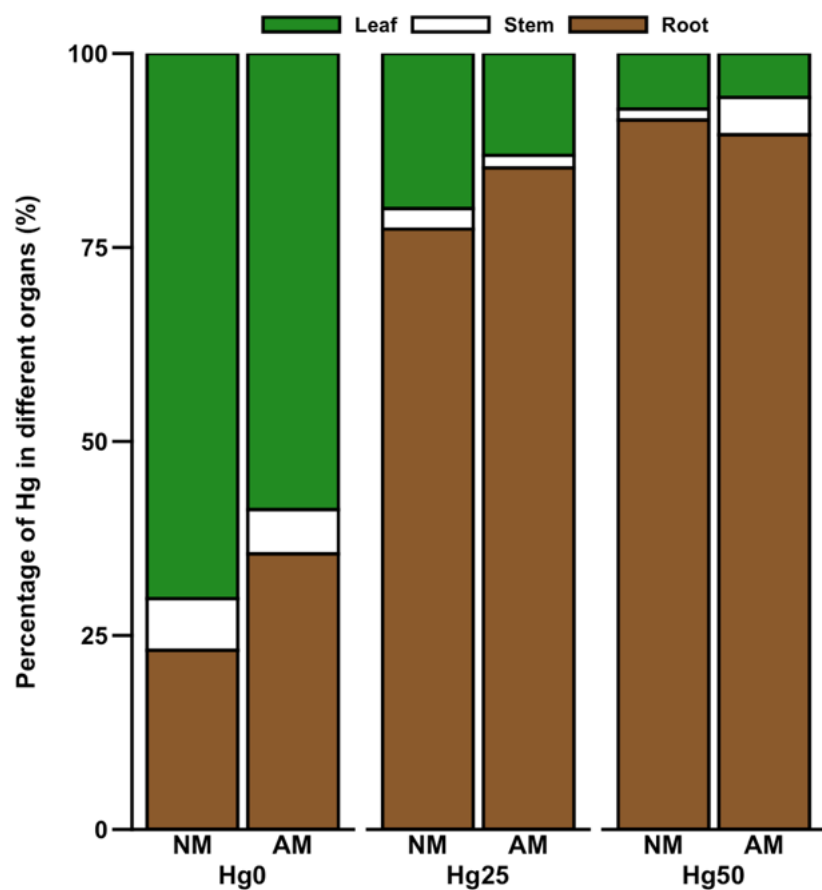


Figure 2

Percentage allocation of mercury (Hg) concentration in different organs of non-mycorrhizal (NM) and mycorrhizal (AM) plants exposed to three different Hg levels (Hg0, Hg25, Hg50)

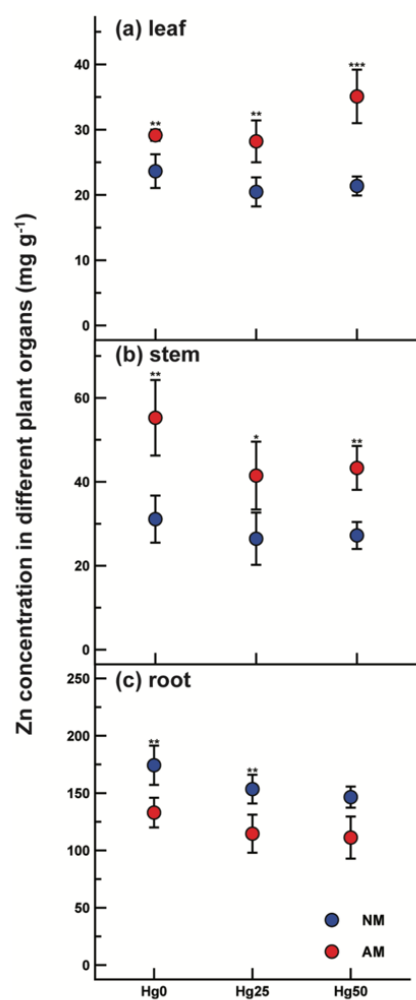


Figure 3

Zinc (Zn) concentration in leaves (a), stems (b) and roots (c) of non-mycorrhizal (NM) and mycorrhizal (AM) plants exposed to three different mercury (Hg) levels (Hg0, Hg25, Hg50). Asterisks denote the significance level (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$)

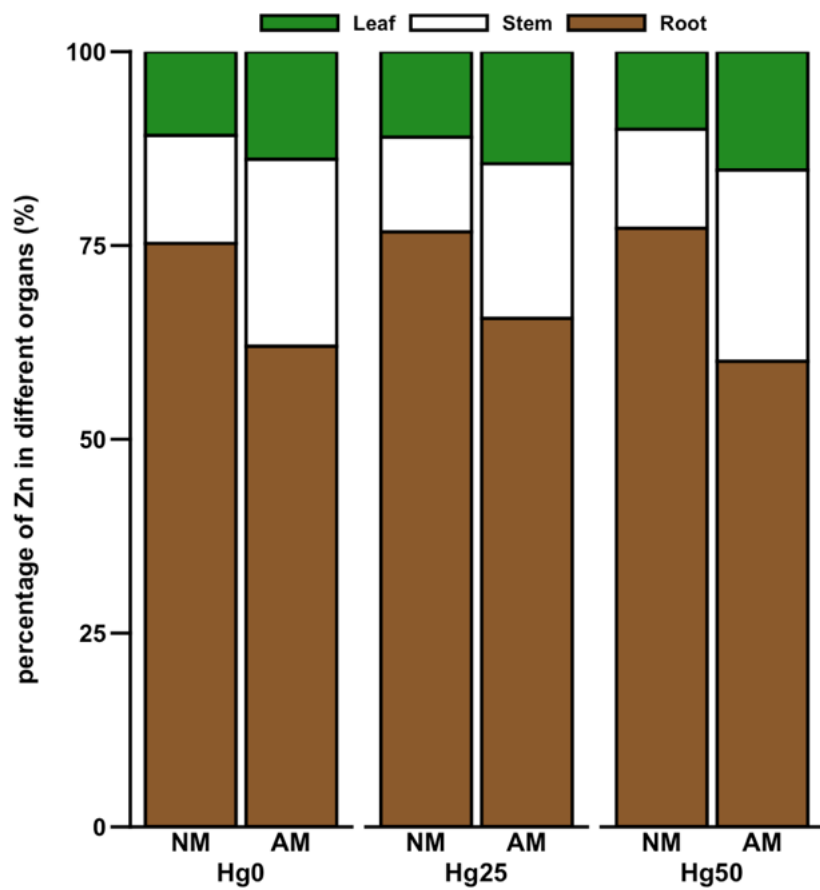
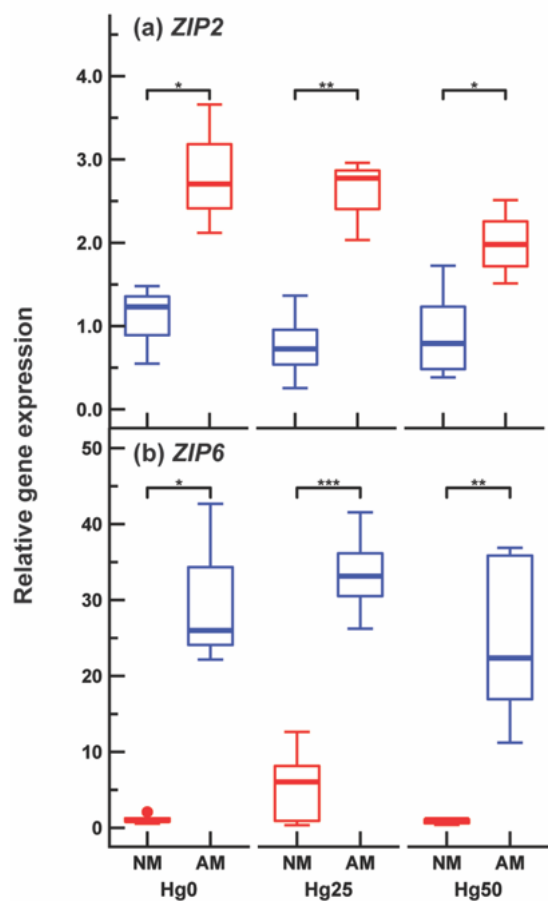


Figure 4

Percentage allocation of zinc (Zn) concentration in different organs of non-mycorrhizal (NM and mycorrhizal (AM) plants exposed to three different mercury (Hg) levels (Hg0, Hg25, Hg50)



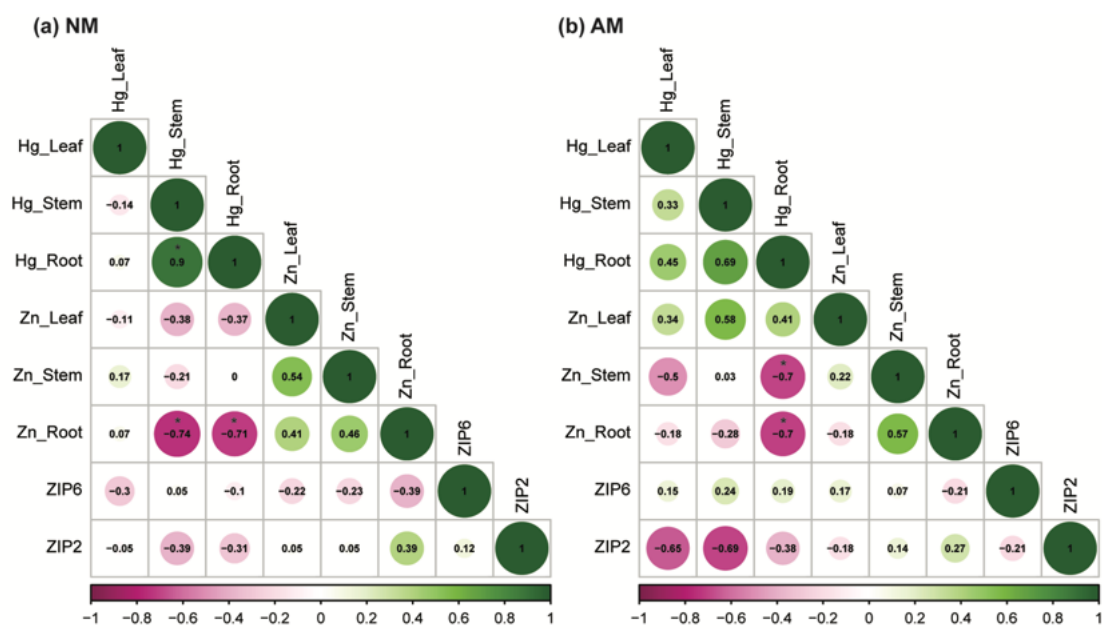


Figure 6

Correlations among measured parameters in non-mycorrhizal (NM) and mycorrhizal (AM) treatments. Numbers show the correlation coefficient. Different colors and size of circles denote the strength of correlations. The asterisk (*) indicates a significant correlation ($P < 0.05$)

Supplementary Files

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