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RESEARCH ARTICLE

Plant response to lead in the presence or absence EDTA in two sunflower genotypes (cultivated *H. annuus* cv. 1114 and interspecific line *H. annuus*×*H. argophyllus*)

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Abstract The aim of the present work was to study the response of two sunflower genotypes (cultivated sunflower *Helianthus annuus* cv. 1114 and newly developed genotype *H. annuus*×*Helianthus argophyllus*) to Pb medium-term stress and the role of exogenously applied EDTA in alleviating Pb toxicity in hydroponics. Plant growth, morpho-anatomical characteristics of the leaf tissues, electrolyte leakage, total antioxidant activity, free radical scavenging capacity, total flavonoid content, and superoxide dismutase isoenzyme profile were studied by conventional methods. Differential responses of both genotypes to Pb supplied in the nutrient solution were recorded. Pb treatment induced a decrease in the relative growth rate, disturbance of plasma membrane integrity, and changes in the morpho-anatomical characteristics of the leaf tissues and in the antioxidant capacity, which were more pronounced in the cultivated sunflower *H. annuus* cv. 1114. The new genotype demonstrated higher tolerance to Pb when compared with the cultivar. This was mainly due to increased photosynthetically active area, maintenance of plasma membrane integrity, permanently high total antioxidant activity, and free radical scavenging capacity as well as total flavonoid content. The addition of EDTA into the nutrient solution led to limitation of the negative impact of Pb ions on the above parameters in both genotypes. This could be related to the reduced content of Pb in the roots, stems, and leaves, suggesting that the presence of EDTA limited the uptake of Pb. The comparative analysis of the responses to Pb treatment showed that the deleterious effect of Pb was more pronounced in the cultivated sunflower *H. annuus* cv. 1114. The new genotype *H. annuus*×*H. argophyllus* was more productive and demonstrated higher tolerance to Pb medium-term stress, which could indicate that it may possess certain mechanisms to tolerate high Pb concentrations. This character could be inherited from the wild parent used in the interspecific hybridization. The ability of EDTA to prevent Pb absorption by the plants could underly the mechanism of limiting of the negative impact of Pb ions. Hence, EDTA cannot be used to enhance Pb absorption from nutrient solution by sunflower plants for phytoremediation purposes.

Keywords Antioxidant activity · EDTA · Lead (Pb) toxicity · Leaf anatomy · Relative growth rate (RGR) · Sunflower

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Introduction

Lead (Pb) pollution of soil has been one of the global environmental concerns over the past decades. Sources of Pb contamination include Pb mines, battery manufacture, the burning of leaded fuels, and application of sludge to agricultural land, leading to an accelerated release of Pb into the environment, which is a serious threat to the health of

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children and wildlife (EPA 2005). Pb is present in soils in unavailable forms for plant uptake due to its strong binding affinity with soil constituents and precipitation as carbonates, hydroxides, and phosphates (McBride 1994).

In order to increase Pb bioavailability in soils and facilitate the phytoextraction of the metal from contaminated sites and the translocation from root to shoot, application of EDTA has been proposed (Komárek et al. 2007). Its effectiveness is based on the ability to solubilize soil-bound Pb for root uptake due to its high chemical affinity for Pb (log *K*s=17.88) (Martell et al. 2001). Plants are capable of transporting, accumulating, and translocating Pb as an EDTA–Pb complex from the medium to the above-ground plant parts (Tian et al. 2011). Non-selective apoplastic root uptake of EDTA–Pb complexes (Hernández-Allica et al. 2007) and their translocation via the xylem vessels to the shoot has been reported (Vassil et al. 1998; Saifullah et al. 2009). EDTA-enhanced uptake and root-to-shoot translocation of Pb have been observed in many plants (Blaylock et al. 1997; Huang et al. 2008; Ruley et al. 2006).

Inconsistency in the literature exists on the role of EDTA in Pb uptake and accumulation, which may be attributed to different experimental conditions. When plants were grown on soil, EDTA is suggested to increase the desorption of Pb from the soil matrix to soil solution (Ruley et al. 2006; Liu et al. 2007), thus enhancing its uptake by plants. However, there are data showing that when plants are cultivated in EDTA-containing nutrient solution, EDTA chelates Pb and decreases the effectiveness of roots in taking up the EDTA–Pb complexes from the solution, thus reducing the uptake of Pb by the roots (De la Rosa et al. 2007; Huang et al. 2008). It remains still unclear whether the EDTA–metal complexes are directly absorbed by the roots or a dissociation of the complex occurs prior to its uptake (Saifullah et al. 2009).

Free protonated EDTA may lead to phytotoxicity and pose potential risks to the environment (Kim et al. 2003). As shoot Pb accumulation has been shown to peak at equimolar ratios of EDTA to Pb in solution without any phytotoxicity symptoms (Hernández-Allica et al. 2007), EDTA and Pb should be supplied at equimolar concentrations to the nutrient solution, thus ensuring the formation of 100 % EDTA–Pb complex (Sarret et al. 2001).

It has been reported that cultivated sunflower (*Helianthus annuus* L.) is a high biomass-producing oil crop, which can be used as a bioindicator of environmental pollution with Pb ions (Krystofova et al. 2009) and can accumulate significant amounts of Pb when applied in combination with EDTA to Pb-contaminated soils (Sinegani and Khalilikhah 2008).

In the present study, cultivated sunflower *H. annuus* cv. 1114 and the new genotype *H. annuus*×*Helianthus argophyllus* grown in a hydroponic system were used in order to elucidate the effects of ionic Pb and chelated Pb (EDTA–Pb) on plant growth, uptake of Pb, Ca, and Fe, major leaf anatomical characteristics, electrolyte leakage, and antioxidant defense system.

Materials and methods

Plant material and growth conditions

Seeds of sunflower plants (cultivated *H. annuus* cv. 1114 and interspecific line *H. annuus*×*H. argophyllus*) were germinated on wet filter paper. The line was obtained by interspecific hybridization between cytoplasmic male sterile *H. annuus* cv. 1114 ($2n=2x=34$) and wild annual silver-leaf sunflower *H. argophyllus* Torr. & Gray ($2n=2x=34$) (Vassilevska-Ivanova and Tcekova 2005). The 5-day-old seedlings were transferred to 1,200-ml pots containing aerated nutrient solution in a greenhouse under natural light. The basic nutrient solution contained (μM): 200 $Ca(NO_3)_2{\cdot}4H_2O$, 100 $MgSO_4{\cdot}7H_2O$, 400 KNO_3, 300 NH_4NO_3, 10 Fe–EDTA, 5 $NaH_2PO_4{\cdot}H_2O$, 8 H_3BO_3, 5 $MnSO_4{\cdot}H_2O$, 0.16 $CuSO_4{\cdot}5H_2O$, 0.38 $ZnSO_4{\cdot}7H_2O$, 0.06 $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$ (pH4.3). $NaH_2PO_4{\cdot}H_2O$ concentration was maintained at a minimum level to prevent precipitation of Pb. Part of the plants received this nutrient solution (control plants), while the rest were exposed to a solution supplemented with 0.1 mM Pb in the presence or absence of an equimolar concentration of EDTA (0.1 mM). The sources of Pb and EDTA were Pb $(NO_3)_2$ and Na_2EDTA, respectively. Plants were harvested for analysis 10 days after treatment. Four plants from the two cultivars were included in each experimental group (control, Pb treated, and EDTA–Pb treated). The whole experiment was repeated three times independently.

Plant growth analysis

Growth was assessed by the relative growth rate (RGR) measured as the increase in the fresh weight of roots, stems, and leaves at day 10 after the onset of treatment. The RGR was calculated according to the formula:

$$\mathrm{RGR} = (\ln\ W_2 - \ln\ W_1)t^{-1},$$

where W_1 and W_2 represent the fresh weights at the beginning and the end of the time interval t (days), respectively.

Elemental analysis

Samples for elemental analyses (roots, stems, and leaves) were dried at 65 °C to a constant weight and ashed at 550 °C. Ash (0.1 g) was dissolved in 20 % HCl. The

concentrations of Pb, Ca, and Fe in the solution were determined using an inductively coupled plasma atomic emission spectrometer (Liberty-II, Varian, Austria). Concentrations of all elements were calculated on a dry weight basis of each plant organ.

Electrolyte leakage analysis

The electrolyte leakage (EL) test was conducted according to Yamori et al. (2005). One gram of tissue was cut into segments (4 cm^2) placed in test tubes containing 15 ml of bidistilled H_2O for 36 h at 25 °C. The conductivity of the supernatant (initial EL−C_1) was measured with an electro-conductivity meter. The tubes were placed in a boiling-water bath for 60 min, and the electrical conductivity was obtained after attaining equilibrium at 25 °C (final EL−C_2). The EL was calculated using the following equation: EL (%)=$C_1/C_2 \times 100$.

Light and scanning electron microscopy

Leaf segments from the middle of the fully developed second leaf (10 mm^2) were processed following the protocol described by Doncheva et al. (2009). Briefly, the samples were fixed in 5 % (*v*/*v*) glutaraldehyde in 0.1 M Na cacodilate buffer (pH7) for 2 h at room temperature and postfixed with 1.3 % (*w*/*v*) OsO_4 in the same buffer. Infiltration and embedding were performed using Durcupan ACM (Fluka, Sigma-Aldrich). Semithin sections (1–2 μm), cut from the Durcupan-embedded material with glass knives, were mounted on glass slides, stained with fuchsin and methylene blue, and examined under a Nikon Eclipse 50 light microscope (Japan). The morphometric analysis of the leaf structures was done by measuring five representative semithin cross-sections, which were recorded by a digital camera attached to the microscope.

Several fixed and dehydrated samples were critical-point dried with CO_2, coated with 15 nm thin gold, and examined with a JEOL JSM 35 scanning electron microscope (Japan). The stomatal aperture index (width/length ratio) was calculated by measuring 90 stomatal pores for each treatment.

Data on morphometric analysis and stomatal aperture measurements were obtained using ImageJ software (http://rsb.info.nih.gov/ij/).

Native PAGE analysis and detection of SOD isozymes

Soluble proteins were extracted from finely ground leaf material with 0.1 M K-phosphate buffer (pH7.8) and 0.05 M Tris–HCI buffer (pH7.2) containing 6 mM cystein hydrochloride, 6 mM ascorbic acid, and 0.5 M sucrose. The crude extracts were centrifuged at 15,000×*g* for 20 min at 4 °C, and the supernatants were used for electrophoretic analyses. Isoforms of superoxide dismutase (SOD) were separated by native polyacrylamide gel electrophoresis (PAGE) using 7.5 % polyacrylamide gel according to Davis (1964). Equal amounts of protein (50 μg) were loaded on each lane. SOD activity was localized by photochemical nitro blue tetrazolium staining (Harris and Hopkinson 1976). The three types of SOD (Mn-, Cu/Zn -, and Fe-SOD) were identified by a selective inhibition with 5 mM KCN and 5 mM H_2O_2 (Bridges and Salin 1981). The activities of SOD isoforms were quantified by converting the stained area and intensity into relative units by gel scanning using ImageJ software (http://rsb.info.nih.gov/ij/).

Preparation of leaf ethanol extracts

Finely ground leaf material (1.0 g) was extracted with 80 % ethanol. The homogenate was centrifuged at 12,000×*g* for 30 min at 0–4 °C, and the supernatant was used for analyses of antioxidant activity, free radical scavenging capacity, and total flavonoid content.

Free radical scavenging activity assay

The free radical scavenging activity was measured according to Brand-Williams et al. (1995). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was used as the source of free radicals. Briefly, a freshly prepared DPPH reagent (1.99 ml) and extract solution (0.01 ml) were mixed, and the absorbance was measured at 515 nm. For the blank, methanol was used. The free radical scavenging activity was calculated from a standard curve made with known concentrations of Trolox.

Ferric-reducing antioxidant power assay

Total antioxidant activity was investigated using the ferric-reducing antioxidant power (FRAP) assay according to Benzie and Strain (1999). This procedure involved the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) in a blue colored complex in the presence of bioactive compounds (antioxidants). Briefly, freshly prepared FRAP reagent and extract solution (0.05 ml) were mixed, and the absorbance was measured at 593 nm using FRAP solution as a blank. The antioxidant potential of samples was calculated from a standard curve plotted using known concentrations of $FeSO_4 \cdot 7H_2O$.

Determination of total flavonoid content

Total flavonoid content was determined using the method of Lamaison and Carnet (1990) with modifications. An aliquot (1 ml) of the extract was mixed with the same volume of 2 % $AlCl_3 \cdot 6H_2O$ (*w*/*v*). The absorbance was measured at 430 nm against 80 % ethanol as a blank. Total flavonoid

content was determined using a standard curve made with known concentrations of rutine.

Statistical analysis

Statistical analysis of data was made using IBM SPSS Software, version 19. Treatments (control, Pb, and EDTA–Pb) and cultivars (cultivated *H. annuus* cv. 1114 and the interspecific line *H. annuus*×*H. argophyllus*) were used as independent factors in a series of two-way ANOVA. The dependent variables were all measured quantitative parameters. Data are presented as means with SE obtained from the number *n* of repeated measurements varying for the different quantitative parameters. The means were compared by Waller–Duncan test and were considered different at $P \leq 0.05$.

Results

Plant growth

Treatment with 0.1 mM Pb decreased the growth rate of the sunflower plants tested (Fig. 1). RGR of the leaves, stems, and roots of *H. annuus* L. cv. 1114 decreased by 17, 30, and 52 %, respectively, compared with the controls. Treatment with Pb reduced RGR to a lesser extent in the new genotype *H. annuus*×*H. argophyllus*, the differences being not significant. RGR of the stems and roots of the cultivated *H. annuus* L. cv. 1114 was significantly increased after the addition of EDTA as compared with Pb treatment alone ($P \leq 0.01$).

Concentration of Pb, Ca, and Fe

In general, the exposure of the sunflower plants to Pb medium-term treatment led to higher concentrations of Pb in the roots than in the above-ground parts of both the cultivar and the interspecific line (Fig. 2a, d). The highest Pb concentration was measured in the roots of the interspecific line. The presence of EDTA in the nutrient solution resulted in a significant reduction of Pb concentrations in the plant organs of both the cultivar and the interspecific line ($P \leq 0.001$).

Our results showed that Pb treatment affected the uptake of Ca (Fig. 2b, e). Ca concentrations in the leaves and stems of *H. annuus* L. cv. 1114 were decreased due to Pb treatment by 25 and 38 %, respectively, with a significant difference ($P \leq 0.05$), while in the roots, it remained unchanged compared to the control (Fig. 2b). The addition of EDTA increased the content of Ca in the leaves and stems compared to Pb treatment alone (Fig. 2b). However, Ca concentrations in the plant organs of the new genotype *H. annuus*×*H. agrophyllus* remained almost unchanged in the leaves and stems during both treatments (Pb and EDTA–Pb), whereas in the roots, the concentration of Ca was decreased only upon Pb treatment (Fig. 2e).

The concentrations of Fe in the leaves, stems, and roots of the cultivar were reduced after Pb treatment ($P \leq 0.01$) (Fig. 2c). The addition of EDTA lowered further Fe concentrations in the cultivar, the highest decrease being observed in the roots (Fig. 2c). In the interspecific line, treatment with Pb reduced the concentration of Fe in the stems ($P \leq 0.01$) (Fig. 2f). The addition of EDTA decreased the concentration of Fe in the roots compared to Pb treatment alone ($P \leq 0.001$).

Leaf anatomy

A comparative analysis of the effects of Pb levels on the morpho-anatomical parameters of the leaves was further performed (Table 1). Pb treatment led to a significant decrease in leaf thickness, the upper and lower epidermis, mesophyll thickness, as well as the area per palisade and spongy cells in *H. annuus* cv. 1114 ($P \leq 0.05$), whereas in the interspecific line, the mesophyll thickness was increased, thus leading to thicker leaves ($P \leq 0.05$). The area per palisade mesophyll cell was also enhanced (Table 1). The addition of EDTA restored the morpho-anatomical parameters in the interspecific line and alleviated the negative Pb effect on the leaf anatomy of the cultivar.

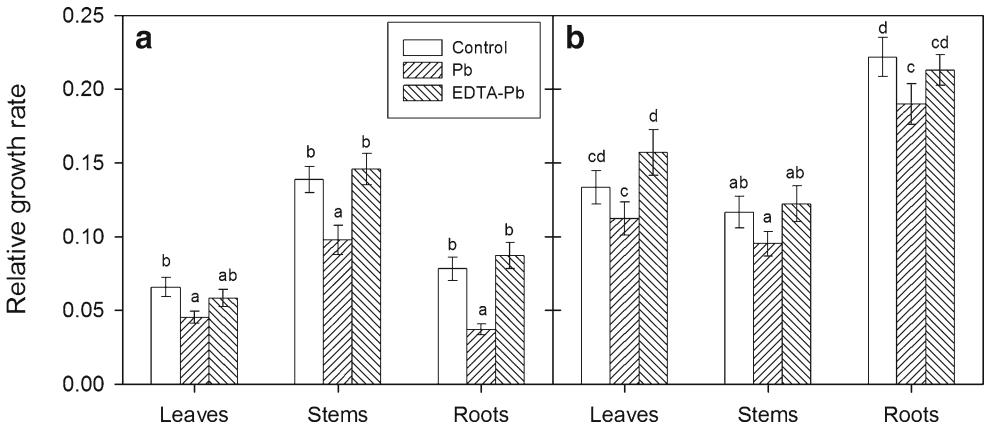

Fig. 1 Relative growth rate (RGR) of the cultivated *Helianthus annuus* cv. 1114 (**a**) and the interspecific line *Helianthus annuus*×*Helianthus argophyllus* (**b**) after treatment with 0.1 mM Pb or 0.1 mM EDTA+0.1 mM Pb for 10 days. Data are means±SE (n=12). *Different letters* indicate significant differences (at $P \leq 0.05$) among treatments and the two sunflower genotypes separately for leaves, stems, and roots

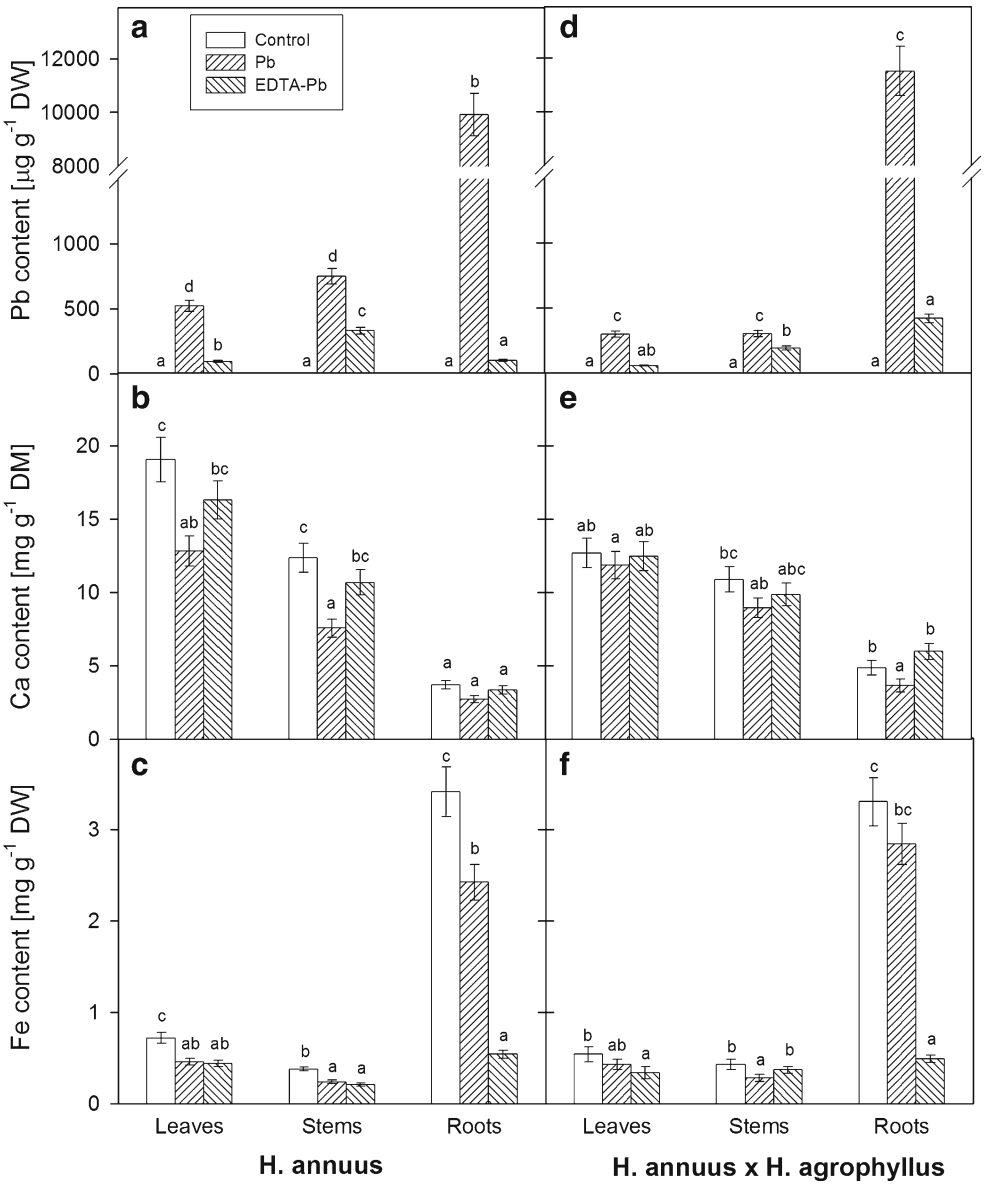

Fig. 2 Total concentration of Pb (**a**, **d**), Ca (**b**, **e**), and Fe (**c**, **f**) in sunflower leaves, roots, and stems of the cultivated *Helianthus annuus* cv. 1114 and the interspecific line *Helianthus annuus*×*Helianthus argophyllus* after treatment with 0.1 mM Pb or 0.1 mM EDTA+ 0.1 mM Pb for 10 days. Data are means±SE (n=12). *Different letters* indicate significant differences (at $P \leq 0.05$) among treatments and the two sunflower genotypes separately for leaves, stems, and roots

The SEM analysis of the abaxial side of the leaves showed that stomata were normal in appearance, and they were located at the level of the rest epidermal cells in the leaves of both the cultivar and the interspecific line (Fig. 3). The average stomatal aperture under control conditions was higher in *H. annuus* cv. 1114 as compared with the new genotype *H. annuus*×*H. argophyllus* (Fig. 4). The stomatal aperture decreased significantly when the cultivated sunflower plants were exposed to Pb treatment ($P \leq 0.001$). In contrast, stomatal aperture remained almost unchanged due to both Pb and EDTA–Pb treatments in the interspecific line. The negative effect of Pb treatment on the stomatal aperture in the leaves of *H. annuus* cv. 1114 was prevented in the presence of EDTA, the increase in stomatal aperture compared to Pb treatment alone being statistically significant ($P \leq 0.001$).

Our results showed long, sharp-pointed, nonglandular trichomes growing on the abaxial surface of the epidermis of control leaves in both the cultivated *H. annuus* cv. 1114 and the new genotype *H. annuus*×*H. argophyllus* (Fig. 3a, d). They were composed of several single, elongated cells arranged in rows and narrowed at the apex. On the surface of some nonglandular trichomes, wall protuberances were visible (Fig. 3d). After exposure to Pb, the abaxial parts of the leaves of both the cultivar and the new genotype were covered by numerous nonglandular trichomes of different length, which were made up of one or several cells (Fig. 3b, e). In addition, the base of the trichomes was swollen. The glandular trichomes were found mostly in Pb-treated plants, and they were composed of stalks and spherical heads (Fig. 3b). The density of trichomes was higher in *H. annuus*

Table 1 Morphometric assessment of leaves (transverse sections) of sunflower plants (cultivated *Helianthus annuus* cv.1114 and the interspecific line *Helianthus annuus*×*Helianthus argophyllus*) after treatment with 0.1 mM Pb and 0.1 mM EDTA+0.1 mM Pb

Parameters Variants	Total leaf thickness (μm)	Mesophyll thickness (μm)	Area per cell PP (μm^2)	Area per cell SP (μm^2)	Upper epidermis height (μm)	Lower epidermis height (μm)
H. annuus cv.1114						
Control	264 ± 8^{a}	213 ± 10^{a}	758 ± 20^{a}	788 ± 14^{a}	32.6 ± 1.3^{c}	16.2 ± 1.9^{bc}
0.1 mM Pb	200 ± 7^{b}	158 ± 11^{b}	497 ± 17^{b}	734 ± 23^{d}	26.8 ± 2.5^{b}	10.7 ± 1.1^{a}
0.1 mM EDTA+0.1 mM Pb	238 ± 9^{ab}	193 ± 9^{ab}	887 ± 42^{c}	733 ± 21^{d}	27.8 ± 3.9^{bc}	17.1 ± 1.0^{bc}
H. annuus×*H. agrophyllus*						
Control	435 ± 13^{c}	391 ± 20^{c}	$1{,}909\pm56^{d}$	$1{,}166\pm31^{c}$	23.6 ± 0.9^{ab}	14.0 ± 1.0^{ab}
0.1 mM Pb	494 ± 14^{d}	449 ± 16^{d}	$2{,}048\pm71^{e}$	973 ± 42^{b}	18.4 ± 0.3^{a}	17.2 ± 1.0^{bc}
0.1 mM EDTA+0.1 mM Pb	429 ± 15^{c}	381 ± 18^{c}	$1{,}999\pm36^{d}$	$1{,}028\pm24^{b}$	22.2 ± 0.5^{ab}	19.9 ± 1.5^{c}

Data are the means±SE (n=12). Different letters indicate significant differences (at $P\leq0.05$) among treatments and the two cultivars for each measured parameter separately

PP palisade parenchyma, *SP* spongy parenchyma

cv.1114 (Fig. 3b). A decrease in the number of trichomes was recorded in EDTA-Pb treated plants (Fig. 3c, f).

Electrolyte leakage

Treatment with Pb increased the electrolyte leakage in the leaves of *H. annuus* cv.1114 compared with the control (8 %), and the difference was significant ($P\leq0.01$). The addition of EDTA induced less electrolyte leakage in the leaves of *H. annuus* cv.1114 than the Pb treatment ($P\leq0.05$). However, no significant changes were noted in the electrolyte leakage of the interspecific line *H. annuus*×*H. argophyllus* under both Pb and EDTA–Pb treatments compared with the control (Fig. 5).

Antioxidant capacity

The antioxidant capacity of the leaves was assessed based on the total antioxidant activity, free-radical scavenging activity, and flavonoid content (Table 2). The total antioxidative capacity in controls was higher in the interspecific line compared with the cultivar. Treatment with Pb led to a statistically significant ($P\leq0.001$) increase in the total antioxidant activity, free-radical scavenging activity, and flavonoid content in the leaves of *H. annuus* cv. 1114. The combined treatment with EDTA–Pb led to a decrease in the total antioxidant capacity in the cultivar with a significant difference ($P\leq0.05$) compared to Pb treatment alone. In contrast, these parameters remained almost unchanged under both Pb treatment alone and the combined treatment with EDTA–Pb in the leaves of the new genotype.

In the leaves of control *H. annuus* cv. 1114 plants, five SOD isoforms were detected using nondenaturing PAGE (Fig. 6a). As judged by their sensitivity towards KCN and H_2O_2, they were identified as Mn-SOD (named Mn-SOD II), Fe-SOD (named Fe-SOD III), and three Cu/Zn-SOD (named Cu/Zn-SOD I, Cu/Zn-SOD II, and Cu/Zn-SOD III isoforms in order of increasing electrophoretic mobility) (Jamal et al. 2007; Fernandez-Ocana et al. 2011). On the other hand, two Cu/Zn-SODs (named Cu/Zn-SOD I and Cu/Zn-SOD III) and Fe-SOD III were identified in the control plants of the new genotype *H. annuus*×*H. argophyllus* (Fig. 6b), which was similar to results obtained by Fambrini et al. (1997) for some hybrid sunflower lines. Treatment with Pb induced two new SOD isoforms (Mn-SOD I and Fe-SOD I) and the disappearance of Fe-SOD III in the leaves of *H. annuus* cv.1114, whereas two new Mn-SOD isoforms (Mn-SOD I and Mn-SOD II) were identified in the profile of the new genotype. The combined EDTA–Pb treatment resulted in changes in the profile of SOD isoforms in *H. annuus* cv.1114 when compared with Pb treatment alone including disappearance of the bands corresponding to Mn-SOD I and Fe-SOD I. In addition, the activity of Fe-SOD III was restored as in the control. In the SOD profile of EDTA–Pb-treated leaves of the interspecific line, the band corresponding to Mn-SOD I was reduced to trace amounts, whereas the rest bands remained unchanged.

Discussion

In the present paper, using the cultivated *H. annuus* cv. 1114 and the newly developed genotype *H. annuus*×*H. argophyllus* grown in a hydroponic system, we assessed plant responses to Pb ions either alone or in the presence of EDTA based on a number of physiological, morphological, and biochemical parameters. We have extended some previous studies (Geebelen et al 2002; De la Rosa et al. 2007; Huang et al. 2008; Tian et al. 2011), indicating that Pb phytotoxicity can be alleviated in the presence of EDTA.

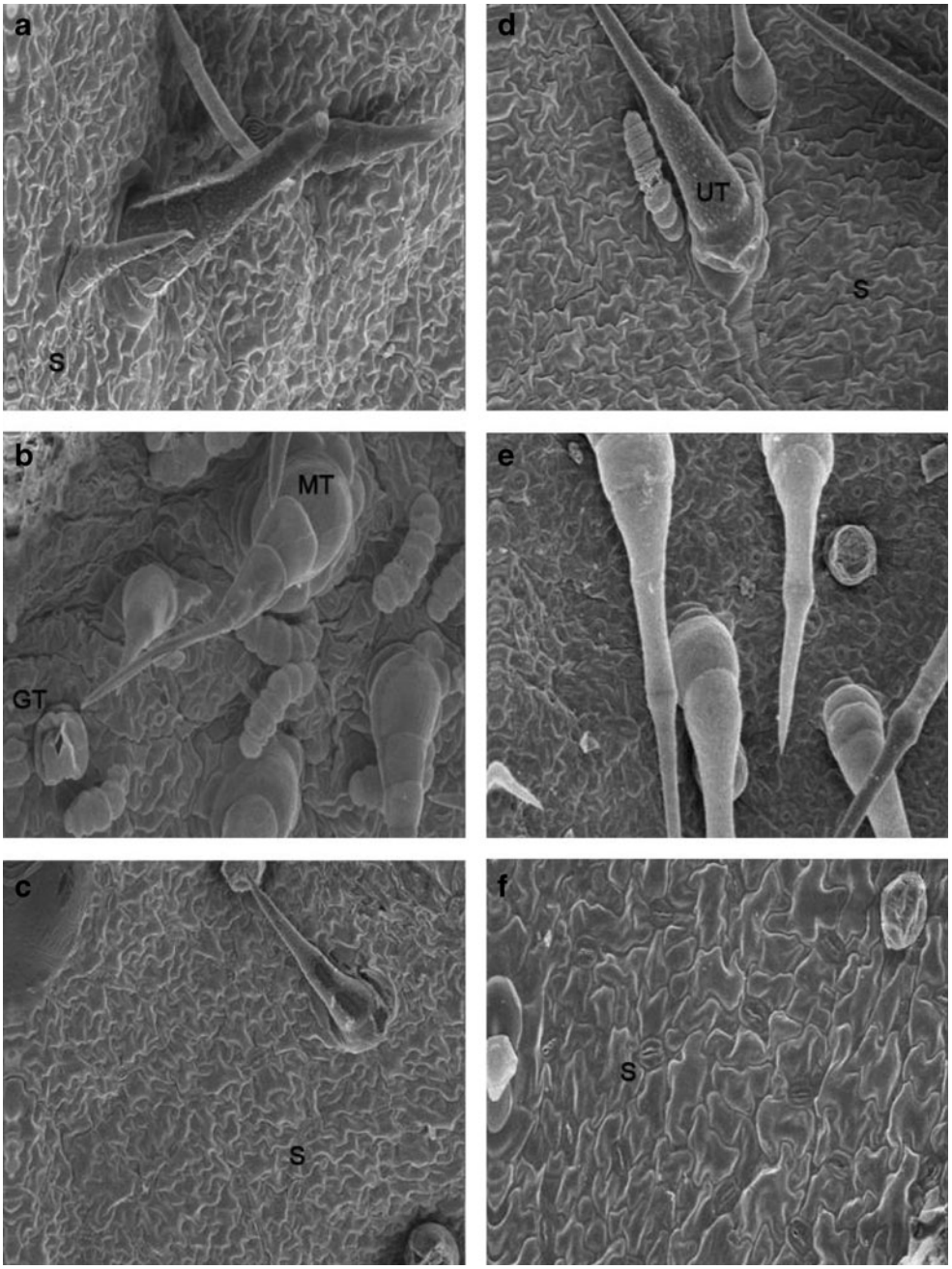

Fig. 3 SEM micrographs of leaf surface in the cultivated *Helianthus annuus* cv. 1114 and the interspecific line *Helianthus annuus*×*Helianthus argophyllus* after treatment with 0.1 mM Pb or 0.1 mM EDTA+0.1 mM Pb for 10 days (**a**–**f**). *Helianthus annuus* cv. 1114: **a** control, **b** 0.1 mM Pb and **c** 0.1 mM EDTA+0.1 mM Pb; *Helianthus annuus*×*Helianthus argophyllus*: **d** control, **e** 0.1 mM Pb, and **f** 0.1 mM EDTA+0.1 mM Pb (×600). *S* stomata, *GT* grandular trichomes, *MT* nongrandular trichomes, *UT* unicellular trichomes

The investigated sunflower genotypes largely differed in their responses to Pb in hydroponic solution. The relative growth rates (RGRs) of the plant organs were much more affected by Pb in the cultivated *H. annuus* cv. 1114 than in the new genotype *H. annuus*×*H. argophyllus* (Fig. 1). The accumulation of Pb in the plant organs of both genotypes (Fig. 2a, d) showed that the higher Pb resistance of new genotype *H. annuus*×*H. argophyllus* cannot be attributed to a lower Pb concentration in the roots of this line (Fig. 2a, d) but rather to internal tolerance mechanisms. Our data are consistent with the increased accumulation of Pb in *Atriplex halimus* roots and their ability to tolerate high Pb concentrations (Manousaki and Kalogerakis 2009).

It has been reported that Pb induces a production of reactive oxygen species (Liu et al. 2009), which in turn leads to activation of an antioxidant system in *Jatropha curcas* seedlings and cuttings (Shu et al. 2012). The increase in membrane permeability under Pb (Fig. 5) indicated that Pb caused oxidative damage in the leaves of the cultivated sunflower plants. In our study, the interspecific line possessed a higher antioxidant capacity both in controls and Pb-treated leaves (Table 2). In contrast, *H. annuus* cv. 1114 having lower constitutive antioxidant potential requires an activation of the antioxidant capacity to counteract oxidative bursts in the Pb-treated leaves. This was confirming by increasing the total antioxidant activities, the amount of

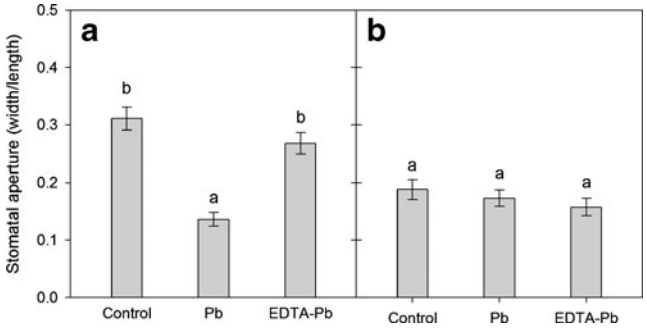

Fig. 4 Stomatal aperture (width/length) in the cultivated *Helianthus annuus* cv. 1114 (**a**) and the interspecific line *Helianthus annuus*× *Helianthus argophyllus* (**b**) after treatment with 0.1 mM Pb or 0.1 mM EDTA+0.1 mM Pb for 10 days. Data are means±SE (n= 12). *Different letters* indicate significant differences (at $P \leq 0.05$) among treatments and the two sunflower genotypes

flavonoids and the scavenging activities on free radicals (Table 2). The data are in line with the stability in the Fe-SOD isoenzyme pattern observed in the interspesific line under Pb treatment, in contrast with the rearrangement of the Fe-SOD pattern in the cultivar (Fig. 6a, b). Treatment with Pb induced the emergence of a new Mn-SOD I isoform in *H. annuus* cv.1114 (Fig. 6a) and two new Mn-SOD isozymes (Mn-SOD I and II) in the leaves of the interspecific line (Fig. 6b). These data are supported by the results of Fernandez-Ocana et al. (2011) proving that sunflower has two mitochondrial Mn-SOD genes, and their expression could act as an early signal (within the first 1–8 h of treatment) in the prevention of oxidative damage during an environmental stress. Plants with expressed Mn SODs have an increased protection ability against abiotic stress (Alsche et al. 2002).

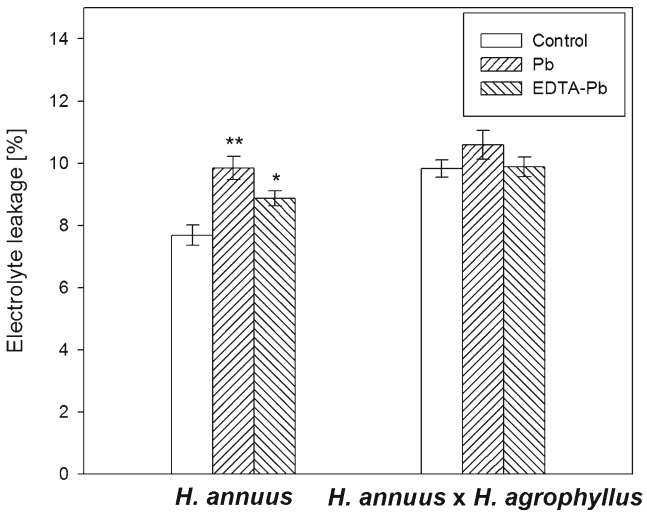

Fig. 5 Electrolyte leakage in leaves of cultivated *Helianthus annuus* cv. 1114 (**a**) and the interspecific line *Helianthus annuus*×*Helianthus argophyllus* (**b**) after treatment with 0.1 mM Pb or 0.1 mM EDTA+ 0.1 mM Pb for 10 days. Data are means±SE (n=12). Results are significantly different from the corresponding controls at *$P \leq 0.05$ and **$P \leq 0.01$

Treatment with Pb led to a significantly decreased Ca concentration in the leaves and the stems (Fig. 2b, e) only in the cultivated sunflower plants. Calcium plays a vital role in plants by acting as a messenger to regulate plant growth and development, provides membrane stability and stress tolerance, and imparts signaling specificity during biological responses (Marschner 1995; Hirschi 2004). This may be interpreted in terms of the role of Ca^{2+} in controlling membrane structure and function getting bound to phospholipids, thus stabilizing lipid bilayers and eventually providing structural integrity to cellular membranes (Hepler 2005). On this basis, we could explain the increased electrolyte leakage in the leaves of Pb-treated *H. annuus* L. cv. 1114 (Fig. 5) containing smaller amount of Ca (Fig. 2b). Electrolyte leakage was also used as a marker to assess plant tolerance to different kinds of stress like drought (Roy et al. 2009), salinity (Collado et al. 2010), and Cu and Zn toxicity (Xu et al. 2008).

The sunflower genotypes revealed evident differences in their leaf anatomy (Table 1). In control plants, the leaf thickness, the proportion of the leaf volume occupied by mesophyll, as well as the area per palisade and spongy parenchyma cell were greater in the new genotype. The anatomy of a leaf is an important parameter in the assessment of plant responses to air pollution (Gostin and Ivanescu 2007) and metal toxicity (Doncheva et al. 2009). The exposure to Pb decreased the thickness of the leaves, the mesophyll layer, and the spongy area per cell in the leaves of *H. annuus* cv. 1114. A similar effect was detected in in Pb-treated soybean plants (Weryszko-Chmielewska and Chwil 2005). Furthermore, the morphological alterations in some cell organelles were found to reflect the toxicity level and tolerance under Pb stress (Jiang and Liu 2010). In contrast, a Pb-induced increase in the morpho-anatomical parameters was found in the leaves of the interspecific line. Leaf thickness is closely related to the effectiveness of the phytosinthetic performance and plant growth as it determines, at least partially, the amount of the absorbed light and the rate of CO_2 diffusion (Garnier et al. 1999). The increased leaf thickness and mesophyll layer may be interpreted as a mechanism for maintaining a functionally active photosynthetic area confirmed by a high photosynthetic activity (data not shown), thus contributing to the higher Pb tolerance of the new genotype.

Stomata play a significant role in sensing the changes in the surrounding environment (Hetherington and Woodward 2003). Treatment with Pb resulted in stomata closure in *H. annuus* cv. 1114 but not in the interspecific line (Figs. 3 and 4). Stomata closure may be induced by an increased hydrogen peroxide production resulting from an imballanced signaling pathway (Fotopoulos et al. 2008), which could occur in Pb-treated *H. annuus* cv. 1114 plants. Trichomes have a substantial storage capacity to act as a sink during detoxification of toxic heavy metals and xenobiotics by functioning via

Table 2 Total antioxidant activity, free radical scavenging activity and total flavonoid content in the leaves of cultivated *Helianthus annuus* cv. 1114 and the interspecific line *Helianthus annuus*×*Helianthus argophyllus* after treatment with 0.1 mM Pb or 0.1 mM EDTA+0.1 mM Pb

Parameters Variants	Antioxidant activity (μmol FRAP g^{-1} FW)	Free radical scavenging activity (μmol Trolox g^{-1} FW)	Total flavonoids content (mg rutin g^{-1} FW)
H. annuus cv.1114			
Control	3.808±0.009^{b}	0.895±0.002^{a}	1.837±0.007^{a}
0.1 mM Pb	4.941±0.016^{c}	1.298±0.003^{d}	2.486±0.006^{d}
0.1 mM EDTA+0.1 mM Pb	3.400±0.004^{a}	0.907±0.003^{ab}	2.261±0.004^{b}
H. annuus×*H. agrophyllus*			
Control	5.621±0.006^{e}	1.205±0.003^{c}	2.537±0.009^{e}
0.1 mM Pb	5.825±0.008^{ef}	1.273±0.003^{cd}	2.414±0.007^{c}
0.1 mM EDTA+0.1 mM Pb	5.431±0.004^{de}	1.224±0.000^{c}	2.396±0.006^{c}

Data are means±SE (n=9). Different letters indicate significant differences (at $P \leq 0.05$) among treatments and the two cultivars for each measured parameter separately

glutathione conjugation and sequestration in the vacuole (Gutiérrez-Alcalá et al. 2000). Besides, they have considerable potential in increasing heavy metal accumulation for phytoremediation purposes (Dominguez-Solis et al. 2004). An increase in the number and swelling of the base of trichomes was recorded in the leaves of the Pb-treated sunflower plants (Fig. 3b, e), suggesting an increased capacity for detoxification of Pb ions. The role of trichomes in plant adaptation mechanisms to reduce Pb toxicity was also reported by Weryszko-Chmielewska and Chwil (2005) for soybean leaves.

In our study, the application of EDTA–Pb in the nutrient solution resulted in a significant limitation of the Pb phytotoxic effect. This led to improvement of plant growth (Fig. 1), an increase in Ca concentrations in all organs (Fig. 2b, e), a decrease in oxidative stress (Table 2 and Fig. 6), as well as a recovery effect of the leaf electrolyte leakage (Fig. 5) and the leave anatomy (Table 1 and Figs. 3 and 4). On the other hand, the addition of EDTA dramatically decreased the Pb uptake by the plants and its translocation within in the plants of both genotypes (Fig. 2a, d). This result suggests that the EDTA present in the nutrient

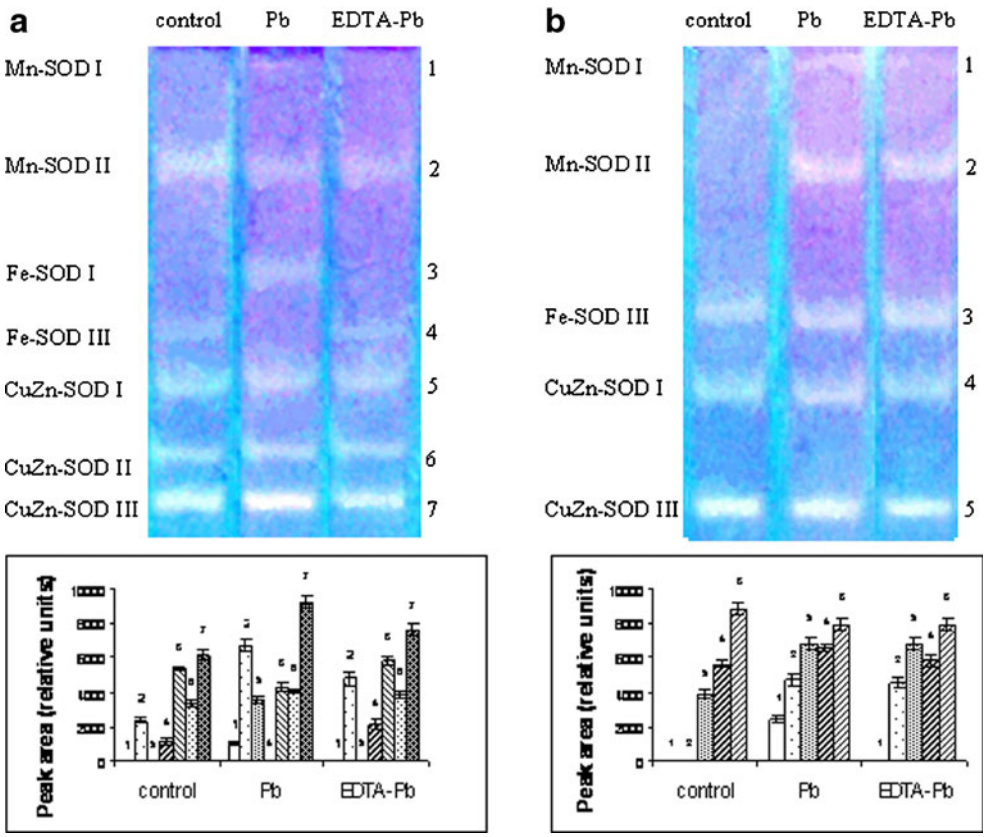

Fig. 6 Identification of SOD isoenzymes in leaves of cultivated *Helianthus annuus* cv. 1114 (**a**) and the interspecific line *Helianthus annuus*×*Helianthus argophyllus* (**b**) after treatment with 0.1 mM Pb or 0.1 mM EDTA+0.1 mM Pb for 10 days. Isoenzymes of SOD were separated by native PAGE and quantified by converting the stained area and intensity into relative units

solution impedes the absorption of Pb ions by the plants. The protective effect of EDTA against Pb toxicity could be due to fact that binding toxic-free Pb ions reduces plant exposure to their influence.

Conclusion

In the present work, the comparative analysis of the responses of sunflower plants to Pb medium-term stress showed that the deleterious effects of Pb were more pronounced in the cultivated sunflower *H. annuus* cv. 1114 grown in a hydroponic system. The newly developed genotype *H. annuus*×*H. argophyllus* was more productive and with a higher tolerance to Pb phytotoxicity, which could indicate that it may possess certain mechanisms to tolerate high Pb root concentrations. This character could be inherited from the wild parent used in the interspecific hybridization. The ability of EDTA to alleviate Pb toxicity in both genotypes may be due to the prevention of Pb absorption by the roots. Hence, EDTA cannot be used to enhance Pb absorption from nutrient solution by hydroponically grown sunflower plants for phytoremediation purposes. Therefore, in agreement with Liu et al. (2007), it can be concluded that the EDTA-assisted technique is more suitable for Pb-contaminated soil.

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