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REGULAR ARTICLE

Copper uptake kinetics in hydroponically-grown durum wheat (*Triticum turgidum durum* L.) as compared with soil's ability to supply copper

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Abstract This study investigated (a) net Cu uptake kinetics in durum wheat (*Triticum turgidum durum* L.) exposed to free Cu^{2+} activities in solution ranging from 0.4 to 2,420 nM and (b) the relative importance of plant uptake and soil's ability to supply Cu^{2+} to the roots. Plant Cu flux showed a hyperbolic shape, enabling to estimate the Michaelis–Menten kinetic parameters (F_{max} and K_M) for durum wheat. Plant Cu flux was then compared with soil Cu flux as assessed by the Diffusive Gradient in Thin film technique on seven soil samples. This comparison suggested that the rate-limiting process of Cu bioavailability to durum wheat would be plant uptake kinetics in most contaminated soils with the exception of moderately contaminated, calcareous soils. However, theoretical considerations targeted soil's ability to supply Cu as the rate-limiting process in most soils for Cu (hyper-) accumulator plants with requirement larger than that of common crop species.

Keywords Bioavailability · Influx · Kinetics · Metal · Plant · Rhizosphere

Introduction

The determination of metal uptake kinetics is a necessary step toward the modelling of soil–plant transfer in nutrient-poor or contaminated environments (Nowack et al. 2006). For instance, the Barber-Cushman approach was applied to predict plant uptake of a range of metals such as cadmium (Cd), manganese (Mn) and zinc (Zn) (Mullins et al. 1986; Adhikari and Rattan 2000; Sadana and Claassen 2000; Sterckeman et al. 2004).

In the case of copper (Cu), Seuntjens et al. (2004) simulated plant Cu uptake using kinetic parameters F_{max} and K_M determined for rice (Lindon and Henriques 1992). However, the choice of kinetic parameters used for this purpose is questionable as

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there is little available literature on Cu uptake kinetics and these are not environmentally relevant. Most of kinetic parameters were indeed calculated from experiments carried out with free Cu^{2+} activities, $\{Cu^{2+}\}$, varying between 1.6 and 500 µM (Cathala and Salsac 1975; Veltrup 1976; Bowen 1987), which far exceed $\{Cu^{2+}\}$ usually found in soils even when heavily contaminated (Sauvé et al. 1997; Vulkan et al. 2000). Furthermore, some other studies determined kinetic parameters on the basis of total Cu concentrations in nutrient solution (Nielsen 1976; Lindon and Henriques 1992), while free Cu^{2+} is usually considered as the major Cu species that plants are able to take up. Only Antunes and Hale (2006) recently carried out a more relevant investigation of Cu uptake in durum wheat (*Triticum turgidum durum* L.), i.e. accounting for Cu speciation in solution with $\{Cu^{2+}\}$ from the sub-nanomolar to the micromolar range. However, the experimental procedure used, i.e. only root Cu accumulation in darkness was considered, was rather designed to investigate the effect of diffusive limitations on root Cu uptake than the actual plant uptake kinetics.

In comparison, uptake kinetics of Cd and Zn were recently described for a range of plant species from agricultural crops to metal hyperaccumulator (Hart et al. 1998, 2002; Lombi et al. 2001). In these studies, uptake fluxes were determined on intact plants with metal activities from the nanomolar to micromolar range and by distinguishing the metabolic influx into root symplasm from the passive loading of metal in root apoplasm. Nevertheless, all these experiments were carried out on short-time scales (20 min) and therefore did not account for any longer-term regulation of uptake by internal plant nutrient status (Reid and Liu 2004). In the range of metal toxicity, some modifications of uptake kinetics are expected to occur (Briat and Lebrun 1999). In the particular case of Cu, it was also noted that Cu induced rhizoderm ruptures in cowpea (*Vigna unguiculata* L.) after 12 h of exposure to 1.1 µM Cu^{2+} (Kopittke et al. 2008). This result highlights the need to carry out uptake experiments over several days.

Consequently, relevant kinetic parameters for Cu uptake in intact plants are currently lacking and could thus restrict the ability of numerical models to predict soil–plant transfer of Cu. This study therefore aimed at determining the kinetic parameters ($F_{\max}$ and K_M) of Cu uptake in durum wheat at optimal and toxic ranges of $\{Cu^{2+}\}$. The uptake experiment was carried out over 8 d to account for the impact of a potential Cu phytotoxicity. Finally, the relevance of kinetic parameters for soil–plant transfer was assessed by comparing the respective importance of the theoretical plant Cu flux and the soil's ability to supply Cu^{2+} at the root surface on a set of seven soil samples showing a wide range of pH and total Cu content.

Material and methods

Plant growth

Durum wheat seeds (cv. Acalou) were germinated for 3 d in the dark on filter paper moistened with $CaCl_2$ 600 µM and H_3BO_3 2 µM. The growth chamber parameters were set at (day/night): 25/20°C, 75/70% of relative humidity and 450 µmol photons m^{-2} s^{-1} light intensity. Seedlings were then transferred in 35-dm^3 tanks (20 pairs of seedlings per tanks) for four additional days. From the second week, seedlings were exposed for an additional 2-week to a complete nutrient solution (µM): $Ca(NO_3)_2$ 2000, KNO_3 2000, $MgSO_4$ 1000, KH_2PO_4 500, NaFe(III)EDTA 100, H_3BO_3 10, $MnCl_2$ 2, $ZnSO_4$ 1, $CuCl_2$ 1 and Na_2MoO_4 0.05. The nutrient solution was renewed every second day.

At the end of this pre-culture stage, 20 homogeneous pairs of plants were selected on the basis of their whole fresh biomass. Five pairs of plants were harvested to determine Cu content and plant root parameters (see below) prior to exposure to a range of Cu concentrations. The remaining 15 pairs of plants were transferred for another 8-d period of exposure in 15 complete nutrient solutions with the same composition as above (with however KH_2PO_4 reduced to 50 µM) and containing Cu concentrations ranging from 2.6 to 95 µM. The experiment was thus conducted without replication (except for control plants). Solution pH was set at 6.0 (±0.1) and buffered with 1 mM MES (2-morpholinoethanesulphonic acid). Free Cu^{2+} activities, $\{Cu^{2+}\}$, in the nutrient solution were buffered with EDTA (100 µM), supplied as NaFe(III) EDTA (as a source of Fe), to reach 0.4 to 2,420 nM. Free Cu^{2+} activities were calculated with the aqueous speciation model PHREEQC (version 2.14.3.2411) and the MINTEQ database of thermodynamic constants (Appendix). Nutrient solutions were renewed daily about 6 h after the beginning of the day-period to limit nutrient depletion as well as root-induced alkalisation and exudation of Cu-chelating compounds, especially phytosiderophores which are usually secreted from 3 to 6 h after the onset of light (Reichman and Parker 2007). Solution pH was measured daily just before renewal with a combined glass-electrode (6.0234.110, Metrohm, Switzerland). Total Cu concentration was also measured in each initial solution and after 1, 4 and 8 d of plant growth in the solutions (before renewal) by flame atomic absorption spectrometry (Varian SpectraAA-600, Australia). The quality of speciation modelling was checked by measuring $\{Cu^{2+}\}$ on five initial nutrient solutions with a Cu ion-selective electrode (Cu-ISE; DX-264, Mettler Toledo, USA) and a double-junction reference electrode (Inlab-302, Mettler Toledo). ISE measurements were calibrated between p$\{Cu^{2+}\}$ 9.0–5.2 in 10^{-4}M Cu solutions buffered with iminodiacetic acid and potassium phthalate according to the procedure described by Sauvé et al. (1995). Before measurements, ISE was polished for 30 s with a 3-µm Al_2O_3 strip then successively soaked for 5 min in H_2SO_4 0.025 M and Na_4EDTA 0.1 M.

After 8 d of exposure, the 15 pairs of plants (which were at the tillering stage) were harvested. Plant shoots and roots were separated. Roots were divided in three equivalent sub-samples in order to measure total and symplasmic Cu content as well as root length. Shoots and roots used to measure total Cu content were directly oven-dried at 105°C, while root sub-samples used for symplasmic Cu and length measurements were stored at −20°C before analyses. The five pairs of control plants were handled similarly.

Plant measurements

After thawing, root length was measured on stained roots using a scanner (Epson expression 10000 XL) with a two light sources procedure and the WinRHIZO software (version 2005 b, Regent Instruments, Canada), as previously detailed by Bravin et al. (2009). After root length measurements, root samples were oven-dried at 105°C for dry biomass determination. Relative root length (*RRL*) calculation was adapted from Kinraide et al. (2004) as followed:

$$RRL = 100 \times (RL - RL_{\min})/(RL_{\max} - RL_{\min}) \qquad (1)$$

where *RL* is the root length for a given Cu exposure, $RL_{\min}$ and $RL_{\max}$ the minimal and maximal root length measured among the range of exposure to Cu.

Copper rhizotoxicity was assessed by fitting the *RRL* vs. $\{Cu^{2+}\}$ relationship with the Hill's equation:

$$RRL = \{Cu^{2+}\}^{-a}/\left(\{Cu^{2+}\}^{-a} + EC_{50}{}^{-a}\right) \qquad (2)$$

where a is the Hill number and EC_{50} the $\{Cu^{2+}\}$ in solution resulting in a 50% reduction in the maximal *RRL*. The Excel™ macro REGTOX (version 7.0.5; Vindimian 2001) was used to compute fitting parameters (a and EC_{50}), EC_{25}, EC_{75}, EC_{90} and the 99% non-parametric confidence intervals for EC_{25}, EC_{50}, EC_{75} and EC_{90}.

Another set of frozen root sub-samples was used to measure symplasmic Cu after the extraction of apoplasmic Cu with HCl on thawn roots. The extraction procedure was previously detailed and tested by Chaignon et al. (2002), based on the earlier work of Iwasaki et al. (1990). Iwasaki et al. (1990) had shown in the first place that HCl did not extract root symplasmic Cu, providing the extraction was not too long and conducted under mildly acidic conditions. Chaignon et al. (2002) later checked that freezing and thawing of roots before the extraction and HCl concentrations (10^{-3} and 10^{-2} M for short periods of time) used during the extraction were not affecting the integrity of root plasma membrane, in order to avoid any overestimation of Cu binding in root apoplasm. Although the desorption efficiency of the extraction procedure was not assessed here, a large underestimation of apoplasmic Cu was unlikely to occur as a large part of root Cu was recovered in the apoplasm (see below in the Results section). Roots were finally oven-dried at 105°C for dry biomass determination.

Copper concentration in shoots and roots (for total and symplasmic Cu determination) was determined after grinding and digestion as detailed by Bravin et al. (2009). The accuracy of Cu determination was checked by including blanks and reference materials of maize shoots (*Zea mays* L., V 463, Bureau InterProfessionnel d'Etudes Analytiques, France) and olive leaves (*Olea europaea* L., n° 62, Community Bureau of References, Commission of the European Communities) in the procedure.

Plant Cu flux (F_{plt}, ng Cu m^{-2} root surface area s^{-1}) was calculated for each Cu exposure according to

Mullins and Sommers (1986) formula, accounting for an exponential root growth:

$$F_{\mathrm{plt}} = (m - m_{\mathrm{C}})/(S - S_{\mathrm{C}}) \times k \tag{3}$$

$$k = \ln(S/S_{\mathrm{C}})/t \tag{4}$$

where m_{C} and m are the mass of Cu (ng) accumulated in the whole plants after pre-culture (control) and 8-d exposure to Cu, S_{C} and S the root surface area (m^2) after pre-culture and 8-day exposure to Cu, k the growth constant (s^{-1}) and t the duration of exposure to Cu (8 d). In a preliminary experiment, we checked that an exponential function can be applied properly to model root growth with or without Cu rhizotoxicity.

Kinetic parameters of Cu uptake by durum wheat were determined from the Michaelis–Menten equation:

$$F_{plt} = F_{\max} \times \left\{\mathrm{Cu}^{2+}\right\}/\left(K_{\mathrm{M}} + \left\{\mathrm{Cu}^{2+}\right\}\right) \tag{5}$$

where $F_{\max}$ is the maximal flux of Cu in plant (ng Cu m^{-2} s^{-1}) and K_{M} the $\{Cu^{2+}\}$ at 1/2 $F_{\max}$ usually standing for the affinity of Cu for transporters in the plasma membrane of root cells and/or binding sites in root apoplasm. Michaelis–Menten equation was fitted on plant Cu flux accounting for Cu in root apoplasm (uptake flux) or not (absorption flux). Parameters of Michaelis–Menten equation and its statistical significance were calculated with Statistica (version 7, Statsoft) using the Marquardt–Levenberg algorithm. The level of statistical significance was represented by ** and *** for $P \leq 0.01$ and $P \leq 0.001$, respectively.

Soil measurements

Seven topsoil samples collected in former vineyards of Southern France were selected to cover a large range of pH_{CaCl2} (4.1–7.8). Soil samples were either strongly acidic (A and B), slightly acidic (C and D) or calcareous (E, F and G) (Table 1). For each pH category, soil samples were either little (B, D and G) or moderately (A, C and F) Cu-contaminated. An additional highly Cu-contaminated, calcareous soils (E) was also included. Additional soil physical and chemical properties can be found in Michaud et al. (2007) and Bravin (2008).

Soil extract with 0.01 M $CaCl_2$ (1:10 soil:solution ratio) is usually suggested as a reasonable surrogate for the determination of total concentration and speciation of Cu in soil solution, while in $CaCl_2$ extracts total Cu concentration tends to be slightly under-estimated and $\{Cu^{2+}\}$ slightly over-estimated in comparison with measurements made directly in soil solution (Sauvé et al. 1997; Kalis et al. 2007). Consequently, ISE measurements were carried out on 0.01 M $CaCl_2$ extracts (1:10 soil:solution ratio) to estimate $\{Cu^{2+}\}$ in soil solution. Single measurement of $\{Cu^{2+}\}$ (no replicate) was performed on each soil as a preliminary experiment on a larger set of soil samples showed that $p\{Cu^{2+}\}$ deviation was lower than 5% (results not shown). Diffusive gradient in thin film (DGT) technique was also deployed on each soil sample in triplicate to assess Cu flux in soil (Table 1). The procedures followed for ISE and DGT measurements were already detailed by Bravin (2008, p. 84–85). DGT measurements were computed as a flux (F_{DGT}, ng Cu m^{-2} s^{-1}):

$$F_{\mathrm{DGT}} = m/(A\,t) \tag{6}$$

where m is the mass of Cu (ng) accumulated in the resin, A the active surface area of DGT unit (3.14 cm^2) and t the deployment time (17 h). The thickness of the diffusive layer, including the diffusive gel and the filter membrane, was equal to 0.093 cm.

Results

Chemical stability of nutrient solutions over time

The major parameters driving $\{Cu^{2+}\}$ in nutrient solutions were total Cu concentration and pH. Total Cu concentration appeared rather stable over time with maximum changes lower than 30% and 20% for exposure treatments 1–5 ($[Cu]_{Tot} < 20.3\,\mu M$) and 6–15 ($[Cu]_{Tot} > 24.7\,\mu M$), respectively (results not shown). These variations were attributed to both plant Cu uptake and decrease in solution volume due to water uptake.

Despite the addition of MES, alkalisation occurred and pH increased up to 7.3 in the less Cu-concentrated solutions (results not shown). In the most Cu-concentrated solutions, pH increase was lower likely due to the buffering ability of freshly precipitated Fe oxyhydroxides and a lower root biomass (see below). Such alkalisation induced a large decrease in $\{Cu^{2+}\}$ (one or two orders of magnitude) and outlined the difficulties to maintain

Table 1 Free Cu^{2+} activity, theoretical plant Cu flux (F_{Tplt}) and DGT Cu flux (F_{DGT}) in seven soil samples with varying pH and total Cu content. F_{Tplt} was calculated by considering the uptake kinetic parameters determined for (a) durum wheat in the present experiment ($F_{Tplt\text{-}1}$; see Table 3), (b) durum wheat with a plausible unbiased K_M ($F_{Tplt\text{-}2}$; K_{M*} = 1 nM) or (c) an expected Cu (hyper-) accumulator plant ($F_{Tplt\text{-}3}$; F_{max*}=170 ng Cu $m^{-2}s^{-1}$ and K_{M*}= 1 nM) and $\{Cu^{2+}\}_{CaCl2}$ measured in each $CaCl_2$-soil extract (see Discussion section for rationale about F_{max*} and K_{M*} values). Values in parentheses stand for mean standard errors (n=3)

Soil samples		pH_{CaCl2}	Total Cu mg kg^{-1}	$\{Cu^{2+}\}_{CaCl2}$ nM	$F_{Tplt\text{-}1}$ ng Cu m^{-2} s^{-1}	$F_{Tplt\text{-}2}$	$F_{Tplt\text{-}3}$	F_{DGT}
A	2[a]	4.1	184	5091	42	43	170	64 (17)
B	2[b]	4.3	88	2853	42	43	170	51 (6)
C	8[b]	5.6	165	33	12	42	165	43 (14)
D	9[b]	5.8	53	31	11	42	165	5.5 (0.5)
E[c]	30[b]	7.5	1030	10	5	39	155	123 (37)
F[c]	26[b]	7.6	163	5	2	36	142	22 (2)
G[c]	23[b]	7.8	56	5	3	36	142	2 (2)

[a] Correspondence with soil samples reported in Bravin (2008, p. 93)

[b] Correspondence with soil samples reported in Michaud et al. (2007)

[c] Calcareous soil samples

stable chemical conditions in nutrient solutions exposed to plant with large root biomass as stressed by Kopittke and Menzies (2006). As nutrient solutions were renewed daily, results were however discussed on the basis of the initial composition of nutrient solutions at each level of exposure.

Plant Cu content and toxicity

Plant Cu content increased with increasing $\{Cu^{2+}\}$ in nutrient solutions, showing a hyperbolic pattern with a saturation shape for both shoots and roots (Fig. 1). Copper contents ranged from 9 to 53 mg kg^{-1} in shoots (Fig. 1a) and from 25 to 1,485 mg kg^{-1} in roots with 53–81% bound to the apoplasm (Fig. 1b). Copper content in shoots increased almost linearly with the increase in Cu content in the root symplasm (results not shown). However, Cu translocation from root symplasm to shoots, as deduced from shoot to root symplasm Cu content ratio, decreased 20-fold when $\{Cu^{2+}\}$ increased up to 50 nM, then remained steady for higher $\{Cu^{2+}\}$ (results not shown).

Several symptoms of Cu phytotoxicity were observed. Interveinal chlorosis consistent with Fe deficiency occurred for $\{Cu^{2+}\}$ in solution equal or larger than 550 nM. Shoot and especially root biomasses were also affected and decreased with increasing $\{Cu^{2+}\}$ in solution (Fig. 2a). Although no replication was performed at each $\{Cu^{2+}\}$, the relative root length (*RRL*) was clearly affected by Cu in the low range of exposure as *RRL* decreased by up to 44% for Cu content in the root apoplasm lower than 500 mg kg^{-1} (Fig. 3a) or $\{Cu^{2+}\}$ in solution lower than 50 nM (Fig. 3b, see the insert). Above these thresholds, the *RRL* still decreased with increasing Cu content in the root apoplasm and, more particularly, with increasing $\{Cu^{2+}\}$ in solution (Fig. 3a and b). The *RRL* vs. $\{Cu^{2+}\}$ relationship could be fitted to Hill's equation (Eq. 2) to determine rhizotoxicity thresholds (Fig. 3b). This revealed that $\{Cu^{2+}\}$ in solution of 15, 63, 268 and 1,140 nM reduced the *RRL* by 25, 50, 75 and 90%, respectively, compared to the maximal root length measured (Table 2). Visual symptoms of rhizotoxicity were also observed (Fig. 2b, c and d). The increasing exposure of plants to $\{Cu^{2+}\}$ in solution induced an increase in lateral root formation. Nevertheless, the growth of these lateral roots appeared strongly restricted and almost completely inhibited at the highest $\{Cu^{2+}\}$ (Figs. 2c and d).

Cu uptake kinetics in plant

Copper fluxes in plant, accounting for the apoplasm pool (uptake flux) or not (absorption flux), could be fitted with the Michaelis–Menten equation (Fig. 4). In both cases, experimental data exhibited a hyperbolic pattern with a sharp and almost linear increase in Cu fluxes for $\{Cu^{2+}\}$ lower than 100 nM, followed by a saturation shape up to 2,400 nM $\{Cu^{2+}\}$. The kinetic parameters (F_{max} and K_M) deduced from the Michaelis–

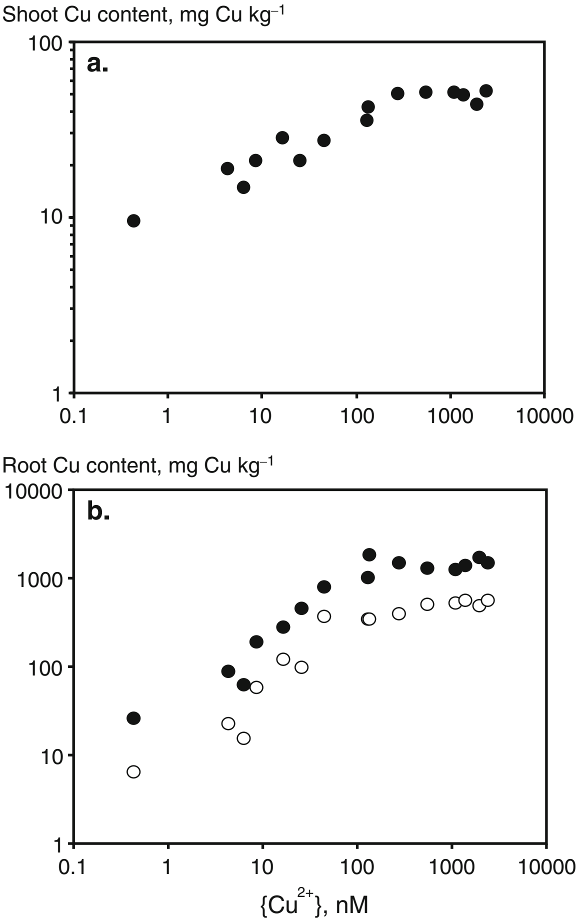

Fig. 1 Shoot (**a**) and (**b**) Cu contents as a function of {Cu^{2+}} in solution. In graphic b, closed symbols stand for total root Cu content, i.e. accounting for Cu in root apoplasm, while opened symbols stand for Cu content in root symplasm only

Menten equation are given in Table 3. It is noteworthy that accounting for the apoplasm pool involved a 2.3-fold increase in F_{max} and a 1.5-fold decrease in K_M, highlighting the affinity of root apoplasm for Cu. As uptake and absorption kinetics were measured over several days of exposure, the measured fluxes obviously corresponded to net Cu fluxes, i.e. integrating influx into the roots and efflux from the roots to the solution.

Comparison between theoretical plant Cu fluxes and DGT Cu flux

The kinetic parameters specifically determined for durum wheat (Table 3) enabled to calculate the theoretical plant Cu flux (F_{Tplt}) in seven soil samples, based on $\{Cu^{2+}\}_{CaCl2}$ measured in $CaCl_2$-soil extracts with ISE (Table 1) and Eq. 5. These theoretical plant fluxes can be then compared with corresponding soil fluxes (F_{DGT}), i.e. soil's ability to supply Cu^{2+} at the root surface, as assessed by DGT (Table 1). Such a comparison enables to assess whether soil re-supply kinetic or plant uptake kinetic is the rate-limiting process controlling Cu bioavailability to plants. This comparison is made in Fig. 5 where both the DGT Cu flux as a function of $\{Cu^{2+}\}_{CaCl2}$ and the Michaelis–Menten model for Cu uptake kinetics in durum wheat (solid line) are plotted. Soil Cu flux was lower than F_{Tplt} only for the slightly acidic, little contaminated soil (D). The largest differences between F_{Tplt} and F_{DGT} was observed for the moderately (F) and highly (E) contaminated, calcareous soils where F_{Tplt} was 10- and 26-fold lower than F_{DGT}, respectively.

Due to uncertainty on the K_M value (see Discussion section for rationale), a similar comparison between F_{Tplt} and F_{DGT} was also performed by recalculating the plant uptake kinetics with a plausible “unbiased” K_{M*} value (K_{M*}=1 nM) while keeping F_{max} unchanged (Fig. 5, dotted line). In this case, F_{DGT} was lower than F_{Tplt} not only for the slightly acidic, little contaminated soil (D), but also for the little and moderately contaminated, calcareous soils (F and G). Plant Cu flux was lower than F_{DGT} only for the strongly acidic soils (A and B) and the highly contaminated calcareous soil (E), while F_{Tplt} and F_{DGT} were comparable for the slightly acidic, moderately contaminated soil (C).

This kind of theoretical comparison also enables to investigate plant Cu requirements that differ from that of durum wheat. A last calculation of the plant uptake kinetics was therefore performed by considering a new F_{max} value (F_{max*}=170 ng Cu m^{-2}s^{-1}), about 4-fold larger than F_{max} value determined for durum wheat (see Discussion section for rationale), and K_{M*} (Fig. 5, dashed line). In this last case, F_{DGT} was lower than F_{Tplt} for all soils except for the highly contaminated, calcareous soil (E) where F_{Tplt} and F_{DGT} were comparable.

Discussion

Activity-dependent kinetics and the associated parameters, F_{max} and K_M, are needed for the modelling of metal uptake by plants. However, in the case of Cu,

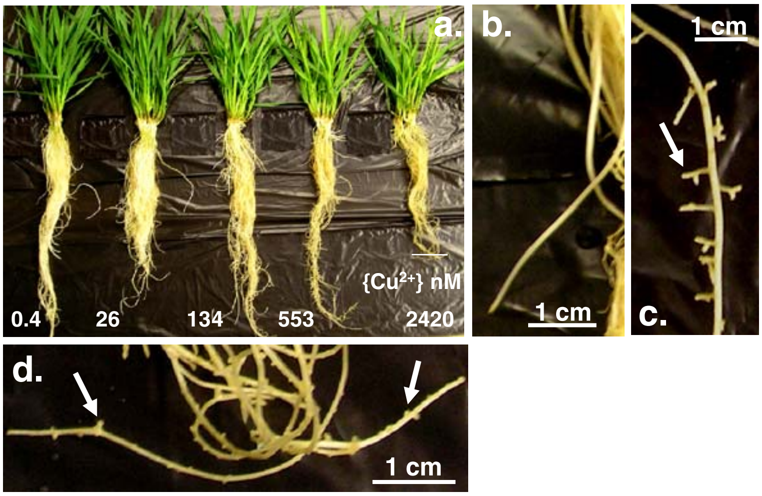

Fig. 2 Copper toxicity for durum wheat grown for 8 d in nutrient solutions at various $\{Cu^{2+}\}$. Photographs of whole plants harvested after 8 d of exposure to $\{Cu^{2+}\}$ (**a**) and root morphology for 0.4 (**b**), 553 (**c**) and 2,420 (**d**) nM $\{Cu^{2+}\}$, respectively. *Arrows* (**c** and **d**) indicate abnormal lateral root formation

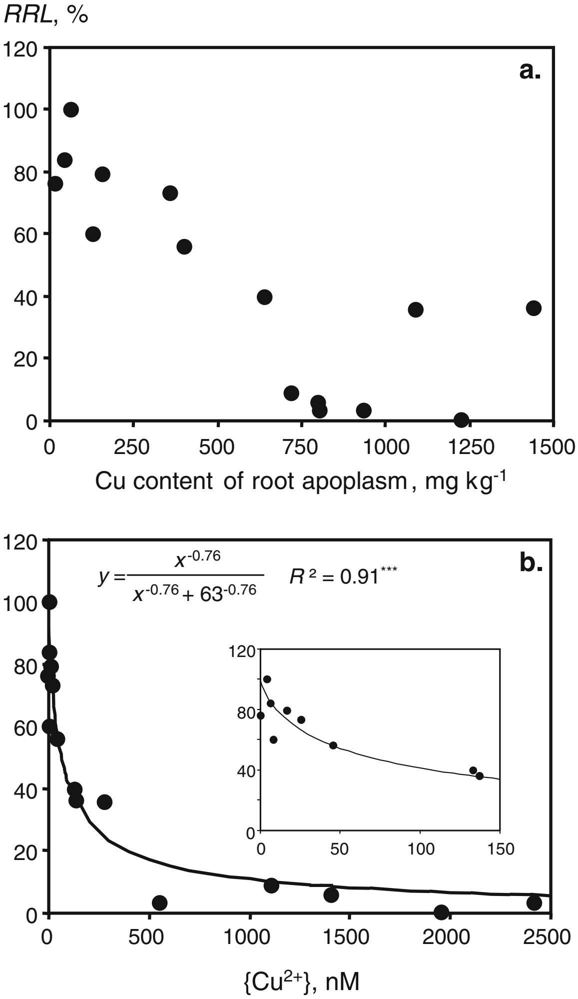

Fig. 3 Relative root length (*RRL*) as a function of Cu content of root apoplasm (**a**) and $\{Cu^{2+}\}$ in solutions (**b**). Curve and equation in (**b**) were obtained by fitting the data to Hill's equation (Eq. 2)

the reliability and the environmental relevance of kinetic parameters reported in the literature are questionable. In the present study, such parameters were determined for durum wheat in a more relevant range of $\{Cu^{2+}\}$, 0.4–2,420 nM. The interest of these kinetic parameters for plant uptake modelling was also further addressed by comparing the theoretical plant uptake flux to the ability of soil to supply Cu^{2+} at the root surface.

Copper phytotoxicity for environmentally relevant $\{Cu^{2+}\}$

Compared with previous studies about uptake kinetics of Cu by plants (Cathala and Salsac 1975; Veltrup 1976; Bowen 1987), the range of $\{Cu^{2+}\}$ investigated in the present work was several orders of magnitude lower and thus more environmentally relevant with respect to typical $\{Cu^{2+}\}$ found in soils, including contaminated soils, usually ranging from 10^{-12} to 10^{-5} M (Sauvé et al. 1997; Vulkan et al. 2000). Even though relatively low Cu concentrations were used in the present study, strong Cu phytotoxicity symptoms were observed, altering the growth of the whole plant.

Copper contents of 9 to 20 mg kg^{-1} in shoots and 25 to 90 mg kg^{-1} in the whole roots when plants were exposed to $\{Cu^{2+}\}$ lower than 10 nM suggest that $\{Cu^{2+}\}$ in the nanomolar or sub-nanomolar range is non-toxic to durum wheat. This is support by Michaud et al. (2008) who suggested that Cu content of about 100–150 mg kg^{-1} is the critical level in the

Table 2 Free Cu^{2+} activity (nM) involving a 25% (EC_{25}), 50% (EC_{50}), 75% (EC_{75}) and 90% (EC_{90}) reduction in the relative root length for plants growing in solutions with $\{Cu^{2+}\}$ in the range 0.4–2,420 nM. Values in parentheses stand for the 99% non-parametric confidence intervals

EC_{25} nM	EC_{50}	EC_{75}	EC_{90}
15 (7–23)	63 (30–81)	268 (101–353)	1,140 (267–1,804)

Table 3 Kinetic parameters of Michaelis–Menten equation for Cu uptake and absorption fluxes in durum wheat. Uptake flux accounts for Cu in root apoplasm, while absorption flux does not. Standard errors are indicated in parentheses

	F_{max} ng Cu m^{-2} s^{-1}	K_M nM	R^2
Absorption flux	18.6 (0.9)***	127 (27)***	0.96***
Uptake flux	43 (3)***	86 (23)**	0.94***

whole roots for the occurrence of Cu rhizotoxicity to durum wheat. Nevertheless, root length was altered by $\{Cu^{2+}\}$ as low as 15 nM which induced a 25% reduction in root elongation, while $\{Cu^{2+}\}$ of about 60 nM reduced root elongation by 50% (Table 2). These values appeared lower than phytotoxic thresholds previously reported in the literature. Kopittke et al. (2007) mentioned a 10% reduction in root or shoot biomasses of cowpea for 1,000 nM $\{Cu^{2+}\}$, while Michaud et al. (2008) reported a 50% reduction in root elongation of durum wheat for 600 nM $\{Cu^{2+}\}$. However, Cu^{2+} phytotoxicity for a given $\{Cu^{2+}\}$ is known to increase with increasing pH (Zhao et al. 2006). Kopittke et al. (2007) and Michaud et al. (2008) experiments were carried out at pH 4.5 and 5.5 respectively, which may explain the lower phytotoxicity measured in comparison with our experiment at pH 6. The visual symptoms of rhizotoxicity, i.e. an increased number of poorly developed lateral roots with increasing $\{Cu^{2+}\}$ (Fig. 2c and d), were previously mentioned by Kopittke and Menzies (2006) and Kopittke et al. (2007) for cowpea. These authors further interpreted the reduction of root elongation of cowpea by an increased rigidity of root cell wall in which Cu was accumulated. Such accumulation led to cell rupturing in the elongation

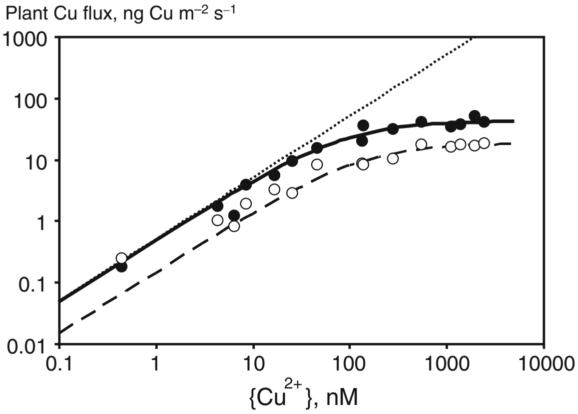

Fig. 4 Activity-dependent kinetics of net Cu fluxes in intact plants. Closed and opened symbols stand for uptake and absorption fluxes, respectively, given that uptake accounts for Cu in root apoplasm while absorption does not. Experimental data were fitted to Michaelis–Menten equation (Eq. 5) for uptake (*solid line*) and absorption (*dashed line*) (see Table 3 for corresponding model parameters). The *dotted line* stands for the diffusion limitation flux that likely drives the plant uptake flux in EDTA-buffered solutions at low $\{Cu^{2+}\}$ (i.e. $\{Cu^{2+}\} < K_M$), according to the reaction layer concept (thickness of the reaction layer, μ=65 μm) (see Discussion section for rationale, Eq. 7)

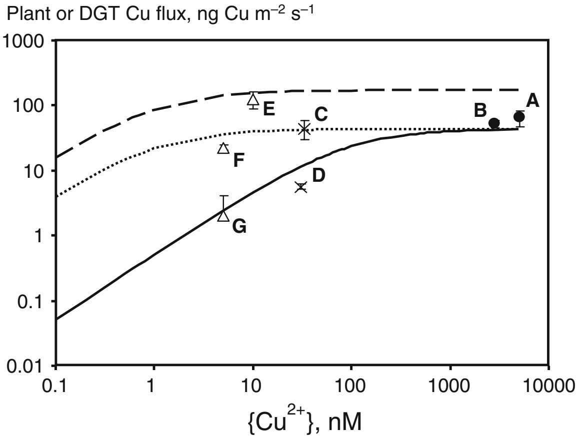

Fig. 5 Comparison between the theoretical uptake fluxes of Cu in plants (lines) and the ability of seven soil samples to supply Cu^{2+} at the root surface as measured by DGT (data points). Solid line stands for the uptake flux of Cu in durum wheat determined in the present study (F_{max}=43 ng Cu $m^{-2}s^{-1}$; K_M= 86 nM). Dotted line stands for the "unbiased" uptake flux of Cu in durum wheat, taking into account a plausible unbiased K_M (F_{max}=43 ng Cu $m^{-2}s^{-1}$; K_{M*}=1 nM). *Dashed line* stands for the expected uptake flux of Cu in plants showing large Cu requirements, i.e. with a large F_{max} (F_{max*}=170 ng Cu $m^{-2}s^{-1}$; K_{M*}=1 nM). See Discussion section for the rationale about F_{max*} and K_{M*} values. Closed circles stand for non calcareous soils with pH<5 and crosses stand for non calcareous soils with pH>5. Opened triangles stand for calcareous soils

zone for $\{Cu^{2+}\}$ around 1 µM, in a similar way as for the more documented case of Al rhizotoxicity (Kopittke et al. 2008). These alterations of root structure could be responsible for nutrient deficiencies such as iron deficiency, consistent with the interveinal chlorosis observed in our study and elsewhere (Kopittke and Menzies 2006; Michaud et al. 2008). They may also dramatically impact uptake kinetics. This conclusion therefore justifies carrying out kinetic experiments over longer durations than couples of minutes or hours in order to account for plant adaptative response to growth conditions.

Copper uptake kinetics in durum wheat

The activity-dependent kinetics of Cu absorption in durum wheat, which strictly speaking excludes the portion of root Cu adsorbed onto the cell walls (apoplasm), showed a hyperbolic shape with a saturable component for $\{Cu^{2+}\}$ above 500 nM (Fig. 4). Hart et al. (1998, 2002) and Lombi et al. (2001) also described a saturable component for the absorption of Cd and Zn by several plant species. This result suggests that Cu absorption in durum wheat is metabolically driven by transport protein(s) in the plasma membrane of root cells. Compared with other metals such as Zn and Fe, transporter(s) involved in Cu absorption into roots of graminaceous species is (are) poorly described. Although Cu can efficiently compete with Fe to form metal-phytosiderophore complex (Reichman and Parker 2005), Cu-phytosiderophore absorption mediated by *yellow stripe1* transporter may be of limited significance in the primary absorption of Cu by graminaceous species (Roberts et al. 2004; Yruela 2009). Alternatively, Cu absorption in durum wheat could be mediated by the high-affinity Cu^{+} transporter COPT1 and the Cu^{2+} transporter ZIP2 as recently described for the non graminaceous species *Arabidopsis thaliana* (Puig et al. 2007; Burkhead et al. 2009; Yruela 2009). When considering the Cu uptake flux in durum wheat, i.e. including the apoplasmic Cu in roots, the shape of the kinetics also exhibited a saturable component with a higher maximal flux (F_{max}). The difference between the uptake and the absorption kinetics was steady for $\{Cu^{2+}\}$ above 500 nM. This suggests the saturation of the Cu binding capacity of root apoplasm, reaching about 1 g Cu kg^{-1} dry root biomass (results not shown). This is in line with former results of Antunes and Hale (2006) who observed a saturation trend of Cu accumulation in the roots of 11 day-old durum wheat exposed to $\{Cu^{2+}\}$ in the micromolar range.

Compared with the literature on Cu uptake kinetics in plants (accounting for apoplasmic pool or not), the maximal fluxes (F_{max}) determined on short-term experiments (from 20 min to 4 h) were 7- to 1,400-fold larger than the F_{max} value determined for durum wheat in the present experiment (Cathala and Salsac 1975; Veltrup 1976; Bowen 1987). Veltrup (1976) reported for *Hordeum distichum* L. a 20-fold decrease in F_{max} when measured over 2 h instead of 20 min and an additional 2-fold decrease in F_{max} when measured over 24 h. This suggests that F_{max} parameter was highly sensitive to the duration of uptake experiments, which could be related to the time required for physiological adaptation of plants to environmental constraints such as metal toxicity. Conversely, K_M values found in the literature did not seem to be related to the duration of the experiments. In contrast, it varied considerably with the range of tested Cu^{2+} concentrations, ranging from 1.6 up to 500 µM (Cathala and Salsac 1975; Veltrup 1976; Bowen 1987). In these former studies, tested Cu^{2+} concentrations were all extremely high compared with concentrations reported to occur in soils, even in highly contaminated soils, and their environmental relevance is thus questionable. We decided to investigate a narrower range of concentrations, more likely to be experienced by soil-grown plants, which may explain why we found smaller K_M values than those reported earlier. Our kinetic parameters could be compared more reliably to those achieved by Antunes and Hale (2006) for durum wheat exposed to $\{Cu^{2+}\}$ ranging from 0.03 to 830 nM. Although this study mainly addressed the Cu binding properties of root apoplasm rather than the determination of true uptake kinetic parameters, we can estimate F_{max} and K_M values equal to about 30–100 ng Cu $m^{-2}s^{-1}$ and 0.2 nM, respectively. This F_{max} value is therefore comparable to ours while K_M is 430-fold lower. The large discrepancy in the K_M value could be partly explained by differences in the experimental design. Antunes and Hale (2006) used younger durum wheat seedlings of only 11-day-old and exposed the plants to Cu^{2+} in the darkness.

The Cu kinetic parameters determined for durum wheat can also be compared with those reported for

Cd uptake as plants usually take up very small amounts of Cd as for Cu. Investigating Cd uptake in durum wheat for environmentally relevant exposure, Hart et al. (1998, 2002) obtained $F_{\max}$ values which were 6- to 8-fold larger than those found for Cu in our experiment. This discrepancy could be explained by either the occurrence of different uptake pathways (transporters) for Cd and Cu or by the shorter duration (20 min) of their experiments as already discussed above. Conversely, K_M values of 40–70 nM obtained by these authors for Cd were in close agreement with our results for Cu. The new set of Cu uptake kinetic parameters that we determined in durum wheat therefore appeared more environmentally relevant than those available in the literature. Nevertheless, it should be kept in mind that the determination of such kinetic parameters is partly dependent on plant species/genotypes (Lombi et al. 2001), plant age (Chen et al. 2008) and experimental conditions such as the composition of the nutrient solution.

For the latter, Degryse et al. (2006) recently showed that plant uptake kinetics of a range of metals including Cu was driven in the low concentration range by diffusion limitations that occurred during the supply of free metals to roots, even when free metal activities are buffered with chelators such as EDTA. Although the determination of $F_{\max}$ value remains driven by physiological processes, K_M value is therefore likely overestimated by several orders of magnitude, i.e. the affinity of metals to roots is substantially underestimated, in most studies due to diffusion limitations (Degryse et al. 2009b). In chelator-buffered solutions, the diffusion limitation flux, F_{diff} (ng Cu m^{-2} s^{-1}), can be estimated using the reaction layer concept as follows:

$$F_{\text{diff}} = 10^3 \times (D/\mu) \times \left\{\text{Cu}^{2+}\right\} \times M_{\text{Cu}} \tag{7}$$

where D is the diffusion coefficient of free Cu^{2+} in solution ($5\ 10^{-10}\text{m}^2\text{s}^{-1}$), μ the thickness (m) of the reaction layer and M_{Cu} the molar mass (63.5 g mol^{-1}) of Cu. The reaction layer is the zone close to the root surface where the dissociation rate of the metal-chelator complex is not fast enough to re-supply the pool of free metal readily taken up by the root, thereby leading to the depletion of free metal activity towards the root surface. For a more detailed discussion about this concept, readers should refer to Degryse et al. (2006). Within the low $\{\text{Cu}^{2+}\}$ range, i.e. when $\{\text{Cu}^{2+}\} < K_M$, the Michaelis–Menten model (Eq. 5) can be reduced to a linear model:

$$F_{\text{plt}} = F_{\max} \times \left\{\text{Cu}^{2+}\right\}/K_M \tag{8}$$

Assuming that diffusion limitations occurred in this low $\{\text{Cu}^{2+}\}$ range, F_{plt} becomes equal to F_{diff} and, consequently, μ can be calculated by combining Eqs. 7 and 8:

$$\mu = 10^3 \times D \times M_{\text{Cu}} \times K_M/F_{\max} \quad \Rightarrow \quad \mu \approx 65\mu\text{m} \tag{9}$$

The corresponding model for F_{diff} is depicted in the Fig. 4. The order of magnitude thus calculated for μ supports the occurrence of diffusion limitations in our EDTA-buffered solutions (Degryse et al. 2009a). Recently, Degryse et al. (2009a, b) suggested “unbiased” K_M value equal to about 1 nM for Zn and Cd uptake by *Spinacia oleracea* L.. As the range of the rate constants for water loss by metal aqua-complexes ($\text{M(H}_2\text{O)}_6{}^{2+}$) are fairly similar for Cd, Cu and Zn (Morel and Hering 1993), this suggests that the “unbiased” K_M value characterising Cu uptake kinetics in durum wheat is also likely close to 1 nM, i.e. about 80-fold lower than the value determined in the present work (Table 3). Although the study of Antunes and Hale (2006) was not properly designed to determine uptake kinetic parameters of Cu in durum wheat, their findings even suggested a K_M value in the sub-nanomolar range. In this study, the authors used NTA to buffer $\{\text{Cu}^{2+}\}$. The lability of Cu-NTA complex is more than 50-fold larger than that of Cu-EDTA complex (Degryse et al. 2006), which could partly explain the 430-fold lower K_M value than that we found in the present study.

Comparison of Cu uptake kinetics in plants and soil's ability to supply Cu

Most of the few studies dealing with the modelling of soil–plant metal transfer concluded that soil's ability to supply metals at the root surface was usually lower than plant requirement and consequently that soil supply was the rate-limiting process of metal phytoavailability (Mullins et al. 1986; Adhikari and Rattan 2000; Sterckeman et al. 2004). As a result, former model predictions of metal uptake in plants were poorly sensitive to uptake kinetic parameters in comparison with soil parameters. In the case of Cu, the uptake kinetic parameters determined for durum wheat suggest that plant requirement are very low, which is consistent with the low concentrations of Cu usually found in plants (compared with other micronutrients). Thus, it appeared relevant to compare the theoretical plant uptake flux of Cu (F_{Tplt}) to soil Cu flux (F_{DGT}), i.e. soil's ability to supply Cu^{2+} at the root surface as assessed with the DGT technique. This comparison was made in Fig. 5 on a set of seven soil samples exhibiting a wide range of pH and total Cu content, by plotting both the DGT Cu flux as a function of $\{\mathrm{Cu}^{2+}\}_{\mathrm{CaCl2}}$ and different Michaelis–Menten models for Cu uptake kinetics in plants. Three sets of Cu uptake kinetics were considered in Fig. 5 with either (a) the uptake kinetic parameters determined for durum wheat ($F_{\max}$ and K_{M}; solid line), (b) uptake kinetic parameters of durum wheat modified to account for an "unbiased" K_{M} ($F_{\max}$ and $K_{\mathrm{M}*}$; dotted line) or (c) expected uptake kinetic parameters for a plant with a large Cu requirement, i.e. with a large $F_{\max}$ ($F_{\max*}$ and $K_{\mathrm{M}*}$; dashed line). Consequently, if F_{DGT} is lower than F_{Tplt}, i.e. if data points (F_{DGT} vs. $\{\mathrm{Cu}^{2+}\}_{\mathrm{CaCl2}}$) are below the Michaelis–Menten curve (Fig. 5), soil metal supply is expected to be the rate-limiting process. Conversely, if F_{Tplt} is equal or lower than F_{DGT}, plant uptake flux is expected to be the rate-limiting process.

When using the uptake kinetic parameters of durum wheat determined in the present study, it is noteworthy that F_{DGT} was lower than F_{Tplt} only for the little Cu-contaminated, slightly acidic soil (D, Table 1). This is consistent with the large increase in F_{DGT} noted either in strongly acidic soils (pH<5) at a given level of soil contamination or with increasing total soil Cu (Table 1; Bravin 2008). It is also striking that the ability of soil to supply Cu^{2+} was 10- and 26-fold larger than F_{Tplt} for the two Cu-contaminated, calcareous soils (E and F, Table 1), in spite of the very low $\{\mathrm{Cu}^{2+}\}$ measured in such calcareous soils. The importance of F_{DGT} in contaminated calcareous soils in comparison with plant uptake could be attributed to both the ability of dissolved organic matter (DOM) to mobilise Cu from the soil solid-phase to the solution at high pH and the lability of organically-bound Cu in solution. Consequently, this comparison suggests that the rate-limiting process for durum wheat would be plant uptake kinetics rather than soil Cu supply flux in most soils. Exceptions could occur for little contaminated, slightly acidic soils that exhibit a poor ability to supply Cu^{2+} in solution compared with plant requirement.

However, as discussed above, the K_{M} value determined herein for Cu uptake kinetics in durum wheat is likely overestimated and thus deserves further discussion. A similar comparison between F_{Tplt} and F_{DGT} was performed by recalculating the Michaelis–Menten curve with a plausible "unbiased" K_{M} value, $K_{\mathrm{M}*}$, set at 1 nM while keeping $F_{\max}$ unchanged (Fig. 5, dotted line). It was not surprising to observe that F_{DGT} remained larger than F_{Tplt} for the strongly acidic soils as plant uptake kinetics is drastically limited by internal plant regulations at such high $\{\mathrm{Cu}^{2+}\}$ in solution, corresponding to the saturation component of the Michaelis–Menten model. Conversely, $K_{\mathrm{M}*}$ had a huge impact for calcareous soils as F_{DGT} became lower than F_{Tplt} both in weakly and moderately contaminated, calcareous soils. When considering a more suitable K_{M} value, these results lead to conclude that for plant species with Cu requirement similar to that of durum wheat, i.e. non (hyper-) accumulator plants including the most common crop species (Chaignon et al. 2002, 2003, 2009; Faucon et al. 2007; Bravin et al. 2009), plant uptake kinetics would be the rate-limiting process in most of contaminated soils. Exceptions could occur for moderately contaminated, calcareous soils. These conclusions support those of Degryse et al. (2009a) who recently investigated the relation between soil DGT fluxes and plant uptake for metals. These authors suggested that plant uptake of Zn and Cu would be limited by soil re-supply mostly in moderately or uncontaminated soils at high pH. On the other hand, as regards to the impact of K_{M} value on the nature of the rate-limiting process, this also justifies strengthening our efforts to determine more accurately and broadly metal uptake kinetics in plants.

To check the impact of larger Cu requirements by plants, as would occur in e.g. (hyper-) accumulator species, on the nature of the rate-limiting processes, Michaelis–Menten was recalculating using $K_{\mathrm{M}*}$ and a larger $F_{\max}$, $F_{\max*}$, set at 170 ng Cu $\mathrm{m}^{-2}\mathrm{s}^{-1}$ (Fig. 5, dashed line). The $F_{\max*}$ value was chosen on the basis of the 4-fold increase in $F_{\max}$ observed for Cd uptake when comparing durum wheat (Hart et al. 1998, 2002) with the hyperaccumulator species *Thlaspi caerulescens* ecotype Ganges (Lombi et al. 2001). Except for the highly contaminated, calcareous soils, F_{DGT} was lower than F_{Tplt} for all soils including the two strongly acidic soils. Plant species with a high Cu requirement, such as Cu (hyper-) accumulator, would therefore be expected to deplete Cu^{2+} in the rhizosphere, i.e. Cu phytoavailability would mainly depend on soil's ability to supply Cu^{2+}, except when grown in highly contaminated soils. Such calculations may explain why DGT measurements were the best predicator of shoot Cu content in the indicator, accumulator species *Lepidium heterophyllum* Benth which was shown to be able to accumulate very large amount of Cu in its shoots (up to about 800 to 900 mg kg^{-1}; Zhang et al. 2001), while DGT measurement was a lesser predicator than total soil Cu of shoot Cu content in bread wheat, which is presumably a plant species with a low Cu requirement (Nolan et al. 2005) in as much as durum wheat and the most common crop species.

However, these comparisons between F_{Tplt} and F_{DGT} should be interpreted with caution. Firstly, although F_{Tplt} can be larger than F_{DGT} at the scale of the whole root system, the conclusion could be more balanced at the scale of root fragment as the physiological activity of root fragments greatly varied along the root axis (Puig et al. 2007). In comparison, F_{DGT} is much more homogeneous over the whole DGT surface area. As for plant uptake kinetics that can greatly differ for different plant species and/or experimental conditions (see the above discussion), soil metal flux as measured by DGT can be also altered by a range of abiotic and biotic factors as already discussed by Degryse et al. (2009a). Among these, DGT measurement largely depends on the thickness of the diffusive layer as F_{DGT} increases with decreasing thickness of the diffusive layer (Lehto et al. 2006). In our experiment, the thickness of the diffusive layer was arbitrarily set at 0.9 mm according to Zhang et al. (2001). The deployment time of DGT can also affect measurements as F_{DGT} was found to decrease as much as 5-fold after several days (Nowack et al. 2004). The advection flux of metal due to plant water uptake (i.e. mass flow) was also neglected in the present calculations. However, Oliver and Barber (1966) and Lehto et al. (2006) estimated that mass flow would significantly contribute to Cu and Zn uptake in plants, especially when grown in soils with a poor ability to re-supply free metal ions to soil solution, i.e. with a low buffering capacity and/or a slow metal desorption kinetics. Our calculations also do not account for root distribution in soil and the consequent competition between roots for Cu uptake (Barber 1995), which may result in altered $\{Cu^{2+}\}$ in the vicinity of neighbouring roots. Finally, other rhizosphere processes could greatly alter metal chemistry at the soil–root interface, such as pH changes and exudation of metal-complexing compounds (Hinsinger and Courchesne 2008; Bravin et al. 2009). The present study therefore provides new but still incomplete knowledge of the potential rate-limiting processes driving Cu phytoavailability. Additional processes listed above should be accounted for in a more integrative way in future studies.

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