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Rapid screening of *Medicago truncatula* germplasm for mercury tolerance at the seedling stage

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ABSTRACT

The development of novel phytoremediation strategies to mitigate mercury contamination, an increasingly important worldwide threat, could be enhanced by identifying Hg-tolerant legume cultivars. A fast method was thus developed to screen a germplasm collection of the model legume *Medicago truncatula* for Hg tolerance, testing 258 accessions at the seedling stage in a miniaturized hydroponic system and using root growth as an indicator of tolerance. To validate the results of the screening, the Hg-tolerant cultivars identified were analyzed in a referenced hydroponic growth assay. The different growth parameters measured in this assay were then subjected to a factorial analysis (principal component analysis method) in order to establish indices of tolerance, which further supported the rapid method of screening. Accordingly, four Hg-tolerant *M. truncatula* cultivars were selected that might serve as source material for genetic improvement, as model cultivars to study Hg tolerance in legumes and/or to develop soil phytoremediation approaches.

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1. Introduction

Mercury is normally present in soil at low concentrations, ranging from 0.01 to 0.5 $\mu g\,g^{-1}$ (Bowen, 1979). However, in addition to natural Hg accumulation, human activities like mining, the application of fertilizers and pesticides, and chlor-alkali processing plants, are leading to severe Hg contamination problems worldwide (Patra and Sharma, 2000). This not only constitutes a major risk to the environment but also to human health. Bioavailable Hg species are readily taken up from the soil and water by different organisms, thus entering the food chain and leading to serious toxicity problems (Peralta-Videa et al., 2009).

Certain plants can tolerate high levels of heavy metals, and even accumulate them in great quantities without significant phytotoxic effects (Baker et al., 1994). Phytoremediation is an approach that attempts to take advantage of these properties by using such plants to clean up polluted soils, representing a cheaper and more environmentally friendly strategy than traditional physical and chemical treatments. Phytoremediation of heavy metals from contaminated soils can be achieved by reducing metal mobility and bioavailability through phytostabilization, involving the absorption, accumulation, adsorption and/or precipitation by roots, and phytoextraction, which involves the accumulation of the metal mainly in the shoots and leaves of tolerant plants (Jabeen et al., 2009; Krämer, 2005; Zhao and McGrath, 2009).

The molecular mechanisms underlying Hg toxicity and tolerance in plants have been widely investigated (Ortega-Villasante et al., 2005, 2007; Shen et al., 2011; Zhou et al., 2007, 2008, 2009, 2012). However, few studies have sought to identify non-transgenic, Hg-tolerant plants that can be efficiently used in Hg-phytoremediation. Some natural Hg-tolerant ecotypes have been described (Freitas et al., 2004; Massa et al., 2010; Molina et al., 2006; Pérez-Sanz et al., 2012), and recently, the legume plants *Medicago polymorpha* and *M. orbicularis* were found in highly Hg-contaminated soils (Nonnoi et al., 2012).

The growth of metal-tolerant pioneer plants can increase the organic matter content and improve metal-degraded soils, an approach that has been proposed as a restoration strategy in polluted areas (Arocena et al., 2010). *Medicago truncatula* is a forage legume that can fix atmospheric nitrogen through its symbiosis with rhizobial bacteria, and since Hg-tolerant rhizobia have been reported in Hg-contaminated soils (Nonnoi et al., 2012; Ruiz-Díez et al., 2012), *M. truncatula* may represent a suitable pioneer plant. Moreover, this species has been widely used to study different aspects of legume biology, and its close genetic relationship with alfalfa (*M. sativa*) makes it an appropriate source of genes for genetic improvement and an ideal model to study tolerance mechanisms in this major legume crop (Choi et al., 2004).

Tolerance to metals is the initial criterion to select plants suitable for effective phytoremediation of metal-contaminated sites, and rapid screening tests that use accurate indicators are

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fundamental. Selected tolerant legumes could grow and colonize Hg-contaminated soils allowing efficient phytoremediation, restoring soil fertility and accelerating ecological succession. Some screening methods for plant tolerance to different heavy metals have already been described (Dos Santos Utmazian et al., 2007; Sledge et al., 2005; Wang et al., 2011; Wieshammer et al., 2007). Here, a miniaturized hydroponic system was developed to identify Hg-tolerant cultivars of *M. truncatula* from a germplasm collection at the seedling stage. The screening method was validated in a referenced hydroponic system, and growth parameters were identified that may serve as valid indicators of Hg-tolerance in *M. truncatula*.

2. Material and methods

2.1. Plant material and germination

Two-hundred and fifty eight accessions of *M. truncatula* were obtained from the National Plant Germplasm System of the United States Department of Agriculture (USDA). The seeds were scarified with sandpaper and sterilized in 50% (v/v) commercial bleach for 15 min, then they were rinsed (4 × 20 min) with sterile water. Seeds were imbibed in sterile water at 4 °C overnight and then germinated on 1% agar/water in Petri dishes in growth chamber conditions (25/19 °C, 16/8 h) for 48 h in darkness.

2.2. Screening for mercury tolerance

Seedlings were transferred to the so-called Fåhraeus slide hydroponic system, as described previously (Heidstra et al., 1994; Ortega-Villasante et al., 2005) with some modifications. The seedlings were placed in holes perforated in a Fompex® sheet that held the aerial part out of the nutrient solution in Schieferdecker glass staining containers (250 mL). The bottom of the glass container was covered with aluminum foil and the seedlings were acclimatized for 24 h prior to Hg treatment.

A modified Hoagland nutrient solution was used (2.02 g/L KNO_3, 0.68 g/L KH_2PO_4, 0.182 g/L $CaCl_2{\cdot}2H_2O$, 0.615 g/L $MgSO_4{\cdot}7H_2O$, 0.109 g/L K_2SO_4, 0.205 g/L Hampiron: Rhône Poulenc), and 1.35 mL of a solution containing: 11 g/L H_3BO_3, 6.2 g/L $MnSO_4{\cdot}H_2O$, 10 g/L KCl, 1 g/L $ZnSO_4{\cdot}7H_2O$, 1 g/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.5 g/L $CuSO_4{\cdot}5H_2O$ and 0.5 mL/L H_2SO_4.

For the screening assay, seedlings of Hg-sensitive *M. truncatula* cv. Parabinga were exposed to different Hg concentrations (0–7 μM $HgCl_2$) for different times (48, 72 and 96 h) under growth chamber conditions (180 μmol photon m^{-2} s^{-1}, 25/19 °C, 16/8 h photoperiod, 65% relative humidity). The relative root growth (RRG) of the seedlings was used as an indicator of metal tolerance, measuring the root length of the seedlings before and after treatment (Δ root $length_{Hg}$), and expressing the increase in length relative to that of control seedlings grown in the absence of Hg (Δ root $length_C$):

$$RRG = \left(\frac{\Delta\ \text{root length}_{Hg}}{\Delta\ \text{root length}_{C}}\right) \times 100$$

In each condition (0 and 4 μM $HgCl_2$, 48 h) 10–30 seedlings were analyzed per variety.

2.3. Screening validation

For validation, seedlings were placed in growth pouches (CYG Seed Germination Pouches, Mega International, Minneapolis) (Shvaleva et al., 2010) containing 50 ml of nutrient solution (5 seedlings per pouch), and they were acclimatized for 24 h prior to exposure to Hg. To set up the experimental conditions for the validation assay, *M. truncatula* cv. Parabinga was used. Several Hg concentrations (0–40 μM $HgCl_2$) were assayed under growth chamber conditions (180 μmol photon m^{-2} s^{-1}, 25/19 °C 16/8 h photoperiod, 65% relative humidity). In subsequent experiments, seedlings were exposed to 20 μM $HgCl_2$ for 12 days, analyzing 5–10 pouches, each containing five plants, per variety and treatment. The number of leaves, the root length, and the root and shoot fresh and dry weights were measured for control plants and those exposed to Hg, and the relative parameters (RX) were calculated as follows:

$$RX = \left(\frac{\text{Parameter } X_{Hg}}{\text{Parameter } X_{C}}\right) \times 100$$

2.4. Mercury analysis

Mercury concentration was measured according to Millán et al. (2006) and Sierra et al. (2012) using an atomic absorption spectrometer specifically designed for Hg determination (Advanced Mercury Analyser -AMA254- LECO Company). This equipment directly measures solid samples without a need of sample chemical pre-treatment and with a detection limit of 0.01 ng of Hg. Certified reference materials were used to determine the accuracy and precision of the measurements (CRM) and validate the applied methods. These reference materials were CLAY-2 Number CRM051-050 (Clay soil, 4.080 ± 0.099 mg kg^{-1}) and CRM 051 (soil from USA contaminated area, 29.90 ± 5.96 mg kg^{-1}). The mean value determined for 10 measurements using the AMA-254 equipment was 4.166 ± 0.096 mg kg^{-1}; and 29.53 ± 0.48 mg kg^{-1}, respectively. At a 95% confidence level, no significant differences were detected between the certified value and the experimental one, so this method was considered to be accurate for total Hg determination. When samples showed a high Hg content, out of equipment range limit (>600 ng), they were pre-processed by an acidic digestion following the EPA 3052 method in an ETHOS 1 microwave (Milestone SRL). Recovery percentage for certified reference material (CRM 051) was 99%.

2.5. Statistical analyses and tolerance index

All statistical analyses were performed using IBM SPSS Statistics 20 software (SPSS Inc., Chicago, IL, USA). To determine whether the number of cultivars tested was appropriate, the frequency distribution of relative root growth was determined. The root lengths determined in the screening assay were analyzed by ANOVA ($p < 0.05$; $n \geq 10$), and cultivars that displayed no change or a significant increase in growth following exposure to Hg in comparison to control plants were considered potentially Hg-tolerant. The relative parameters and the root:shoot ratios obtained from the validation assay were also analyzed by ANOVA ($p < 0.05$) and Fisher LSD tests were applied for pair-wise comparison of the relative parameters between cultivars. An analysis of variance was performed using the MANOVA ($p < 0.05$) procedure, considering the different accessions as replicates of potentially tolerant or sensitive plants.

A factorial analysis using a principal component analysis method (PCA) was carried out for each of the different relative parameters to identify the major components of variability. The components identified (PC1 and PC2) were represented in a scatter plot to analyze associations. A tolerance index was calculated for each cultivar using the following equation;

$$TI = \left[\frac{\sum(RX \times LVX_{PC1})}{100}\right] \times \%Var_{PC1} + \left[\frac{\sum(RX \times LVX_{PC2})}{100}\right] \times \%Var_{PC2}$$

in which RX represents the relative parameter *X*, LVX_{PC} the loading value for RX in one PC, and $\%Var_{PC}$ the percentage of the total variance explained by that PC. The normalized tolerance index (*n*TI)

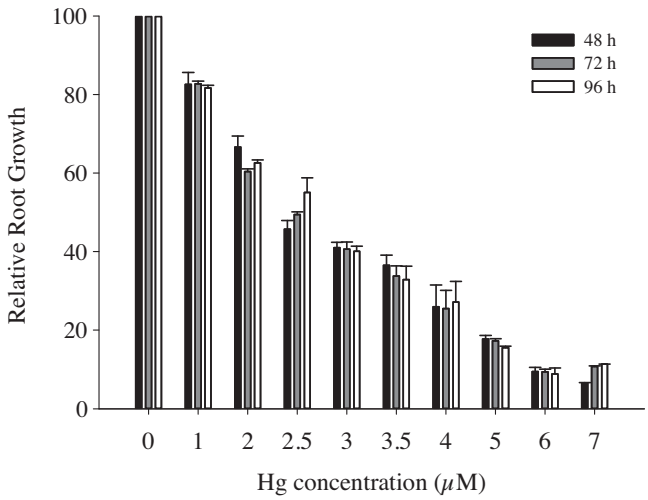

Fig. 1. Relative root growth of *M. truncatula* cv Parabinga seedlings exposed to different Hg concentrations for varying times. Mean values are shown (n = 10–30) and the bars indicate the standard errors.

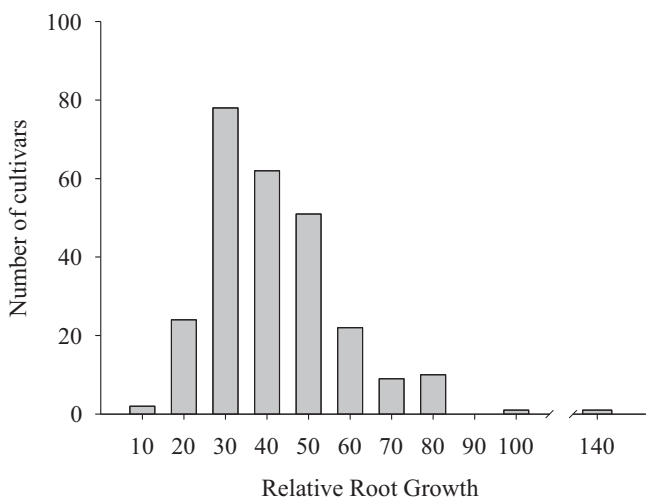

Fig. 2. Frequency distribution of the relative root growth for *M. truncatula* seedlings from 258 cultivars.

was calculated by dividing by the maximum TI obtained for the cultivars analyzed.

3. Results

3.1. Screening seedlings for mercury tolerance

The appropriate metal concentration and length of exposure in screening assays were determined based on the root growth response of Hg-sensitive *M. truncatula* cv. Parabinga. Exposure to between 0 and 7 μM $HgCl_2$ for 48, 72 and 96 h was assayed (Fig. 1) and mercury had a detrimental effect on seedling root growth that augmented as the concentration of $HgCl_2$ increased. At the selected concentration (4 μM $HgCl_2$) and length of exposure (48 h), mercury exerted a clear effect on this cultivar without completely arresting root growth (RRG = 30%).

Seedlings of 258 cultivars obtained from the National Plant Germplasm System, USDA, were grown for 48 h in the presence or absence of Hg (4 μM) and root growth was measured before and after exposure, calculating the RRG for each cultivar (Supplementary Table 1). The results of the screening followed a normal frequency distribution (Fig. 2), indicating that the assay conditions

Table 1
The relative root growth (RRG) and the increase in root length in the presence or absence of Hg for six *M. truncatula* cultivars.

Cultivar	RRG	Δ root length (cm)	
		Hg	Control
W6 6019	141.7*	1.70 ± 0.23	1.20 ± 0.11
W6 6107	100.0	1.60 ± 0.31	1.60 ± 0.23
W6 5992	79.4	1.80 ± 0.30	2.26 ± 0.60
W6 6023	77.9	1.32 ± 0.20	1.70 ± 0.50
W6 6020	65.3	1.06 ± 0.32	1.63 ± 0.15
W6 6042	58.1	0.50 ± 0.08	0.86 ± 0.37

Standard deviations are indicated.
* Significant differences between treatments (one-way ANOVA, $p \leq 0.05$, $n \geq 10$).

and the number of cultivars tested were adequate. Most cultivars displayed poor RRG, indicating strong sensitivity to Hg, with the RRG values ranging from 10% to 50% in 217 cultivars, with values above 75% in 6 cultivars, and with only two accessions exhibiting RRG values ≥100% (Fig. 2).

An analysis of variance (ANOVA; $p < 0.05$; $n \geq 10$) of the increase in root length for each *M. truncatula* accession revealed that there were no significant differences in root growth in the presence or absence of Hg in five cultivars, and one cultivar, W6 6019, exhibited significantly greater root growth in the presence of Hg, with a RRG value of 141.7% (Table 1).

A correlation analysis was performed comparing the results obtained here with those reported previously on aluminum tolerance for the same cultivars (Sledge et al., 2005). No correlation was found between aluminum and mercury tolerances (data not shown).

3.2. Screening validation

To determine whether the miniaturized hydroponic system was an appropriate method to screen for Hg tolerance in *M. truncatula*, the results were validated using another hydroponic system in growth pouches. *M. truncatula* cv. Parabinga was used to assay the growth in this system. Several Hg concentrations were tested (0–40 μM $HgCl_2$) and as expected, the metal affected all growth parameters measured in a dose and time dependent manner (data not shown). To carry out the assays, a Hg concentration of 20 μM was selected as an appropriate concentration with which to treat plants for 12 days, non-lethal conditions that produced substantial differences in all growth parameters.

To validate the screening results, the four potentially Hg-tolerant cultivars with the highest significant RRG values were selected (Table 1), as well as four apparently Hg-sensitive cultivars with RRG values ranging from 16% to 50% (Supplementary Table 1). The morphological parameters of these cultivars were measured (number of leaves, root length, root and shoot fresh and dry weights) and the relative values were calculated to exclude the possibility of genetic variability among different cultivars. For three of the sensitive cultivars, the relative number of leaves (RNL) was significantly lower following exposure to Hg, and this parameter was significantly higher for one tolerant accession (W6 6019), which produced twice the number of leaves in conditions of Hg stress (Table 2). All the tolerant cultivars had RNL values ≥100%, and pair-wise comparisons revealed that these values were significantly higher than those of the sensitive cultivars, for which RNL values remained below 85%. RRG was significantly lower for all cultivars in the presence of Hg than in control conditions, except for the sensitive cultivar W6 6142 for which the differences in the presence or absence of Hg were not significant. In terms of the relative root fresh weights (RRFW), Hg stress failed to produce a significant difference in one sensitive cultivar and three of the tolerant cultivars, although the three tolerant cultivars had

Table 2
Relative parameters of tolerant and sensitive cultivars grown in hydroponic pouches in the presence or absence of Hg.

	Cultivar	RNL	RRG	RRFW	RRDW	RSFW	RSDW
Tolerant	W6 6019	200.00 ± 0.00* a	77.60 ± 5.65* b	112.13 ± 3.45 a	106.84 ± 4.53 a	111.11 ± 4.04 ab	116.40 ± 4.54 ab
	W6 6107	100.00 ± 0.00 b	73.94 ± 2.46* b	94.28 ± 12.15 a	122.29 ± 1.92 a	130.95 ± 6.87* a	130.10 ± 5.06 a
	W6 5992	100.00 ± 0.00 b	68.87 ± 3.34* b	99.30 ± 2.69 a	114.48 ± 1.87 + a	103.15 ± 3.92 ab	114.93 ± 2.02* ab
	W6 6023	102.50 ± 2.50 b	73.10 ± 1.57* b	76.52 ± 1.75* b	102.06 ± 4.64 a	119.55 ± 4.55* ab	127.82 ± 4.41* a
Sensitive	PI 516950	84.54 ± 2.73c	76.39 ± 6.53* b	91.04 ± 2.32 b	95.34 ± 1.38 b	94.27 ± 3.55 c	102.92 ± 4.57 ab
	W6 6142	80.64 ± 4.03* c	93.61 ± 3.86 a	70.55 ± 3.28* bc	60.33 ± 3.96* c	75.82 ± 2.14* d	81.36 ± 5.94 b
	PI 384634	70.08 ± 3.42* d	51.98 ± 8.73* c	68.11 ± 7.46* c	95.21 ± 2.43 b	80.12 ± 3.00 cd	84.20 ± 0.81 b
	PI 199257	74.07 ± 0.93* cd	40.31 ± 1.62* d	36.93 ± 4.37* d	75.56 ± 11.80 c	77.28 ± 4.35* d	84.52 ± 7.99 b

RNL, relative number of leaves; RRG, relative root growth; RRFW, relative root fresh weight; RRDW, relative root dry weight; RSFW, relative shoot fresh weight; RSDW, relative shoot dry weight.
Standard errors are indicated.
The different letters indicate significant differences between cultivars for each parameter (LSD test, $p < 0.05$).
* Significant differences between treatments for one cultivar (one-way ANOVA, $p < 0.05$, $n = 5–10$).

Table 3
Mercury accumulation in roots and shoots of *M. truncatula* tolerant and sensitive cultivars grown in hydroponic pouches.

	Cultivar	[Hg] (mg kg^{-1})	
		Root	Shoot
Tolerant	W6 6019	155.1 ± 6.5	7.49 ± 0.93
	W6 6107	1355.3 ± 33.3	28.93 ± 2.17
	W6 5992	455.9 ± 11.1	19.63 ± 2.97
	W6 6023	1046.5 ± 8.1	37.11 ± 4.68
Sensitive	PI 516950	1536.8 ± 6.1	128.40 ± 15.19
	W6 6142	1784.0 ± 30.3	124.10 ± 10.48
	PI 384634	987.7 ± 25.9	144.80 ± 22.2
	PI 199257	591.8 ± 7.9	94.75 ± 11.91

Standard deviations are indicated.

significantly higher RRFWs than the sensitive accessions. The relative root dry weight (RRDW) of all four tolerant cultivars was over 100%, significantly higher than that of the sensitive cultivars. Similarly, the relative shoot fresh weight (RSFW) of all tolerant cultivars was above 100%, and pair-wise comparisons revealed that all tolerant cultivars had significantly higher RSFWs than any of the sensitive cultivars. A similar tendency was also observed following pair-wise comparison for relative shoot dry weights (RSDW), which was not significant in all cases. The root:shoot ratios were not significantly affected by Hg stress (data not shown). No significant differences were observed between cultivars, with the exception of tolerant accession W6 5992, which exhibited a higher ratio (0.56) than the rest of the cultivars (0.26–0.40), both in the absence and in the presence of Hg. No other obvious phenotypic or anatomical differences were observed between sensitive and tolerant cultivars.

Hg concentration was analyzed in roots and shoots of treated plants (Table 3). Hg accumulation was higher in roots than in shoots for all cultivars. The potentially tolerant cultivars showed lower Hg concentrations in shoots than the sensitive cultivars. Among the tolerant cultivars W6 6107 and W6 6023 accumulated the highest levels of Hg both in roots and in shoots. W6 6019 was the cultivar with the lowest Hg accumulation in both organs.

To compare the potentially Hg-tolerant and Hg-sensitive cultivars, the data were combined and each cultivar was considered as a replicate within its tolerance category for statistical analyses. This approach allowed to identify common patterns in the response of tolerant and sensitive cultivars, and to select the parameters best suited to assess Hg tolerance (or toxicity). The average relative parameters were all below 90% for the sensitive cultivars, while the RNL, RRDW, RSFW and RSDW values were over 110% for tolerant cultivars, indicating improved plant growth in the presence of Hg. Indeed, all the relative parameters were significantly higher (30–40%) for tolerant versus sensitive cultivars, except for the RRG, for which the increase was not significant (Fig. 3).

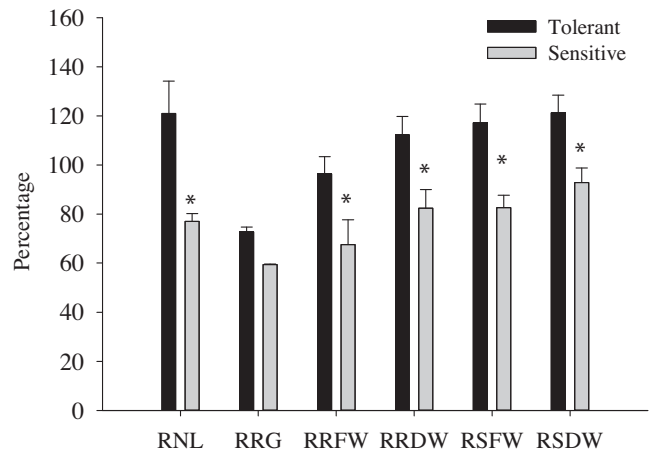

Fig. 3. Average values of the relative parameters for tolerant and sensitive cultivars grown in hydroponic pouches. RNL, relative number of leaves; RRG, relative root growth; RRFW, relative root fresh weight; RRDW, relative root dry weight; RSFW, relative shoot fresh weight; RSDW, relative shoot dry weight. Bars indicate standard errors. The asterisks indicate significant differences between tolerant and sensitive cultivars (MANOVA, $p \leq 0.05$, $n = 5–10$).

3.3. Principal component analysis and tolerance index

A factorial analysis (PCA method) was applied to the different relative parameters to identify those that accounted for the major component of variability (Fig. 4). The PCA revealed two components that accounted for 73.57% of the total variance. On one hand, PC1 accounted for 48.77% of the explained variance, with the following eigenvalues:

$$PC1 = RNL \times 0.918 + RRG \times (-0.068) + RRFW \times 0.809 + RRDW \times 0.204 + RSFW \times 0.801 + RSDW \times 0.860$$

While the second component accounted for 24.80% of the total variance, with the following eigenvalues:

$$PC2 = RNL \times 0.010 + RRG \times (-0.708) + RRFW \times 0.090 + RRDW \times 0.761 + RSFW \times 0.455 + RSDW \times 0.440$$

Two different groups were identified by plotting the components against one another, corresponding to potentially tolerant and sensitive cultivars (Fig. 4). These groups were mainly associated with PC1, and the tolerant cultivars exhibited higher values than the sensitive cultivars for this loading component (Fig. 4). The analysis also identified the relative number of leaves and the relative shoot fresh weight (eigenvalues 0.918 and 0.860, respectively)

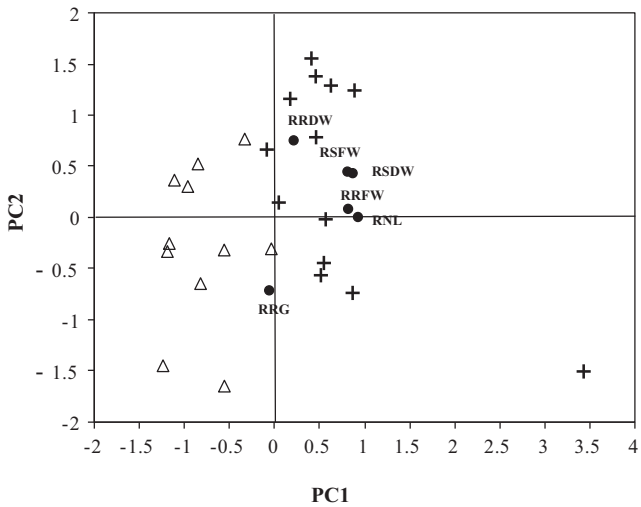

Fig. 4. Scatter plot of the two principal components obtained from PCA that differentiate two groups, (+) tolerant and (Δ) sensitive cultivars ($n = 25$), and the relative parameters according to the eigenvalues (•).

Table 4
Normalized tolerance indices for tolerant and sensitive *M. truncatula* cultivars. The *n*TI was calculated based on all the relative parameters, while the *n*TI-mv included the relative number of leaves and relative shoot fresh weight. The RRG values from the screening are indicated for reference.

	Cultivar	*n*TI	*n*TI-mv	RRG (screening assay)
Tolerant	W6 6019	1.00	1.00	141.7
	W6 6107	0.77	0.72	100.0
	W6 5992	0.72	0.66	79.4
	W6 6023	0.63	0.71	77.9
Sensitive	PI 516950	0.56	0.51	49.5
	W6 6142	0.40	0.38	22.0
	PI 384634	0.52	0.43	19.7
	PI 199257	0.52	0.50	16.1

as the main contributors to the maximum principal component variation.

Using the PCs identified by the PCA, an index of Hg tolerance was generated and normalized to a range of 0–1. A ranking from the most Hg-tolerant to the most sensitive cultivars was obtained using the normalized tolerance index *n*TI (Table 4), which coincided with that of the RRG measurements obtained from the screening of seedlings of tolerant cultivars. This confirmed W6 6019 to be the most Hg-tolerant cultivar of the different accessions tested. While the *n*TI values for tolerant cultivars were higher than 0.60, those calculated for sensitive accessions were lower, although they did not correlate with the RRG values from the initial screening. When the *n*TI values were recalculated using only the RNL and RSDW from PC1, the resulting values were higher for tolerant than for sensitive cultivars. Moreover, although the derived tolerance ranking was not identical to that obtained from the initial screening, the results validated the screening and indicated that high RRG values at the seedling stage appear to predict a good development and high biomass of the aerial part of the plant.

4. Discussion

Legumes are of great interest for the phytoremediation of soils contaminated with heavy metals and they are promising candidates for the successful re-vegetation of ecosystems degraded by mining activities (Dary et al., 2010). Forage legumes present certain advantages, in that they grow rapidly, produce high yields of biomass and are easy to harvest. The aim of this work was to develop a reliable method to analyze a *M. truncatula* germplasm for Hg tolerance in order to rapidly screen a large number of genotypes at the seedling stage using an approach that requires little space and produces minimal waste. Various methods have been described to assess heavy metal tolerance in *Medicago* spp. in soils, hydroponic systems and cell culture (Narasimhamoorthy et al., 2007; Sledge et al., 2002; Zhou et al., 2007). However, the conditions in a hydroponic system can be more carefully controlled and metals are more stable in a nutrient solution than in soils, preventing changes in their concentration. Several different parameters have been used as indicators of metal tolerance in plants, including photosynthetic activity, oxidative stress, nutrient content (Ortega-Villasante et al., 2007; Peralta-Videa et al., 2002, 2004; Yadav, 2010) and in particular, parameters associated with root growth (Bidar et al., 2007; Esteban et al., 2008; Hakmaoui et al., 2006; López et al., 2005; Peralta et al., 2001; Peralta-Videa et al., 2002, 2004; Prasad, 1995; Sledge et al., 2005). Indeed, inhibition of root elongation is one of the most rapid effects induced in conditions of metal stress (Ortega-Villasante et al., 2005; Sabreen and Sugiyama, 2008; Sandalio et al., 2001; Schützendübel et al., 2001).

The present screening indicated that, in general, *M. truncatula* was sensitive to Hg. However, metal tolerance can be detected by screening a sufficient number of cultivars (Sledge et al., 2005; Wang et al., 2011), and some potentially Hg-tolerant cultivars were identified. In order to find common patterns of tolerance to different metals, a correlation analysis was performed comparing the results obtained here with those reported previously for the same cultivars on aluminum tolerance (Sledge et al., 2005). The absence of a significant correlation might indicate that Al and Hg tolerance mechanisms are different and specific, although it does not exclude the possibility of certain common responses.

Tolerance and toxic effects are dependent on the developmental status of the plant (Foy et al., 1978; Peralta-Videa et al., 2004) and thus, to determine whether testing seedlings was appropriate to identify Hg-tolerant cultivars, the screening results were validated in a referenced hydroponic system previously used to study the tolerance of *Medicago* spp. to heavy metals and salinity (Coba de la Peña et al., 2010; Shvaleva et al., 2010; Verdoy et al., 2006). By testing the four cultivars with the most tolerant RRG and four sensitive cultivars, the results of the initial screening were validated. Indeed, all potentially tolerant cultivars presented RNL, RSFW, RSDW and RRDW values above 100%, suggesting that the plants tolerated Hg stress, and that they even grew better in the presence of this heavy metal. All the parameters measured were more significantly altered in the sensitive cultivars than in the potentially tolerant cultivars, corroborating the results of the initial screening. Interestingly, the RRG values did not differ significantly between the tolerant and sensitive cultivars. Parameters related to shoot growth are considered more appropriate to define metal tolerance in mature plants (Dos Santos Utmazian et al., 2007; Nihei et al., 2005; Shiyab et al., 2009), and correlations have been reported between shoot growth and metal tolerance (Sabreen and Sugiyama, 2008).

The power of root growth as a tolerance indicator was reduced in the pouch system assay, as this parameter was more affected than others by Hg stress, and the differences between tolerant and sensitive cultivars were less significant. However, RRG was confirmed as a valid parameter to assess tolerance at the seedling stage, as the cultivars with the highest Hg tolerance according to this parameter displayed good aerial development in the growth pouch assay.

To further validate the screening method, a factorial analysis (PCA method) was performed that supported the validity of this method by identifying two distinct groups of cultivars, those that were potentially tolerant or sensitive to Hg stress. Efforts have been made to find good indicators and indices of metal tolerance. Root and shoot length have been used to generate two different tolerance indices for copper and cadmium (Hakmaoui et al., 2006), while

tolerance indices were developed for cadmium and zinc based on root and leaf biomass production (Dos Santos Utmazian et al., 2007). To categorize tolerance, an index based on the components obtained from the PCA was developed. The calculated index separated tolerant from sensitive cultivars, and generated the same tolerance ranking as that obtained from the initial screening for tolerant cultivars. This was not the case for sensitive cultivars, probably due to experimental error resulting from the limited root growth of the seedlings from sensitive cultivars. Only slight variations were observed when only the main variables were used (relative number of leaves and relative shoot dry weight), suggesting that those two parameters constitute good indicators of Hg tolerance in the hydroponic pouch assay. The validation of these results and the classification obtained with the tolerance indices support the suitability of screening methods based on RRG values at the seedling stage.

Mercury accumulation was analyzed in roots and shoots of tolerant and sensitive cultivars, and diverse Hg levels were found, which did not correlate with Hg tolerance. This result was not unexpected, as metal tolerance and metal hyperaccumulation are genetically independent traits (Maestri et al., 2010). Nonetheless, Hg concentrations in shoots of tolerant cultivars were consistently lower than those found in shoots of sensitive cultivars. Some of the tolerant cultivars could find application in Hg phytostabilization and be used to limit spread of the contaminant in affected soils.

The findings demonstrate that screening in a miniaturized hydroponic system is a suitable method to identify Hg-tolerant *M. truncatula* cultivars at the seedling stage, using RRG as an indicator of metal tolerance. This method did allow a rapid screening of a large number of accessions, providing results that were confirmed in a growth pouch validation assay. A tolerance index calculated based on the PCA results corroborated the interim classification (tolerant or sensitive). The parameters RNL and RSDW were the best indicators of Hg tolerance in the validation assay and did allow easy selection of tolerant cultivars. Further studies will be required to determine whether the selected tolerant cultivars can be used to define the mechanisms of Hg tolerance in *M. truncatula*, as source material for genetic improvement, or for use in Hg phytoremediation strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2013.03.004.

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