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Title page

Title:

Morphological and functional responses of a metal-tolerant sunflower mutant line to a copper-contaminated soil series

Short title: Morphological and functional responses of sunflower to Cu excess

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ABSTRACT

The potential use of a metal-tolerant sunflower mutant line for biomonitoring Cu phytoavailability, Cu-induced soil phytotoxicity and Cu phytoextraction was assessed on a Cu-contaminated soil series (13-1020 mg Cu kg^{-1}) obtained by fading a sandy topsoil from a wood preservation site with a similar uncontaminated soil. Morphological and functional plant responses as well as shoot, leaf and root ionomes were measured after a 1-month pot experiment. Hypocotyl length, shoot and root dry weight (DW) yields, and leaf area gradually decreased as soil Cu exposure rose. Their dose-response curves (DRC) plotted against indicators of Cu exposure were generally well fitted by sigmoidal curves. The half-maximal effective concentration (EC_{50}) of morphological parameters ranged between 203 and 333 mg Cu kg^{-1} soil, corresponding to 290-430 µg Cu L^{-1} in the soil pore water, and 20±5 mg Cu kg^{-1} DW in the shoots. The EC_{10} for shoot Cu concentration (13-15 mg Cu kg^{-1} DW) coincided to 166 mg Cu kg^{-1} soil. Total chlorophyll content and total antioxidant capacity (TAC) were early biomarkers (EC_{10}: 23 and 51 mg Cu kg^{-1} soil). Their DRC displayed a biphasic response. Photosynthetic pigment contents, e.g. carotenoids, correlated with TAC. Ionome was changed in Cu-stressed roots, shoots and leaves. Shoot Cu removal peaked roughly at 280 µg Cu L^{-1} in the soil pore water.

Highlights:

▸ Leaf area, hypocotyl length, root and shoot dry weight yields correlated with total Cu concentration in the soil and the soil pore water.

▸ Total chlorophyll content and antioxidant capacity in the second leaf pair earlier sensed Cu excess than morphological parameters.

▸ Shoot Cu removal peaked at 280 µg Cu L^{-1} in the soil pore water.

Keywords: antioxidant capacity; biomarker; carotenoid; *Helianthus annuus* L.; phytotoxicity; phytoremediation

Abbreviations: ABTS: 2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate), AOA: antioxidant activity, APX: ascorbate peroxidase, AsA: ascorbate, Carot: carotenoid content, CEC: cation exchange capacity, Chl *a*: chlorophyll a content, Chl *b*: chlorophyll b content, ChlTOT: total chlorophyll content, CuSH: shoot Cu concentration, CuSPW: total Cu concentration in the soil pore water, CuTOT: total soil Cu, DHAR: dehydroascorbate reductase, DMA: dimethylamine, DMF: N,N-dimethylformamide, DPPH: 2,2-diphenyl-1-picrylhydrazyl, DRC: dose-response curve, DW SH: shoot dry weight yield, DW RT: root dry weight yield, EC: effective concentration, EL: epicotyl length, FW: fresh weight, FRAP: ferric reducing antioxidant potential, GR: glutathione reductase, GSH: reduced glutathione, GSSG: oxidized glutathione, HL: hypocotyl length, IRT: iron-regulated transporter, MDHAR: monodehydroascorbate reductase, NA: nicotianamine, OM: organic matter, ORAC: oxygen radical absorption capacity, ROS: reactive oxygen species, SL: stem length, SOD: superoxide dismutase, S/R: shoot dry weight yield: root dry weight yield ratio, TAC: total antioxidant capacity, TE: trace element, TEAC: Trolox equivalent antioxidant capacity, TF: translocation factor, TLA: total leaf area, ZIP: zinc- and iron-regulated transporter

1. INTRODUCTION

Copper is a pivotal micronutrient for plants through numerous metabolic processes, e.g. electron transfer reactions of respiration (cytochrome c oxidase, alternate oxidase) and photosynthesis (plastocyanin), detoxification of superoxide radicals (Cu-Zn superoxide dismutase), lignification of plant cell walls (laccases), perception of the plant hormone ethylene, carbohydrate metabolism, and phenolic compound production in response to pathogens (Hänsch and Mendel 2009; Palmer and Guerinot 2009; Shi et al. 2011; Ravet and Pilon 2013). Plants regulate Cu homeostasis by controlling its uptake through the expression and stability of Cu transporters, e.g. COPper Transporter family (COPT) (Yuan et al. 2011; Hötzer et al. 2012; Peñarrubia et al. 2015). At higher impregnation than the cellular Cu homeostasis (5-20 µg Cu g^{-1} DW), Cu excess can impact many physiological processes and induce toxicity symptoms (e.g. biomass reduction, root growth inhibition, bronzing, chlorosis, Fe, Zn and P uptake reduction, chloroplast integrity loss, etc.) (Yruela 2009; Marschner 2011). One driver of Cu toxicity is its contribution to reactive oxygen species (ROS) formation likely through Fenton and Haber-Weiss reaction (Sharma and Dietz 2009; Nehnevajova et al. 2012). ROS can peroxide lipids and oxide proteins and guanine (Verdoni et al. 2001; Mendoza-Soto et al. 2012).

To better assess the phytotoxicity of Cu-contaminated soils, chemical indicators of soil Cu exposure must be complemented with plant assays combining morphological endpoints and biomarkers (Vangronsveld and Clijster 1994; Lequeux et al. 2010; Mocquot et al. 1996; Verdoni et al. 2001; Meers et al. 2006). Biochemical biomarkers such as antioxidant responses are more sensitive to metal excess in such plant tests (Mocquot et al. 1996; Hartley-Whitaker et al. 2001; Meers et al. 2006; Qi et al. 2006; Lyubenova et al. 2009; Korpe and Aras 2011; Nehnevajova et al. 2012). However, the Cu concentration in plant parts may show contradictory results depending on organ and plant species (Cuypers et al. 2000, 2002; Boojar and Goodarzi 2007; Thounaojam et al. 2012). Several methods are used to estimate total antioxidant capacities in plant extracts, e.g. DPPH, TEAC, FRAP, and ORAC assays (Prior et al., 2005; MacDonald-Wicks et al. 2006; Dudonne et al. 2009). One relevant method is TEAC, which uses the $ABTS^{+\bullet}$ radical scavenging capacity (Re et al., 1999; Dudonne et al., 2009). However its potential to assess in a routine way the phytotoxicity of metal-contaminated soils, and notably the Cu-contaminated ones under phytomanagement, with various plant species, is not investigated.

Sunflower (*Helianthus annuus* L.) is a promising candidate to phytomanage Cu-contaminated soils (Mench et al. 2010; Kolbas et al. 2011; Herzig et al. 2014; Kidd et al. 2015). On one hand, morphological and physiological traits of young and adult sunflower plants are responding to Cu excess allowing its use for biomonitoring (Lin et al. 2003; Madejon et al. 2003; Kolbas et al. 2011; 2014; Nehnevajova et al. 2012). On the other hand, several sunflower mutant lines obtained by chemical mutagenesis phytoextract more metals (Cu, Zn, Cd, and Pb) than their mother lines in field conditions (Nehnevajova et al., 2009; Kolbas et al. 2011; Herzig et al. 2014), and show an increased antioxidant status at high metal

exposure (Nehnevajova et al. 2012). The use of such metal-tolerant sunflowers to improve plant assays and to appraise phytoextraction option for Cu-contaminated soils was explored (Kolbas et al. 2014). In a previous Fluvisol series, soil Cu contamination however was imposed by its spatial variability across the sampled field plots. Consequently, total Cu concentrations in the soil and soil pore water corresponding to effective concentrations (EC_{10}, EC_{50}) for morphological parameters of a sunflower mutant line exposed to Cu excess were not accurately determined. Moreover biochemical endpoints such as TAC were not considered. This pot experiment aimed at appraising the morphological and functional responses of the same metal-tolerant sunflower mutant on a soil series obtained by mixing a sandy Cu-contaminated soil from a wood preservation site and an uncontaminated soil of the same type in various proportions, i.e. the fading technique, allowing a steady increase in soil Cu exposure. Such soil series, more realistic than hydroponics and spiked soils, is generally useful to gain dose-effect relationships for determining both upper critical metal(loid) concentrations for plant parameters and related effective metal(loid) concentrations in the soil and soil pore water (Verdoni et al. 2001; Japenga et al. 2007; Marchand et al. 2016). The following questions were addressed:

(1) How the morphological and functional parameters of a metal-tolerant sunflower mutant are changing across a soil series with a steady increase in Cu exposure? Which sunflower parameters are more relevant to assess the phytotoxicity of Cu-contaminated soils?

(2) What are the earlier Cu effective concentrations, i.e. EC_{10} and EC_{50}, for these plant parameters and the corresponding values for soil Cu exposure?

(3) What is the potential shoot Cu removal of this sunflower mutant depending on soil Cu exposure?

2. MATERIALS AND METHODS

2.1 Soil series

The sandy coarse Cu-contaminated soil (UNT soil, Fluvisol – Eutric Gleysols, World Reference Base for soil resources, 1020 mg Cu kg^{-1} soil) was sampled in the 0-25 cm soil layer at the P1-3 sub-site (plot #31, Kolbas et al. 2011) of a wood preservation site, Saint Médard d'Eyrans SW France (Tab. 1). The uncontaminated soil (CTRL soil, 13 mg Cu kg^{-1}), with the same soil type, was sampled (0-25 cm) in a field plot cultivated with maize at the Couhins INRA experimental farm, located 18 km from the contaminated site (Villenave d'Ornon, Gironde, France). Both soils were sieved at 5 mm and air-dried. The soil series was made by carefully mixing (run-over-run) the UNT soil with the CTRL soil in a ratio from 0:100% to 100:0% with a 10% step (Tab. 2). Soil samples (1 kg DW) were placed in plastic pots (1.3 L) to consist 11 treatments (in quadruple) labeled from C_0 to C_{100}.

One Rhizon MOM moisture sampler (Eijkelkamp, The Netherlands) was inserted with a 45^o angle into each potted soil. Soils were watered with deionised water, maintained daily at 70% of field capacity (10% of air-dried soil mass), and allowed to react, notably regarding the microbial communities, for one month prior sowing. For all soils, soil pore waters (10 mL) were collected three times with a week interval and kept at 4°C prior to analysis to make a 30-mL sample. The pH in pore water was measured (Hanna instruments, pH 210, combined electrode Ag/AgCl - 34) and elements were analyzed by ICP-AES (Varian Liberty 200).

2.2 Plant growth and morphological parameters

Sunflower seeds of Mutant 1 line [M6 (6^{th} generation), 1/67-35-190-04] obtained by chemical mutagenesis using ethyl methane sulfonate (Nehnevajova et al. 2009), showing the best results in previous phytoextraction field experiments (Lyubenova et al. 2009; Kolbas et al. 2011), was used. Sunflowers were sowed in each potted soil (n=4) 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, and 65% relative humidity (ISO 2012). Pots were arranged in a fully randomized block design and watered daily with deionized water (50% water holding capacity of soil). The soils were fertilized twice, i.e. just before the start of plant cultures and two weeks after, with a modified Hoagland n°2 nutrient solution supplying no Fe and other trace metals (Hewitt 1966; Kolbas et al. 2014). The experiments were carried out in two batches: one to study the morphological parameters and the ionomes of plant parts, and the second to investigate the functional plant parameters.

Sunflower plants were collected after 1 month, at growth stage B3/B4 when the 2^{nd}-pair leaves reached 4-cm length (Terres Inovia 2017). Shoots and roots were collected for each plant (roots being firstly carefully washed with tap water, then rinsed with distilled water, and blotted on filter paper), weighted (FW), and visible symptoms were recorded. Fresh weight biomasses and morphological parameters were measured, i.e. SL, HL, EL, and TLA (scanner EPSON Expression 10000 XL, software WINFOLIA). Plant parts thereafter

were rinsed in distilled water, oven-dried at 50°C for 48h, and shoot and root DW yields and water content were determined.

The Chl *a*, Chl *b*, Chl TOT and total carotenoids were extracted with DMF from the 2nd pair leaves (L2) and their foliar contents were computed from measurements of the extracts at 470, 647 and 664.5 nm (spectrophotometer CARY 100 Scan, Lagriffoul et al. 1998).

2.3 Trolox equivalent antioxidant capacity (TEAC)

The antioxidant capacity of plant extracts was determined using the ABTS radical cation ($ABTS^{+\bullet}$) decolorization assay modified by Ozgen et al. (2006), which is based on the reduction of ABTS radicals by extractable antioxidants of plant samples.

2.3.1 Chemicals

All reagents, i.e. 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), PBS (phosphate-buffered saline) (pH=7.4), potassium persulfate, sodium acetate trihydrate, and acetic acid (glacial) were purchased from Sigma-Aldrich (France).

2.3.2 Sample preparation

Fresh weighed aliquots (0.2 g) of L2 leaves were sampled at noon and immediately ground, using a benchtop homogenizer (Polytron PT 35/4.00, Kinematica GMBH, Luzern - Switzerland) in 4 mL PBS. The homogenate was left at 4C° for 10 min, then centrifuged (12000 g) for 30 min at 4°C, and the supernatant was used for measurements.

2.3.3 Spectrophotometric analysis

The stock solution of ABTS radical cation was produced by dissolving 75 mg ABTS and 12 mg of potassium persulfate in distilled water in 20 mL flask and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The $ABTS^{+\bullet}$ working solution was diluted in PBS (pH=7.4) to an absorbance of 0.7±0.02 at 734 nm (spectrophotometer CARY 100 Scan). Its absorbance ($A_{control}$) was controlled before the measurement of plant samples. 100 µL of plant extract (supernatant) were mixed with 3 mL of $ABTS^{+\bullet}$ solution, and the sample absorbance (A_{sample}) was read after 10 min at 734 nm. All solutions were daily prepared and used. All determinations were carried out in triplicate. The antioxidant capacity in plant samples was expressed in relative (a) and absolute (b) values:

(a) the scavenging inhibition capacity of ABTS·in the extract, expressed in percent, was computed from the following equation: % inhibition = $[(A_{control}-A_{sample})]/(A_{control})]*100$;

(b) a calibration curve was determined using Trolox (1.25-250 µg mL^{-1}), and the total antioxidant capacity (TAC) in plant samples was quantitatively expressed in µg Trolox equivalent mL^{-1} leaf extract and in µg Trolox equivalent g leaf FW^{-1}.

2.4 Ionome of plant tissues

Plant samples were ground in a titanium mill (Retsch MM200), and weighed aliquots (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 Inter-Professionnel 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%. The translocation factor (TF) was calculated on the basis of foliar and root element concentrations: $TF = C_{leaves} / C_{root}$

2.5 Statistical analysis

A principal component analysis (PCA) was performed for plant parameters and indicators of soil Cu exposure. The degree of co-linearity of soil properties was determined using the Pearson correlation coefficient test. One-way analysis of variance (ANOVA test) was performed to evaluate differences in soil and plant parameters across the soil series. Normality and homoscedasticity of residuals were met for all tests. Post-hoc Tukey's HSD tests were used to assess mean multi-comparison. Differences were considered significant for p-value <0.05. Mean values followed by the same letter are not different at the 5% level (SNK test using foreign and agricolae packages). A PCA and one-way ANOVA were carried out for the ionome of plant parts. Pearson correlation coefficients between plant parameters, foliar ionomes, and total Cu in soil and soil pore water were determined.

The DRC - package was used for modeling the dose-response curves between plant parameters and indicators of Cu exposure (Knezevic et al. 2007). A symmetric model was used for the DW SH, DW RT, HL and TLA parameters, with a four-parameter log-logistic equation: **Y = c + (d-c/1+exp[b(log X-log E)])**

where, Y is the response variable, c is the lower limit, d is the upper limit, X is the Cu dose (e.g. total soil Cu), E is the Cu dose required for 50% response (e.g., an effective concentration, EC_{50}, required to halve the shoot biomass), and b is the slope of each curve.

A hormesis model was used for SL, TAC, and photosynthetic pigment contents, with the addition of term f in the numerator, but in that case, the effective concentrations could not be determined on the whole DRC:

Y = c + (d+fX-c/1+exp[b(log X-log E)])

The effective concentrations at 10, 50 and 90% levels (EC_{10}, EC_{50}, and EC_{90}) were computed. All statistical analyses were performed using R software (version 2.14.1, R Foundation for Statistical Computing, Vienna, Austria).

3. RESULTS AND DISCUSSION

3.1 Soil parameters and Cu exposure

Total soil Cu varied between 13 and 1020 mg Cu kg^{-1} (Tab. 2) and its values were better distributed than in the previous soil series sampled in field plots (Kolbas et al. 2014). In the soil pore water, the pH values ranged from 5.93 to 7.15 and total Cu increased from 0.19 to 0.80 mg L^{-1} (2.99-12.6 µM Cu). Total Cu in the soil and the soil pore water were correlated ($r^2 = 0.6$). Similar ranges (in mg Cu L^{-1}) were reported in the soil pore water of topsoils from the same wood preservation site, i.e. 0.37-1.78 (Bes et al. 2010), 0.13-0.92 (Kolbas et al. 2014), and 0.22-0.68 (Oustrière et al. 2017) with influences of soil pH, CuTOT and dissolved organic matter (DOM). Total Cu in soil pore water can vary from 0.009 to 16.8 mg L^{-1}, the percentage of free Cu^{2+} in total soluble Cu ranging from 0.02 to 96% depending on pore water pH and, to a lesser extent, on dissolved organic C (Vulkan et al. 2000). Total Cu in pore waters collected in field conditions can range between 2 - 104 µg Cu L^{-1} (0.03-1.64 µM) in uncontaminated soils and between 25 - 27,100 µg Cu L^{-1} (0.39-426 µM) in contaminated soils (Moreno-Jimenez et al. 2011).

3.2 Plant parameters

The PCA graph for the plant parameters and indicators of soil Cu exposure roughly explained 77% of the total variance (axis #1 – 57.81%, axis #2 – 19.15%) and showed several parameter groups (Fig. 1a and b). Morphological and functional parameters were separated by both axes (Fig. 1c and d; Tab. S1). Axis #1 corresponded to changes in Cu concentrations in the soil, the soil pore water and shoots, which opposed to most morphological parameters (Fig. 1, 2a, b and d). Axis #2 mainly matched with biochemical parameters and SL (Fig. 1b, 2c, e and f). The S/R ratio, showing the biomass allocation, was related to Cu concentrations in soil and plant parts, but its significance in PCA was low, as well as those of relative water content and TAC which were more correlated with photosynthetic pigment contents. The SL was located at an independent median position. In contrast, the HL negatively correlated with Cu exposure and positively with other morphological parameters. Correlation analysis (Fig. 1b) and one-way ANOVA on the axis #1 and #2 coordinates of PCA showed a sigmoid curve and three soil groups, i.e. C_0 to C_{20}, C_{30}, C_{40} to C_{100} for the axis #1 (Fig. 1c). The axis #2 was characterized by more complex patterns and included four soil groups: (1) C_0, C_{40} and C_{50}; (2) C_{10}, C_{20} and C_{80}; (3) C_{30}, C_{60} and C_{70}; and (4) C_{90} and C_{100} (Fig. 1d).

3.2.1 Morphological responses

The CuSPW and CuTOT strongly correlated with the majority of morphological parameters, i.e. TLA, HL, DW SH, DW RT and their ratio (S/R), except for SL and EL (Tab. S3). As Cu exposure increased, the values of these parameters were gradually reduced (e.g. DW SH, Fig. 2a). Decreases in shoot and root DW yields, and leaf area well fitted a symmetric sigmoidal DRC (Fig. 2), demonstrating that a linear response of such parameters to soil Cu exposure is not relevant for this metal-tolerant sunflower mutant line (Markert et al.

1997). The S/R ratio strongly depended on Cu exposure (R=0.89; Tab. S3), confirming previous findings (Kolbas et al. 2014).

Effective concentrations are listed in Tab. 3. Roots were earlier impacted by Cu excess than shoots, i.e. EC_{50} for root DW yield was 203 mg Cu kg^{-1} soil (290 µg Cu L^{-1} in soil pore water) whereas EC_{50} for shoot biomass was 333 mg Cu kg^{-1} soil (432 µg L^{-1} in soil pore water). The curve slope decreased more sharply for roots than for shoots (Fig. 2a and b). This was explained by preferential Cu accumulation in sunflower roots (see below), in agreement with Lin et al. (2003), Jones et al. (2016) and Cicatelli et al. (2017). In comparison, using the shoot length of *Lolium perenne* L., EC_{10}, EC_{25} and EC_{50} of total soil Cu (in mg kg^{-1}) as phytotoxic Cu thresholds were 327, 735 and 1144 (Verdejo et al. 2015). Root and shoot growth reductions were likely due to (1) Cu-induced oxidative stress and its consequences and (2) changes in cellular nutrient homeostasis related to reduced nutrient uptakes by damaged roots (Mocquot et al. 1996; Cuypers et al. 2002; Nehnevajova et al. 2012; Luo et al. 2016; Rizwan et al. 2016). Copper is acquired by roots from the soil via high-affinity Cu+ transporters of the COPT family of transmembrane proteins, which are pivotal to maintain Cu homeostasis, while endogenous Fe, Mn, and Zn concentrations in plants may influence their expression (Yuan et al. 2011). An enzyme cascade, e.g. ascorbate peroxidase, glutathione reductase, catalase, superoxide dismutase, glutathione S-transferase, glutathione peroxidase, dehydroascorbate reductase, glutathione reductase, glyoxalase I and glyoxalase II, act in synergy for efficient protection against ROS-damage in addition to detoxification, chelation and compartmentation of Cu excess (Hossain et al. 2012; Luo et al. 2016). Copper storage in roots likely limits Cu export through the symplast, xylem loading and long-distance transport with chelators, such as nicotianamine (Burkhead et al., 2009).

Several morphological parameters had significant changes but their use in phytoindication required non-linear models: e.g. SL increased at relatively low Cu exposure and peaked at C_{50} (416 mg Cu kg^{-1} soil, Fig. 2c) whereas shoot and root DW yields were halved; this may reflect a re-allocation of resources and the stem role as a conducting element rather than a sink for Cu accumulation. The less sensitive morphological parameters were HL (Fig. 2d) and SL as its EC_{10} was 355 mg Cu kg^{-1} soil (Tab. 3, Fig. 2c). The particular response of the hypocotyl to Cu excess confirmed previous findings (Lin et al. 2003).

3.2.2 Functional responses

3.2.2.1. Photosynthetic pigment contents

Cu excess induces changes in pigment compositions and ultrastructure of chloroplast, decreases net photosynthesis rate, reduces ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) efficiency, and affects electron transport and PSII activities (Saglam et al. 2016). Total chlorophyll content in sunflower leaves varied from 70 mg to 384 mg m^{-2} (Fig. 2e). The carotenoid content ranged between 6.2 mg and 41.2 mg m^{-2} and Chl*a*/Chl*b* ratio between 1.3 and 2.9. The contents of photosynthetic pigments followed a complex curve with two peaks corresponding to 214 and 1020 mg Cu kg^{-1} (Fig. 2e). As total soil Cu increased in the 100-200

mg Cu kg^{-1} range, the chlorophyll and carotenoid contents rose. This increase in photosynthetic pigments may balance the reduced photosynthesis, notably the potential Cu-induced reduction in CO_2 fixation through decrease in RUBISCO activity and/or content (Saglam et al. 2016). In this interval of soil Cu contamination, foliar Cu concentration (13-21 mg kg^{-1}, Tab. 4) remained just below its upper critical threshold for sunflower (Tab. S2). The potential damages caused by this slight increase in Cu exposure may be quenched by the antioxidant defenses, as suggested by the TAC peak (Fig. 2f). In the 315-517 mg Cu kg^{-1} soil range, corresponding to 20-40 mg Cu kg^{-1} in the leaves (Tab. 4), both shoot DW yield and total chlorophyll content decreased (Fig. 2) and interveinal chlorosis developed especially on young leaves, likely due to impacts on chlorophyll biosynthesis, decrease in foliar Mg and Fe concentrations (Tab. 4) and ultrastructural changes in chloroplasts (Singh et al. 2004; Feigl et al. 2015; Saglam et al. 2016). Excess Cu in shoots can lead to lipid peroxidation, reduce network of thylakoid membranes in the chloroplast and predispose photosystem II to photoinhibition by outcompeting iron (Pätsikkä et al. 2002; Yruela 2009). In the chlorophyll biosynthesis, Cu excess can affect the protochlorophyllide reductase and Mg-chelatase activities (Liotenberg et al. 2015). Magnesium activates more than 300 enzymes and is involved in many physiological processes during plant growth, including its function as central atom of chlorophyll and in protoporphyrin IX Mg-chelatase, S-adenosyl-L-methionine:Mg-protoporphyrin IX methyltransferase, and Mg-protoporphyrin IX monomethyl ester oxidative cyclase (Guo et al. 2016). Cu in excess may substitute to Mg in chlorophyll molecules, thus reducing photosynthesis (Küpper et al. 2003; Gerola et al. 2011), and may displace Mg required for chlorophyll biosynthesis. Here, foliar Cu concentrations were significantly negatively correlated with foliar Mg and Mn concentrations (Tab. 4 and S3), however both remained in their range for optimal plant growth in vegetative parts, i.e. 1.5–3.5 g Mg and 10-30 mg Mn kg^{-1} (Marschner 2011; Guo et al. 2016).

The content of chlorophyllous pigments well correlated with foliar Fe concentration (R=0.70-0.78, Tab. S3). Foliar chlorosis caused by Cu/Fe antagonism in Cu-stressed plants is documented (Pätsikka et al. 2002; Yruela 2009). Here, foliar Fe concentrations decreased from 56 to 29 mg kg^{-1} as foliar Cu concentration increased from 10 to 40 mg kg^{-1} (Tab. 4) but both parameters did not correlate across the whole soil series (Tab. S3) as foliar Fe concentration increased after 718 mg Cu kg^{-1} soil because the shoot biomass, including the stem and hypocotyl, fell down (Fig. 2a).

Over 617 mg Cu kg^{-1} soil and 40 mg Cu kg^{-1} in the leaves (Tab. 4), total chlorophyll content increased again (Fig. 2e) and foliar Fe concentration as well (Tab. 4). This symptom, so-called “leaf bronzing”, would have two reasons. The first is the decreased leaf size and simultaneously the increase in cell number per leaf surface unit. Here, the linear increase in chlorophyll content at high Cu exposure (Fig. 2e) matched with the decrease in shoot biomass (Fig. 2a). While the foliar content of chlorophyllous pigments increased, their total foliar amounts (TLA × ChlTOT) remained the same, confirming previous findings (Gruber et al. 2009). The second reason would be the increased concentrations of atypical pigments and

cellular metabolites, such as Cu-chlorophyll, anthocyanins, and carotenoids, followed by the inhibition of cell expansion by Cu (Kato and Shimizu 1987; Gerola et al. 2011). The restructured Cu-chlorophylls are unsuitable for photosynthesis, have antioxidant function, and senescent plants remain dark green (Küpper and Kroneck 2005). This sunflower mutant line can produce a higher carotenoid amount than other cultivars when grown on metal-contaminated soils (Nehnevajova et al. 2012), having a potential advantage against metal-induced oxidative stress. The direction of structural adaptations in photosynthetic leaf apparatus depends on plant species and the Cu level. Oregano plants exposed to 0.3-25 µM Cu g^{-1} soil showed a linear decrease in chlorophyll content and a compensatory mesophyll thickening (Panou-Filotheou et al. 2001). For sunflower, such responses were only observed in the 214-517 mg Cu kg^{-1} soil range (Fig. 2e) corresponding to 20-40 mg Cu kg^{-1} in the leaves. Increase in Chl*a*/Chl*b* ratio may indicate the reduction of the grana structure (Küpper et al. 2003) when the synthesis of the photosystem cores takes metabolic preference over the synthesis of the light-harvesting complex II (Pätsikkä et al. 2002; Mijovilovich et al. 2009). Here, the Chl*a*/Chl*b* ratio significantly negatively correlated with foliar Cu concentration (Tab. S3).

b. Antioxidant plant responses

Leaf TAC (in µg Trolox equivalent g^{-1} leaf FW) increased three times from 50 in C_0 plants to 155 in C_{10} plants, and then, gradually fell down for C_{50} plants growing at 517 mg Cu kg^{-1} soil (Fig. 2f). Over 617 mg Cu kg^{-1} soil (i.e. 40 mg Cu kg^{-1} in leaves), leaf TAC slightly increased again as ChlTOT and varied in the 80-100 range (Fig. 2f). Such complex response to increasing soil Cu exposure may reflect the combination of various factors, i.e. increase in CuSPW, the development of oxidative stress, reduction in shoot biomass (as for photosynthetic pigments) and carbohydrate synthesis (Marschner 2011). Leaf TAC displayed a similar DRC and well correlated with chlorophyll (r=0.72) and carotenoid (r=0.80) contents (Tab. S3), raising questions about their interaction with antioxidant responses for this sunflower mutant (Nehnevajova et al. 2012). The leaf TAC was more sensitive than the chlorophyll content (EC_{10}: 23 and 51 mg Cu kg^{-1}, respectively; Tab. 3), confirming previous findings (Sun et al. 2010). Due to its DRC (inverted U-shape), leaf TAC was not correlated to the indicators of soil Cu exposure (e.g. CuTOT and CuSPW) and CuSH on the whole soil series (Tab. S3). For characterizing soil Cu phytotoxicity, use of leaf TAC as endpoint must be combined with either the shoot or foliar Cu concentrations (Fig. 2f, Tab. 4).

Copper excess in plants generally increases the activity of antioxidant enzymes (e.g. GPOD, APX, CAT and SOD), notably in roots, and changes the concentrations, redox status and cellular compartmentation of related metabolites (e.g. GSH/GSSG, AsA/DMA) and enzyme activities (DHAR, MDHAR, and GR) involved in the ascorbate–glutathione cycle, which may affect cellular homeostasis and redox potential (Mocquot et al. 1996; Cuypers et al. 2000; Nehnevajova et al. 2012; Thounaojam et al. 2012; Peñarrubia et al. 2015). Decrease

in leaf TAC in sunflowers between 214 and 517 mg Cu kg^{-1} soil may reflect a progressive decline in antioxidant enzymatic system, AsA and GSH concentrations, and reducing power in cells as Cu-induced chlorosis developed and damages to the PSII reaction centre and electron transport increased (Thomas et al. 2013). Copper excess can inhibit the activity of certain antioxidant enzymes, e.g. SOD, by replacing another element such as Fe and Mn in their active site but also by changing their structure (Perry et al. 2010).

3.2.3 Shoot, leaf and root ionomes and mineral masses

Cu: Shoot Cu concentrations (CuSH, in mg Cu kg^{-1} DW) progressively increased from 7.3 ±1.2 for C_0 plants to 49.8 ± 12 for C_{70} plants (corresponding to 718 Cu kg^{-1} in soil and 480 µg Cu L^{-1} in soil pore water) and thereafter levelled off at higher soil Cu exposures (Fig. 2g), which likely reflected impacts on roots. CuSH strongly correlated with total Cu in soil and soil pore water (0.73 and 0.83, respectively; Tab. S3), confirming previous findings (Kolbas et al. 2011, 2014; Nehnevajova et al. 2012; Rivelli et al. 2012). Foliar Cu concentrations (mg Cu kg^{-1} DW) ranged from 10 to 55.4 (Tab. 4).

Root Cu concentrations were higher than shoot and foliar Cu concentrations (Fig. 2, Tab. 4). Root Cu concentration can indicate soil Cu phytoavailability in controlled conditions (Chaignon et al. 2003), but this endpoint often has shortcomings for characterizing soil phytotoxicity due to the iron plaque trapping metals on root surface and root contamination with substrate particles unremoved after washing. Therefore, shoot and foliar Cu concentrations are more use to determine upper critical threshold values (Macnicol and Beckett 1985; Verdejo et al. 2015; Tab. S2).

Plants in uncontaminated conditions require 5 - 20 mg Cu kg^{-1} DW in the shoots and 6 - 100 mg Cu kg^{-1} DW in the roots depending on the species (Kabata-Pendias and Pendias 2011; Marschner 2011). Upper critical threshold values for shoot and root Cu concentrations of several plant species grown in hydroponics, pot and field conditions are presented in Tab. S2. Maximum upper critical threshold value reported for foliar Cu concentration in sunflower is 70 mg kg^{-1} but in hydroponics for plantlets (Lin et al. 2003), while it reached 36 mg kg^{-1} in shoots for 1-month sunflower plants grown in potted soils from field plots (Kolbas et al. 2014, Tab. S2). Both values framed the plateau reached by the shoot Cu concentration in our experiment, which is usually lower than foliar Cu concentration due to the dilution caused by the lower Cu concentration of stem biomass. For the soil series sampled in field plots, CuTOT varied between 21 and 1170 mg kg^{-1}, CuSPW ranged between 0.22 and 0.76 mg L^{-1}, and shoot Cu concentration was in the 6-36 mg Cu kg^{-1} range (Kolbas et al. 2014).

Based on decrease in shoot DW yield (Fig. 2a), the EC_{10} for shoot Cu concentration was in the 13-15 mg Cu kg^{-1} DW range. The EC_{50} often used to characterize Cu-tolerance reached 25 mg Cu kg^{-1} DW (i.e. 0.46 mg Cu L^{-1} in the soil pore water), which matched with previous findings for *H. annuus* (Rivelli et al. 2012), *Zea mays* (Mocquot et al. 1996), *Lactuca sativa* (Verdejo et al. 2016) and *Chloris gayana* (Sheldon and Menzies 2005) (Tab. S2). Such half maximal effective concentrations in plant tissues and soil pore water can be

used for investigating in a routine way the soil phytotoxicity in initial and residual risk assessments and the effectiveness of remediation options and amendments for Cu-contaminated soils (Ali et al. 2002; Kopittke et al. 2009; Verdejo et al. 2016).

Sunflower generally accumulates Cu mainly in roots, with a relatively low root-to-shoot Cu translocation (Madejon et al. 2003; Kolbas et al. 2014). Here, differences between accumulation capacities of roots and leaves progressively increased as Cu excess rose, which significantly reduced the translocation factor (TF) from 0.095 for the C_0 plants to 0.028 for the C_{90} plants (Tab. 4). The excluder strategy of this sunflower mutant line is confirmed. Roots can fix Cu on the epidermal Fe plaque and cell walls, bound to pectins and glycoproteins, and manage Cu absorption and translocation by activating efflux pumps and COPT Cu transporters (Batty et al. 2000; Boojar and Goodarzi 2007; Pilon 2011). Other mechanisms promote detoxification and sequestration in root cells, mostly in the primary cortex, from the parenchymal cells to endodermal barrier, and the xylem parenchyma, e.g. production of Cu-complexing compounds, which can be divided into two main groups: metallothionein-like compounds and phytochelatins and other thiols (Sanchez-Pardo et al. 2014; Ravet and Pilon, 2013; Printz et al. 2016).

Changes in plant biomass and tissues Cu concentration across the soil series can influence the mineral mass of elements in the shoots (e.g. here referred to shoot Cu removal as the product of DW SH × CuSH). For this sunflower, shoot Cu removal peaked up to 200 µg Cu $plant^{-1}$ between 114 - 416 mg Cu kg^{-1} soil, and then decreased at higher Cu exposures to reach a plateau around 100-120 µg Cu $plant^{-1}$ (Fig. 2h).

Other elements

Copper excess led to imbalances in foliar nutrient concentrations, their intensity depending on Cu exposure (Tab. 4; Fig. S1). Foliar Ca, Mg, Mn and Zn concentrations were highly related to foliar Cu concentration, total soil Cu, and Cu in pore water (Tab. 4; Tab. S3). Foliar Ca concentration was positively correlated with foliar Cu concentration (Tab. S3). The reverse occurred for Mg, Mn and Zn, despite increase in their total soil content across the soil series (Tab. 1). This may affect enzyme activities having Mg, Mn or Zn as co-factors in plant parts, e.g. cytochrome c oxidase in the mitochondrial respiratory chain. By comparison, in the Cu-contaminated soil series from field plots, both shoot Ca and Mg concentrations were enhanced as soil Cu exposure increased (Kolbas et al. 2014). Copper excess in the cytosol of root cells causing the OH● generation and their interaction with the cytosolic OH●-binding site of plasma membrane cation channels may activate Ca^{2+}-influx channels, for allowing root growth and prospection for element acquisition, but also open K^+ efflux channels and prevent auxin redistribution, which inhibit root elongation (Printz et al. 2016).

As foliar Cu concentration increased from 10 to 31 mg kg^{-1} (Tab. 4) and roots less developed (Fig. 2b), foliar Fe concentration was roughly halved from 56 to 29 mg kg^{-1}. In contrast, the root-to-leaf Zn transfer increased (Tab. 4 and Fig. S1) even though the Zn concentration in the soil pore water did not differ significantly (Tab. 2). Consequently the

shoot Zn : shoot Fe concentration ratio (expressed in mmol kg^{-1} for both metals) increased from 0.6 to 1.4 in the 0.15-0.6 mmol Cu kg^{-1} range for shoot Cu concentration. The development of Fe deficiency and oxidative stress can induce many molecular mechanisms to restore the cellular Fe homeostasis, which likely result in higher root-to-shoot Zn transfer (Sinclair and Krämer 2012). Under Cu excess, Cu and Fe are key-players in both root and shoot processes and may compete not only in the epidermal cells but also for the intercellular and intracellular transport in roots (Shi et al. 2011; Printz et al. 2016). Cu acquisition/redistribution and Fe homeostasis are linked (Ravet and Pilon 2013). Regarding Cu translocation, the membrane protein AtYSL2 involved in the maintenance of Fe homeostasis can transport Cu-nicotianamine complexes (Printz et al. 2016). Lignification/inhibition of the root cell elongation may impact inducible Fe and Zn membrane transporters essential for Zn and Fe uptake (e.g. ZIP, IRT, etc.) (Palmer and Guerinot 2009). The expression of ZIP2 and ZIP4 able to mediate the transport of divalent cations is influenced by Cu availability (Printz et al. 2016).

Changes in element uptake and distribution in plant parts induced by Cu excess were integrated by changes in translocation factor. For Zn, K and P, TF values increased, whereas they decreased for Mg, Mn, and B (Tab. 4, Fig. S1). The TF curve of Mg (Fig. S1b) mimicked the DRC pattern of photosynthetic pigments (Fig. 2e) and leaf antioxidant activity (Fig. 2f)). Mg is a key player in chlorophyll biosynthesis, carbon partitioning from shoots to roots, and cytoplasmic pH regulation. Copper can substitute Mg in chlorophyll molecules, disrupting the normal course of metabolic processes (Küpper et al. 2003). Both cellular Ca and Mg homeostasis can help to quench oxidative stress in Cu-stressed plants and alleviate metal toxicity (Kinraide et al. 2004; Yruela 2009).

The Mn TF value transiently increased for the C_{10} and C_{20} plants (Tab. 4; Fig. S1) which matched with increase in total soil Mn (Tab. 1), leaf TAC (Fig. 2e), total chlorophyll content (Fig. 2) and shoot Cu removal (Fig. 2h). Foliar P concentration was less reduced than root P concentration, and therefore the TF value for P increased (Tab. 4, Fig. S1). This may have consequences as notably P-type ATPases belong to a large superfamily of ATP-driven pumps involved in the transmembrane transport of many cations across cell membranes, e.g. P-type ATPases (HMA) 5 to 8 (Burkhead et al. 2009). As COPT2 is delivering Cu to the multi-copper oxidases LPR1 and LPR2 (low phosphate roots 1 and 2), this may interact in phosphate sensing and root growth response to low phosphate, whereas ethylene due to Cu stress responses may be involved in various external and internal plant adaptations to limitation of nutrients including P (Printz et al. 2016).

CONCLUSIONS

- On this soil series with total soil Cu ranging from 13 to 1020 mg Cu kg^{-1} soil, biochemical parameters of the sunflower mutant were early endpoints based on the EC_{10}, with a high signal intensity. Between 13 and 517 mg Cu kg^{-1} in the soil (194-505 µg Cu L^{-1} in soil pore water, 3.05 – 7.94 µM), their dose-response curves peaked indicating both the rise of

oxidative stress and impacts on the chlorophyllous pigments. Morphological parameters of sunflower showed simpler DRC.

- Based on changes in shoot and root DW yields, the EC_{50} value reflecting Cu tolerance of this sunflower mutant line was in the 203-333 mg Cu kg^{-1} soil range, corresponding to 280-460 µg Cu L^{-1} in soil pore water (4.40 -7.24 µM).
- Shoot Cu removal peaked at 214 mg Cu kg^{-1} soil (280 µg Cu L^{-1} in soil pore water, 4.4 µM). The potential Cu phytoextraction was significantly higher on the 114-416 mg Cu kg^{-1} soil range, and it decreased at higher total soil Cu. Non-enzymatic, antioxidant status quantified by TAC, total chlorophyll, and foliar Mn and Zn concentrations peaked also for such soil Cu exposures.
- Over 617 mg Cu kg^{-1} soil, the dose-response curves for foliar antioxidant activity and the contents of photosynthetic pigments, mirrored by the development of a leaf bronzing, were likely explained by the inhibition of cell expansion and changes in leaf histology with smaller, compacted cells.

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Table 1. Soil physico-chemical parameters

Parameters*	**unit**	**CTRL**	**UNT**
Sand	g kg^{-1}	742	858
Silt	g kg^{-1}	216	83
Clay	g kg^{-1}	42	59
organic C	g kg^{-1}	7.05	8.42
Total N	g kg^{-1}	0.531	0.562
C/N	-	13.3	15
OM	g kg^{-1}	12.2	14.6
pH		7.1	6.2
CEC	cmol kg^{-1}	2.71	3.21
Olsen-extractable P**	g kg^{-1}	0.067	0.029
K	g kg^{-1}	3.97	8.24
Ca	g kg^{-1}	8.2	24.1
Mg	g kg^{-1}	0.24	0.996
Na	g kg^{-1}	0.55	2.24
Al	g kg^{-1}	7.3	18.6
Ni	mg kg^{-1}	2	5.56
Fe	g kg^{-1}	2.66	7.13
Mn	mg kg^{-1}	50	208
Cu	mg kg^{-1}	**13**	**1020**
Zn	mg kg^{-1}	13.2	38.5
Cd	mg kg^{-1}	0.116	0.067
Cr	mg kg^{-1}	9.85	24.6
As	mg kg^{-1}	-	8.5
Co	mg kg^{-1}	-	2.01
Mo	mg kg^{-1}	0.21	0.40
Pb	mg kg^{-1}	1.3	26.2
Tl	mg kg^{-1}	0.14	0.29
Se	mg kg^{-1}	-	0.17
Sb	mg kg^{-1}	-	0.37

*All soil analyses were performed at the INRA Laboratoire d'Analyses des Sols (LAS, Arras, France) using standard methods (INRA LAS 2014). CTRL: uncontaminated soil; UNT: untreated contaminated soil. ** extracted by the Olsen method.
Each soil sample was made from the collection of 10 sub-samples (6 kg FW each, 0-25 cm soil layer) on a circle and combined in a final sample of 60 kg FW.

Table 2. Total soil Cu, and mineral composition (in mg L^{-1}) and pH of soil pore water across the soil series

Soils	CTRL (%)	UNT (%)	CuTOT** (mg kg^{-1})	Al	B	Ca	Cu	Fe	Mg	Mn	P	K	Zn	pH
C_0	100	0	13	0.176**a** ±0.079	0.136**b** ±0.007	77.6**c** ±8.6	0.194**e** ±0.011	0.103***** ±0.097	10.3**a** ±6.5		0.86**bc** ±0.28	14.1**c** ±4.0	0.0053* ±0.0046	7.15**a** ±0.03
C_{10}	90	10	114	0.064**ab** ±0.016	0.161**b** ±0.006	111.0**abc** ±19.1	0.332**de** ±0.061	0.001***** ±0.001	10.3**a** ±1.7		0.29**c** ±0.04	30.0**bc** ±6.1	0.0037* ±0.006	7.03**ab** ±0.07
C_{20}	80	20	214	0.044**b** ±0.004	0.152**b** ±0.031	115.0**abc** ±22.5	0.289**de** ±0.053	0.015***** ±0.026	14.6**a** ±4.0		1.16**bc** ±0.20	30.9**bc** ±8.6		6.79**abc** ±0.16
C_{30}	70	30	315	0.106**ab** ±0.038	0.373**a** ±0.022	106.7**abc** ±15.5	0.459**cd** ±0.025	0.059***** ±0.029	13.6**a** ±1.8	0.0076*	1.77**bc** ±0.40	84.2**a** ±16.3	0.0062* ±0.0108	6.62**abc** ±0.20
C_{40}	60	40	416	0.066**ab** ±0.029	0.168**b** ±0.029	92.2**abc** ±23.3	0.426**cd** ±0.040	0.028***** ±0.048	9.3**a** ±0.6		1.76**bc** ±0.36	21.6**bc** ±5.6	0.0033* ±0.0057	6.60**bc** ±0.21
C_{50}	50	50	517	0.118**ab** ±0.047	0.203**ab** ±0.021	84.2**bc** ±11.3	0.505**bcd** ±0.034	0.088***** ±0.051	10.5**a** ±1.7		4.43**bc** ±0.45	44.3**abc** ±7.3	0.0132* ±0.0151	6.32**cd** ±0.10
C_{60}	40	60	617	0.069**ab** ±0.009	0.223**ab** ±0.041	116.2**abc** ±10.2	0.497**bcd** ±0.028	0.026***** ±0.026	14.1**a** ±2.3	0.0086*	5.26**bc** ±0.26	43.5**abc** ±6.7	0.0089* ±0.0090	6.48**cde** ±0.23
C_{70}	30	70	718	0.064**ab** ±0.011	0.220**ab** ±0.029	120.8**abc** ±19.5	0.486**bcd** ±0.047	0.018***** ±0.016	15.3**a** ±3.1	0.0111	3.64**bc** ±0.43	47.9**abc** ±9.7	0.0033* ±0.0057	6.35**cde** ±0.26
C_{80}	20	80	819	0.046**b** ±0.015	0.201**ab** ±0.037	154.3**a** ±11.2	0.707**ab** ±0.045	0.019***** ±0.018	13.1**a** ±1.0		5.86**b** ±0.51	40.4**abc** ±2.8	0.0047* ±0.0081	6.30**cde** ±0.30
C_{90}	10	90	919	0.017**b** ±0.007	0.216**ab** ±0.017	120.3**abc** ±21.6	0.635**abc** ±0.026	0.046***** ±0.008	10.6**a** ±1.9		12.02**a** ±1.93	68.9**ab** ±11.9		6.05**de** ±0.29
C_{100}	0	100	1020	0.092**ab** ±0.007	0.322**ab** ±0.022	141.7**ab** ±17.6	802**a** ±0.058	0.001***** ±0.001	15.3**a** ±2.2	0.0076*	4.19**bc** ±0.36	63.6**abc** ±8.5	0.0141* ±0.003	5.93**e** ±0.09

Mean values (n=3) followed by the same letter are not different at the 5% level; <dl (below detection limit: Mn <0.005; Zn<0.001 mg L^{-1});* one replicate below the detection limit (n<3); (mg kg^{-1}): total soil Cu

Table 3. Effective concentrations (EC, expressed in total soil Cu, mg Cu kg^{-1}) at 10%, 50% and 90% for several parameters of sunflower plants, calculated using the DRC-package.

Plant parameters	EC		
	10	50	90
Shoot DW yield (DW SH)	166	333	668
Root DW yield (DW RT)	74	203	552
Total leaf area (TLA)	201	335	559
Stem length (SL)*	355	407	485
Total chlorophyll content (Chl TOT)*	51	329	442
Total antioxidant capacity (TAC)*	23	301	436
Shoot Cu concentration (CuSH)*	83	322	583

* computed on the C_0-C_{60} soil series, with total soil Cu varying from 13 to 517 mg Cu kg^{-1}

Table 4. Ionome of sunflower leaves and roots and transfer factor (TF)

Elements		Al	B	Ca	Cu	Fe	Mg	Mn	P	K	Zn
Soils		mg kg^{-1}	mg kg^{-1}	g kg^{-1}	mg kg^{-1}	mg kg^{-1}	g kg^{-1}	mg kg^{-1}	g kg^{-1}	g kg^{-1}	mg kg^{-1}
C_0	leaves	12.5 A	53.5 ABC	24.2 C	10.0 E	56.5 AB	6.5 AB	37.3 A	4.3 A	29.5 A	38 A
	±SD	1.2	5.2	3.6	1.2	4.2	0.7	1.7	0.4	4.3	5.3
	roots	2130 abc	18.8 a	7 b	105.1 d	2466 ab	2.4 abc	49.2 bc	5.1 a	19.3 a	72.1 a
	±SD	155.4	3.6	0.3	25.8	1057.9	0.3	10.3	0.5	2.9	13.1
	TF	0.006	2.84	3.5	0.095	0.023	2.7	0.76	0.84	1.5	0.53
C_{10}	leaves	16.1 A	53.2 ABC	24.6 C	13.1 DE	46.2 AB	7 A	31.6 ABC	2.7 AB	24.5 A	37 A
	±SD	2.0	6.8	2.3	2.5	4.3	1.1	6.6	0.4	10.6	1.6
	roots	1318 c	17.8 a	12.8 ab	225 cd	1009 b	1.8 c	27 c	3 ab	13.7 ab	52.8 ab
	±SD	53.9	2.8	1.2	22.7	123.8	0.1	4.1	0.5	4.4	7.6
	TF	0.012	2.99	1.9	0.058	0.046	3.9	1.17	0.91	1.8	0.7
C_{20}	leaves	30.7 A	61.4 A	26.2 BC	21.3 CDE	54.7 AB	5.8 ABC	35 AB	2.1 AB	26 A	46.7 A
	±SD	2.8	6.2	2.4	3.2	6.7	0.7	5.4	0.4	4.9	4.6
	roots	2059 abc	21.6 a	8 ab	550 bcd	1623 ab	2 bc	36.8 bc	2.1 b	15.8 a	48 abc
	±SD	157.5	2.6	0.6	93.8	111.3	0.5	4.2	0.3	4.7	2.9
	TF	0.015	2.84	3.3	0.039	0.034	2.9	0.95	1	1.7	0.98
C_{30}	leaves	15.7 A	54.9 ABC	24 C	20.8 CDE	42.1 AB	5.6 ABC	30.2 ABCDE	1.7 B	26.5 A	49.3 A
	±SD	3.4	2.4	3.7	1.9	6.6	1.1	12.2	0.4	7.7	6.6
	roots	1971 abc	23.5 a	9.8 ab	673 abc	2172 ab	2.9 abc	48.8 bc	1.9 b	10.9 ab	42.3 bcd
	±SD	213.5	4.8	2.7	177.3	459.6	0.1	11.0	0.1	2.8	5.
	TF	0.008	2.33	2.4	0.031	0.019	1.9	0.62	0.94	2.4	1.17
C_{40}	leaves	15.4 A	57.1 AB	24.9 BC	29 BCDE	32.7 B	4.1 BC	29.9 ABCDE	1.88 B	25.7 A	53.1 A
	±SD	1.4	7.8	2.3	5.9	4.3	1.0	5.1	0.4	10.7	10.8
	roots	1591	24.7	12	810	1914	3.9	51.7	1.73	5.5	31.3
	±SD	262.3 bc	2.4 a	1.5 ab	117.9 ab	227.3 ab	0.3 abc	6.6 bc	0.4 b	0.9 b	7.4 bcde
	TF	0.01	2.31	2.1	0.036	0.017	1.03	0.58	1.08	4.7	1.7
C_{50}	leaves	19 A	53.7 ABC	26.4 BC	31 BCD	29.3 B	3.9 C	30.9 ABCD	2.18 AB	19.7 A	47.2 A
	±SD	2.5	5.3	1.5	2.1	3.6	0.3	5.4	0.4	1.7	8.4
	roots	2028 abc	25.3 a	14.2 a	904 ab	2127 ab	4.6 a	55.4 abc	1.85 b	4.4 b	24.9 cde
	±SD	220.9	3.3	1.3	250.7	247.4	0.5	6.4	0.2	0.5	4.4
	TF	0.009	2.12	1.86	0.035	0.014	0.85	0.56	1.18	4.48	1.89
C_{60}	leaves	16.0 A	56.7 AB	29.2 ABC	40 ABC	29.8 B	3.6 C	25.8 ABCDE	2.32 AB	18.9 A	50.4 A
	±SD	4.2	5.5	2.0	2.5	0.8	0.4	5.9	0.2	3.6	4.4
	roots	2159 abc	25.4 a	10.6 ab	959 ab	2415 ab	4 abc	63.5 ab	1.51 b	4.11 b	28.6 bcde
	±SD	212.7	5.7	1.9	60.2	786.5	0.3	13.0	0.1	0.4	6.5
	TF	0.007	2.23	2.74	0.042	0.012	0.91	0.41	1.53	4.58	1.76

Mean values (n=3) followed by the same letter are not different at the 5% level (leaves: capital letters; roots: lowercase letters); one replicate below the detection limit.

Table 4: to continue

Elements		Al	B	Ca	Cu	Fe	Mg	Mn	P	K	Zn
Soils		mg kg^{-1}	mg kg^{-1}	g kg^{-1}	mg kg^{-1}	mg kg^{-1}	g kg^{-1}	mg kg^{-1}	g kg^{-1}	g kg^{-1}	mg kg^{-1}
C_{70}	leaves	27.1 A	41.1 ABC	31 ABC	47.0 AB	49.4 AB	4.5 ABC	17 CDE	2.4 AB	17.7 A	41.9 A
	±SD	3.5	9.0	5.2	7.6	5.3	0.3	5.3	0.3	6.0	2.0
	roots	2269 ab	22.5 a	10.8 ab	947 ab	2518 ab	4.1 ab	66.5 ab	1.57 b	4.2 b	22.3 de
	±SD	471.6	3.6	1.1	101.0	165.0	0.5	7.5	0.2	0.3	3.2
	TF	0.012	1.83	2.87	0.05	0.02	1.1	0.25	1.53	4.21	1.88
C_{80}	leaves	16.3	33.8	33.8	39	66.1	4	14.8	2.73	19.4	44.9
	±SD	4.3 A	9.0 C	3.6AB	2.7ABC	9.7AB	1.5BC	2.5DE	0.1AB	3.4A	12.1A
	roots	2200 abc	21.1 a	12.5 ab	1035 ab	2351 ab	4.1 ab	67.9 ab	1.75 b	5.29 b	18.6 de
	±SD	267.5	4.9	3.2	203.1	988.0	0.9	10.2	0.5	2.0	5.2
	TF	0.007	1.6	2.7	0.038	0.028	0.99	0.22	1.56	3.67	2.41
C_{90}	leaves	3.2*	34.6 BC	36.3 A	40 BCDE	41.9 AB	3.3 C	13.4 E	2.6 AB	18.71 A	40.9
	±SD	*	2.1	3.5	2.7	3.5AB	0.4C	0.7E	0.5AB	2.9A	7.0A
	roots	2977 a	19.4 a	10 ab	1056 a	3061 a	4.02 abc	87.5 a	1.63 b	5.37 b	22.9 de
	±SD	750.2	2.8	1.1	151.3	250.6	0.9	17.7	0.1	1.0	5.6
	TF	0.001	1.78	3.62	0.028	0.014 A	0.82 C	0.15	1.6	3.49	1.78
C_{100}	leaves	29.7 A	34 C	36.1 A	55.4 E	92.9 A	3.7 C	18.6 BCDE	3.7 AB	19.87 A	61.9 A
	±SD	6.7	5.0	3.5	6.6	35.0	0.4	4.8	0.6	2.7	6.7
	roots	2145 abc	20.9 a	9.7 ab	1000 ab	2111 ab	3.3 abc	62 abc	1.59 b	4.31 b	16.8 e
	±SD	378.8	3.1	1.2	87.2	242.8	0.5	11.7	0.5	0.4	2.5
	TF	0.014	1.62	3.71	0.055	0.044	1.13	0.3	2.32	4.61	3.69
**	leaves			19-24	3-12	50-200	1.5-3	10-30		29-38	20-100

Mean values (n=3) followed by the same letter are not different at the 5% level (leaves: capital letters; roots: lowercase letters); one replicate below the detection limit **concentrations for optimal plant growth

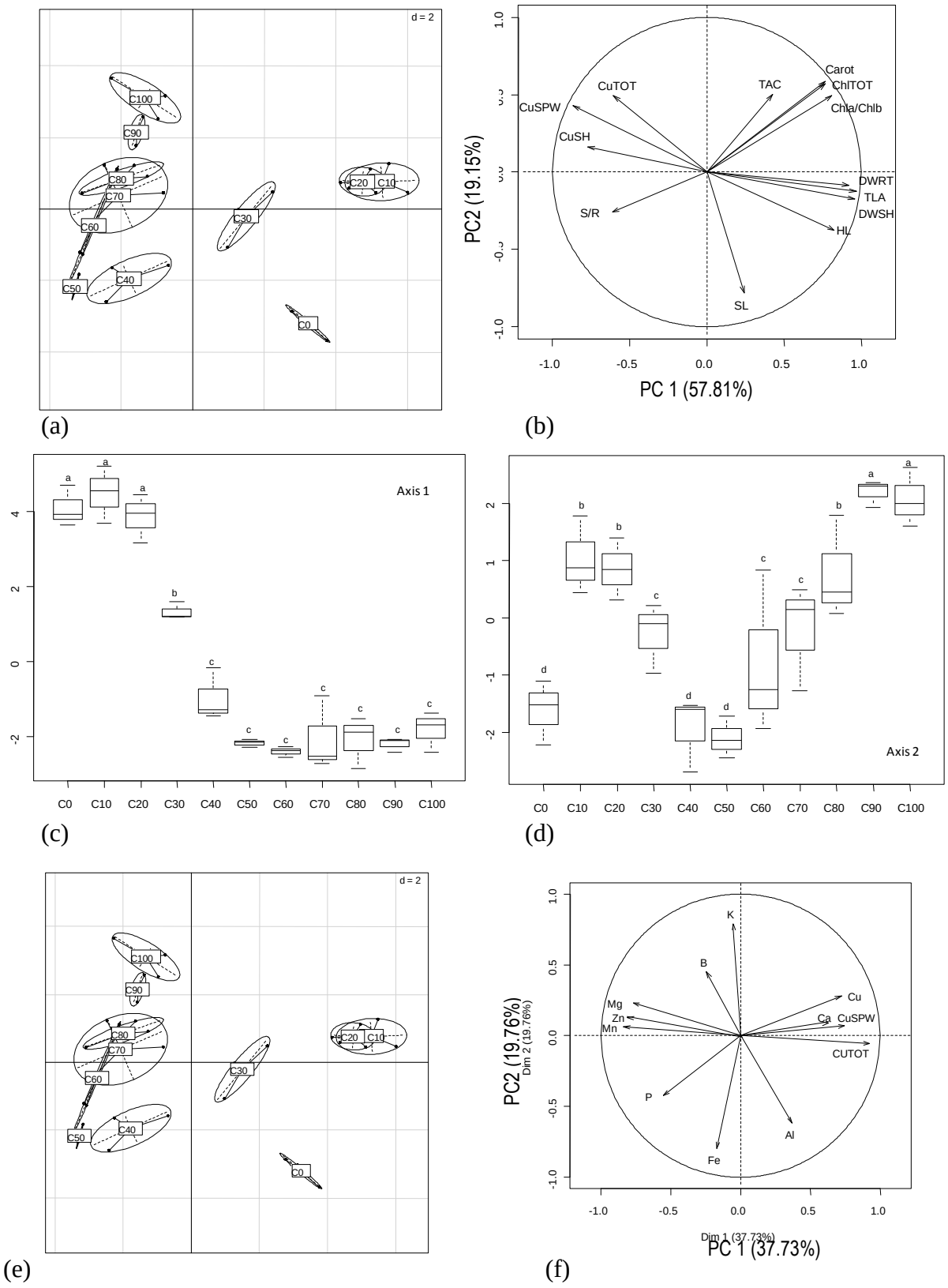

Figure 1. Principal Component Analysis (PCA) for parameters of the sunflower plants and soil Cu exposure: (a) 2D plane of principal axis and (b) correlation circle of plant parameters and indicators of soil Cu exposure; (c) the class distribution for PC axis #1; (d) the class distribution for PC axis #2; (e) 2D plane of principal axis and (f) correlation circle of shoot ionome and indicators of soil Cu exposure

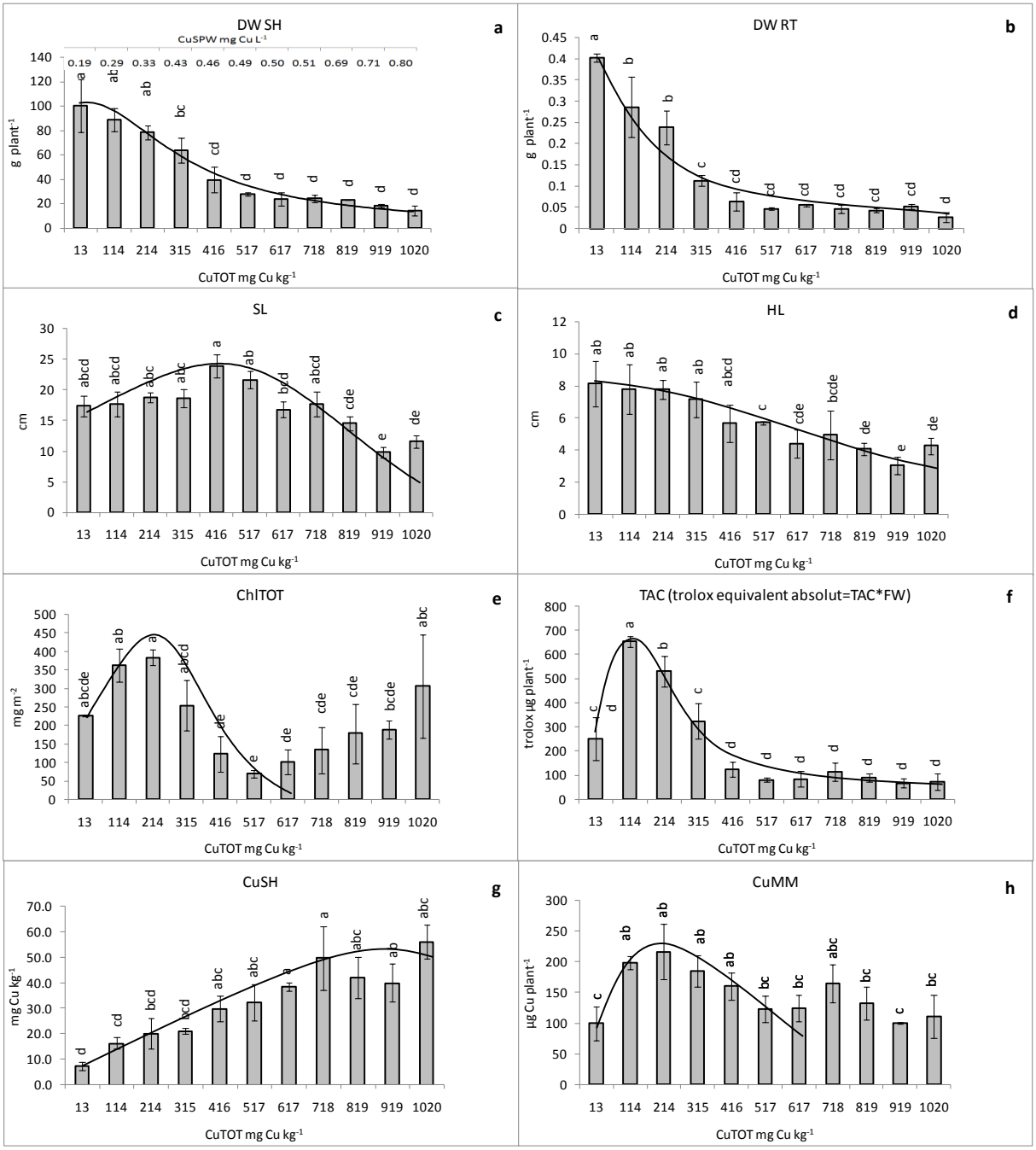

Figure 2. Phenotypic responses of sunflower plants.
(a) shoot DW yield; (b) root DW yield; (c) stem length; (d) hypocotyl length; (e) total chlorophyll content (f) total antioxidant capacity in leaves (µg Trolox equivalent g leaf FW^{-1} x leaf FW yield) ; (g) shoot Cu concentration; and (h) shoot Cu removal.
The curves for plant parameters were obtained using the DRC-package; for figures e, f, and h modelling was made only in the 13-617 mg Cu kg^{-1} soil range
Symbols represent means ± standard errors (n=4); means followed by the same letter are not different at the 5% level. X axis: CuTOT, total soil Cu (mg Cu kg^{-1} soil DW)

Supplementary materials

Table S1. Variable contributions to axes for Principal Component Analysis (PCA) of the sunflower and soil Cu exposure parameters

Factor	Correlation	P value
Dimension 1		
TLA	0.966	<0.001
DW SH	0.951	<0.001
DW RT	0.916	<0.001
HL	0.825	<0.001
Chl*a*/Chl*b*	0.821	<0.001
ChlTOT	0.779	<0.001
Carot	0.774	<0.001
TAC	0.433	<0.001
S/R	-0.602	<0.001
CuTOT	-0.687	<0.001
CuSPW	-0.745	<0.001
CuSH	-0.856	<0.001
Dimension 2		
Carot	0.569	<0.001
ChlTOT	0.552	<0.001
CuSPW	0.510	<0.001
TAC	0.543	<0.001
Chl*a*/Chl*b*	0.480	<0.001
TAC	0.477	<0.001
CuTOT	0.442	<0.001
HL	-0.395	<0.001
SL	-0.795	<0.001

Table S2. Upper critical threshold Cu concentrations reported for plant species (in mg Cu kg^{-1} DW).

Upper critical concentrations			Plant species	Experiment	Soil type	Cu contamination	References
Leaves	**Shoot**	**Roots**					
	26	290	*Chloris gayana*	H		0.2 – 7 µM, Cu-resin	Sheldon and Menzies 2005
70		138	*Helianthus annuus*	H		copper sulfate 10 µM – 1mM	Lin et al. 2003
25.6			*Helianthus annuus*	P	clay loam	Cu sulfate spike (400 mg/kg)	Rivelli et al. 2012
20-30	15-20		*Helianthus annuus*	F	sandy	wood preservation site	Kolbas et al. 2011
	36	1233	*Helianthus annuus*	P	sandy	wood preservation site	Kolbas et al. 2014
	21	620	*Helianthus annuus*	P	sandy	wood preservation site	*this study*
		41	*Vigna mungo*	P		Cu spike	Kalyanaraman and Sivagurunathan 1993
		337	*Zea mays*	H		Cu sulfate 0-80 µM	Ouzounidou et al. 1995
21		26	*Zea mays*	H		Cu sulfate 0.01-10 µM	Mocquot et al. 1996
	21	174	*Zea mays*	H		Cu sulfate 0.5-157 µM	Ali et al. 2002
	55	3060	*Phragmites australis*	H		Cu sulfate 0.5-157 µM	Ali et al. 2002
	15	2300	*Triticum aestivum*	H		Cu sulfate 0-50 µM	Taylor and Foy 1985
	10	134	*Triticum aestivum*	F		Porphyry Cu mineralisation	Cook et al. 1997
	20	66	*Vigna unguiculata*	H		Cu sulfate 0-3 µM	Kopittke et al. 2011
	11-39	128-705	*Triticum durum*	F	calcareous/non-calcareous	former vineyard soils	Michaud et al. 2007
	39		*Lolium perenne*	P	clay loam to sandy	mining	Verdejo et al. 2015
	21		*Lactuca sativa*	P	clay loam to sandy	mining	Verdejo et al. 2016

P: Pot experiment; H: Hydroponics; F: field plot

Table S3. Pearson correlation coefficients between plant parameters, foliar element concentrations, and total Cu in soil and soil pore water for 4-week old sunflower plants.

	Morphological parameters							Biochemical parameters						Foliar element concentrations									
	TLA	SL	HL	EL	DW SH	DW RT	S/R	Chl*a*	Chl*b*	Chl*a*/Chl*b*	ChlTOT	Carot	TAC	Al	B	Ca	Cu	Fe	Mg	Mn	P	K	Zn
SL	0.31^{ns}																						
HL	**0.93****	0.55^{ns}																					
EL	-0.40^{ns}	0.67*	-0.19^{ns}																				
DW SH	**0.99****	0.33^{ns}	0.94**	-0.38^{ns}																			
DW RT	**0.95****	0.16^{ns}	**0.84****	-0.51^{ns}	**0.95****																		
S/R	-0.82**	-0.43^{ns}	-0.76**	0.11^{ns}	**-0.84****	**-0.76****																	
Chl*a*	0.65*	-0.23^{ns}	0.55^{ns}	-0.69*	0.62*	0.58^{ns}	-0.30^{ns}																
Chl*b*	0.52^{ns}	-0.35^{ns}	0.41^{ns}	-0.71*	0.48^{ns}	0.45^{ns}	-0.14^{ns}	**0.98****															
Chl*a*/*b*	**0.77****	-0.16^{ns}	0.62*	-0.67*	0.73*	0.70*	-0.45^{ns}	**0.93****	**0.86****														
ChlTOT	0.63*	-0.26^{ns}	0.52^{ns}	-0.69*	0.59^{ns}	0.55^{ns}	-0.26^{ns}	**0.99****	**0.99****	**0.92****													
Carot	0.63*	-0.23^{ns}	0.52^{ns}	-0.67*	0.59^{ns}	0.55^{ns}	-0.30^{ns}	**0.99****	**0.96****	**0.93****	**0.99****												
TAC	0.32^{ns}	-0.06^{ns}	0.31^{ns}	-0.36^{ns}	0.29^{ns}	0.17^{ns}	-0.18^{ns}	0.76**	0.74**	0.62*	**0.76****	**0.80****												
Al	-0.63*	-0.04^{ns}	-0.53^{ns}	0.21^{ns}	-0.63*	-0.62*	0.69*	-0.35^{ns}	-0.29^{ns}	-0.34^{ns}	-0.34^{ns}	-0.31^{ns}	0.02^{ns}										
B	0.05^{ns}	0.55^{ns}	0.20^{ns}	0.29^{ns}	0.09^{ns}	0.08^{ns}	-0.15^{ns}	-0.39^{ns}	-0.42^{ns}	-0.43^{ns}	-0.40^{ns}	-0.42^{ns}	-0.17^{ns}	0.19^{ns}									
Ca	**-0.84****	-0.38^{ns}	**-0.78****	0.24^{ns}	**-0.83****	**-0.77****	**0.80****	-0.57^{ns}	-0.41^{ns}	**-0.74****	-0.53^{ns}	-0.58	-0.41^{ns}	0.41^{ns}	0.10^{ns}								
Cu	**-0.87****	-0.25^{ns}	**-0.74****	0.23	**-0.87****	**-0.84****	**0.88****	-0.40^{ns}	-0.26^{ns}	-0.60*	-0.37^{ns}	-0.39^{ns}	-0.04^{ns}	**0.76****	0.10^{ns}	**0.74****							
Fe	0.42^{ns}	-0.40^{ns}	0.28^{ns}	**-0.80****	0.39^{ns}	0.49^{ns}	0.03^{ns}	0.71*	**0.70*****	**0.78****	0.71*	0.70*	0.39^{ns}	0.11^{ns}	-0.32^{ns}	-0.37^{ns}	-0.16^{ns}						
Mg	**0.88****	0.36^{ns}	**0.91****	-0.28^{ns}	**0.89****	**0.82****	**-0.76****	0.43^{ns}	0.33^{ns}	0.49^{ns}	0.41^{ns}	0.38^{ns}	0.14^{ns}	-0.69*	0.20^{ns}	-0.60*	**-0.75****	0.19^{ns}					
Mn	**0.84****	0.43^{ns}	**0.86****	-0.26^{ns}	**0.85****	**0.88****	-0.61*	0.32^{ns}	0.21^{ns}	0.43^{ns}	0.30^{ns}	0.27^{ns}	-0.08^{ns}	-0.49^{ns}	0.35^{ns}	-0.61*	-0.66*	0.32^{ns}	**0.85****				
P	0.64*	-0.23^{ns}	0.49^{ns}	**-0.76****	0.64*	**0.81****	-0.32^{ns}	0.53^{ns}	0.49^{ns}	0.56^{ns}	0.52^{ns}	0.49^{ns}	0.01^{ns}	-0.33^{ns}	0.16^{ns}	-0.39^{ns}	-0.43^{ns}	0.69*	0.54*	0.72*			
K	-0.11^{ns}	0.55^{ns}	0.08^{ns}	0.59^{ns}	-0.09^{ns}	-0.27^{ns}	-0.10^{ns}	-0.39^{ns}	-0.38^{ns}	-0.50^{ns}	-0.39^{ns}	-0.41^{ns}	0.12^{ns}	0.06^{ns}	0.67*	0.23^{ns}	0.17^{ns}	-0.65*	0.15^{ns}	-0.07^{ns}	-0.40^{ns}		
Zn	**0.77****	0.25^{ns}	**0.75****	-0.43^{ns}	**0.77****	**0.85****	-0.43^{ns}	0.41^{ns}	0.33^{ns}	0.48^{ns}	0.39^{ns}	0.36^{ns}	0.03^{ns}	-0.30^{ns}	0.39	-0.50^{ns}	-0.52*	0.51^{ns}	0.71*	**0.94****	**0.85****	-0.16^{ns}	
CuSPW	**-0.92****	-0.61	**-0.95****	0.08^{ns}	**-0.94****	**-0.85****	**0.89****	-0.37^{ns}	-0.22^{ns}	-0.49^{ns}	-0.34^{ns}	-0.35^{ns}	-0.18^{ns}	0.58*	-0.33^{ns}	**0.78****	**0.83****	-0.10^{ns}	**-0.88****	**-0.85****	-0.46^{ns}	-0.17^{ns}	-0.72*
CuTOT	**-0.84****	-0.65	**-0.92****	0.09^{ns}	**-0.86****	**-0.81****	0.75*	-0.32^{ns}	-0.19^{ns}	-0.40^{ns}	-0.29^{ns}	-0.29^{ns}	-0.15^{ns}	0.42^{ns}	-0.47^{ns}	**0.75****	**0.73****	-0.16^{ns}	**-0.83****	**-0.91****	-0.54*	-0.18^{ns}	**-0.81****

Significance level: ^{ns} Not significant, * P<0.05, **P<0.01 (in bold)

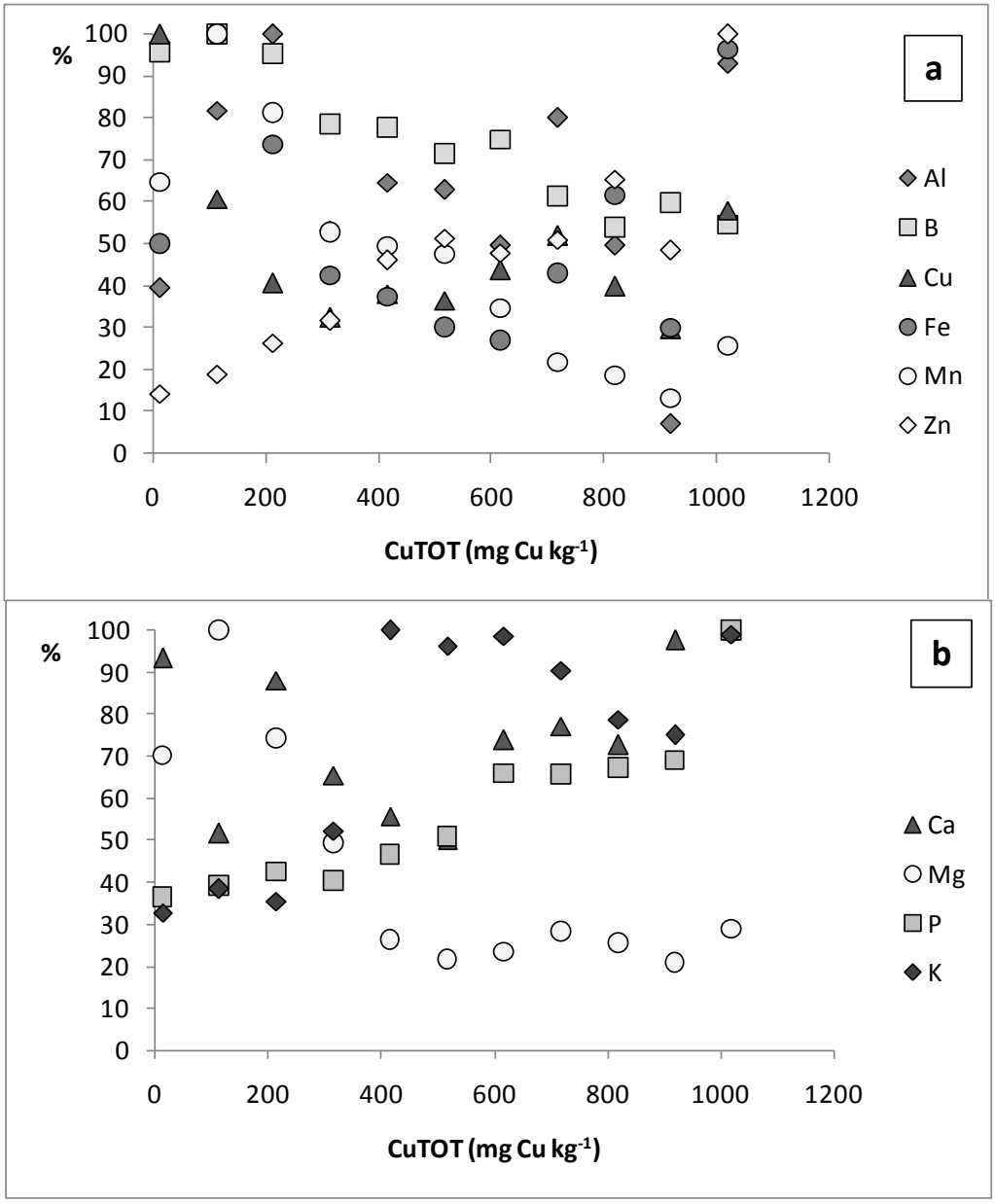

Figure S1. Changes in transfer factor (TF) for (a) micro- and (b) macro-nutrients in sunflower plants across the soil series (in % relative to maximum value). X axis – total soil Cu