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REGULAR ARTICLE

Phenotypic seedling responses of a metal-tolerant mutant line of sunflower growing on a Cu-contaminated soil series: potential uses for biomonitoring of Cu exposure and phytoremediation

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Abstract
Background and aims The potential use of a metal-tolerant sunflower mutant line for both biomonitoring and phytoremediating a Cu-contaminated soil series was investigated.
Methods The soil series (21–1,170 mg Cu kg^{-1}) was sampled in field plots at control and wood preservation sites. Sunflowers were cultivated 1 month in potted soils under controlled conditions.
Results pH and dissolved organic matter influenced Cu concentration in the soil pore water. Leaf chlorophyll content and root growth decreased as Cu exposure rose. Their EC_{10} values corresponded to 104 and 118 μg Cu L^{-1} in the soil pore water, 138 and 155 mg Cu kg^{-1} for total soil Cu, and 16–18 mg Cu kg^{-1} DW shoot. Biomass of plant organs as well as leaf area, length and asymmetry were well correlated with Cu exposure, contrary to the maximum stem height and leaf water content.
Conclusions Physiological parameters were more sensitive to soil Cu exposure than the morphological ones. Bioconcentration and translocation factors and distribution of mineral masses for Cu highlighted this mutant as a secondary Cu accumulator. Free Cu^{2+} concentration in soil pore water best predicted Cu phytoavailability. The usefulness of this sunflower mutant line for biomonitoring and Cu phytoextraction was discussed.

Keywords Chlorophyll · *Helianthus annuus* L · Phytoextraction · Phytotoxicity · Soil pore water · Shoot Cu removal

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Highlights A metal-tolerant sunflower mutant can be used for monitoring and phytoremediating soil Cu contamination at a wood preservation site.

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Abbreviations

BCF	Bioconcentration factor
Carot	Carotenoid content
CCA	Chromated copper arsenate
CEC	Cation exchange capacity
Chl	Chlorophyll
CTRL	Control soil
Cu-ISE	Copper ion selective electrode
ChlTOT	Total chlorophyll content

CuMM	Shoot Cu removal
CuRT	Root Cu concentration
CuSH	Shoot Cu concentration
CuSPW	Total Cu concentration in the soil pore water
CuTOT	Total soil Cu
DL	Dolomitic limestone
DMF	N,N-dimethylformamide
DOM	Dissolved organic matter
DW	Dry weight
DWSH	Shoot DW yield
EC_{10}	in a graded dose response curve, the concentration of a compound where 10 % of its maximal effect is observed
EMS	Ethyl methanesulphonate
INRA LAS	French National Institute for Agricultural Research - Laboratory of soil analysis Arras France
ISE	Ion selective electrode
LA	Leaf asymmetry
OM	Organic matter
RPI	Relative parameter index
RTEI	Relative treatment efficiency index
SL	Stem length
TE	Trace element
TF	Translocation factor
TI	Tolerance index
TLA	Total leaf area
TOC	Total organic carbon
UNT	Untreated soil
WC	Water content

Introduction

Anthropogenic soil contamination by Cu can result in serious negative consequences, such as damages on ecosystems, agricultural productivity, contamination of water resources and health risks for animals (Adriano 2001). In France, 838 Cu-contaminated sites are referenced by the national authority (Basol 2013). In many cases, the use of Cu-based preservatives (e.g. Cu sulphate and CCA) is involved. At wood preservation sites, wood washings often result in Cu-contaminated topsoils (Bes and Mench 2008; Mench and Bes 2009). Copper bioavailability is influenced by many factors, notably soil type, total soil Cu, composition of soil pore water (i.e. CuSPW, Cu^{2+}, Ca^{2+}, Mg^{2+}, pH, DOM, ionic strength, and redox potential), and runoff parameters (Gunkel et al. 2003; Luo et al. 2006; Thakali et al. 2006; Chaignon et al. 2009; Forsberg et al. 2009; Bravin et al. 2010a). Copper phytotoxicity is mainly due to the existence of two readily interconvertible oxidation states that makes Cu highly reactive and a catalyst of the formation of free radicals through the Haber-Weiss reaction in plants with subsequent metal-induced oxidative signalling and damage to cells at level of lipids, membranes, nucleic acids, and proteins (Yruela 2009; Smeets et al. 2013). Excess Cu interferes with important cellular processes such as photosynthesis, pigment synthesis, plasma membrane permeability and other metabolic mechanisms, causing a strong inhibition of plant development (Kuepper et al. 2009; Palmer and Guerinot 2009; Jung et al. 2012; Smeets et al. 2013).

Biomonitoring is a part of the initial risk assessment of TE-contaminated soils. It is also used for evaluating the efficacy of remediation options implemented to either remove contaminants or minimize pollutant linkages of TE-contaminated soils (Adriano et al. 2004). Various bioindicators have been used for assessing ecotoxicity of Cu-contamination (da Silva et al. 2010; Fritsch et al. 2010; Leduc et al. 2008; Maderova et al. 2011; Marchand et al. 2011). In terrestrial ecosystems, plants are sensitive organisms to metal exposure and accumulation, due to a large contact surface with the soil and the atmosphere. Responses of vegetative structures are widely examined at various scales, ranging from molecular compounds to plant individuals, population, and community (Bes et al. 2010; Ernst and Peterson 1994; Hernandez and Pastor 2008; Korpe and Aras 2011). Phenotypic responses of plants to excess Cu can be divided into two main groups: (1) biometrical or structural (i.e. morphological and anatomical) and (2) physiochemical or functional (i.e. physiological and (bio)chemical) responses (Ernst and Peterson 1994; Lagadic et al. 1997). Sensitivity of parameters used to asses phenotypic responses into these groups differ according to soil contamination levels (Meers et al. 2006). In most cases, physiochemical biomarkers are more sensitive, e.g. primarily protein and DNA (genotoxic effects) damages are detected (Korpe and Aras 2011; Mendoza-Soto et al. 2012; Mocquot et al. 1996; Qi et al. 2006). In addition, morphological parameters alone are considered not sensitive enough to fully assess potential phytotoxic effects in moderately contaminated soils (Meers et al. 2006).

For metal phytoextraction, beside hyperaccumulators, secondary metal-accumulators, notably non-food crops,

deserve attention due to their significant aerial biomass and financial opportunities from plant-based feedstock (Mench et al. 2010; Vamerali et al. 2010; Vangronsveld et al. 2009; Vassilev et al. 2004). Plants with high shoot biomass, shoot TE concentrations related to TE exposures in the growing media, and high shoot TE removals, are relevant candidates for both biomonitoring TE-contaminated soils and TE phytoextraction. Sunflower (*Helianthus annuus* L.) is a potential candidate for coupling both objectives as (1) its morphological and physiological traits, mainly at seedling stage, reflect TE exposure (Lin et al. 2003; Madejon et al. 2003; Nehnevajova et al. 2012), and (2) it provides financial returns through oilseed and biomass production and can be included in a sustainable crop rotation promoting soil functions, nutrient cycles, microbial community and other ecosystem services, with either no or acceptable residual pollutant linkages (Faessler et al. 2010; Nehnevajova et al. 2009; Tahsin and Yankov 2007). In contrast to transgenic plants, which use is restricted to laboratory scale in many countries, non-genetically modified plants can be tested for improving shoot TE removals under field conditions. Mutant lines, obtained by seeds exposure to EMS, have been selected for higher shoot metal concentrations (Nehnevajova et al. 2005, 2009, 2012). Such sunflower mutants accumulate more metals (Zn, Cd, Pb, and Cu) than their motherlines at high metal exposure in field conditions (Kolbas et al. 2011; Nehnevajova et al. 2009). Characterization of the seedling responses of such mutants to increasing exposure of metals such as Cu and comparison with field dataset could calibrate a plant test with a wider responsive range, able to better discriminate initial and residual soil phytotoxicities and the feasibility of metal phytoextraction.

This study aimed at assessing phenotypic seedling responses of a metal-tolerant sunflower mutant line growing on a soil series with increasing total soil Cu, sampled in field plots at a wood preservation site and a control site. Soil physico-chemical parameters and composition of soil pore water were measured to characterize soil Cu exposure. Relationships between biometrical and physio-chemical parameters of 4-week-old seedlings and parameters of soils and soil pore water were investigated for determining relevant plant endpoints for biomonitoring. Dose-effect relationships were proposed for potential application in plant testing and predicting potential use of this mutant line for phytoextraction in Cu-contaminated soils.

Material and methods

Site and field trial

The wood preservation site (about 10 ha, Saint-Médard d'Eyrans, Gironde, SW France; N 44°43.353, W 000°30.938) has been used for over a century (Mench and Bes 2009). Topsoils (0–0.25 m, Fluviosol) are sandy, i.e. 85.8 % sand, 5.9 % clay, 8.3 % silt, 1.6 % OM, and C/N 17.2, with a low CEC (3.5 cmol kg^{-1}), and display a high spatial variability for total soil Cu and Cu in soil pore water, mainly reflecting long-term used of Cu-based salts and washings of treated timbers (Bes et al. 2010; Mench and Bes 2009). Distribution of soil Cu in physical and operationally defined soil fractions as well as impacts on soil enzyme activities were reported by Lagomarsino et al. (2011).

The field trial dedicated to aided phytoextraction at the sub-site P1-3 consists in four blocks and 31 field plots (2 m^2) with total soil Cu (CuTOT) in topsoil (0–25 cm) varying between 163 and 1,170 mg Cu kg^{-1} (Table 1; Kolbas et al. 2011). CuTOT exceeded the median and upper whisker background values (in mg Cu kg^{-1}) of French sandy soils, i.e. 3.2 and 8.4, and of topsoils in the Aquitaine region, France, i.e. 13.9 and 55.8 (Kolbas et al. 2011). Total soil contents (in mg kg^{-1}) varied between 4.8 and 8.6 for As, 4.7–6.0 for Ni, 15.8–22.5 for Cr, 35–98 for Zn, 17–23 for Pb, 0.1–0.2 for Cd, and 1.5–2.0 for Co. For these elements, background levels of French sandy soils were only slightly exceeded for Zn (48 mg kg^{-1}) in 14 plots, but total soil As, Co, Cd, Cr, Ni, Pb, and Zn did not differ between the plots and globally Cu was the main contaminant in plot topsoils (Kolbas et al. 2011).

As sunflower cultivars were unable to grow in the untreated plots with high CuTOT and based on a pot experiment (Bes and Mench 2008), compost made from poultry manure and pine bark chips (5 % w/w, Orisol, Cestas, France) and dolomitic limestone (0.2 % w/w, Prodical Carmeuse, Orthez, France) were incorporated into the topsoils (0–25 cm) of three blocks (i.e. block #1: plots #1 to #10, block #2: plots #11 to #20, and block #3: plot #21 to #30, hereafter referred to OMDL plots) in March 2008 (Kolbas et al. 2011). Block #4 remained untreated and was considered as a single plot (UNT #31), whereas each amended block was divided in 10 plots (2 m^2). An uncontaminated plot (2 m^2, CTRL-plot #32) with similar alluvial sandy soil type was managed in a kitchen garden located at 18 km from the site

Table 1 Total soil Cu and physico-chemical properties of soil pore waters

Block/plot		Soil	Soil pore water						
		CuTOT mg kg^{-1}	CuSPW mg L^{-1}	Cu^{2+} µM L^{-1}	pCu^{2+}	Cu ratio %	pH	DOM OD cm^{-1}	TOC mg C L^{-1}
block 1* (OMDL)	Mean	315.5**b**	0.243**c**	0.075**c**	7.14**a**	2.14**a**	7.25**b**	0.793**a**	123.6**a**
	SD	45.1	0.065	0.026	0.15	0.95	0.07	0.263	41.8
	Min-Max	239–384	0.171–0.397	0.038–0.124	6.90–7.41	0.94–3.46	7.16–7.39	0.50–1.40	76–212
block 2* (OMDL)	Mean	334.7**b**	0.225**c**	0.049**cd**	7.31**a**	1.43**b**	7.14**bc**	0.747**a**	107.7**a**
	SD	92.3	0.043	0.016	0.11	0.71	0.14	0.113	13.7
	Min-Max	163–518	0.161–0.324	0.039–0.064	7.19–7.40	1.15–2.08	6.95–7.36	0.55–0.95	92–131
block 3* (OMDL)	Mean	972.3**a**	0.764**a**	0.424**b**	6.41**b**	3.51**b**	6.95**c**	0.728**a**	127.5**a**
	SD	132.5	0.088	0.009	0.07	0.29	0.17	0.111	18.6
	Min-Max	753–1170	0.591–0.926	0.153–0.669	6.17–6.82	1.42–4.92	6.69–7.24	0.56–0.90	106–160
block #4** (UNT plot #31)	Mean	1016**a**	0.548**b**	4.177**a**	5.38**c**	49.08**a**	5.93**d**	0.249**b**	43.9**c**
	SD	110	0.047	0.895	0.09	11.28	0.08	0.031	3.7
	Min-Max	909–1130	0.512–0.602	3.37–5.14	5.29–5.47	39.6–61.6	5.82–5.99	0.21–0.27	39–45
CTRL** (plot #32)	Mean	21.0**c**	0.150**c**	0.036**d**	7.44**a**	1.56**b**	7.90**a**	0.795**a**	60.8**b**
	SD	2.65	0.017	0.003	0.004	0.15	0.07	0.037	2.5
	Min-Max	16–22	0.132–0.163	0.033–0.039	7.41–7.48	1.38–1.69	7.79–7.92	0.75–0.82	59–64

Values are means ± SD (*n=10, **n=3); different letters stand for statistical significance at the 0.05 level with the Tukey HSD test

CuTOT total soil Cu, *CuSPW* total Cu concentration in soil pore water, *Cu^{2+}* free copper ion content in soil pore water, *Cu ratio* Cu^{2+} vs. CuSPW, *pH* pH in soil pore water, *DOM* dissolved organic matter in soil pore water, *OD* optical density, *TOC* total organic carbon in soil pore water

(Gradignan, France). Across the 32 field plots, soil pH ranged from 7.0 (UNT) to 7.54 (CTRL), CEC from 31 (UNT) to 194 mmol kg^{-1} (CTRL), and total organic matter from 14.5 (UNT) to 78 g kg^{-1} (CTRL). Soil pH, CEC and total organic matter in OMDL plots varied in the ranges 7.18–7.29, 47–48 mmol kg^{-1}, and 25.1–28.4 g kg^{-1}, respectively.

Soils and soil pore waters

Three soil samples (3 kg soil FW) were collected in each plot (n=32) with a stainless spade from the 0–25 cm soil layer and combined to make a composite soil sample, which was air-dried, sieved (<5 mm, nylon mesh), and manually homogenized. Trace element concentrations in soils were determined with standard methods and a quality scheme by INRA LAS (2013), Arras, France, i.e. inductively coupled plasma/atomic emission spectroscopy (ICP-AES) for metals after wet digestion in HF and $HClO_4$, and hydride-generation for As after wet digestion in H_2SO_4/HNO_3 (2/1) with V_2O_5 at 100 °C (3 h). Two certified reference materials, i.e. BCR No. 141 (calcareous loam soil) and BCR No. 142 (light sandy soil) from the Bureau Communautaire de Référence, were used by INRA LAS in the quality scheme.

Soil samples (1 kg air-dried) were placed in plastic pots (1.3 L) (in triplicates), watered with deionised water, and daily maintained at 70 % of field capacity (10 % of air-dried soil mass). One Rhizon MOM moisture sampler (Eijkelkamp, The Netherlands) was inserted with a 45° angle into each potted soil. Soil pore water (10 mL) were collected three times with 1 week of interval and kept at 4 °C prior to analyse pH (Hanna instruments, pH 210, combined electrode Ag/AgCl - 34) and Al, Cu, B, Na, K, Fe, Mn, P, and Zn (ICP-AES, Varian Liberty 200). As total soil As and Cr were low in all sampled soils (Kolbas et al. 2011), these elements were analyzed in soil pore water only for several selected samples. Free copper (Cu^{2+}) ions were determined by a Cu-ISE (Fischer Bioblock) after calibration (Buck and Cosofret 1993; Luo et al. 2006). During the measures, nitrogen bubbling was maintained into soil pore waters notably to remove carbon dioxide and oxygen and to reduce the oxidation of soluble compounds. The free Cu activity, i.e. the concentration of free Cu ions corrected with the Debye-Huckel theory activity coefficient—is reported in pCu^{2+} defined as the negative $\log_{10}$ of the molar quantity of Cu^{2+} ions activities and, therefore, pCu^{2+} are unitless. The content of dissolved organic matter (DOM) in soil pore waters was quantified by both spectrophotometry (spectrophotometer CARY 100 Scan, 340 nm, Baker et al. 2008) and analysis of total organic carbon (TOC) (Hiper TOC Analyzer Thermo scientific, HiPerTOC 2004).

Biotest and plant analysis

The sunflower mutant 1 line [M6 (6th generation), 1/67-35-190-04], obtained by chemical mutagenesis using EMS (Nehnevajova et al. 2005, 2009) and previously assessed at field scale vs. commercial cultivars (Kolbas et al. 2011), were sowed in each potted soil (four seeds per pot, in triplicates) in a climatic chamber, with the following conditions: 14 h light/10 h darkness regime, 150 µmol m^{-2} s^{-1}, 25 °C/22 °C, 65 % relative humidity (ISO 2005). Pots were arranged in a fully randomized block design on a table and watered daily with deionized water (50 % water holding capacity). The soils were fertilized twice, i.e. just before starting plant culture and 2 weeks after with a modified Hoagland n°2 nutrient solution (Hewitt 1966) supplying no Fe and other trace metals.

Plants were collected after 1 month at growth stage 2.4 (CETIOM 1995) when the 2nd pair of leaves reached 4 cm length. Roots were carefully washed in deionized water and gently blotted. Shoots and roots were weighed (FW), rinsed in distilled water, oven-dried at 50 °C for 48 h, and then DW yield and WC were determined. Other biometrical parameters were measured, i.e. stem and leaf length, leaf area and asymmetry (scanner EPSON Expression 10000 XL, software WINFOLIA, Kryazheva et al. 1996). Root microstructures were observed (binocular PERFEXSCIENCES with camera moticam 2000 and lamp Motic MLC-150c). Chlorophyll a and b and total carotenoids were extracted from the 2nd leaf pair (1 cm^2, in duplicates) with DMF and their foliar contents computed from measurements at 470, 647 and 664.5 nm of the extracts (spectrophotometer CARY 100 Scan, Lagriffoul et al. 1998). Plant samples were ground in a titanium mill (Retsch MM200). Weighed aliquots of plant materials (0.5 g DW) were wet digested under microwaves (Marsxpress, CEM) with 5 mL supra-pure 14 M HNO_3 and 2 mL 30 % (v/v) H_2O_2 not stabilized by phosphates. Certified reference material (maize V463 BIPEA - Bureau InterProfessionnel d'Etudes Analytiques, France) and blank reagents were included in all series. Element concentrations in digests were determined by ICP-AES

(Varian Liberty 200). All elements were recovered (>95 %) according to the standard values and standard deviation for replicates (n=3) was <5 %.

To quantify the impact of excess Cu and efficiency of OMDL amendment on sunflower growth, several indexes were computed: (1) the Relative Treatment Efficiency Index ($RTEI=(P_t-P_c)/(P_t+P_c)$, Marchand et al. 2010) to compare the OMDL soils with the UNT soil; (2) the Relative Parameter Index ($RPI(\%)=100\times(P_t-P_{min})/(P_{max}-P_{min})$, Bravin et al. 2010b) which determines the relative increase in values compared with the total amplitude of increase, and (3) the tolerance index ($TI(\%)=100\times P_t/P_{max}$, Ke et al. 2007), where P_t - value of the plant parameter in a treatment ; P_c - value of the plant parameter in the control; P_{min} and P_{max} - minimum and maximum values of the plant parameter.

The translocation factor ($TF=C_{shoot}$ / C_{root}), bioconcentration factor (BCF, $BCF_{shoot}=C_{shoot}$ / C_{soil}, $BCF_{root}=C_{root}$ / C_{soil}), and shoot Cu removal, i.e. so-called also mineral mass (CuMM, $CuMM_{shoot}=C_{shoot}\times DW_{shoot}$) computed with shoot Cu concentration (mg kg^{-1} DW) and shoot biomass (g DW ha^{-1}), were calculated to assess the metal phytoextraction efficiency (Kolbas et al. 2011; Li et al. 2010); C_{shoot} and C_{root} are the metal concentrations in the sunflower shoots and roots, respectively, C_{soil} is the metal concentration in the soil, and DW_{shoot} is the shoot DW yield.

Statistical analysis

Statistical analysis (one way ANOVAs) was performed to evaluate differences in (1) parameters of soils and soil pore waters and (2) biometrical plant parameters depending on blocks. Post hoc Tukey's HSD tests were conducted to assess multi-comparison of mean values. Differences were considered statistically significant at $p<0.05$. A maximum likelihood factor analysis was also conducted on soil, soil pore water, and plant parameters (biometrical and physiochemical). Pearson correlation coefficients (linear regression) between soil and plant parameters were calculated (significance level, $p<0.05$). Stepwise regression AIC (Akaike Information Criterion), with subset regressions (r^2 adjusted) on the parameters of soils and soil pore water was used to predict the relevant parameters for phytoextraction studies: pCu^{2+}, shoot DW yield, CuSH and CuMM. Best combination of a minimum number of factors and the maximal r^2 adjusted were expressed by multivariate equations (Eq. 1–5). Exposure concentrations (EC_{10} and EC_{50}) of Cu respectively leading to 10 % and 50 % changes of plant parameters were calculated using linear regression and drc (dose–response curve) package (Knezevic et al. 2007). All statistical analyses were performed using R software (version 2.14.1, R Foundation for Statistical Computing, Vienna, Austria).

Results

Soils and soil pore waters (Table 1)

Total soil Cu (in mg kg^{-1}) ranged from 21 (CTRL soil) to 1,170 (plot #30) and mean values were higher in blocks #3 and #4 than in blocks #1 and #2. Total Cu concentration in soil pore water (CuSPW, in mg Cu L^{-1}) varied between 0.15 (CTRL soil) and 0.93 (plot #21), its mean values increasing from 0.22±0.04 (block #2) to 0.76±0.09 (block #3) in contaminated soils and being similar in blocks #1 and #2. The OMDL incorporation did not affect total soil Cu when blocks #3 and #4 (UNT) were compared, but it increased CuSPW. Free Cu ions (Cu^{2+}) and CuSPW in blocks #1 and #2 showed similar trends. Concentrations for Cu^{2+} (in μM) were lower in block #1 and #2 soils than in block #3 soils and peaked in the UNT soil. Due to both high OM content and low total soil Cu in the CTRL soil, the Cu^{2+}concentration in soil pore water was the lowest. Based on total soil Cu, the OMDL treatment reduced the Cu^{2+}concentration of soil pore water in amended soils. This was particularly significant in block #3 compared with UNT. The free Cu ions:CuSPW ratio (in %) in soil pore water ranged from 0.94 to 4.92 (mean value: 2.37) in OMDL soils and peaked to 49.1 in the UNT soil. Most soil Cu parameters were correlated (Fig. 1, Circle 1). Soil parameters placed at the figure centre were relatively independent, i.e. DOM, TOC, Na, B, As, and P in soil pore water. pCu^{2+} (ISE) decreased from 7.52 to 5.38 (Table 1) and was better correlated with CuTOT and CuSPW than Cu^{2+} (Table 2, Fig. 1).

The pH of soil pore water increased from 5.82 (UNT) to 7.39 (plot #5) in contaminated soils and leveled up to 7.9 in the CTRL soil. This parameter was in decreasing order CTRL>block #1=block #2>block #3>UNT (Table 1) and highly correlated with the pCu^{2+} concentration (R=0.78; Table 2). It was less relevant to predict CuSPW (R=−0.52). The ratio of free Cu^{2+} ions in the soil pore water exponentially decreased as its pH rose

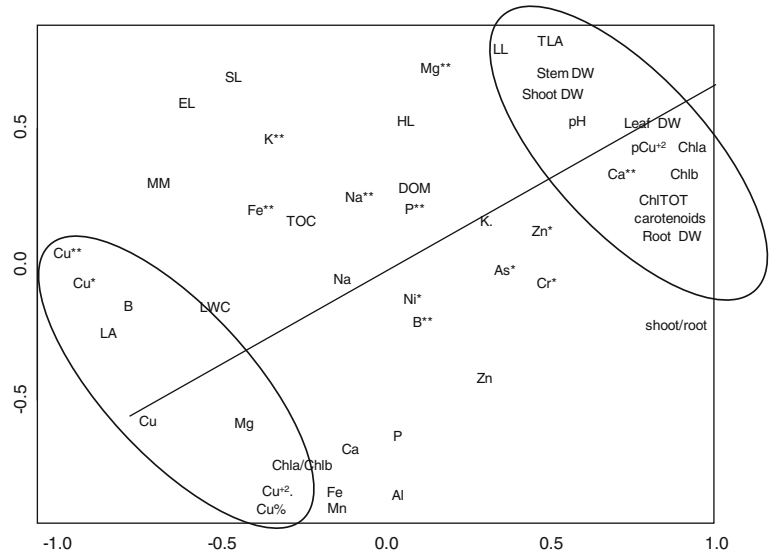

Fig. 1 Maximum likelihood factor analysis performed on soil, soil pore water and plant parameters. *DOM* dissolved organic matter, *EL* epicotyl length, *HL* hypocotyl length, *LA* leaf asymmetry, *LL* leaf length, *LWC* leaf water content, *MM* mineral mass of Cu in shoot, *SL* stem length, *TLA* total leaf area, *TOC* total organic carbon, * - total element in the soil, ** - total element in the soil pore water, without * - shoot concentration for each element. Root metal concentrations were no shown because they were highly correlated with total soil metals and may not necessarily reflect the plant physiological processes

($Y=2\ 10^{+11}x^{-12.9}$, $R^2=0.59$, Table 1). The DOM values in the soil pore water determined by both TOC and spectrophotometry methods were well correlated ($R=0.8$; $p<0.001$, Table 2), except a slight discrepancy for the CTRL soil with a higher value for the spectrophotometry method (Table 1). All amended blocks roughly had a 2.5–3 times higher DOM concentration (108–127 mg C L^{-1}) than UNT (44 mg C L^{-1}) (Table 1). Spectrophotometric DOM values were significantly negatively correlated with free Cu ions ($R=-0.51$, $p=<0.01$) and the Cu^{2+}:CuSPW ratio ($R=-0.49$; $p<0.01$) (Table 2). However, TOC values were less related to Cu^{2+} ($R=-0.37$; $p<0.05$).

Modeling of Cu exposure based on soil parameters

Adjusted R^2 for pCu^{2+} in simple regression with pH in soil pore water was 0.58 (Fig. 2a). It increased to 0.82 for pCu^{2+} in multiple regression with pH in soil pore water and CuSPW (Fig. 2a, line 1): $pCu^{2+}=0.84+0.78*pH$ -

Table 2 Pearson's correlation coefficients between soil and soil pore water parameters (bivariate correlations)

	CuTOT	CuSPW	pCu^{2+}	Cu^{2+}	Cu ratio%	pH	DOM
CuTOT							
CuSPW	0.92^{***}						
pCu^{2+}	-0.80^{***}	-0.82^{***}					
Cu^{2+}	0.37^{NS}	0.34^{NS}	-0.75^{***}				
Cu ratio%	0.20^{NS}	0.15^{NS}	$^{-}0.60^{**}$	0.98^{***}			
pH	-0.53^{**}	-0.52^{**}	0.78^{***}	-0.78^{***}	-0.69^{***}		
DOM	-0.22^{NS}	-0.09^{NS}	0.41^{*}	-0.51^{**}	-0.49^{**}	0.46^{**}	
TOC	0.16^{NS}	0.24^{NS}	0.10^{NS}	-0.37^{*}	-0.43^{*}	0.17^{NS}	0.80^{***}

Significance level: ^{NS} Not significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$

CuTOT total soil Cu, *CuSPW* total Cu concentration in soil pore water, Cu^{2+} free copper ion concentration in soil pore water, *Cu ratio* Cu^{2+} vs. CuSPW, *pH* pH in soil pore water, *DOM* dissolved organic matter in soil pore water, *TOC* total organic carbon in soil pore water

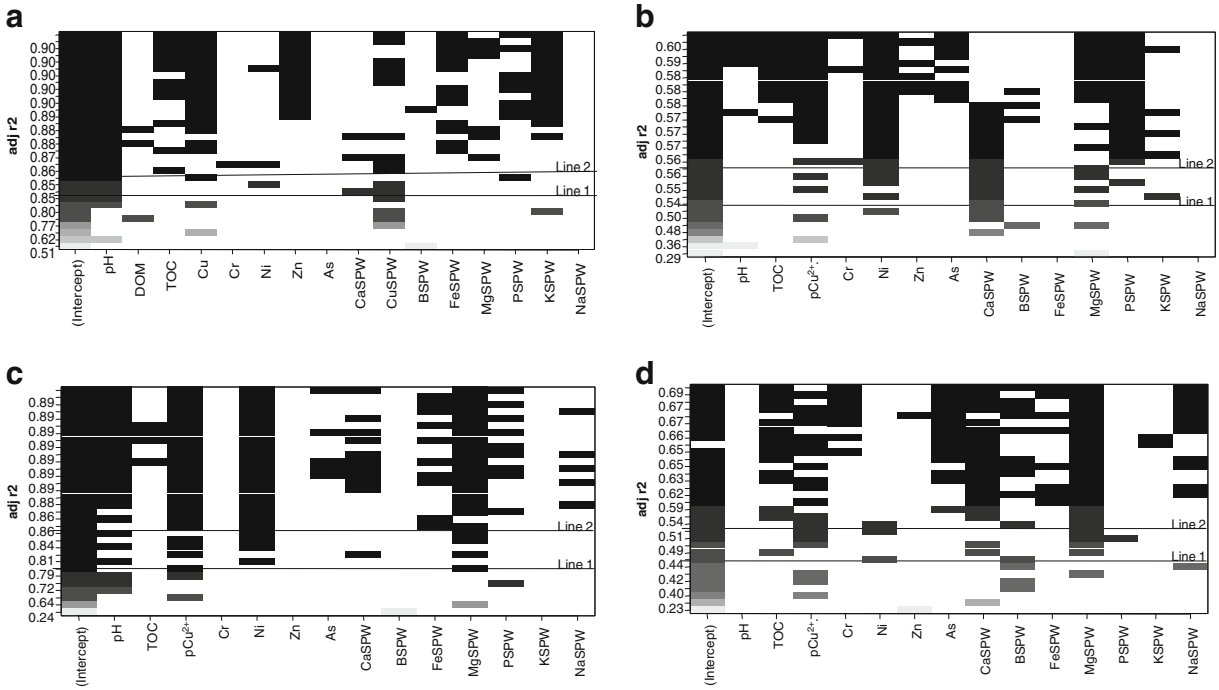

Fig. 2 Stepwise regression AIC (Akaike Information Criterion) with subset regressions (y-axis: adjusted r^2, adjr2; the greyscale indicated the adjr2 levels) between **a** pCu^{2+}, **b** shoot DW yield, **c** shoot Cu concentration, **d** shoot Cu removal, and the physico-chemical parameters of soil and soil pore water. Line 1 – for two variables in the regression; line 2 – for three variables in the regression. CaSPW, BSPW, FeSPW, MgSPW, PSPW, KSPW and NaSPW are respectively the total concentrations of Ca, B, Fe, Mg, P, K and Na in the soil pore water

1.11*CuSPW (Eq. 1, R^2=0.82, p value<0.0001). The CuTOT had less importance for predicting pCu^{2+} (R^2=0.80, p<0.0001). The best combination with three soil factors was achieved with pH, TOC and CuSPW (Fig. 2a, line 2): pCu^{2+}=2.56+0.63*pH+0.0033*TOC−1.30*CuSPW, (Eq. 2, R^2=0.86, p value<0.0001).

Plant parameters and their relationships with soil parameters

Morphological and physiological parameters varied in response to Cu exposure (Table 3). Correlations of plant phenotypic traits with soil Cu parameters are listed in Table 4. The soil, soil pore water, and plant parameters were projected on a plane according to their respective correlations (Fig. 1). The majority of plant parameters grouped in Circle 2, and were correlated negatively with total soil Cu and positively with pCu^{2+}, except stem, epicotyl and hypocotyl lengths, and leaf WC. Based on the whole dataset, pCu^{2+}, free Cu^{2+} ions, and the free Cu ions:CuSPW ratio well correlated with many plant parameters, i.e. n=13, 12, and 10 significant correlations with $R_p \geq |0.5|$, respectively (Table 4, Fig. 3) demonstrating that these indicators of labile Cu pool can predict the phytotoxicity of studied Cu-contaminated soils. CuTOT, pH in soil pore water, and CuSPW were, in a lesser extent, also frequently correlated with phenotypic traits, i.e. n=9, 9, and 8 significant correlations, respectively.

Root and shoot DW yields

Increase in total soil Cu reduced root growth and length, notably for the UNT plants, and induced changes in the structure of root system such as an increase in lateral root formation. Roots of block #3 plants and especially of UNT plants displayed a brownish color and were short, thick, and highly branched ('barb-wire' or 'coralloid' roots), with a reduced formation of root hairs. Excess Cu indicated by low pCu^{2+} in soil pore water reduced root DW yield, and thus whole plant biomass, before to negatively impact shoot DW yield (Table 3, Fig. 2). Root DW yield of UNT plants was seven folds lower than that of CTRL plants whereas this ratio was

Table 3 Morphological and physiological parameters of 1 month-old sunflower plants

Block #		Parameters											
		Stem length cm	Hypocotyl length cm	Epicotyl length cm	Leaf area cm^2	Leaf asymmetry	Leaf length cm	Leaf WC %	Root DW g $plant^{-1}$	Shoot DW g $plant^{-1}$	shoot DW/ root DW	Chl TOT mg m^{-2}	Carot mg m^{-2}	Chl*a*/ Chl*b*
Block 1*	Mean	9.90**b**	4.80**a**	3.78**b**	46.05**b**	0.055**b**	5.70**ab**	49.0**b**	1.14**a**	2.40**ab**	2.09**b**	338**a**	45.8**a**	3.33**b**
	SD	1.05	0.77	0.57	12.32	0.007	0.55	11.9	0.30	0.50	0.98	57	6.2	0.10
Block 2*	Mean	10.50**ab**	5.00**a**	4.21**b**	52.42**b**	0.057**b**	6.33a**b**	50.4**b**	1.00**a**	2.64**ab**	2.58**ab**	339**a**	45.6**a**	3.36**b**
	SD	1.38	0.88	0.51	12.68	0.008	0.79	11.2	0.29	0.61	1.17	46	5.4	0.07
Block 3*	Mean	12.24**a**	4.65**a**	5.65**a**	42.10**b**	0.081**a**	5.60**b**	57.3**ab**	0.47**b**	2.00**b**	4.34**a**	115**b**	20.9**b**	3.36**b**
	SD	1.10	0.68	0.48	8.53	0.011	0.64	8.2	0.17	0.49	0.79	28	4.7	0.05
UNT**	Mean	3.50**c**	2.22**b**	0.82**c**	8.24**c**	0.075**a**	2.71**c**	73.9**a**	0.20**c**	0.59**c**	2.94**ab**	6.9**c**	6.2**c**	3.99**a**
	SD	1.08	0.57	0.33	0.56	0.005	0.04	4.6	0.07	0.13	0.91	2.4	1.6	0.05
CTRL**	Mean	8.69**b**	4.00**ab**	3.17**b**	74.08**a**	0.036**c**	7.12**a**	49.4**b**	1.43**a**	3.38**a**	2.36**b**	381**a**	44.9**a**	3.03**c**
	SD	2.87	1.35	0.88	15.46	0.006	0.71	15.4	0.48	0.70	0.97	30	2.6	0.05

Replicates for mean values (*n=10, **n=3); different letters stand for statistical significance at the 0.05 level with the Tukey HSD test

5.7 folds for the shoot DW yield (Table 3). Correlations of root DW yield with indicators of soil Cu exposure (i.e. CuTOT, CuSPW, and pCu^{2+}) were stronger than those of shoot DW yield (0.79 and 0.56; 0.81 and 0.51; 0.76 and 0.62, respectively, Table 4). Shoot DW yield is however more easy to determine in bioassay and useful to compute shoot Cu removal by seedlings, which might be an indicator of the phytoextraction potential at field scale. Beside pCu^{2+}, Mg and Ca concentrations in soil pore water (MgSPW and CaSPW) influenced the modelling of shoot DW yield (Figs. 1 and 2b line 1): DWSH=0.269+0.029 * CaSPW+0.114 * MgSPW (R^2=0.51; p value<0.0001)

The root and shoot relative indexes varied with Cu exposure and amendment incorporation (Table 6). Blocks #3 and #4 (UNT) had similar CuTOT, but OMDL enhanced the tolerance index (TI) of shoots in average by 42 % with a 17 %–59 % range, whereas TI for the roots increased only by 20 % with a 13 %–33 % range (Table 6). The RTEI confirmed that the OMDL treatment promoted plant growth, notably shoots compared to roots in block #3 (0.54 and 0.41, respectively). Plants in blocks #1 and #2 showed higher TI than in block #3, reaching about 70 % for roots and shoots, that merely reflected both a lower total soil Cu and decreased Cu exposure induced by OMDL. Similar trends were observed for other indices (Table 3). Changes in the shoot DW:root DW ratio indicated which plant part was more affected by increasing Cu exposure. In control plants, this ratio was rather low (2.36±0.97, Table 3). As total soil Cu increased, it peaked to 4.34±0.79 for block #3 plants showing that roots were more impacted, but fell down in the UNT plants (2.94±0.91) (Table 1).

Maximum stem height and leaf parameters

As free Cu ions increased in soil pore water, i.e. pCu^{2+}<7, stem elongation and thinning occurred (Fig. 3), although maximum stem height was not linearly correlated with total soil Cu. Plants from block #3 were higher than control plants (12.2±1.1 cm and 8.7±2.9 cm, respectively), but plants from blocks #1 and #2 and the control had similar stem height (in cm, 9.9, 10.5, and 8.7, respectively) (Table 3). The epicotyl length was more correlated with Cu exposure than the hypocotyl length (0.31 and −0.19, respectively, Table 4). At high Cu^{2+} concentrations in soil pore water (pCu^{2+}<6), the stem was shorter (Fig. 3) and presented a reddening (purple coloration). The shoot

Table 4 Pearson's correlation coefficients between soil and plant parameters (bivariate correlations), and effective concentration (EC_{10}, EC_{50}), obtained by linear and drc-modelling

Parameters		Soil and soil pore water								CuTOT (mg kg^{-1})		CuSPW (μg L^{-1})		pCu^{2+}	
		CuTOT	CuSPW	pCu^{2+}	Cu^{2+}	Cu ratio	pH	DOM	TOC	EC_{10}	EC_{50}	EC_{10}	EC_{50}	EC_{10}	EC_{50}
Plant	SL	0.36^{*}	0.44^{*}	-0.07^{NS}	-0.47^{**}	-0.60^{**}	0.12^{NS}	0.27^{NS}	0.40^{*}	*nm*	*nm*/745	*nm*	**608**/671	*nm*	*nm*
	HL	-0.15^{NS}	-0.11^{NS}	0.31^{NS}	-0.50^{**}	-0.53^{**}	0.32^{NS}	0.25^{NS}	0.21^{NS}	*nm*	*nm*	*nm*	*nm*	*nm*	*nm*
	EL	0.53^{**}	0.60^{**}	-0.19^{NS}	-0.36^{NS}	-0.50^{**}	-0.01^{NS}	0.28^{NS}	0.49^{**}	**912**/*nm*	*nm*	**728**/*nm*	*nm*	*nm*	*nm*
	TLA	-0.44^{*}	-0.41^{*}	0.55^{**}	-0.61^{**}	-0.54^{**}	0.56^{**}	0.14^{NS}	-0.19^{**}	**395**/*nm*	*nm*	**312**/*nm*	*nm*	*nm*/6.52	**6.93**/5.83
	LA	0.40^{*}	0.35^{NS}	-0.56^{**}	0.74^{***}	0.71^{***}	-0.60^{**}	-0.19^{NS}	0.07^{NS}	**282**/*nm*	**954**/*nm*	*nm*	*nm*	**6.7**/*nm*	**5.7**/*nm*
	LL	-0.37^{*}	-0.36^{*}	0.64^{**}	-0.73^{***}	-0.68^{**}	0.62^{**}	0.32^{NS}	0.05^{NS}	*nm*	*nm*	*nm*	*nm*	**6.5**/6.4	*nm*/5.37
	DW RT	-0.79^{***}	-0.81^{***}	0.76^{***}	-0.50^{**}	-0.35^{NS}	0.65^{**}	0.15^{NS}	-0.21^{NS}	**155**/252	**677**/739	**118**/96	**590**/420	**7.3**/7.3	**6.53**/6.50
	DW SH	-0.56^{**}	-0.51^{**}	0.62^{**}	-0.61^{**}	-0.53^{**}	0.58^{NS}	0.20^{NS}	-0.14^{NS}	**323**/*nm*	**717**/*nm*	**261**/*nm*	**607**/*nm*	**7.04**/6.86	**5.15**/5.85
	WC	0.41^{*}	0.44^{*}	-0.53^{**}	0.48^{**}	0.41^{NS}	-0.43^{*}	-0.10^{NS}	0.07^{NS}	**620**/*nm*	*nm*	**538**/*nm*	*nm*	*nm*	**6.4**/*nm*
	S/R	-0.69^{**}	-0.75^{***}	-0.60^{**}	-0.24	-0.10^{NS}	0.46^{**}	0.15^{NS}	-0.10^{NS}	**193**/428	**966**/1050	**143**/383	**718**/800	**7.28**/*nm*	**6.32**/*nm*
	ChlTOT	-0.89^{***}	-0.91^{***}	0.89^{***}	-0.58^{**}	-0.42^{NS}	0.63^{**}	0.14^{NS}	-0.17^{NS}	**138**/383	**691**/730	**104**/156	**524**/442	**7.35**/7.40	**6.67**/6.65
	Carot	-0.88^{***}	-0.91^{***}	0.89^{***}	-0.58^{**}	-0.42^{NS}	0.60^{**}	0.15^{NS}	-0.15^{NS}	**151**/380	**759**/789	**114**/160	**571**/500	**7.33**/7.55	**6.60**/6.62
	Chl*a*/Chl*b*	-0.17^{NS}	-0.13^{NS}	0.51^{**}	-0.74^{***}	-0.76^{***}	0.42^{*}	0.39^{*}	0.53^{**}	*nm*	*nm*	*nm*	*nm*	**7.29**/*nm*	**6.58**/*nm*
	CuSH	0.52^{**}	0.45^{**}	-0.81^{***}	0.98^{***}	0.93^{***}	-0.83^{***}	-0.52^{**}	-0.32^{NS}	*nm*/642	**454**/*nm*	*nm*(333)	**344**/*nm*	*nm*/6.84	**4.01**/*nm*
	CuRT	0.77^{***}	0.70^{***}	-0.93^{***}	0.86^{***}	0.75^{***}	-0.79^{***}	-0.49^{**}	-0.21^{NS}	**158**/230	**608**/773	**108**/223	**465**/*nm*	**6.8**/7.58	*nm*/6.82
	CuMM	0.79^{***}	0.76^{***}	-0.82^{***}	0.53^{**}	0.39^{*}	-0.71^{***}	-0.41^{*}	-0.14^{NS}	*nm*/219	**258**/*nm*	*nm*/128	**192**/*nm*	**7.64**/*nm*	*nm*/7.17
$R_p \geq \lvert 0.5 \rvert$		9	8	13	12	10	9	1	1						

Significance level: ^{NS} Not significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$. $R_p \geq |0.5|$ number of plant parameters correlated with each soil parameter

Bold font – modelling using the linear regression/regular font – modelling using drc-package (Knezevic et al. 2007), *nm* – not modelled

Chl*a*/Chl*b* ratio of chlorophyll *a* content /chlorophyll *b* content, *CuTOT* total soil Cu, *CuSPW* total Cu concentration in soil pore water, Cu^{2+} free copper ion concentration in soil pore water, *Cu ratio* Cu^{2+} vs. CuSPW, *DOM* dissolved organic matter in soil pore water, *DW RT* root dry weight biomass per plant, *DW SH* shoot dry weight biomass per plant, *EL* epicotyl length, *HL* hypocotyl length, *LL* leaf length, *pH* pH in soil pore water, *S/R* DW SH/DW RT, *TOC* total organic carbon in soil pore water

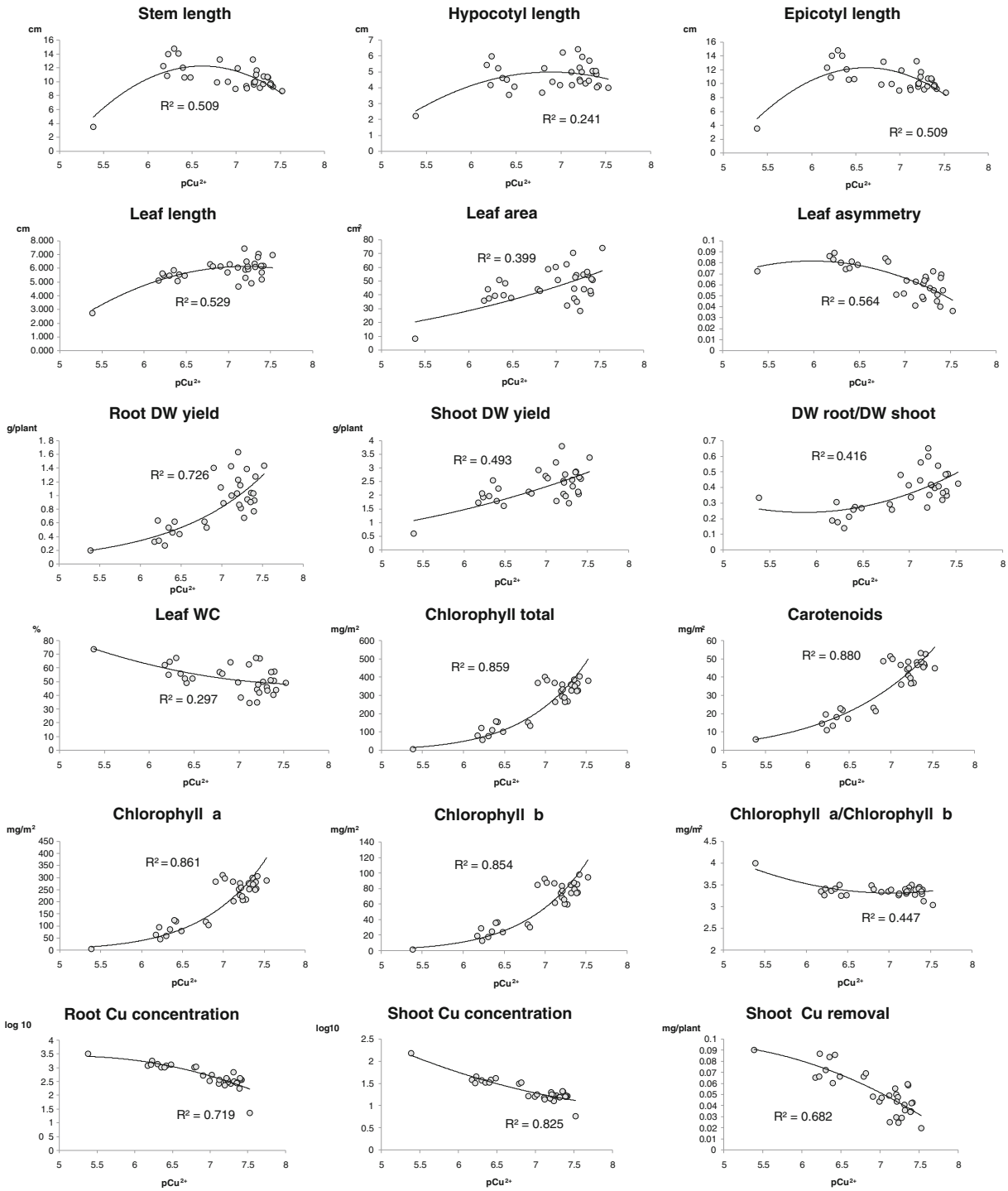

Fig. 3 Relationships between phenotypic traits of 1 month-old sunflower plants and soil Cu exposure assessed by pCu^{2+}. For shoot and root Cu concentrations, values (in mg kg^{-1} DW) were log10 -transformed

growth of plants cultivated on the UNT soil was stopped at the hypocotyl stage (3.5±1.1 cm, Table 3).

Visual symptoms of Cu phytotoxicity, i.e. wilting, foliar chlorosis, bronzing, necrosis, and leaf asymmetry,

were recorded as Cu exposure increased. Leaf length, area, and symmetry were significantly reduced as Cu^{2+} increased in the soil pore water (Table 3) and were better correlated with this parameter (−0.73, −0.61, and 0.74, respectively) than with CuTOT and CuSPW (Table 4; Figs. 1 and 2). In control plants, leaf water content (WC) remained close to 50 % (Table 3). Leaf water content slightly negatively correlated with pCu^{2+} (r=−0.53; Table 4; Fig. 1), and as Cu exposure rose, WC varied between 49 % and 57 % for plants cultivated in the OMDL soils and significantly peaked up to 74 % for the UNT plants (Table 3, Fig. 3).

ChlTOT widely ranged from 381 mg m^{-2} in control leaves to 7 mg m^{-2} in UNT leaves (Table 3). Chlorophylls significantly decreased (50 folds) more than carotenoids (7 folds) (Table 3; Fig. 3). Changes in photosynthetic pigments were highly correlated with soil Cu parameters, e.g. ChlTOT up to R=−0.91 with CuSPW (Table 4) and R=0.86 with pCu^{2+}, and well fitted by a power function (Fig. 3). They correlated with soil pH in a lesser extent, i.e. 0.63 for ChlTOT and 0.6 for Carot. The Chl *a*/Chl *b* ratio did not depend on CuTOT and CuSPW (Table 4). However, increases in Cu^{2+}concentration and the Cu^{2+}:CuSPW ratio in soil pore water greatly reduced foliar Chl *b* content (r=0.74 and 0.76, respectively, Table 4, Fig. 2).

Copper concentrations in plant tissues and effective concentrations

Mean values of root Cu concentrations ranged from 355 to 1,233 mg kg^{-1} for the OMDL plants, with 177 (plot # 20) and 1,768 mg Cu kg^{-1} (plot # 22) as minimum and maximum values, and peaked up to 3,272 mg Cu kg^{-1} for the UNT plants (Table 5). Root Cu concentration and soil Cu parameters were strongly correlated, i.e. CuTOT (r=0.77), CuSPW (r=0.70), and pCu^{2+} (r=−0.93) (Table 4). Root Cu concentration increased according to a second degree polynomial function as pCu^{2+} diminished from 7.5 to 6.2 (Fig. 3). Mean values of shoot Cu concentrations for the OMDL plants varied between 16 and 36 mg kg^{-1}, with 12.7 (plot #10) and 44.8 (plot #22) as minimum and maximum values, and were lower than for the UNT plants (154 mg kg^{-1}, Table 5). Shoot Cu concentration less depended on CuTOT (r=0.52), but well correlated with Cu^{2+} concentration in the soil pore water (r=0.98, Table 4). Shoot Cu concentration rose and well fitted a second degree polynomial function as pCu^{2+} decreased from 7.5 to 6.2 (Fig. 3). Besides usual correlations with both pH and pCu^{2+} in soil pore water (Table 4), shoot Cu concentration depended also on Mg in soil pore water (Fig. 2c line 1, CuSH=436−53.6 * pCu^{2+} + 3.98 * MgSPW (R^2=0.81, p<0.0001).

Shoot DW yield responded to shoot Cu concentration with an exponential equation ($y=2.972\ e^{-0.01x}$, r=0.7). Shoot DW yield was plotted with indicators of soil Cu exposure (Fig. 3) and the computed EC_{10} and EC_{50} values (Table 4) corresponded to 323 mg and 717 mg Cu kg^{-1} in soil. Changes in shoot DW yield for block #1 and #2 plants were also partly explained by changes in other soil parameters (i.e. pH, OM, etc.) after OMDL incorporation (data not shown). The indicators of Cu uptake (TF and BCF_{shoot}) plotted with CuTOT were well fitted by a hyperbolic equation (r=0.79 for BCF_{shoot} and 0.69 for TF) but their significance decreased over 300 mg Cu kg^{-1} soil. The BCF_{shoot} value varied between 0.04 (UNT) to 0.28 (CTRL) (Table 5). The TF value peaked in the control soil (0.26) and was the lowest in block #3 (0.029) according to increase in root Cu concentration (Table 5). The TF for Cu showed a 10-fold decrease when total soil Cu exceeded 500 mg kg^{-1}, and then remained at a steady value for higher CuTOT in the OMDL plots (Table 5). For the UNT plants, TF value increased 1.6-fold compared to block #3 plants due to higher shoot Cu concentration.

Ionome of plant parts (Table 5)

Shoot Fe concentration varied in the 48–69 mg kg^{-1} range for the control and OMDL plants but peaked to 354 mg Fe kg^{-1} in the UNT plants due to their lower shoot DW yield (Table 3). As Cu exposure rose, shoot K concentration increased, whereas root K concentration decreased, except in the UNT plants for which both were reduced likely due to K leakage from highly Cu-stressed roots. Magnesium concentrations were enhanced in both shoot and roots in relation to Cu exposure (R=0.93 for shoots). The correlation between shoot Cu and Zn concentrations (R=0.75) resulted from a cluster effect, as the composition of UNT plants opposed to other ones, and it faded without the UNT data. Shoot B and Cu concentrations were positively correlated (R=0.9) on all the pCu^{2+} range (Fig. 1). Shoot Ca concentration was lower at both low (UNT) and high (CTRL) pCu^{2+} in soil pore water (Tables 1 and 5).

Table 5 Ionome of sunflower shoots and roots, translocation factor (TF), shoot bioconcentration factor (BCF), and shoot Cu removal (CuMM)

Block #		Elements (mg kg^{-1} DW)											Cu BCF	CuMM g Cu $plant^{-1}$
		Al	B	Ca	Cu	Fe	Mg	Mn	P	K	Na	Zn		
block 1*	shoot	10**c**	40**c**	24186**a**	16**c**	63**b**	5636**b**	46**b**	2340**b**	36592**b**	1**b**	52**b**	0.051	0.039**c**
	roots	4357**B**	44**B**	7681**B**	359**C**	4455**B**	3247**BC**	125**B**	2201**B**	28017**AB**	562**B**	76**AB**	1.073	
	TF	0.002	0.918	3.149	0.045	0.014	1.736	0.370	1.063	1.306	0.002	0.677		
block 2*	shoot	19**c**	42**c**	23534**a**	17**c**	69**b**	4701**b**	45**b**	1992**b**	40947**b**	2b	45**b**	0.051	0.045**c**
	roots	3509B	21**B**	7098**B**	355**C**	3994**B**	2869**C**	118**B**	1980**B**	31506**A**	461**B**	70**AB**	1.063	
	TF	0.005	1.975	3.316	0.048	0.017	1.638	0.383	1.006	1.300	0.003	0.643		
block 3*	shoot	30**b**	56**b**	23962**a**	36**b**	58**b**	7027**ab**	47**b**	2063**b**	48072**a**	2**b**	50**b**	0.037	0.072**b**
	roots	3349B	24**B**	7433**B**	1233**B**	4209**B**	3987**B**	118**B**	1860**B**	23304**B**	592**AB**	53**B**	1.269	
	TF	0.009	2.321	3.224	0.029	0.014	1.763	0.400	1.109	2.063	0.004	0.958		
UNT**	shoot	139**a**	128**a**	28105**b**	154**a**	354**a**	8742**a**	86**a**	8588**a**	32784**c**	176**a**	188**a**	0.152	0.115**a**
	roots	11069**A**	71**A**	16661**A**	3273**A**	11517**A**	8989A	301**A**	4636**A**	9534**C**	761**A**	99**A**	3.221	
	TF	0.013	1.802	1.687	0.047	0.031	0.973	0.285	1.852	3.439	0.231	1.898		
CTRL**	shoot	18**b**	35**c**	19677**a**	6**d**	48**b**	3428**c**	22**c**	2989**b**	37841**b**	1**b**	24c	0.286	0.020**d**
	roots	1583**C**	18**B**	9063**B**	22**D**	1801**C**	2412C	46**C**	3784**A**	35915**A**	198**C**	71**AB**	1.048	
	TF	0.011	1.925	2.171	0.260	0.027	1.421	0.485	0.790	1.054	0.003	0.343		

Values are means (* n=10, ** n=3); different letters stand for statistical significance at the 0.05 level with the Tukey HSD test

Shoot Cu removal (Table 5)

Shoot Cu removal varied (in mg Cu plant^{-1}) from 0.02 (CTRL) to 0.115 (UNT). Its value was more increased in amended soils (block #3) than shoot Cu concentration (1.6 for CuSH and 4.3 folds for CuMM, respectively). Shoot Cu removal highly paralleled soil Cu parameters (e.g. pCu^{2+}) and pH in soil pore water (−0.82 and −0.71, respectively) (Table 4 and Fig. 1). As shoot DW yield, shoot Cu removal was multivariable and its modelling using only soil parameters showed a low significance (Fig. 2d). The best three-term equation included pCu^{2+}, Mg in soil pore water, and total soil Ni (NiTOT): CuMM=0.24−0.027 * pCu^{2+} + 0.0048 * MgSPW+ 0.0099 * NiTOT (r^2=0.54, p<0.001).

Discussion

Cu exposure in the soil series

Sustainable phytomanagement of Cu-contaminated soils needs to assess ecological risks before, during and following their remediation, and to evaluate the potential of new phytoremediation options. In situ and *ex situ* (in potted soils) collection of soil pore water may be a routine, cost-effective way to monitor root exposure to metals and predict phytotoxic risks (Moreno-Jimenez et al. 2011) as well as impacts on microbes (Maderova et al. 2011). Concentrations in pore waters collected in field conditions ranged between 2 and 104 μg Cu L^{-1} in uncontaminated soils and 25–27,100 μg Cu L^{-1} in contaminated soils, and at two Cu-contaminated sites, corresponding to historic neighbouring industrial legacy, Cu in pore water reached 25–47 μg L^{-1} (Merton Bank, UK) and 49–1,190 μg L^{-1} (Prescot, UK) (Moreno-Jimenez et al. 2011). In S. Fergus sandy Cu-contaminated soils (total soil Cu: 43–2,710 mg kg^{-1}; pH 5.2–6.2), Cu in pore water varied between 30 and 4,050 μg L^{-1} (Maderova et al. 2011). Data of our soil series (total soil Cu: 21–1,170 mg kg^{-1}, CuSPW 150–837 μg Cu L^{-1}, Table 1) fitted into these intervals, with wider ranges for Cu^{2+} in soil pore water and total soil Cu than for pH, TOC, and CuSPW (Table 1). The linear relationship between CuSPW and total soil Cu (Table 2) became weaker at high CuTOT, confirming previous findings (Inaba and Takenaka 2005; Kolbas et al. 2011). This may reflect the influence of other soil factors, e.g. soil pH, OM, other element contents, etc. Soils well-aged with respect to metals and organic residues are suitable for resolving questions of metal solubility dependence on OM and pH (Datta and Young 2005; Thakali et al. 2006). Here, soil Cu contamination has been built during more than 50 years, and compost has been added 2 years before our plant testing. Adding compost into the soil caused Cu complexation with OM, notably the coarse compost fraction, at the P1-3 sub-site (Lagomarsino et al. 2011), and it decreased CuSPW after 1 month into a potted Cu-contaminated soil (2,600 mg Cu kg^{-1}; pH 6.25) from the same wood preservation site (Bes and Mench 2008). Liming close to neutrality would promote such complexation and increase Cu binding to (hydr)oxides and clays with subsequently a decrease in water-soluble Cu fraction (Lagomarsino et al. 2011). This was confirmed by roughly a 10-fold decrease of Cu^{2+} in pore water when comparing OMDL-treated soils of block #3 and the UNT soil, but CuSPW was enhanced despite increased soil pH (Table 1). This suggested Cu mobilisation by DOM (Beesley and Dickinson 2011). DOM in pore waters ranged between 30 and 125 mg C L^{-1} in an unpolluted urban soil amended with a green compost and reached 25–50 mg C L^{-1} in a brownfield soil (Moreno-Jimenez et al. 2011). Composted amendments increased DOM in soil pore water to 100–300 mg C L^{-1} and co-mobilised Cu (Beesley and Dickinson 2011). Our values are similar, i.e. 39.6 (UNT) - 172 mg C L^{-1} (block #1), highest values reflecting high OM content in the CTRL soil and compost addition into the OMDL soils (Table 1). Soil OM and DOM in the soil solution react with Cu, and their complexes modify Cu solubility, chemical species, and resupply from soil solid phases (Ashworth and Alloway 2007; Temminghoff et al. 1997). Based on DGT (diffusion gradients in thin film), the replenishment capacity for Cu was reduced in the OMDL soils of block #3 although the initial solubility was higher (Pang and Puschenreiter, 2013, personal communication) suggesting OM fractions may differently affect Cu bioavailability. The stability of Cu-OM complexes and their different dissociation rates related to the ligand functional groups may regulate Cu mobility and bioavailability from soil to roots (Thakali et al. 2006; Degryse et al. 2009). Soluble low molecular weight compounds, from mineralized OM and rhizodeposition, could increase Cu solubility, whereas high molecular weight compounds (e.g. humic acids, coarse compost fraction) may sorb Cu. Dissociation rates of soluble Cu-OM complexes may

also influence Cu root uptake by Cu transporters of the COPT (COPper Transporter)/Ctr (Copper transporter) protein family (Jung et al. 2012). This may explain higher correlations of shoot DW yield and of shoot and root Cu concentrations with pCu^{2+} than CuTOT and CuSPW (Table 4).

Soil pH and OM are key-players mutually dependent for Cu in the processes of precipitation, sorption by and distribution in soil fractions, and operational mobility (Clemente et al. 2010; Maderova et al. 2011). At pH 6.6, CuSPW would be mostly in the Cu-DOM form (>99 %, Temminghoff et al. 1997). This was validated in both CTRL and OMDL soils but not in the UNT soil (Table 1). Exponential decrease of the ratio of free Cu^{2+} ions with increasing pH and DOM in the soil pore water (Table 1) likely reflected Cu binding by OM in the solid phase and DOM (Carrillo-Gonzalez et al. 2006; Luo et al. 2006; Sauvé et al. 1997). Modelling of pCu^{2+} as indicator of Cu exposure (Eq. 1 and 2) confirmed the influence of pH, OM and CuSPW as in previous models (McBride et al. 1997; Sauvé et al. 1997; Sauvé 2003).

Plant parameters

In overall, the increase of free Cu ions in soil pore water was stressing the physiology of sunflower seedlings, which influenced their morphological parameters (Table 4; Fig. 3). Oxidative stress and Cu-induced changes in chloroplast ultrastructure and nutrient homeostasis at sub-cellular level explained leaf chlorosis and necrosis (Mocquot et al. 1996; Palmer and Guerinot 2009; Smeets et al. 2013). Across the tested plant parameters, photosynthetic pigment contents were the most sensitive to excess Cu in the soil and soil pore water, e.g. EC_{10} of ChlTOT and Carot corresponded to 7.35 and 7.33 for pCu^{2+}, 104 and 114 μg Cu L^{-1} for CuSPW, and 138 and 151 mg Cu kg^{-1} soil for CuTOT, respectively (Table 4). The ratio Chl*a*:Chl*b* had also an early response (7.29 for pCu^{2+}). Total chlorophyll content and Chl*a*:Chl*b* ratio were changed in Rousos et al. (1989), but net photosynthesis and aboveground biomass were not affected. Arellano et al. (1995) and Luna et al. (1994) reported depressed growth, the breakdown of chlorophyll and carotenoids, and a reduced photosynthetic capacity at high Cu exposures. High Cu concentrations may destroy thylakoid membranes via lipid peroxidation and especially affect photosystem II (Patsikka et al. 2002; Yruela 2009). Leaf chlorosis can block the activity of ribulose, 1–5, bisphosphate carboxylase-oxygenase and CO_2 fixation and interfere at the photosystem level, inducing a higher sensitivity to photoinhibition (Cook et al. 1997; Cuypers et al. 2000; Patsikka et al. 2002). The reduction of the grana structure is consistent with the increased Chl*a*:Chl*b* ratio and may indicate that the synthesis of the photosystem cores takes metabolic preference over the synthesis of the light-harvesting complex II (Patsikka et al. 2002; Rivelli et al. 2012). A reduction of photosynthetic surface area and content of photosynthetic pigments generally decrease the intensity of photosynthesis and the carbohydrate accumulation, and finally the plant biomass, as confirmed in Table 3 and Fig. 3.

Morphological parameters, which are integrative responses, were less sensitive than physiological parameters to excess Cu based on EC values (Table 4). Changes in the root system of Cu-stressed sunflower confirmed previous reports (Bravin et al. 2010a; Lequeux et al. 2010). Root biomass was the most sensitive (Table 4 and Fig. 3), its EC_{10} value corresponding to 7.3 for pCu^{2+}, 155 mg Cu kg^{-1} soil for CuTOT and 118 μg Cu L^{-1} for CuSPW, whereas shoot yield was reduced over 323 mg Cu kg^{-1} soil (CuTOT) and 261 μg L^{-1} (CuSPW) (Table 4). This may be due to Cu retention in roots and the influence of seed reserves. The ratio (shoot DW:root DW) had also higher EC_{10} values than roots, i.e. CuTOT: 193 mg Cu kg^{-1} soil, CuSPW: 143 μg L^{-1}, and pCu^{2+} 7.28 (Table 4). The root system is plastic and excess Cu inhibits primary root growth and simultaneously stimulates lateral root formation due to changes in mineral profile, hormonal status, mitotic activity, cell membrane viability, H_2O_2 concentration, and lignin deposition (Jiang et al. 2000; Lequeux et al. 2010). Damages on roots likely reduce nutrient and water uptake, causing shoot growth reduction and changes in ionome of plant parts (Lequeux et al. 2010).

The RTEI, RPI and TI values (Table 6) confirmed that free Cu ions preferentially influence root biomass, and less shoot yield (Song et al. 2004). At similar total soil Cu, Cu in soil pore water was more bound by DOM in the OMDL soils of block #3 than in the UNT soil (Table 1), root better developed (Table 6), and root physiological activity, notably Cu-induced peroxidase activity (Mocquot et al. 1996), would be less impacted by Cu. This may partly explain, higher shoot Cu removal by sunflower at field scale after incorporation of OMDL into Cu-contaminated soils (Kolbas et al. 2011).

Moderate shoot Cu concentrations in plants of block #1 and #2 caused stem elongation and etiolation with a

Table 6 Relative Treatment Efficiency Index (RTEI), Relative Parameter Index (RPI), and Tolerance Index (TI) for root and shoot DW yields (g plant^{-1}) depending on soil treatments

Block	RTEI	RPI (%)	TI (%)
Roots			
block #1	0.70	74	79
block #2	0.67	64	69
block #3	0.41	22	33
UNT	0	0	13
Shoots			
block #1	0.61	65	71
block #2	0.63	73	78
block #3	0.54	51	59
UNT	0	0	17

hormesis effect as defined by Calabrese and Blain (2009) (EC_{50} corresponding to 671 μg L^{-1} for CuSPW). Stem reddening at pCu^{2+} below 6 suggested an increase in anthocyanins and the antioxidant status of plants (Posmyk et al. 2009). High shoot Cu concentrations contribute to disturb the mitosis (Jiang et al. 2004; Liu et al. 2009). Leaf asymmetry (LA) integrates this breakdown affecting leaf and individual development (Parsons 1992). Our data (Table 4 and Fig. 3) supported LA as a relatively early routine biomarker to detect chemically stressed plant and to assess the environment quality, even though its relationship with contaminant exposure may be influenced by other limiting factors (Kryazheva et al. 1996; Ambo-Rappe et al. 2008).

Water stress damages photosynthetic apparatus, inhibits plant photosynthesis, influences enzyme activities, and induces oxidative stress damaging proteins, membrane lipids and other cellular components (Rivelli et al. 2012; Waraich et al. 2011). In uncontrolled water regime, water content (WC) decreases in metal-stressed plants (Barcelo and Poschenrieder 1990). Depending on plant metal content and water regime, WC may be affected by reduced water movement and stomatal closure (Alaoui-Sosse et al. 2004). Excess Cu reduces the water potential and transpiration rates and enhances diffusive resistance and relative WC of cauliflower (Chatterjee and Chatterjee 2000). On the correlation map (Fig. 1), WC was rather independent of other plant parameters and its EC_{10} value corresponding to 620 mg kg^{-1} for CuTOT and 538 μg L^{-1} for CuSPW reflected a low sensitivity to Cu excess (Table 4).

Plant composition and shoot Cu removal

Concentration of Cu in plant tissues generally mirrors root Cu exposure, but the relationship depends on plant species and plant parts (Poschenrieder et al. 2001). As Cu increases in the substrate, the relative Cu concentration rises in roots, is reduced in aerial parts, and remains constant in the hypocotyl of sunflower (Forsberg et al. 2009; Lin et al. 2003). Relationships between root and shoot Cu concentrations (data log-transformed) and pCu^{2+} were well fitted by quadratic curves (Fig. 3), which agreed with Panou-Filotheou and Bosabalidis (2004) and suggested to use this sunflower mutant as bioindicator for assessing Cu exposure and phytotoxicity of Cu-contaminated soils. As sunflower cultivars and most plant species, this sunflower mutant displayed higher Cu concentrations in roots than in shoots and TF values below 1 (Table 5) (Alaoui-Sosse et al. 2004; Navari-Izzo et al. 2006; Song et al. 2004). Root Cu concentrations may however reflect Cu co-precipitation on the Fe-root plaque, incomplete desorption during the washing procedure and storage in the root ultrastructure, and must be used with caution as indicator of soil Cu exposure (Dickinson et al. 2009; Panou-Filotheou and Bosabalidis 2004). Fellet et al. (2007) reported higher TF (0.124) and BCF_{shoot} (0.19) values in sunflower than our data (Table 5), likely due to higher total soil Cu (1,589–1,943 mg Cu kg^{-1}). Elevated TF values for some elements (i.e. P, K, Mg, and Ca) in Cu-stressed plants compared with controls (Table 5) confirmed their relationships with the shoot yield, but reports suggested their role in controlling oxidative stress in plant cells (Cook et al. 1997; Thakali et al. 2006). Functional responses of

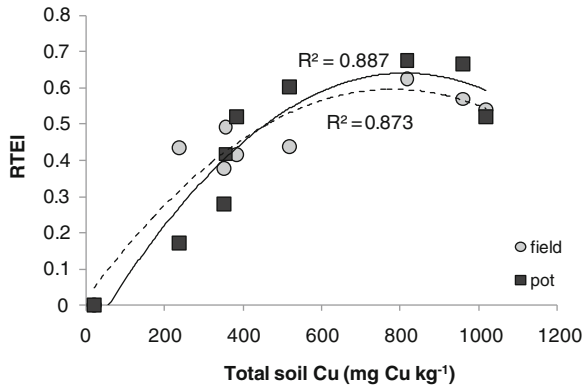

Fig. 4 Comparison of RTEI index for shoot Cu removal in field and pot experiments. (data for shoot Cu removals in the field experiment obtained in Kolbas et al. 2011)

plants to Cu exposure generally are earlier indicators than structural ones (Meers et al. 2006; Mocquot et al. 1996). This was confirmed by our results (Table 4). The EC_{10} values of the most sensitive parameters, i.e. chlorophyll and carotenoid contents (Table 4), corresponded to 16–18 mg Cu kg^{-1} DW in shoots. Such values matched with the upper critical threshold values (20–30 mg Cu kg^{-1} DW) reported in leaves and shoots of many plants (Macnicol and Beckett 1985; Mocquot et al. 1996).

Secondary metal accumulators with high biomass production for feedstock can be used for metal phytoextraction and providing financial opportunities from the biomass valorization (Mench et al. 2010; Vassilev et al. 2004). Here, similar RTEI values showed that shoot Cu removals in this bioassay predicted those obtained in the field plots in 2008–2009 (Fig. 4). This will be useful to figure potential shoot Cu removal by this sunflower mutant at field scale, even though it may depend on annual climatic conditions and further OM incorporations into the Cu-contaminated soil.

Sunflower accumulates metals mainly in roots (Madejon et al. 2003; Singh et al. 2004), even though some reports claimed its ability to translocate Pb, Cd, and Zn in aerial parts (Adesodun et al. 2010; Faessler et al. 2010). Shoot Cu and Zn concentrations were more correlated accounting for the UNT soil (Table 5), which agreed with Nehnevajova et al. (2009). The synergy between shoot B and Cu concentrations in relation with soil Cu contamination confirmed previous studies (Santra et al. 1989).

Potted plant test is a routine way to assess the phytotoxicity of contaminated soils and amendment effectiveness for improving soils (Bes and Mench 2008). Dissonances can however occur between controlled and field conditions (Bravin et al. 2010b; Kidd et al. 2009; Warne et al. 2008). For this soil series and compared to field conditions (Kolbas et al. 2011), discrepancies concerned shoot WC, shoot Cu concentration (e.g. for this sunflower grown in block #3 soils, 36 mg and 85 mg kg^{-1} in pots and plots, respectively), and soil Cu exposure leading to plant mortality, likely because water supply and evapotranspiration were not limited in pots. Exposure time and influence of environmental factors were also lower in controlled conditions.

Total soil Cu in this soil series was mainly distributed in the 21–400 mg kg^{-1} (n=20) and the 800–1,170 mg kg^{-1} ranges (n=10), with less values (n=2) in the 400–800 mg kg^{-1} one. Consequently, dose-effect relationships were a bit weakened by a clustering effect (Fig. 3), leading to a slight uncertainty for the EC_{10} and EC_{50} values of several plant parameters (e.g. SL, WC, and TLA, Table 4). An option to further improve such modeling of dose-effect relationships requested for a routine way will be to work on an aged soil series with a better distribution of soil Cu exposure, which can be obtained notably with the so-called fading method (Japenga et al. 2007).

Conclusion

A metal-tolerant sunflower mutant line was grown in pots on a Cu-contaminated soil series collected in field plots, with pCu^{2+} ranging from 5.38 to 7.52 in the soil pore water. Chlorophyll and carotenoid contents, root DW yield, and leaf asymmetry had the lowest EC_{10} values to detect an adverse effect of excess Cu. Strong relationships between phenotypic traits of sunflower and indicators of labile Cu pool in the soil such as Cu^{2+} in pore water suggested to use this mutant line as bioindicator for assessing soil Cu exposure, phytotoxicity of Cu-contaminated soils, and potential shoot Cu removal. The relevance of soil pore water to assess the phytotoxicity of Cu-contaminated soils and of seedling responses to orient their phytomanagement at field scale deserves further attention based on additional soil series.

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